The Effects of Crude Oil Contamination on the Reproduction of Freshwater Turtles

A Thesis
Submitted to the Faculty
of
Drexel University
by
Barbara Allen Bell
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
June 2005
Dedications

For Joseph Richard Cox
Acknowledgments

I express deep gratitude for my committee members, Drs. James R. Spotila, Susan Kilham, Michael O’Connor, Anthony Steyermark, Stephen McDow, and Aleister Saunders for their expert help during this process. I wish to thank Dick Nugent, Gary Stolz, Kate McManus, Mike McMenamen and other U.S. Fish and Wildlife Service personnel at the John Heinz National Wildlife Refuge for their permission to study turtles at the refuge and their enthusiasm for and assistance with this project. I also thank Dr. Harold Avery, Dr. Paul Sotherland and Dr. Katherine Goddard-Doms for their invaluable assistance. I thank Dr. Ronald Brooks for his insight, as well as Dr. Jennifer Elwood, Dr. Betsy Rich and Dr. Walter Bien. I thank Dr. Justin Congdon for the contribution of data from E.S. George Reserve in Michigan and insight to chapter 4. I would like to acknowledge the hard work of Drexel graduate students Vincent Saba, Susanna Clusella, Anika McKessey, Amanda Curtin, Annette Seig, Bibi Santidrian, Karen Klein, John Wnek, Shaya Honarvar, Kim Magrini and Bryan Wallace, as well as the 50+ Drexel undergraduate students without whose help this project would not have been possible. Special thanks to Robin Van Meter for her assistance and contribution of data to Figure 9. Infinite thanks to Meghan Davis for her patient support and encouragement and to my parents for the same. This research was funded by the Sun Oil Company, the ERM Foundation and the Drexel University L. Drew Betz Chair of Environmental Science.
Table of Contents

LIST OF TABLES.............................................................................................................. vii
LIST OF FIGURES........................................................................................................... viii
ABSTRACT.................................................................................................................... xi

CHAPTER 1. INTRODUCTION ...................................................................................... 1
  Bibliography ............................................................................................................. 6

CHAPTER 2. EFFECTS OF EXPOSURE TO CRUDE OIL ON FERTILITY OF EGGS AND REPRODUCTIVE OUTPUT OF FRESHWATER TURTLES ............................... 8
  Introduction .............................................................................................................. 8
  Hypotheses tested.................................................................................................. 10
  Materials and Methods .......................................................................................... 11
    Mark-Recapture and Egg Collection ................................................................. 11
    Calculation of relative clutch mass and fertility ............................................... 12
  Data Analysis ........................................................................................................... 13
  Results ...................................................................................................................... 15
    Fertility .................................................................................................................. 15
    Relative clutch mass ............................................................................................ 15
  Discussion .............................................................................................................. 17
    Fertility .................................................................................................................. 18
    Relative clutch mass ............................................................................................ 20
  Summary ................................................................................................................. 23
  Bibliography ......................................................................................................... 25

CHAPTER 3. THE EFFECT OF MATERNAL OIL EXPOSURE ON SURVIVAL, STAGE AT DEATH, AND EMERGENCE SUCCESS OF EMBRYOS ................................. 36
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bibliography</td>
<td>73</td>
</tr>
<tr>
<td>CHAPTER 5. OIL EXPOSURE CAUSED DEFORMITIES IN EMBRYOS AND HATCHLINGS OF TURTLES IN THE JOHN HEINZ NATIONAL WILDLIFE REFUGE</td>
<td>91</td>
</tr>
<tr>
<td>Introduction</td>
<td>91</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>93</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>94</td>
</tr>
<tr>
<td>Results</td>
<td>95</td>
</tr>
<tr>
<td>Discussion</td>
<td>97</td>
</tr>
<tr>
<td>Summary</td>
<td>102</td>
</tr>
<tr>
<td>Bibliography</td>
<td>103</td>
</tr>
<tr>
<td>CHAPTER 6. CONCLUSIONS</td>
<td>111</td>
</tr>
<tr>
<td>VITA</td>
<td>114</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1. Change in number of snapping turtle eggs laid from the year of the spill to subsequent 1 or 2 years. ................................................................................................................................. 29

2. Reproductive parameters for painted turtles (C.p.) and snapping turtles (C.s.) from the John Heinz National Wildlife Refuge, 2000-2003. Year 2000 were placed in bold font to emphasize the year of the oil spill. Years with less than 5 clutches examined were placed in italics. GBM = female gross body mass, CS = clutch size, mean egg mass = average mass of egg in a single clutch, CM = clutch mass, RCM = relative clutch mass, SD = 1 standard deviation. ............................................................................................................... 30

3. System for rating severity of deformities in embryos and adults of snapping turtles and painted turtles at the JHNWR. ........................................................................................................ 78

4. Number and percent of deformities of adult painted (Chrysemys picta) and snapping (Chelydra serpentina) turtles from JHNWR 2000-2003. Painted turtles had up to 4 deformities each. ................................................................................................................................. 79

5. Incidence of abnormalities in C. picta and C. serpentina of different age classes from the E.S. George Reserve in Ann Arbor, Michigan, 1975-2003. .................................................... 80
LIST OF FIGURES

1. Mean percent fertility of control and oil exposed snapping turtles from JHNWR 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error. ................................................................. 32

2. Mean percent fertility of control and oil exposed painted turtles from JHNWR 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error of the mean. ................................................................. 33

3. Mean relative clutch mass of oil exposed and control snapping turtles (*Chelydra serpentina*) from the John Heinz National Wildlife Refuge, 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error of the mean. ................................................................. 34

4. Mean relative clutch mass of oil exposed and control painted turtles (*Chrysemys picta*) from the John Heinz National Wildlife Refuge, 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error of the mean. ................................................................. 35

5. Percent of dead embryos out of all fertile eggs for oil exposed versus unexposed snapping turtles and painted turtles from JHNWR, 2000 and 2002. Numbers represent numbers of clutches examined. Error bars represent one standard error of the mean. ........................................................................................................ 55

6. Percent of snapping turtles that died at each stage of death (Yntema, 1968), 2000. “Unk” represents those eggs that were known to be fertile but either did not contain a discernable embryo or contained an embryo too decomposed to place in a single stage. .................................................................................................................. 56

7. Percent of snapping turtle and painted turtle embryos that died during middle, early or late periods of development, 2000. “Unk” signifies eggs that were fertile but decomposed to a point where no embryo was discernable. Numbers represent counts of dead embryos per category. Error bars represent one standard error of the mean. .................................................................................................................. 57

8. Percent embryos emerged from snapping turtle (*C. serpentina*) and painted turtle (*C. picta*) clutches from the JHNWR, 2000 and 2002. Numbers represent number of clutches examined. Error bars represent one standard error of the mean. .............. 58
9. Percentage of each type of embryonic deformity out of all *C. serpentina* eggs with deformed embryos from JHNWR. Percentages were calculated from 4 years of data. 210 embryos and neonates out of 1460 eggs with visible embryos were deformed.

10. Deformities in snapping turtle (*Chelydra serpentina*) hatchlings. All at hatching stage 26 [Yntema, 1968]. A, B and D are hatchlings with lethal deformities, C is a hatchling with a minor deformity. A. “Corkscrew” tail; mandible and maxilla short; prefrontal bone grown backward; cyclops; brain protruding from skull; abnormal pigmentation; kyphosis. B. Bent tail; prefrontal bone grown backward; no eyes; brain protruding from skull; abnormal pigmentation; rear limbs deformed; cervical spine bent 90°. C. Normal (black) vs. abnormal (gray) pigmentation. D. Maxilla and premaxilla not fused; short; eyes slanted forward; right eye would not open wide; abnormal pigmentation; yolk sac still herniated.

11. Percentage of type of each embryonic deformity out of all *C. picta* eggs with deformed embryos from JHNWR. Percentages were calculated from 4 years of data. 84 embryos and neonates out of 155 eggs with visible embryos were deformed.

12. Deformities in painted turtles (*Chrysemys picta*). Staged according to [Mahmoud, 1984]. All turtles had lethal deformities. A. Stage 23 (hatching stage); dwarf. B. Stage 23; dwarf; plastron narrow so yolk opening wide; tail bent; body skewed along anteroventral axis. C. Stage 23; deformed carapace, plastron, rear limbs, head, jaws; no tail; no eyes. D. Detail of head of (C), maxilla and premaxilla not fused; mandible deformed; no eyes; brain covered by skin only. E. Stage 18; dwarf; clear area is brain with fluid buildup; all visible body parts deformed; no tail; ectocardia. F. Stage 18; dwarf; tail bent; no pigment; no carapace or plastron; organs herniated through back; brain protruding; most body parts deformed.


14. A. Severity of deformities in embryonic and hatchling snapping turtles (*Chelydra serpentina*) from the JHNWR 2000-2003. The four year mean shows that the majority of deformities were lethal. B. Severity of deformities in embryonic and hatchling painted turtles (*Chrysemys picta*) from the JHNWR, 2000-2003. The four year mean shows that the majority of deformities were lethal. Numbers represent number of clutches examined. Error bars represent one standard error of the mean.

15. A. Percentage of each type of external deformity out of all adults captured, *C. serpentina*. B. Percentage of each type of external deformity out of all adults captured, *C. picta*.
16. Examples of deformities seen in adult turtles. All deformities are minor. A. *C. serpentina*. Scoliosis, scutes deformed as a result. B. *C. picta*. 3 short toes with toenails, several extra protruding scales. C. *C. picta*. One central toe; abnormal scales D. *P. rubriventris* (hatchling). One extra vertebral scute......................... 88

17. Proportion of abnormalities for turtles from ESGR. A. All adult and juvenile painted turtles 1975-2003. B. Hatchling snapping turtles 2004. .......................................................... 89

18. Prevalence of minor, moderate and lethal deformities among all snapping turtle hatchlings examined at the JHNWR and ESGR. ............................................................... 90

19. Percent deformity of embryos and hatchlings of painted turtles (*C. picta*) and snapping turtles (*C. serpentina*) at the JHNWR, 2000 and 2002. Numbers represent numbers of clutches examined. Error bars represent one standard error of the mean. ................................................................................................................................. 107

20. Types of deformities seen in embryos and hatchlings from oil exposed and control snapping turtles from JHNWR, 2000 and 2002................................................. 108

21. Deformities in snapping turtle embryos and hatchlings from oil exposed females. A, B, C, and E were alive when egg was dissected after full incubation period, D emerged from the egg successfully. B and C were photographed after formalin preservation. A. Dwarf, hatching stage. B. Dwarf, no pigment, deformed jaw and frontal skull, hatching stage. C. Abnormal pigment, curled tail, deformed plastron, upper jaw not developed so eyes and skull deformed, brain exposed, hatching stage. D. Hatchling with curled tail. E. Undersized with facial, pigment and tail abnormalities, hatching stage. F. Hatchling with deformed tail and underdeveloped carapace (rear).......................................................................................................... 109

22. Percent of snapping turtle embryos/hatchlings from JHNWR that fell into each category of severity of deformity for each exposure group, 2000 and 2002. Numbers represent numbers of clutches examined. Error bars represent one standard error of the mean. ................................................................. 110
ABSTRACT
The Effects of Crude Oil Contamination on the Reproduction of Freshwater Turtles
Barbara Allen Bell
James R. Spotila, Ph.D.

A large crude oil spill in the John Heinz National Wildlife Refuge impacted the reproduction of resident turtle populations. The goal of this group of studies was to determine the effect of crude oil exposure on female snapping turtle (*Chelydra serpentina*) and painted turtle (*Chrysemys picta*) fertility, reproductive output, and development of offspring. There was no significant difference in egg fertility between female snapping turtles exposed to oil or control turtles. However, female snapping turtles had significantly lower fertility of eggs in 2002 compared to 2000. There was no difference in reproductive output as measured by relative clutch mass (RCM) between exposure groups or years for snapping turtles or painted turtles. Embryonic death was greater and hatching success lower for snapping turtle clutches from oil exposed females compared to controls in 2000 but not 2002. Most snapping turtle embryos died early in development, and there were significantly more early deaths for oil exposed *Chelydra* than controls. *Chrysemys* had a greater incidence of embryonic deaths than *Chelydra*. Control painted turtles not only had a higher incidence of abnormality than control snapping turtles, but malformations were more severe in the former than the latter. Lethal deformities were dominant in both snapping turtle and painted turtle control embryos in 2000 but not 2002. Tail and shell abnormalities were most common in snapping turtles both years, while dwarfism and shell abnormalities were most common for painted turtles. Oil exposure exacerbated developmental problems in snapping turtles, causing increased incidence and severity of deformity in embryos. Both species
exhibit high rates of embryonic and adult deformity in the refuge. Although the refuge offers many advantages to the resident turtle populations, background pollution places a developmental burden on the life history of these turtles that was exacerbated by exposure to crude oil.
CHAPTER 1. INTRODUCTION

In February of 2000, an underground pipeline ruptured and leaked approximately 758,000 liters of West African Cabinda crude oil at the edge of a 59 ha lake (impoundment) in the John Heinz National Wildlife Refuge (JHNWR) in suburban Philadelphia, Pennsylvania. The lake was covered with 20 cm of ice at the time, but oil facilitated melting in the spill area. The oil recovery team installed booms and dams to contain the oil, and skimmed oil from water and vegetation. Clean up personnel erected a fence around the spill area after the ice melted to prevent animals from entering or leaving the spill area. Sediment analyses indicated that the extent of the contamination was localized to areas close to the rupture site. The surface layer of ice limited the spread of the surface slick (which was floating under the ice) by protecting it from wind dispersion to an approximate 1 ha area (ENTRIX, 2000).

As the oil began to settle to the substrate, turtles left their hibernacula and came to the surface. At the time of the spill, twenty turtles surfaced coated with oil: 5 painted turtles (*Chrysemys picta*), 2 snapping turtles (*Chelydra serpentina*), one state-threatened red-bellied turtle (*Pseudemys rubriventris*) and 12 red-eared sliders (*Trachemys scripta*; two died). These turtles were taken to a local wildlife rehabilitation center for cleaning, and were transferred to my care at Drexel University the same month. I kept these turtles in the laboratory until June 2000 to monitor their recovery.

Crude oil is a mixture of thousands of compounds of varying weights, viscosity and volatility. This complexity leads to multiple toxic effects in organisms. Not only is every type of crude oil different, but batches from the same oil well can differ in composition. In an aquatic spill, weathering alters the mixture as soon as the oil contacts
the atmosphere at the air/water interface. The effects of an oil spill are influenced by the amount released, type of oil and its corresponding characteristics (viscosity, specific gravity, etc.), in addition to physical variables that affect dispersion and weathering. These variables include the effects of currents, wind and sunshine. Heavier components such as asphalts and waxes settle down through the water column, while lighter components begin to evaporate immediately.

Locally, turtles normally hibernate in mud in February. Apparently the turtles were irritated by the oil, and came to the surface into the oil slick. Turtles employ “extrapulmonary respiration” when hibernating: they pull water in and out of their cloaca, and tiny capillaries in a small chamber inside the cloaca (the cloacal bursa) absorb oxygen from the water. The heavy components of the oil settled down onto the mud where the turtles were hibernating, and probably coated and irritated their skin and mucosa, forcing the turtles to the surface.

Polycyclic aromatic hydrocarbons (PAHs) are among the most well-studied of crude oil components and are of particular concern because they persist in the environment for long periods of time, bioaccumulate in organisms, and some are known to be carcinogenic (Wilson and LeBlanc, 2000). They are characterized by two or more fused (sharing a pair of carbon atoms) aromatic (benzene) rings. The PAHs become less soluble in water and more susceptible to oxidation and reduction as molecular weight increases. Low solubility in water causes most PAHs to adsorb to particulate matter and accumulate in tissues of biota. Trace amounts of complex mixtures of PAHs occur in aquatic ecosystems, from both anthropogenic and natural processes (Neff, 1979). A variety of pathways lead to the formation of PAHs, from direct (by bacteria, fungi and
plants) and indirect (synthesized from plant and animal pigments exposed to the reducing environment of anoxic sediment) biosynthesis to burning of fossil fuels (Neff, 1979). Metabolic degradation, photooxidation, sedimentation and evaporation (primarily of the lower molecular weight PAHs) act to reduce amounts of PAHs in the aquatic environment. The PAHs can persist indefinitely if buried in anoxic sediment or other crevices that protect them from degradation (Neff, 1979; Peterson et al., 2003).

Acute toxicity tests can give insight into the acute toxic effects of contaminants but cannot predict the effects of an oil spill on organisms and ecosystems or of sublethal effects of chronic exposure. Some types of weathered crude oil are less toxic than fresh oil when applied directly to duck eggshells (Stubblefield et al., 1995). Macko and King (1980) state that although most crude oils become less toxic after weathering, light Arabian and Iranian crude oils become more toxic. Toxicity of PAHs increases with increasing size to a threshold where they become less bioavailable (i.e. asphaltines) (Carls et al., 2000; Wilson and LeBlanc, 2000). Heavier PAHs such as phenanthrenes and chrysenes are more toxic than lighter components (Falk-Petersen and Kjorsvik, 1987; Wilson and LeBlanc, 2000) and can persist in the environment for years after a spill and persist in tissues longer than other, lighter PAHs (Heintz et al., 1999). Although not as toxic, lighter aromatic compounds are necessary to keep heavier aromatic compounds in solution, and a lack of lighter aromatic compounds may be part of the reason that Hoffman and Gay (1981) reported that weathered oil is less toxic than fresh oil when applied directly to eggshells. Wilson and LeBlanc (2000) state that fresh crude oil is more acutely toxic than weathered due to the presence of the mono- and di-aromatic compounds.
Toxicity of oil can vary with life stage, but the embryo appears to be the most susceptible life stage (Brown et al., 1996; Carls and Rice, 1998; Wilson and LeBlanc, 2000). Determination of sensitivity also depends on the measurement taken. Carls and Rice (1998) found that the larval stage of pollock were the most sensitive in terms of survival to the water soluble fraction of Cook Inlet crude oil when considering the LC$_{50}$ based on concentrations of oil outside the embryos, but embryos in eggs were actually revealed to be the most sensitive life stage when internal concentrations (the amount the embryo or larvae took into yolk and tissues) were taken into account.

Acute effects of oil exposure have been studied more thoroughly than chronic effects on organisms and populations. In terms of reproductive effects on vertebrates, chronic exposure to petroleum leads to developmental abnormalities in embryos (Sharp et al., 1979; Brown et al., 1996), increased embryonic death rates (Brown et al., 1996; Bue et al., 1998; Carls and Rice, 1998) and reduced fecundity of adults exposed to long-term sublethal doses (Rowe et al., 1983). The effects of oil exposure can be passed on through generations. (White et al., 1999) demonstrated that the progeny of an inbred laboratory population of fathead minnows whose parents were exposed to a sublethal concentration of 1 ppb benzo[a]pyrene had significantly reduced survivorship compared to a control.

Snapping turtles and painted turtles are iteroparous, which means they lay multiple clutches within a lifetime. Snapping turtles nest once each year, while painted turtles have been known to sometimes lay two or (rarely) three clutches annually. According to Congdon and Tinkle (1982), most follicle enlargement occurs from late August to October of the year before oviposition, and the remaining energy used to make a clutch of eggs is mobilized from stored lipids in the spring. Lipophilic contaminants
may be passed from mother to egg (Bishop et al., 1994; Pagano et al., 1999), and can adversely affect embryonic development (Bishop, 1991). Bishop et al. (1994) did not find any correlation between body or clutch size measurements and chemical levels in eggs, but they did find significant relationships between body size (a correlate of age) and concentrations of lipophilic compounds in fat and liver of adult snapping turtles. Maternal exposure to crude oil in the months preceding the nesting season may have resulted in the transfer of oil components to egg yolk in turtles in JHNWR.

This dissertation contains results of studies on the effects of crude oil exposure on the reproduction and development of snapping turtles and painted turtles. I did not include data for red-bellied turtles or red-eared sliders because I did not encounter many nesting females of those species and therefore sample sizes were too low for statistical analyses. I will begin with a discussion of the effects of crude oil on fertility and reproductive output (Chapter 2), followed by a treatment of its effect on embryonic death and hatching success (Chapter 3). I will then elucidate data on the prevalence and types of deformities in unoiled turtles at JHNWR (Chapter 4), and then examine the additional effects that crude oil exposure had on normal development (Chapter 5). Chapter 6 will briefly summarize findings from previous chapters.
Bibliography


CHAPTER 2. EFFECTS OF EXPOSURE TO CRUDE OIL ON FERTILITY OF EGGGS AND REPRODUCTIVE OUTPUT OF FRESHWATER TURTLES

Introduction

The persistence of a species or population depends on successful reproduction. Fertility and reproductive output contribute to reproductive success. Reproductive effort, or the amount of physiological resources allocated to reproduction, is difficult to completely quantify. A female’s total reproductive effort for a single season is a result of juggling recently acquired and previously stored energetic resources between courtship, territorial or aggressive behavior, escape from predation, propagule formation and all other physiological needs (such as growth, storage, foraging, etc.). Since reproductive effort cannot be directly measured, it must be examined through correlates such as reproductive output, which can be estimated through relative clutch mass (RCM).

Many reproductive traits, including reproductive output, are associated with body size in many species of turtle (Blueweiss et al., 1978). Body size of turtles is closely correlated with clutch mass, clutch size and egg mass (Iverson, 1977; Congdon and Tinkle, 1982; Congdon et al., 1983; Jackson, 1988; Congdon and van Loben Sels, 1991; Iverson, 1992; but see Steyermark and Spotila, 2001). Larger species of turtles produce lighter clutch masses in relation to body size than smaller species of turtle (Iverson, 1992). Snapping turtles grow larger as they get older, so older snapping turtles have greater abdominal volume available for eggs and may therefore produce more eggs than younger, smaller turtles (Iverson, 1992; Iverson et al., 1993). Larger females can also lay larger eggs due to a larger pelvic opening (Congdon, Gibbons et al., 1983; Tucker, 1997), although there is a stronger correlation between egg size and pelvic opening in smaller species of turtles than in larger species (Gibbons, 1982; Congdon and Gibbons, 1987;
Iverson et al., 1997). Steyermark and Spotila (2001) found that larger female snapping turtles from John Heinz National Wildlife Refuge (JHNWR) laid larger clutches than smaller females - but they did not lay larger eggs. Iverson et al. (1997) suggest that selection may be acting on *Chelydra* to produce a minimum viable egg size and to maximize clutch size.

Because clutch size correlates with body size, one must correct for body size when attempting to compare reproductive output in terms of clutch mass, egg mass or egg size. Relative clutch mass (RCM) is a calculation of the proportion of gravid female body mass comprised of egg mass. Iverson (1992) found that when body size is removed from the analysis, turtles that mature early have a higher annual RCM than those that mature later. RCM can even differ within a species, as researchers found in a population of *Chrysemys picta* in the Nebraska Sandhills where relative clutch mass between years and within individuals that lay multiple clutches in one year varied annually (Iverson and Smith, 1993).

In addition to body size and age, diet (Gibbons and Tinkle, 1969; Moll, 1979), competition, mortality, and environmental predictability can affect reproductive output (Brockelman, 1975). Fertility can be affected by physiological and environmental conditions such as nutrition, hormones, age, mechanical blockage, abnormal gametes, contamination or infection (Shah et al., 2003). Data on reptile fertility are sparse in the scientific literature. Gibbons (1982) found that microhabitat influences or environmental experiences were more influential on clutch size than were age or genetics, and that clutch frequency has more of an influence on annual reproductive output than clutch size in freshwater turtles. Competition for resources remains a factor in reproductive output.
The more successfully a turtle obtains resources, the more energy it will have available to allocate to reproductive effort. For example, Seigel and Fitch (1985) observed a significant increase in the number of snake offspring produced in years with higher resource availability when compared to less environmentally productive years.

In February 2000, an underground pipeline leaked nearly 758,000 liters of Cabinda crude oil into a man-made lake (impoundment) at the John Heinz National Wildlife Refuge, Philadelphia, Pennsylvania. This influx of petroleum added to decades of background contamination from various municipal and industrial sites as well as atmospheric deposition from the neighboring airport and city. Twenty turtles representing four species (snapping turtle, *Chelydra serpentina*, painted turtle, *Chrysemys picta*, state-threatened red-bellied turtle, *Pseudemys rubriventris*, and red-eared slider, *Trachemys scripta*) surfaced through melted ice at the time of the spill. Other turtles were contained inside a fence that oil recovery workers erected around the spill area to prevent biota from spreading the oil. The fence remained around the spill area for over a year. Petroleum exposure affects reproductive success in birds (Ainley et al., 1981; Fry et al., 1986; Irons 1996), fish (Hall and Oris, 1991; Kiceniuk and Khan, 1987) and mammals (Mazet et al., 2001), but the effects of oil on reproductive output and fertility of turtles has not been studied.

**Hypotheses tested**

The goal of this research was to determine if exposure to crude oil affected the fertility and reproductive output of turtles. The first hypothesis tested was that maternal exposure to crude oil would decrease fertility of clutches in snapping turtles (*Chelydra serpentina*) and painted turtles (*Chrysemys picta*) at the JHNWR. The second was that
maternal exposure to crude oil would decrease reproductive output as measured by RCM in the aforementioned species of turtle. Third, I proposed that the initial maternal exposure to crude oil would cause a decrease in number of eggs laid in years subsequent to the oil spill.

**Materials and Methods**

*Mark-Recapture and Egg Collection*

I captured turtles in hoop nets in the fenced spill area and in the opposite end of the lake from March to April of 2000. Turtles exposed to oil during the spill in February were taken to Tristate Bird Rescue and Research, Inc. for critical care and cleaning. They were then transferred to Drexel University in March. None of these turtles laid eggs in 2000. I also trapped, marked and released turtles in the summer of 2001 and 2002. Turtles captured at the time of the spill and those captured inside the fenced area were designated “oil exposed” because they had either been found with oil on them or been held within the oil-exposed area since February. Turtles captured at the opposite end of the lake were designated as controls. It is unlikely that these turtles were exposed to oil because 1. the fence erected around the oil spill area was done soon after the spill and prevented animals from spreading oil and 2. chemical analysis of sediments in the area surrounding the spill indicated that the oil did not extend beyond approximately 1 hectare of the 59-hectare impoundment. I marked turtles by filing or drilling holes in the marginal scutes of the carapace that corresponded to an alphabetic code similar to the numeric code developed by Cagle (1939). I conducted foot patrols of known snapping turtle nesting sites in the JHNWR from May to June 2000-2003. When possible, I
collected eggs from females during oviposition in the field, otherwise, I transported gravid females to the laboratory and induced egg-laying with oxytocin (Ewert and Legler, 1978; 19-21 IU/kg as per Steyermark, pers. communication). I incubated eggs in a Precision™ incubator at 26.5ºC ± 0.5 in 32cm x 18cm plastic boxes with sand maintained at 4% moisture gravimetrically (between -100 and -170 kPa). I replenished moisture in each box three times per week.

Calculation of relative clutch mass and fertility

I determined reproductive output in terms of the proportion of body mass taken up by clutch mass (relative clutch mass, or RCM). I calculated relative clutch mass according to Iverson (1992):

$$\text{RCM} = \frac{\text{clutch mass}}{(\text{gravid body mass} - \text{clutch mass})}$$

I palpated each female after she had completed oviposition to determine if any eggs remained in the oviducts. A calculation of reproductive output requires the mass of each female’s complete clutch. In cases where some egg masses were not available, I calculated the mean egg mass from the rest of the clutch and used that as the estimated mass of each missing data point.

One recaptured oil-exposed female that I collected eggs from in 2000 had to be excluded from the analysis in 2002 even though she technically laid eggs. She appeared to be extremely ill: she was morbidly obese, retaining fluid, and had severe reproductive problems. When she laid eggs, only 7 of them had thin, translucent shells. The rest she laid in a large mound of yolk and albumin.

I determined fertility according to Bell et al. (2003). I classified an egg with a white spot as fertile, even if I could not find an embryo (Blanck and Sawyer, 1981;
Miller, 1985). I dissected eggs that did not form a white spot, or that otherwise appeared dead, and examined them for signs of fertility. I also classified an egg as fertile if an embryo or signs of an embryo, such as remnants of blood or membranes, were present even in the absence of a white spot. I classified an egg as infertile if there was no white spot and the yolk membrane was intact and there was no sign of an embryo. If the yolk membrane was broken, no white spot was present and I did not find an embryo, I classified the egg as unknown fertility. I calculated percent fertility by dividing number of fertile eggs by number of eggs in the clutch. This provided a conservative estimate of fertility because it excluded eggs of unknown status that may have been fertile.

Data Analysis

Although four species of turtles were exposed to the oil spill, painted turtles and snapping turtles were the only groups to produce a large enough sample size of individuals and eggs for statistical analyses. I arcsine-transformed percent fertility data for analysis. I only used snapping turtles with clutches of 15 eggs or more because power analyses indicated that 15 eggs was the minimum number of eggs needed per clutch to show significant differences between clutches. I used painted turtles with clutches of at least 3 eggs in analyses because this was the minimum number of eggs that I determined would accurately estimate trends for Chrysemys, which lays small clutches of 2-11 eggs. The fertility data did not meet the assumptions of analysis of variance (ANOVA) and the sample size of recaptured oil exposed individuals was low (Figure 1) so I could not trust the accuracy of an F statistic generated by a traditional ANOVA. I therefore performed a bootstrap analysis of the data to obtain critical F values under the null hypothesis from a distribution based on actual data. I programmed Matlab to run an ANOVA (on a large
data set created by resampling my actual data) under the alternative hypothesis (that there was a difference in fertility between exposure groups and years) 100,000 times and to generate an F distribution from each F statistic obtained in each run. Because sample size was so low for all categories of all species of turtles, but particularly for oil exposed turtles, I conducted a power analysis to determine if sample size was sufficient to show a true difference between groups. I then conducted a two-way analysis of variance of arcsine-transformed percent fertility with year and degree of oiling as factors, and compared the F values of that analysis to the critical F values of the bootstrap analysis. I did not analyze data from 2001 or 2003 in this analysis due to low recapture rates of oiled turtles in those years. I then compared arcsine-transformed percent fertility between control painted and snapping turtles for all four years using a bootstrap analysis of variance. I also examined the change in number of eggs laid for those snapping turtles for which I had repeat nesting data. My sample size was too low to do so statistically, so I presented those data with descriptive statistics (Table 1).

I used an analysis of variance to compare both gross body mass (GBM) of females and mean egg mass between 2000 and 2002 for snapping turtles or for painted turtles in 2000 only. I arcsine-transformed RCM data prior to analysis. Some turtles had to be excluded from analysis due to lack of body mass data. I compared RCM between oiled and control snapping turtles between years with a two-way ANOVA. I did not have sufficient sample sizes of oil exposed painted turtles in 2001-2003; therefore I performed a one-way ANOVA to compare RCM of oiled and control painted turtles in 2000 only. I also used ANOVA to compare RCM between control snapping turtles from 2000-2003
and control painted turtles from 2000-2003, then compared controls of the two species. I
accepted statistical significance at the 0.05% level.

**Results**

*Fertility*

Mean percent fertility of control female snapping turtles was 98.2% in 2000
(n=12) and 75.0% in 2002 (n=12), while that of oil exposed females was 96% in 2000
(n=9) and 77.6% in 2002 (n=3). The standard error of the transformed data of the oiled
group was more than twice that of the control group for both years (Figure 1). There was
a significant difference in fertility between 2000 and 2002 (df=32, F=7.86, P=0.012) but
not between oiled and control turtles (df=32, F=0.066, P=0.798). Power analysis run via
bootstrap analyses revealed a power of 0.987 (the F statistic generated in each of the
100,000 runs of the bootstrap was greater than the critical value 98.7% of the time) for
year but only 0.078 for degree of oiling. There was a significant difference in fertility
between years (df=46, F=5.36, P=0.002) as well as between species (df=46, F=4.25,
P=0.043) for control snapping turtles and painted turtles (Figure 2). Mean fertility of
controls over four years was higher for painted turtles (97.4%) than snapping turtles
(93.2%).

*Relative clutch mass*

Mean GBM of female snapping turtles studied ranged from 1700 to 7500g over 4
years (Table 2), and was not significantly different between oil exposed and control
turtles in 2000 and 2002 (df=22, F=1.26, P=0.274) or between years (df=1, F=0.367,
P=0.551) (Figure 3). Mean GBM of painted turtles over four years ranged from 243 to
520g, and was not significantly different between oil exposed and control turtles in 2000 (df= 13, F = 0.002, P= 0.964) (Figure 4). Clutch size varied between females and years for both species. Mean egg mass varied from 6.8 to 13.8g for snapping turtles over 4 years, and did not differ significantly between oil exposed and control turtles in 2000 and 2002 (df= 22, F= 0.054, P= 0.818) or between years (df= 2, F= 0.243, P= 0.627). Mean egg mass varied from 3.3 to 7.1g for painted turtles over four years, and did not differ between oil exposed and control turtles in 2000. Clutch mass varied with clutch size and egg mass between years for both species, ranging from 11.3 to 52.1g for painted turtles and 68.4 to 719.5g for snapping turtles.

RCM ranged from 0.062 to 0.125 for control snapping turtles over four years and from 0.025 to 0.128 for oil exposed snapping turtles in 2000 and 2002. RCM of painted turtles ranged from 0.032 to 0.133 for controls over 4 years and 0.073 to 0.144 for oiled turtles in 2000 (the only year with more than 2 oiled turtles). Mean RCM of control snapping turtles was 0.093 in 2000 (n=7) and 0.091 in 2002 (n=10), while that of oil exposed turtles was 0.082 in 2000 (n=6) and 0.073 in 2002 (n=3). Analysis of variance suggested that there was no significant difference between oil exposed and control snapping turtles (df= 22; F= 2.26; P= 0.147) or between years (df= 1; F= 0.152; P= 0.701). Mean RCM of control painted turtles was 0.094 in 2000 (n=8) and 0.114 for oil exposed turtles (n=5). A one-way ANOVA indicated that the difference between oil exposed and control painted turtles in 2000 was not significant (df= 11; F= 1.70; P= 0.219). RCM did not vary among control snapping turtles between years 2000 and 2003 (df= 3; F= 1.38; P= 0.272), nor did it vary among control painted turtles over the same time period (df= 3; F= 0.302; P= 0.824). There was no significant difference in RCM
between control painted turtles and snapping turtles from 2000-2003 (df= 1; F= 3.31e^{-005}; P= 0.995) or between the four years (df= 3; F= 1.066; P= 0.371).

Although sample sizes of recaptured oil exposed turtles were small, the data suggested a trend in the effect of oil exposure on number of eggs laid in subsequent years: snapping turtles exposed to oil laid fewer eggs one to two years after the oil spill than control turtles (Table 1). Unfortunately, missing body mass data prevented calculations of RCM for most of these turtles. Control turtles laid the same number or more eggs than in 2000 (mean = 0.4 more eggs), with one exception, while those exposed to oil laid far fewer eggs in subsequent years (mean = 12.5 fewer eggs).

Discussion

Sample sizes of recaptured oil exposed and control turtles were low for both species for all years, particularly for oiled turtles. The refuge and its turtle populations are very large, which makes it difficult to find individual turtles during patrols of nesting areas. It is possible that some oil exposed turtles were not encountered because they did not produce eggs in subsequent years. Kiceniuk and Khan (1987) found that four of seven female cod chronically exposed to oil did not produce eggs, while all twelve cod in the control group did produce eggs. Oral ingestion of 2ml of weathered Santa Barbara crude oil or application of as little as 0.1mL to breast feathers one month before laying by wedge-tailed shearwaters cause a decrease in number of breeding pairs that laid eggs compared to birds that were not fed oil (Fry et al., 1986). Some oil exposed turtles may have died from direct or indirect toxic effects as well.
Fertility

The lack of a significant difference in fertility between oil exposed and control turtles within years suggests that oil exposure did not affect the fertility of snapping turtles or painted turtles. This lack of difference corroborates findings by Carls et al. (2000), who dosed herring with PAHs. The authors found no correlation between egg fertility and total PAH concentration. However, my results contradict those from other studies on the effects of oil on invertebrates, fish, birds and mammals. Exposure to diesel oil caused a decrease in gametic and nutrient storage cells and an increase in atretic (degenerating) gametes in mussels (Lowe and Pipe, 1986). Cows exposed to large oil fields showed an increased risk of non-pregnancy (Waldner et al., 2001), which can be caused by infertility. Likewise, exposure to petroleum hydrocarbons reduced fertility and fecundity in oligochaete worms, and these effects were positively correlated with concentration (Hauschildt-Lillge, 1982). Furthermore, fertility rate and reproductive potential in subsequent generations of the lowest and highest dose was markedly affected, with a constant decrease in cocoon production to cessation in the fourth generation. Long term, multi-generational effects were more severe than short-term. External application of Prudhoe Bay crude oil to the breast feathers of wedge-tailed shearwaters caused a decrease in number of returning breeding pairs and number of eggs laid the year after treatment, which suggests a long–term effect from a single, small dose (Fry et al., 1986). Apart from the decrease in number of eggs laid by three recaptured oil exposed turtles, I did not see such an effect on turtles in this study. However, this was a relatively short-term study compared to the long life of a turtle, and therefore cannot demonstrate long-term effects of oil exposure on fertility of turtles or their offspring. Turtles, like most
reptiles, mask illness well and take months or even years to show symptoms of or
succumb to illness (Wellehan and Gunkel, 2004).

Fertility decreased for both oil-exposed and unexposed turtles in 2002. This
simultaneous decrease suggests a possible population-wide effect from one or more
unknown environmental factors. There was a significant fish kill due to anoxia in the
Refuge during late summer 2001. I presume that the turtles fed on these dead fish,
because we did not catch many turtles in live traps during the fish kill. It is possible that
they consumed disproportionately more fish that summer than in the preceding year(s).
Fish are high on the food chain and bioaccumulate pollution, and the turtles would have
bioconcentrated contaminants from the fish. An *ad libitum* diet of contaminated fish
might expose them to a greater contaminant load, and therefore greater amount of
contaminants, than they would have experienced on their normal diet. According to
Armstrong (1986), environmental contaminants can affect ovarian function in three ways:
1) through disruption of normal hormone secretion patterns or reproductive behavior; 2)
direct damage to oocytes; or 3) damage to other organs or systems that influence the
normal operation of the hypothalamo-hypophyseal system, which controls gonadotropin
secretion and gonadal function. Gonadotropic hormones play pivotal roles in
reproductive function. Thomas (1988) found that sublethal levels of benzo[a]pyrene that
did not affect the overall health of Atlantic croaker diminished ovarian growth and
production of 17β-estradiol, a steroid hormone responsible for stimulating production of
vitellogenin. Although a contamination influence on fertility in 2002 is possible, natural
environmental and physiological factors such as nutrition and age could have played a
role as well. My sample size for recaptured individuals is low, which can affect the results of analyses, particularly with the large standard deviation found in my data.

*Relative clutch mass*

Exposure to crude oil did not affect reproductive output for those snapping turtles and painted turtles for which I was able to calculate RCM. This is surprising in light of contrary findings by other investigators. The effect of oil on reproductive output has been studied in birds (Macko and King, 1980; Ainley et al., 1981; Fry et al., 1986; Kuletz, 1996; Stubblefield et al., 1995; Irons, 1996;), fish (Hall and Oris, 1991; Johnson et al., 1993), mammals (Mazet et al., 2001) and invertebrates (Hauschildt-Lillge, 1982; Lowe and Pipe, 1986), but not in reptiles. Maternal exposure to anthracene, one of the components of crude oil, resulted in reduced clutch size in fathead minnows (Hall and Oris, 1991). Macko and King (1980) found that a single 200mg oral dose of Bunker C crude oil lowered clutch size of Japanese quail. Maternal ingestion of weathered crude oil reduced egg production and resulted in thin eggshells in mallard ducks in the laboratory (Stubblefield et al., 1995). Application of even 0.1 mL weathered PBCO to female wedge-tailed shearwaters 30 days before egg laying caused a reduction in number of eggs laid and in breeding success (Fry et al., 1986). Mink that orally ingested 500ppm Alaskan North Slope crude oil or bunker C fuel oil showed significantly decreased litter size compared to controls, with fuel oil causing a more marked effect (Mazet et al., 2001). The decrease in clutch size between years for the three oil exposed turtles for which I was not able to calculate RCM suggests that there may indeed have been an effect on reproductive output on some individuals. However, Iverson et al. (1997) did not
find annual variation in RCM despite variation in resource availability, and suggested that RCM may be under genetic control.

Mean egg mass measured for painted turtles in this study were in the same range as that recorded by Ernst (1971) for a Lancaster County, Pennsylvania painted turtle population. Relative clutch mass of JHNWR snapping turtles was greater than the 5.69 reported for another Pennsylvania population (Iverson et al., 1997). Steyermark and Spotila (2001) reported a value for mean egg mass from 24 snapping turtle clutches from JHNWR in 1997 of 11.6g, which is within the range that I calculated for both oil exposed and unexposed turtles in all four years. Mean female mass was lower in 2001 (n=8) and 2002 (n=10) than in 1997 (n=17). Since the mean body mass I calculated in 2000 and 2003 was similar to the mean 5026g that Iverson et al. (1997) reported, I attributed this difference to sampling error. Total clutch size in my study followed a similar pattern of variation between the current study and the 1997 investigation. See Steyermark and Spotila (2001) for a comparison of JHNWR snapping turtle egg mass, clutch size and female mass to other areas in their geographic range.

Multixenobiotic resistance could explain the lack of observed negative effects of oil on fertility and RCM. The turtles used in this study live in an environment that has been heavily contaminated for decades. It is likely that many of the turtles examined in this study were born in and grew to maturity in the refuge or surrounding polluted waters within the past few decades. Cytochrome P450-dependent mixed-function oxidases are responsible for carrying out hydroxylation of PAHs (Armstrong, 1986). As is the case with many metabolites produced by the cytochrome P-450 system, hydroxylated PAHs are often more toxic than the parent compound (Guengerich, 1993). Suppression of
cytochrome P450 would mean decreased production of hydroxylated PAHs. Chronic exposure to pollution can cause a suppression of cytochrome P450 expression. Bello et al. (2001) found that female killifish inhabiting a Superfund site highly contaminated with dioxins did not produce cytochrome P450A 1A1 protein in response to a high dose of the dioxin TCDD while fish from a control site without the contaminant exhibited a strong, dose-dependent response. Male fish from the contaminated site had an induced response to the same dose, but it was markedly lower than that of fish from the control site. The EC$_{50}$ (concentration of contaminant expected to cause a non-lethal effect in 50% of the population studied) for TCDD was 14-fold higher in hepatocytes from killifish from the contaminated site compared to those from the control site, suggesting that the killifish from the contaminated site had a decreased sensitivity to TCDD. The killifish showed decreased sensitivity to other contaminants as well. Altered expression or change in the functioning of receptors can cause chemical resistance (Taylor and Feyereisen, 1996; Hahn, 1998).

All three oil-exposed female snapping turtles for which repeat clutch data were available laid fewer eggs one to two years after oil exposure than in the year of exposure. Although this small sample size can only be suggestive of an effect, (Hall and Oris, 1991) and (Macko and King, 1980) both reported a decrease in number of eggs laid by their vertebrate study organisms after exposure to oil as well. (Irons, 1996) found that productivity of black kittiwake colonies in Prince William Sound, Alaska, decreased after the Exxon Valdez oil spill, and that the proportion of nests at colonies was lower during post spill years than during the spill year and 5 years previous. The number of breeding pairs, however, did not decline after the spill. Productivity did not improve during the 5
post-spill years of the study. Ingestion of Bunker C crude oil caused a reduction in the number of Cassin’s Auklets that laid eggs and in the number of eggs laid by those that did reproduce (Ainley et al., 1981). Gibbons (1982) states that environmental factors and microhabitat effects on an individual are the main influence on clutch size, more so than genetics or body size. I would expect these factors to vary annually for each individual, and to differ between females. Fluctuation in clutch size between years is to be expected, and relative clutch mass data do not suggest any effect of oil exposure on reproductive output in turtles. However, the magnitude of the decrease for two of the three oil-exposed female snapping turtles compared to the small increases, no change, or in one case small decrease in number of eggs laid by control females in subsequent years suggests that oil exposure may have played a role in decreasing clutch size in affected females.

Summary

Exposure to crude oil does not appear to have significantly affected the fertility or relative clutch mass of snapping turtles or painted turtles at JHNWR. This contradicts what many, though not all, authors have reported for other species. The lack of difference may be due to xenobiotic resistance conferred by constant exposure to background pollution. Fertility remained very high for both species in all years tested, although it was lower in 2002 than in 2000. Four years of data on fertility indicated that painted turtles at JHNWR have higher percent fertility on average than snapping turtles. Relative clutch mass did not vary between species, years or exposure groups. However, a
few turtles exhibited a marked decrease in clutch size one or two years after exposure to crude oil.


Table 1. Change in number of snapping turtle eggs laid from the year of the spill to subsequent 1 or 2 years.

<table>
<thead>
<tr>
<th>Exposed or not exposed</th>
<th>Number of years since oil spill</th>
<th>Change in number of eggs laid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not exposed</td>
<td>1</td>
<td>+8</td>
</tr>
<tr>
<td>Not exposed</td>
<td>1</td>
<td>+2</td>
</tr>
<tr>
<td>Not exposed</td>
<td>1</td>
<td>-8</td>
</tr>
<tr>
<td>Not exposed</td>
<td>1</td>
<td>No change</td>
</tr>
<tr>
<td>Exposed</td>
<td>2</td>
<td>-32</td>
</tr>
<tr>
<td>Exposed</td>
<td>2</td>
<td>-31</td>
</tr>
<tr>
<td>Exposed</td>
<td>1</td>
<td>-6</td>
</tr>
</tbody>
</table>
Table 2. Reproductive parameters for painted turtles (C.p.) and snapping turtles (C.s.) from the John Heinz National Wildlife Refuge, 2000-2003. Year 2000 were placed in bold font to emphasize the year of the oil spill. Years with less than 5 clutches examined were placed in italics. There was no data for oil exposed turtles in 2003. GBM = female gross body mass, CS = clutch size, mean egg mass = average mass of egg in a single clutch, CM = clutch mass, RCM = relative clutch mass, SD = 1 standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>n clutches</th>
<th>mean GBM ± SD (g)</th>
<th>range CS</th>
<th>mean CS ± SD</th>
<th>range mean egg mass (g)</th>
<th>mean egg mass ± SD (g)</th>
<th>range CM (g)</th>
<th>mean CM ± SD (g)</th>
<th>range RCM (%)</th>
<th>mean RCM (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control C.p.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>8</td>
<td>412.8 ± 84.4</td>
<td>3 - 9</td>
<td>6.6 ± 1.9</td>
<td>3.3 - 6.6</td>
<td>5.33 ± 1.1</td>
<td>19.0 - 44.4</td>
<td>34.4 ± 10.0</td>
<td>3.8 - 12.3</td>
<td>9.4 ± 2.6</td>
</tr>
<tr>
<td>2001</td>
<td>9</td>
<td>425.1 ± 56.1</td>
<td>3 - 10</td>
<td>5.9 ± 2.2</td>
<td>3.8 - 6.8</td>
<td>5.6 ± 1.0</td>
<td>11.3 - 46.2</td>
<td>34.1 ± 12.9</td>
<td>3.2 - 13.3</td>
<td>8.9 ± 3.6</td>
</tr>
<tr>
<td>2002</td>
<td>11</td>
<td>428.1 ± 56.6</td>
<td>4 - 8</td>
<td>6.2 ± 1.5</td>
<td>4.3 - 6.6</td>
<td>5.7 ± 0.8</td>
<td>17.3 - 52.1</td>
<td>35.5 ± 11.1</td>
<td>4.5 - 12.6</td>
<td>9.0 ± 2.2</td>
</tr>
<tr>
<td>2003</td>
<td>7</td>
<td>457.1 ± 76.0</td>
<td>5 - 8</td>
<td>6.7 ± 1.1</td>
<td>5.1 - 7.0</td>
<td>6.1 ± 0.8</td>
<td>25.3 - 48.6</td>
<td>40.8 ± 7.9</td>
<td>8.1 - 11.5</td>
<td>9.8 ± 1.0</td>
</tr>
<tr>
<td><strong>exposed C.p.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>5</td>
<td>411.0 ± 22.0</td>
<td>5 - 9</td>
<td>7.0 ± 1.6</td>
<td>4.6 - 7.1</td>
<td>6.1 ± 1</td>
<td>29.9 - 48.2</td>
<td>41.7 ± 7.2</td>
<td>7.3 - 14.4</td>
<td>11.4 ± 2.6</td>
</tr>
<tr>
<td>2001</td>
<td>1</td>
<td>439.6</td>
<td>7</td>
<td>7</td>
<td>6.6</td>
<td>6.6</td>
<td>46.3</td>
<td>46.3 ± 11.8</td>
<td>11.8</td>
<td>11.8 ± 11.8</td>
</tr>
<tr>
<td>n clutches</td>
<td>mean GBM ± SD (g)</td>
<td>range CS</td>
<td>mean CS ± SD</td>
<td>mean egg mass ± SD (g)</td>
<td>mean egg mass (g)</td>
<td>range CM</td>
<td>mean CM ± SD (g)</td>
<td>range RCM (%) ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------------------</td>
<td>----------</td>
<td>--------------</td>
<td>------------------------</td>
<td>------------------</td>
<td>---------</td>
<td>------------------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>exposed C.p. 2002</td>
<td>2</td>
<td>434.5 ± 14.9</td>
<td>6-9</td>
<td>7.5 ± 2.1</td>
<td>5.2 - 5.6</td>
<td>5.4 ± 0.27</td>
<td>33.8 - 47.2</td>
<td>40.5 ± 9.49</td>
<td>8.7 - 11.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>exposed C.p. 2003</td>
<td>2</td>
<td>432.5 ± 38.9</td>
<td>7-8</td>
<td>7.5 ± 0.7</td>
<td>4.6 - 5.8</td>
<td>5.2 ± 0.8</td>
<td>32.1 - 46.3</td>
<td>39.2 ± 10.0</td>
<td>8.6 - 11.2 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>control C.s. 2000</td>
<td>7</td>
<td>4726.9 ± 1720.2</td>
<td>18 - 58</td>
<td>34.6 ± 12.4</td>
<td>8.9 - 13.2</td>
<td>11.3 ± 1.7</td>
<td>160.7 - 401.6</td>
<td>6.7 - 9.3 ± 2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control C.s. 2001</td>
<td>8</td>
<td>4240.4 ± 1512.4</td>
<td>13 - 38</td>
<td>27.8 ± 8.7</td>
<td>7.4 - 13.8</td>
<td>11.2 ± 1.9</td>
<td>96.5 - 319.0</td>
<td>6.2 - 8.1 ± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control C.s. 2002</td>
<td>10</td>
<td>4200 ± 1696.9</td>
<td>15 - 48</td>
<td>30.3 ± 10.9</td>
<td>7.8 - 13.1</td>
<td>11.3 ± 2.0</td>
<td>137.2 - 355.0</td>
<td>6.6 - 9.1 ± 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control C.s. 2003</td>
<td>4</td>
<td>5052.5 ± 1397.0</td>
<td>36 - 55</td>
<td>42.3 ± 8.7</td>
<td>8.5 - 12.1</td>
<td>10.8 ± 1.6</td>
<td>324.2 - 461.2</td>
<td>9.2 - 10.0 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>exposed C.s. 2000</td>
<td>6</td>
<td>5448.0 ± 1714.8</td>
<td>10 - 48</td>
<td>36.3 ± 13.6</td>
<td>6.8 - 12.4</td>
<td>11.1 ± 2.1</td>
<td>68.4 - 423.0</td>
<td>2.5 - 8.2 ± 3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>exposed C.s. 2001</td>
<td>2</td>
<td>6169.0 ± 906.5</td>
<td>34 - 42</td>
<td>38.0 ± 5.7</td>
<td>10.2 - 11.0</td>
<td>10.6 ± 0.6</td>
<td>346.9 - 405.1</td>
<td>6.7 - 7.3 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>exposed C.s. 2002</td>
<td>3</td>
<td>5100.0 ± 1300.0</td>
<td>16 - 41</td>
<td>30.7 ± 13.1</td>
<td>11.1 - 12.4</td>
<td>11.9 ± 0.7</td>
<td>166.3 - 509.4</td>
<td>4.8 - 7.3 ± 2.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Mean percent fertility of control and oil exposed snapping turtles from JHNWR 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error of the mean.
Figure 2. Mean percent fertility of control and oil exposed painted turtles from JHNWR 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error of the mean.
Figure 3. Mean relative clutch mass of oil exposed and control snapping turtles (Chelydra serpentina) from the John Heinz National Wildlife Refuge, 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error of the mean.
Figure 4. Mean relative clutch mass of oil exposed and control painted turtles (*Chrysemys picta*) from the John Heinz National Wildlife Refuge, 2000-2003. Numbers represent number of clutches examined. Error bars represent one standard error of the mean.
Chapter 3. THE EFFECT OF MATERNAL OIL EXPOSURE ON SURVIVAL, STAGE AT DEATH, AND EMERGENCE SUCCESS OF EMBRYOS

Introduction

The period from egg laying to emerging is a perilous stage in an embryonic turtle’s life. Successful completion of embryonic development depends on a relatively narrow window of hydric and thermal conditions, as well as yolk investment from the mother, genetic effects, appropriate timing of developing physiological systems, and escape from predation. For example, water potential (the difference in chemical potential between water distributed throughout a substrate and water in a reference state) affects thermal transfer and vapor movement between the nest and its surrounding substrate (Packard et al., 1985). The thermal conductivity of the incubation medium influences heat exchange between the egg and its surroundings (Rimkus et al., 2002), and water exchange in eggs is influenced by changes in egg temperature (Ackerman et al., 1985). Incubation temperature, which can fluctuate as much as nine degrees in natural nests (Packard et al., 1985), determines embryo gender, survival and hatchling size (Brooks et al., 1991; Packard et al., 1999), and can even decrease hatching success if too high (Packard et al., 1987; Steyermark and Spotila, 2001). Hatching success is directly related to moisture regime, so any prolonged period of decreased moisture in the nest environment increases embryonic mortality (Packard and Packard, 1987). Finally, the addition of chemical insults from lipophilic contaminants stored in egg yolk only adds to the long list of challenges to successful development for turtles.

An underground pipeline cracked and leaked nearly 758,000 liters of Cabinda crude oil into an impoundment in the John Heinz National Wildlife Refuge, Philadelphia, Pennsylvania in February 2000. This spill added to background contamination from local...
municipal and industrial sites along the Lower Darby Creek, which feeds the man-made lake (U.S.E.P.A., 2001). Twenty turtles representing four species (painted turtles, *Chrysemys picta*, snapping turtles, *Chelydra serpentina*, red-eared sliders, *Trachemys scripta*, and the state-threatened red-bellied turtle, *Pseudemys rubriventris*) came to the surface coated with oil. Oil recovery workers quickly erected a fence around the spill area to prevent animals from carrying oil from the spill site to other areas of the lake. Many turtles were contained in this fenced area for several months before the nesting season, along with other animals and plants that provided abundant food sources. Therefore, not only the initially oiled turtles, but those resident within the fence were exposed to oil contamination.

In many species across numerous taxa, embryos are more sensitive to environmental toxicants than adults (Sharp et al., 1979; Perera et al., 2003). Although several authors have identified the aromatic fraction of crude oil as the most embryotoxic class of compounds contained therein, the specific components within that class that cause embryonic death are unknown (Neff et al., 1976; Hoffman, 1979; Ellenton, 1982; Walters et al., 1987). Ellenton (1982) proposed that the 2-3 ring aromatic fraction of Prudhoe Bay crude oil (PBCO) and No. 2 fuel oil caused embryonic death based on the fact that they were the most abundant aromatics in the oil. Hoffman (1979) found a different result when he tested individual aromatic components of South Louisiana crude oil (SLCO) on chicken embryos. Although the entire mixture of aromatic compounds proved more embryotoxic than individual classes, 4 ring aromatics were the most toxic individual components. In contrast, Neff et al. (1976) reported that the acute toxicity of PAHs in the marine environment increased with molecular size until the 4 and 5 ring
compounds were reached. However, the latter authors acknowledge that chronic toxicity from these larger molecules with low aqueous solubility could be substantial. These disparate reports from authors using different petroleum products on different organisms in different environments indicate that toxicity of individual components of crude oil is dependent on the species of organism, concentration, vehicle of administration, environment and presence of other contaminants.

Lipophilic contaminants are transferred from female snapping turtles to their eggs, which are reliable indicators of a female’s body burden (Stone et al., 1980; Pagano et al., 1999). Snapping turtle embryos additively accumulate lipophilic contaminants as development progresses (Bishop et al., 1995). By the last ¼ of incubation, embryos in Bishop et al.’s study had accumulated between one third and one half the compounds present in the egg yolk at oviposition. The authors did not report the presence of metabolites of these compounds. On the day of emergence, the average turtle contained 55.2 to 90.5% of all compounds that had been present in the egg at time of oviposition. Therefore, the purpose of this study was to determine if exposure of female turtles to crude oil affected the development and survival of their embryos. I tested two hypotheses in this research: 1. maternal exposure to oil increases embryonic death and therefore decreases emergence success of clutches laid after exposure, and 2. most embryonic death occurs in early stages of development, as in other species of turtles (Bell et al., 2003).
Materials and Methods

Mark-Recapture and Egg Collection

I captured snapping turtles and painted turtles in hoop nets in the fenced spill area and the opposite end of the lake from March to April of 2000. I also trapped, marked and released turtles in the summer of 2001 and 2002. Turtles captured inside the fenced area were designated “oil exposed” because they were either found with oil on them or were chronically exposed to oil within the spill area since February. Turtles captured at the opposite end of the lake were designated as controls. Chemical analyses of sediments in the impoundment indicated that the oil did not spread beyond about 1 ha, and the entire impoundment encompassed approximately 59 ha. I marked turtles by filing or drilling holes in the marginal scutes of the carapace that corresponded to an alphabetic code similar to the numeric code developed by Cagle (1939). I conducted foot patrols of known snapping turtle nesting sites in the JHNWR from May to June 2000-2003. When possible, I collected eggs from females as they were laid in the field, otherwise I transported gravid females to the laboratory and induced oviposition with oxytocin (Ewert and Legler, 1978; 19-21 IU/kg as per Steyermark, pers. communication). I began egg incubation in a Rheem™ environmental chamber at 26.5°C ± 1.0 in 2000, but transferred all eggs to a Precision™ incubator (26.5°C ± 0.5) on June 14, 2000 when the Rheem chamber overheated to 40°C and shut down. I incubated eggs in the Precision™ incubator at 26.5°C ± 0.5 for the rest of the study. Eggs were housed in 32cm x 18cm plastic boxes with sand kept at 4% moisture (-100 to -170 kPa) gravimetrically. I replenished moisture in each box three times per week.

Egg dissection
I incubated eggs that developed a white spot and continued to show signs of embryonic development to full term. I dissected eggs that did not form a white spot or that showed signs of embryonic death such as arrested development (as seen by candling), discoloration, loss of turgidity, or foul smell. I staged snapping turtle embryos according to Yntema (1968) and painted turtle embryos according to Mahmoud et al. (1984). I placed embryos that were too decomposed to identify to a single stage into the categories “early,” “middle” and “late.” The “early” category included stages 0-9 for snapping turtles and 0-7 for painted turtles. The “middle” category included stages 10-18 for snapping turtles and 8-15 for painted turtles. The “late” category included embryos stages 19-26 for snapping turtles and 16-23 for painted turtles. I created an “unknown” category for those eggs that were fertile but no embryo could be seen due to decomposition. I did not combine the “early” and “unknown” categories in snapping turtles because I believed that the “unknown” category included mostly embryos that died at or before blastodisk stage, which is earlier than the majority of embryos included in the “early” stage. I examined each embryo microscopically for deformities. I considered a hatchling to have emerged when it successfully left the eggshell.

Calculations and data analysis

Although four species of turtle were exposed to oil, only two of them (snapping turtles and painted turtles) produced enough clutches from enough individuals to perform statistical analyses. I calculated emergence success as number of hatchlings emerged/number of fertile eggs. I arcsine-transformed all percent data before statistical analyses. I used G tests to examine if there was a significant difference in the proportion of live to dead embryos between snapping turtles and painted turtles (Sokal and Rohlf,
I performed separate G tests for oil exposed vs. control turtles and for data from control turtles in 2000 vs. 2002. A comparison between oil exposed turtle species between the two years was not possible because I only had two oil exposed snapping turtle and painted turtle clutches with dead embryos in 2002. I also used G tests to examine the possible difference in the proportion of embryos falling into “early,” “middle,” “late” and “unknown” categories for snapping turtles in 2000 and 2002 and painted turtles in 2000 only. My small sample size for painted turtles made it challenging to use a G-test with the data divided into the four categories because in that case the calculated expected values for many of the groups were less than 5. I therefore re-categorized stages at death of painted turtles into two groups for analysis: “early + unknown” and “late.” I used one way analysis of variance (ANOVA) to compare arcsine-transformed percent dead embryos between oil exposed and control snapping turtles in 2000 and a one-way bootstrap ANOVA in 2002. I did not perform statistical analyses to compare percent death data between oil exposed and control painted turtles in any year because my sample sizes were too small to accurately represent these populations and most clutches had values near 100% death. I did not perform statistical analyses for percent emergence data because emergence is the opposite of embryonic death, therefore significant relationships between groups that occur with embryonic death data will also be significant with emergence data.

**Results**

The percent of dead embryos did not differ significantly between oil exposed (2000: 95.8%, n=6; 2002: 52.8%, n=2) and control (2000: 92.9%, n=4; 2002: 58.2%,
n=10) painted turtles in 2000 and 2002 (Fig. 5). However, the percent of dead embryos was significantly greater (df= 19, F= 6.47, P= 0.020) for oil exposed snapping turtles (57.0%, n=9) than controls (31.1%, n=12) in 2000 (Fig. 5). There was no significant difference (df=10, F= 4.61, P= 0.516) in percent dead embryos between exposure groups in 2002 (exposed: 7.1%, n=3; control: 3.1%, n=10, Fig 5). G tests indicated that control snapping turtles had a significantly higher ratio of live to dead embryos than painted turtles in 2000 ($G_{adj.}=38.89, X^2=3.84$) and 2002 ($G_{adj.}=84.09, X^2=3.84$). Snapping turtles exposed to oil also had significantly more live to dead embryos than painted turtles in 2000 ($G_{adj.}=28.28, X^2=3.84$).

Embryonic death rates were high for both species in 2000. Death occurred in specific ranges of stages for snapping turtles, aggregating around the late pre-hatching stage and the “unknown” category (Fig. 6). I did not have enough data for painted turtles to perform the same comparison. The “unknown” category contained the most snapping turtle embryos when stages at death were compiled into 4 groups (“early,” “middle,” “late,” and “unknown”) (Fig. 7). There was a significant difference in the proportion of embryos dying among the four categories between embryos from oil exposed and control snapping turtles in 2000 ($G_{adj.}=12.47, X^2=7.815$). Further G tests indicated that there were significantly more early deaths for embryos from oil exposed mothers than controls ($G_{adj.}=10.51, X^2=3.841$). There was no difference between “middle,” “late” and “unknown” categories. A G-test indicated that there was no difference between the proportions of embryos in the two classes of stage at death (“early + unknown” vs. “late”) between embryos from oil exposed and control painted turtles in 2000 ($G_{adj.}=0.069, X^2=3.841$).
Emergence success ranged from 34.6 to 97.1% for clutches from control (n=12) and 4.8 to 91.7% for clutches from oil exposed (n=9) snapping turtles in 2000. In contrast, emergence success for painted turtles clutches in 2000 ranged from 0 to 28.6% for control (n=4) and 0 to 14.3% for oil exposed (n=6). Emergence success improved so much in 2002 for both species that there were not enough data to statistically analyze the effect of stages at death. In snapping turtles, percent emergence ranged from 85.3 to 100% for control (n=10) and 82.4 to 100% for oil exposed (n=3) in 2002. Emergence success was significantly greater for clutches from control snapping turtles compared to those from oil exposed females in 2000 but not 2002 (Fig. 8). There was no statistically significant difference in emergence success of clutches from oil exposed and control painted turtles in either year. Emergence success was significantly greater for snapping turtles than painted turtles in 2000 ($G_{adj} = 38.89, \chi^2 = 3.84$) and 2002 ($G_{adj} = 84.09, \chi^2 = 3.84$).

Discussion

Embryonic mortality rates were so high for all painted turtles that there was no difference between oil exposed and control turtles, nor was there a discernable bias toward early or late deaths. The small sample size of painted turtle embryos that I was able to categorize in a single stage in 2000 made it impossible to detect any differences between stages at death between oil exposed and control turtles.

Embryonic mortality was much greater for painted turtles than for snapping turtles in both years. Painted turtle embryos are smaller than snapping turtle embryos, and Heintz et al. (1999) explained that smaller embryos accumulate PAHs more rapidly.
than larger ones because the absolute uptake rate of PAHs is directly proportional to the surface area:volume ratio. Painted turtle egg yolks had more than twice the percent total lipids (29.80%) found in snapping turtle egg yolks (13.24%) in Nebraska (Rowe et al., 1995). If painted turtle eggs from JHNWR had significantly more percent total lipids than snapping turtles, the former would have the potential to accumulate more lipophilic contaminants per gram of egg mass than the latter. Hoffman (1978) cited the same reason for a difference in the embryotoxicity of South Louisiana crude oil between mallard embryos and chicken embryos.

There was a significant difference in embryonic mortality rates in snapping turtle clutches between 2000 and 2002. Although it is possible that the toxic effect of oil was not present two years after the spill, the higher incidence of embryonic death in 2000 was likely due to the overheating incident in the incubator. Steyermark and Spotila (2001) found higher incidence of embryonic death in eggs incubated at 30.0°C than at lower incubation temperatures, and the incubator in my study reached 40.0°C. Increased embryonic death rates in snapping turtle embryos from oil exposed mothers compared to controls in 2000 corroborate similar findings by other investigators for fish (Carls et al., 1999; Pollino and Holdway, 2002) mammals (Khan et al., 1987; Mazet et al., 2001), birds (Ainley et al., 1981; Lee et al., 1986; Bernatowicz et al., 1996) and invertebrates (Hauschildt-Lillge, 1982). Pollino and Holdway (2002) found a dose-dependent decrease in hatching success for the crimson-spotted rainbowfish as well. Hatching success was lower for clutches of Cassin’s auklets fed 1000 or 600mg of bunker C crude oil or Prudhoe Bay crude oil, but not for those fed 300mg (Ainley et al., 1981). Bald eagle nesting success (number of young produced per nest) decreased in western Prince
William Sound (the location of the Exxon Valdez oil spill with the heaviest oiling of any region) in 1989, the year of the spill (Bernatowicz et al., 1996). The authors attributed this decrease to embryonic death. Eagles in areas that received lighter oiling did not show decreases in nesting success. It is clear from these studies that the embryotoxic effects of crude oil are dose-related, and the data presented in this study suggest that embryos from JHNWR oil exposed mothers received a high enough dose to cause increased embryonic death the year of the spill.

Bishop et al. (1994) hypothesized that lipids for snapping turtle eggs are probably derived largely from daily dietary intake rather than mobilization of stored fats. However, Congdon and Gibbons (1990) indicated that there are two periods of egg growth for painted turtles: mid-summer through late fall and after winter hibernation. Energy for the first period of follicular development is derived from recently harvested food sources (as evidenced by a concomitant increase in stored body lipids), while energy for the second period is derived from stored body lipids (as suggested by the fact that the lipid levels of females decreased almost the same amount as lipids in follicles increased during this period). In either case, the lipophilic contaminants affecting turtles at JHNWR were taken up by the turtles from their diet or by direct ingestion during the spring of 2000. The oil exposed turtles were trapped inside an oil-soaked area for up to four months before they nested, and could have taken up oil from their food or direct contact with the substrate. The contaminated sediment was removed in 2001. Oil-exposed female turtles may have removed oil contaminants from their bodies through xenobiotic metabolism and through lipid deposition in eggs in 2000 and 2001, which
could explain why there was no difference in embryonic death or emergence success between exposure groups in 2002.

Snapping turtle embryos from JHNWR die most frequently either extremely early or just before hatching. Waldner et al. (2001) found that exposure to petroleum products near sour gas-flaring sites was associated with late-term calf death. Steyermark and Spotila (2001) noted that most dead embryos they saw from turtles in this population died late in development. However, the authors did not examine eggs microscopically. They noted that only one half of the eggs in their study showed evidence of embryo formation. Since I found that fertility rates were very high for turtles in this population (see Chapter 2), it is likely that most of the undeveloped eggs they examined actually contained embryos that died very early, which means that in reality most embryos in their study died in the earliest stages of development like the embryos in this study. Bishop (1991) and Bobyn and Brooks (1994) also found that the majority of embryos in unhatched eggs at their contaminated study sites in Canada died in early stages of development that were not visible to the naked eye or late in development. Heintz et al. (1999) found that most natural mortality occurs prior to eyeing in pink salmon reared in uncontaminated conditions. Likewise, the majority of bovine embryonic death occurs in the first 14 days of gestation (Waldner et al., 2001). Although it may seem from the latter two cases that early embryonic death is not uncommon, Packard et al. (1991) found only 6.2% early death in laboratory-incubated painted turtles from Nebraska. If I were to combine the “early” and “unknown” stages for my painted turtles in 2000, nearly 65% of *C. picta* from JHNWR died early. Furthermore, Packard et al. (1991) encountered around 90% hatching success in painted turtles incubated under similar conditions to this study, much
greater than what I encountered. Packard et al. (1987) had 100% hatching success at both 26.0 and 28.5 °C for snapping turtles from the same wildlife refuge in Nebraska that the aforementioned painted turtles were acquired from. These extremely high hatching success values, when compared to those in all four years of this study, suggest that background pollution could be a factor affecting development in turtles at the JHNWR.

Not only was there a higher incidence of embryonic death, there were also significantly more early deaths in snapping turtle embryos from oil exposed mothers than controls in 2000. Khan et al. (1987) found that ingestion of crude oil had the greatest embryotoxic effect on rat fetuses when administered in the first 6 days of gestation. The authors suggested that the critical stages of development susceptible to crude oil toxicity occur early in gestation. Carls and Rice (1988) state that the sensitivity of embryos to external stressors may be greatest at the earliest stages of development “when damage to a few precursor cells will result in extensive damage as the embryo develops.” The authors also state that early embryonic tissues undergo rapid cellular differentiation and rapidly changing structural and functional relationships between cells and cell systems. Brown et al. (1996) observed increased anaphase aberration rates in animals from oil-exposed areas. There may also be chronological differences in permeability of embryos to lipophilic contaminants. For example, Sharp et al. (1979) found that early embryos of killifish were actually more permeable to 14C-naphthalene than later stages.

The sensitivity of early life stages is due primarily to the fact that an embryo requires a balanced set of chromosomes to develop properly (Longwell, 1977). Cells are particularly susceptible to chromosome separation errors and gene-level mutations during meiosis (Longwell, 1977). Stressors in the embryonic environment, exposure to DNA-
damaging agents and chromosomal abnormalities can induce apoptosis (cell death) in the early cleavage stages of development (Betts and King, 2000). Cell death can eliminate cells with chromosomal aberrations, which can be caused by embryonic exposure to contaminants like PAHs (Armstrong, 1986; Shah et al., 2003). A certain amount of cell death is normal in embryonic development, but is detrimental beyond a certain threshold (Betts and King, 2000).

Activity of the cytochrome P450 system may have contributed to interspecies differences in embryonic death rate (Ertl and Winston, 1998) and to the prevalence of late deaths in this population. The cytochrome P450 system, part of the mixed function oxidation suite of enzymes, is responsible for metabolizing xenobiotic compounds. The painted turtle has a particularly high amount of cytochrome P450 compared to other species of reptile, almost twice that of the marsh turtle, *Mauremys caspica* (Ertl and Winston, 1998). If painted turtles have more cytochrome P450 than snapping turtles, they have the potential to break down and thus bioaccumulate greater quantities of metabolites, which can be more toxic than the parent compounds (Guengerich, 1993). It is not known exactly when the mixed function oxidase system first develops in turtles, but the embryo cannot form toxic metabolites without the organs that house the enzymes that metabolize xenobiotic compounds. The snapping turtle embryo does not begin major organ development until about stage 9, but many embryos died much earlier. The fact that many embryos in this study likely died before the formation of the cytochrome P450 system suggests that some early embryonic deaths of turtles in this study may be attributed to lethal genetic mutations or other cellular damage caused by exposure to toxic contaminants. In older embryos, such as those in this study that died late, exposure
to lipophilic contaminants in egg yolk can trigger the cytochrome P450 system. Induction of cytochrome P450 not only causes formation of more of the enzymes, it may down-regulate other P450s with important physiological functions. In this way, it can alter hormonal regulation and disrupt embryonic development (Ertl and Winston, 1998). Embryos that died late may also have accumulated toxic metabolites to a lethal threshold.

Summary

Embryonic death was much more prevalent in painted turtles than snapping turtles at JHNWR. Painted turtles had such a high percent embryonic death and low emergence success that there was no difference between clutches from oil exposed mothers and controls. Maternal exposure to crude oil was correlated with increased embryonic death and decreased emergence success for snapping turtles in the year of the spill. Most snapping turtle embryos, regardless of exposure status, died either very early in development (before formation of a macroscopic embryo) or just before hatching. More embryos from oil exposed females died early in development than control. By 2002 there was no difference in percent dead embryos or percent emerged hatchlings between clutches from oil exposed mothers compared to controls. The amount of embryonic death in both species at JHNWR is higher than that seen at other sites, probably due to the embryotoxic effects of background pollution in this urban environment.
Bibliography


Figure 5. Percent of dead embryos out of all fertile eggs for oil exposed versus unexposed snapping turtles and painted turtles from JHNWR, 2000 and 2002. Numbers represent numbers of clutches examined. Error bars represent one standard error of the mean.
Figure 6. Percent of snapping turtles that died at each stage of death (Yntema, 1968), 2000. “Unk” represents those eggs that were known to be fertile but either did not contain a discernable embryo or contained an embryo too decomposed to place in a single stage.
Figure 7. Percent of snapping turtle and painted turtle embryos that died during middle, early or late periods of development, 2000. “Unk” signifies eggs that were fertile but decomposed to a point where no embryo was discernable. Numbers represent counts of dead embryos per category. Error bars represent one standard error of the mean.
Figure 8. Percent embryos emerged from snapping turtle (C. serpentina) and painted turtle (C. picta) clutches from the JHNWR, 2000 and 2002. Numbers represent number of clutches examined, error bars represent one standard error of the mean.
CHAPTER 4. INCIDENCE AND TYPES OF DEFORMITIES IN CONTROL TURTLES AT THE JOHN HEINZ NATIONAL WILDLIFE REFUGE, PHILADELPHIA, PENNSYLVANIA, USA

Introduction

The position of the John Heinz National Wildlife Refuge (JHNWR) in Philadelphia, Pennsylvania makes the refuge subject to pollution from multiple sources. These sources include 1) runoff from adjacent highways and railroads, 2) atmospheric deposition from the city and the neighboring airport, and 3) seepage from various industrial and municipal sites along the Lower Darby Creek watershed. The JHNWR includes a large, man-made reservoir (impoundment) fed by Lower Darby Creek.

The Darby Creek watershed includes six sites proposed for inclusion on the National Priorities List (NPL), a list of contaminated sites in the United States with priority for further investigation and possible remediation (U.S.E.P.A., 2003). The sites include an oil tank farm, two landfills, a retired sewage treatment plant, a retired municipal incinerator, and an industrial property. Groundwater wells at one of the landfills contained metals and solvents, and both landfills were officially added to the NPL in 2001. The landfills were seeping metals, polychlorinated biphenyls (PCBs) and petroleum byproducts into Darby Creek (U.S.E.P.A., 2001). Water and sediments are deposited into the impoundment in the refuge from Darby Creek. Chemical analyses performed by the EPA from 1973 to 2000 at the landfills revealed the presence of PCBs, polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene and dibenzo(a)anthracene, metals such as arsenic, copper, cadmium, mercury and lead, and assorted volatile and semivolatile organic compounds (U.S.E.P.A., 2003). Many of these materials cause developmental abnormalities in turtles and birds (Anwer and Mehrotra,
1986; Bishop et al., 1991; Bishop et al., 1998; Hoffman and Gay, 1981). The other four areas are either under investigation or under remediation by owners.

Snapping turtles (*Chelydra serpentina*) are well-established bioindicators of environmental contamination (Albers et al., 1986; Bishop, 1990; Bishop et al., 1995; Golet and Haines, 2001; Pagano et al., 1999; Ryan et al., 1986; Stone et al., 1980). Their prevalence in wetland habitats, size, long life span, reproductive habits and large clutch size make them excellent organisms for studies of reproductive toxicity (Bergeron and McLachlan, 1994; Bishop et al., 1991; Bishop et al., 1996; Bishop and Gendron, 1998). They feed at the top of the aquatic food chain and can live for decades, which affords them ample opportunity to accumulate biomagnified toxins. Stone et al. (1980) suggested that they may also be exposed to toxins via dermal absorption from water and sediment and absorption through mucous membranes during cloacal and pharyngeal respiration. Snapping turtle eggs are reliable indicators of a female’s body burden of lipophilic contaminants (Pagano et al., 1999; Stone et al., 1980). Contaminants in painted turtles (*Chrysemys picta*) have not been as thoroughly studied as in snapping turtles (but see Meeks, 1968; Owen and Wells, 1976; Punzo et al., 1979; Reeves et al., 1977; Rie et al., 2000; Yawetz et al., 1997), but their wide geographic range, ease of capture, long life span and abundant numbers make them a viable candidate for toxicity studies.

Some turtles seem to adapt well to urban environments when suitable habitat is available (Neill, 1950; Souza and Abe, 2000; Spinks et al., 2003). Aquatic habitats on the John Heinz Refuge provide abundant food, basking sites and refugia for several species of aquatic turtles (snapping turtle, painted turtle, slider turtle, *Trachemys scripta*, redbellied turtle, *Pseudemys rubriventris*, and stink pot turtle, *Sternotherus odoratus*).
Nesting sites are also abundant in the 1200 acre refuge. I observed large numbers of deformed adults and juveniles among turtles that I captured in the Refuge. Therefore, the objective of this study was to document the occurrence of deformities in natural populations of aquatic turtles inhabiting the JHNWR, determine the extent of deformities in developing embryos and hatchlings, and compare these data to those from less impacted sites.

**Materials and Methods**

*Turtle identification and egg collection*

I collected turtles by hand during the nesting season (2000-2003) or in hoop nets baited with canned sardines and canned corn during spring and summer (2000-2002) and examined each individual for morphological abnormalities. I marked every turtle captured by drilling holes or filing notches in marginal scutes following an alphabetic system similar to the numeric system used by Cagle (1939). I conducted foot patrols of nesting areas in the JHNWR in May and June, 2000-2003. When possible, I collected eggs from females as they were laid in the field, otherwise I transported gravid females to the laboratory and induced oviposition with oxytocin (Ewert and Legler, 1978; 19-21 IU/kg as per Steyermark, pers. communication). I examined each turtle for external deformities.

I incubated eggs in a Precision™ incubator at 26.5°C ± 0.5 in 32cm x 18cm plastic boxes with sand kept at 4% moisture (-100 to -170 kPa) gravimetrically. I replenished moisture in each box three times per week. Eggs that developed a white spot and continued to show signs of embryonic development were incubated full term. I dissected eggs that did not form a white spot or that showed signs of embryonic death
such as arrested development (as seen by “candling”), discoloration, loss of turgidity, or foul smell. I staged snapping turtle embryos according to Yntema, (1968) and painted turtle embryos according to Mahmoud et al. (1984). I examined each embryo microscopically for deformities. Eggs were individually handled only when they exhibited one or more of these signs. I categorized deformities as described in the results.

The relatively uncontaminated E.S. George Reserve (ESGR) in Ann Arbor, Michigan, home to a well-studied population of snapping turtles and painted turtles (Congdon and Gibbons, 1996), was used as a reference area. The ESGR is only subject to contamination via atmospheric deposition. Dr. Justin Congdon provided data on deformities in hatchling and adult snapping turtles and painted turtles at ESGR from 1975 to 2003. Turtles were examined for external deformities. No embryos were examined. He also provided deformity data for neonates from 23 nests hatched in the fall of 2004, which I compared to that for neonates produced from 54 clutches incubated in the laboratory at Drexel University from 2000-2003.

Chemical analyses

The Geochemical and Environmental Research Group (GERG) of Texas A&M University, Texas, U.S.A. performed PAH analyses on 3 sets of 3 pooled eggs from JHNWR control snapping turtle females and lipid samples that I biopsied from subcutaneous fat pads above the rear legs of three JHNWR female snapping turtles. I also obtained PAH analyses on eggs from reference sites in Michigan and Ontario, Canada. I had 1 set of 2 snapping turtle eggs collected from Cedar Lake near Pinckney, Michigan, which is close to the ESGR, and 2 sets of 2 pooled snapping turtle eggs from
Algonquin Park in Ontario, Canada, which is a reference site described in Bishop et al. (1991). I placed eggs singly in solvent-rinsed glass jars two to four weeks after they were laid. Samples remained in a –80°C freezer until they were sent for analysis in 2003.

**Results**

*Deformity rating system*

I developed a system to rate the severity of deformities seen in embryos (Table 1). All types of deformities listed in Table 1 occurred in samples taken over the four years of this study. I divided observed malformations among three categories: minor, moderate and lethal. Minor deformities were those not expected to affect fitness or survival of the individual, such as an extra scute. Moderate deformities may lower likelihood of individual survival, but I had observed adults that survived with such deformities. Examples of moderate deformities seen in adults include unfused maxilla or mandible bones, kyphosis, scoliosis, a single deformed leg and 2 deformed feet. Lethal deformities are those that most likely result in death due to developmental problems or predation of embryos or hatchlings. Such deformities include but are not limited to lack of eyes, brain abnormality, no carapace or plastron or more than one missing or deformed leg. Obviously I did not observe any lethal deformities in adults.
Deformity in embryos

The percentage of each type of deformity in snapping turtle embryos varied from year to year (Fig. 9). The most common deformities among snapping turtle embryos were tail and carapace or plastron (“shell”) malformations. In most cases, these deformities were minor, consisting of bent, short or curled tails (Figures 10A and 10B) and extra or deformed scutes. In a few cases, the tail was missing or the shell was grossly malformed (Fig. 10B) (moderate to lethal deformities). The next most common malformation was a deformed neural tube or spine, usually consisting of kyphosis (a convex spine, as viewed laterally) or scoliosis (a lateral deviation in the spine, as viewed dorsally). The prevalence of the remaining types of deformity was highly variable between years. Dwarfism, spine and skull deformities were more common in 2001 and 2003 than other years. This was largely due to the influence of one female each year that had 100% deformity in embryos. Facial deformities often consisted of unfused maxilla and premaxilla bones, and occasionally missing eyes (Figures 10A and 10B) and short upper and/or lower jaws (Figures 10A and 10D). Some embryos had more than one deformity (Figures 10A and 10B). Deformities of the brain (Figures 10A and 10B), other major organs, pigment (Figures 10A, B and C), scales and edema were relatively rare. From 3-12% of emerged hatchlings had minor deformities.

Painted turtles had more deformities than snapping turtles and the percentage of these deformities varied from year to year (Figure 11). Dwarfism (Figure 12A) and malformations of the shell (Figures 12B and 12C) were the most common type of deformity, followed by skull (Fig. 12D, E and F) abnormalities. Deformities in painted turtles were usually lethal, with the exception of minor shell deformities such as missing
or extra scutes. Most shell deformities fell into the category of “minor.” Fifty percent of painted turtle eggs contained dwarf embryos in 2002 compared to approximately 30% in 2001 and 40% in 2003. There were no dwarf embryos in the three painted turtle clutches examined in 2000. Many embryos had more than one type of deformity (Figures 12D, E and F). Most dwarf embryos had multiple problems such as brain or other organs protruding from the body (Figures 12B and 12E). From 5-20% of emerged hatchlings had minor deformities.

I incubated a total of 35 painted turtle and 55 snapping turtle clutches over four years. I excluded 6 of the painted turtle clutches and 9 of the snapping turtle clutches from analysis of deformity rates due to either small clutch sizes or because the majority of the eggs died too early to see deformities or because most eggs were infertile. A one way ANOVA on the arcsine-transformed data indicated that the percent deformity of clutches was statistically significantly higher (df = 74, F = 23.6, P = 0.000008) every year for painted turtles than for snapping turtles (Fig. 13). The percentage of clutches with at least one deformed embryo was the same for snapping turtles and painted turtles (95%). However, 95% of painted turtle clutches had more than 10% of embryos deformed compared to 58% of snapping turtles. Sixteen out of 29 (55%) painted turtle clutches had 50% or greater deformity compared to 2 out of 46 (4%) snapping turtle clutches. There was significant variation in deformity rates among clutches, ranging from 0 to 100% for both species. With the exception of 2002, the vast majority of deformities in painted turtle embryos were lethal. Moderate deformities were most common for painted turtle embryos in 2002. Severity of deformity was more variable for snapping turtles from year to year, with lethal deformities predominant in 2000 and 2001, but only minor
Deformities predominated in 2002 and 2003. Over the four years of the study, lethal deformities were dominant in both snapping turtle (58.6%, Fig. 14a), and painted turtle embryos (54.8%, Fig. 14b).

Deformity in adults

Deformities in adults of both species were not as severe as those in embryos (Figures 15 and 16). The majority of the deformities were minor, with a few moderate and no lethal abnormalities. Deformed painted turtles occurred more frequently than snapping turtles, and the frequency observed varied among years for both species (Table 4). No deformities were observed in snapping turtle adults in 2000 or 2003. Most adult snapping turtle deformities involved the tail or shell, with a few involving the spine (Fig. 15) and facial area. Most tail deformities involved extra protrusions or other abnormalities at the tip of the tail and most shell deformities involved extra or misshapen scutes. Kyphosis and scoliosis (Fig. 15) were rare. Facial deformities involved a single malformed eye or the jaw, the latter most often in the form of a cleft where the premaxilla and maxilla did not fuse.

Deformities were present in adult painted turtles in all four years, the majority of which involved limbs or shells (Fig. 16). Most deformed limbs involved the feet, which often lacked a number of toes or had a mass of toe-like buds (Figs. 16B and C). As in snapping turtles, most shell abnormalities involved extra or misshapen scutes (Fig. 16D), although some kyphosis, scoliosis and other distortion of the carapace or plastron occurred in small numbers (Fig. 16A). Tail deformities were also common and usually involved extra spines or “buds” at the tip.
Deformities in an uncontaminated reference site

There was a low percentage of abnormalities in juveniles and adults of *C. picta* and *C. serpentina* in ESGR from 1975-2003 (Table 5). Painted turtles had a greater incidence of deformity (14.2% in adults + juveniles) than snapping turtles (6.0% in adults + juveniles). Abnormalities in painted turtles and snapping turtles were predominantly shell anomalies (Fig. 17A and B). There were no tail deformities in painted turtles and no facial deformities in either species from ESGR. Tail deformities were fairly common and facial deformities occurred to a small degree in both species at JHNWR. The prevalence of minor, moderate and lethal deformities of neonates differed significantly ($G_{adj.}=1146.20, \chi^2=5.99$) between the two sites (Figure 18). Minor deformities predominated at both sites, but there were more minor deformities at ESGR compared to JHNWR, and there were fewer moderate deformities seen in hatchlings from ESGR than JHNWR. There were six times as many lethal deformities at JHNWR. A G test indicated that the ratio of minor:lethal deformities was significantly different between the two sites ($G_{adj.}=10.24, \chi^2=3.84$).

Level of Contamination

Lipid samples from JHNWR female snapping turtles had the highest amounts of alkanes and total PAHs of all of the samples, followed by JHNWR egg samples and clean site control egg samples. Alkane levels in lipid samples ranged from 304,544 to 836,806 ng/g. Alkane levels in egg samples ranged from 22,413 to 33,907 ng/g for JHNWR, 23,186 ng/g for Michigan and from 15,332 to 22,528 for Canada. The PAH concentrations followed the same pattern. Concentrations ranged from 2,255 to 4,504
ng/g in lipid samples, 573 to 1,653 ng/g in JHNWR egg samples, 494 ng/g in Michigan egg samples and from 363 to 585 ng/g in Canada egg samples.

Discussion

Painted turtles had a higher rate of embryonic deformity and a greater number of clutches with 50% or greater deformity than snapping turtles. This difference between the two species is surprising because I would expect the more carnivorous snapping turtle to bioaccumulate more contamination than the omnivorous painted turtle (Bishop and Gendron 1998; Meeks 1968). Interspecies differences in accumulation of contaminants occur in turtles as well as in fish and birds (Bishop and Gendron, 1998; Braune and Norstrom, 1989; Meeks, 1968; Meyers-Schöne, et al.,1993; Meyers-Schöne and Walton, 1994). Differences in accumulation of radio-nuclides between C. serpentina and red-eared sliders, Trachemys scripta, depended on type of chemical, food habits and location (Meyers-Schöne et al., 1993). Higher mercury concentrations occurred in snapping turtle tissues than red-eared slider tissues, but red-eared sliders had a higher level of $^{137}$Cesium at the same location, a difference that the authors attributed to food habits. The greater amount of plant material that painted turtles ingest compared to snapping turtles may play a role in the dichotomy of developmental effects between the two species if plants differentially take up certain contaminants, as they did in the case of red-eared sliders and $^{137}$Cesium.

The greater occurrence of embryonic deformity in painted turtles could be due to a greater susceptibility to pollution. Greater susceptibility to pollution could be caused by many factors, such as different rates of cytochrome P450 metabolism, differences in
EROD or Aryl hydrocarbon activity, higher rate of accumulation of contaminants, or lower rate of clearance of contaminants. Painted turtles may accumulate higher levels of one or more harmful contaminants or pass on greater proportions of particular contaminants to their eggs than snapping turtles do. Meeks (1968) observed that the level of DDT in painted turtles’ testes was always high and sometimes exceeded those in fat, while this was not the case for a single snapping turtle assayed. High levels of contaminants in testes could lead to the transfer of damaged sperm or DNA to the egg. The difference in deformity rates between females of the same species is not surprising, because individual differences in metabolism, diet and age probably occur. One female may have a more efficient detoxification system than another, or some individuals may be genetically predisposed to greater genetic damage in the presence of one or more contaminant.

It would be ideal to compare rates of deformities in turtles in this study to rates in turtles from pristine sites. However, there do not appear to be any pristine sites left for comparison. I can compare rates with those for turtles at relatively uncontaminated sites. Rates of embryonic deformity in turtles from JHNWR were much higher than for turtles from relatively uncontaminated reference sites. Deformity rates in snapping turtle embryos and hatchlings from relatively pristine reference sites have been previously examined. Algonquin Provincial Park (APP), Ontario, Canada has been used as a reference site for snapping turtle studies for many years (Bishop et al., 1991, 1996b, 1998; deSolla et al., 2002; Struger et al., 1993). Published deformity rates from APP are much lower than those in snapping turtle embryos from JHNWR, and suggest that pollution at the refuge may affect the development of resident turtles. Bishop et al.
(1991, 1998) reported some of the same types of deformities in snapping turtles from organochlorine pesticide contaminated sites in Ontario that I have seen at JHNWR. The most common deformity in Ontario populations was tail abnormality, which was the most common deformity seen in snapping turtles in JHNWR. Although there were lethal deformities such as dwarfism in Algonquin Park embryos, there were no other severe deformities such as severe spinal deformities and exposed brains. The similarity of some minor deformities, however, suggests that PAHs and organochlorine pesticides affect some developmental pathways in a similar manner.

Incidence of deformities in painted turtles at JHNWR was higher than that of adults and juveniles combined at ESGR. Percent deformity in adult snapping turtles at JHNWR was lower than that at ESGR (Table 2). However, the majority of deformities seen at ESGR were minor shell anomalies. The lack of more serious deformities, such as facial deformities, at ESGR is a stark contrast to the incidence of these abnormalities in both species at JHNWR. Although Dr. Congdon and his colleagues did not examine embryos from ESGR, the high rate of minor deformities in adults and juveniles at this relatively uncontaminated site were often comparable to those of JHNWR adult snapping turtles, although less than those of JHNWR painted turtles. These data suggest that either there is more pollution at ESGR than previously thought, or that deformity rates in painted turtles and snapping turtles are highly variable between locations and that minor deformities may not be uncommon in these species. Chemical analysis of a pooled egg sample from Cedar Lake near the ESGR showed total alkanes and total PAHs similar to those of the least contaminated JHNWR sample, which supports the former hypothesis. The fact that there were significantly more minor deformities and far fewer lethal
deformities at ESGR compared to JHNWR suggests that the former is a less polluted site than the latter, if pollution is the cause of greater deformity rates at JHNWR. However, pollution is not the only cause of embryonic deformity in turtles. Incubation temperatures can cause minor deformities (Packard et al., 1987). The authors found minor deformities in 40% of snapping turtles incubated at 31.0°C, but none at 26.0°C, a temperature very near the 26.5°C that I used. Temperature was constant in the incubators in this study, but that in natural nests can change considerably over the course of incubation (Packard et al., 1985).

Snapping turtle eggs at JHNWR contain much higher levels of PAHs than eggs from Michigan and Canada. However, PAH levels in all locations were only one third those present in fat pads. Total alkane levels were also greater in snapping turtle eggs from JHNWR than from Michigan or Canada, although levels were highest in lipid samples. Either PAHs and alkanes are not in equilibrium between lipid and eggs, or some depuration occurred during incubation.

**Summary**

Snapping turtles and painted turtles in the JHNWR exhibited high rates of embryonic and adult deformity. Painted turtles not only had a higher incidence of abnormality than snapping turtles, but the malformations were more severe in the former than the latter. This difference in prevalence and severity of deformities suggested that painted turtles were more affected by toxins in the refuge than snapping turtles. Rates and severity of deformities in turtles from JHNWR were higher than those of turtles from ESGR, a less contaminated site.
Although the majority of the turtles at JHNWR appear normal, they may be carrying heritable mutations that decrease hatching success and juvenile survival, as suggested by high deformity rates and the fact that undeformed females produce a high percentage of deformed hatchlings. The adverse effect observed in this population at present appears to be in the form of developmental effects, and represents a cost of living in an urban environment. Urbanization and land use pressure will continue to shrink natural habitat in the United States and elsewhere as the human population continues to expand. As human populations encroach upon nature, so does our waste. National Wildlife Refuges, National Parks and other protected areas are becoming increasingly important in wildlife and habitat preservation in the face of this increasing urbanization. The JHNWR is such a place, and is an important refuge for many species of birds and reptiles in the Philadelphia area. Contamination of other wildlife refuges is already a problem (see Beyer et al., 1999; Halbrook and Arenal, 2003), and will become more so in coming decades. These problems need to be addressed if wildlife refuges are to fulfill their intended purpose.
Bibliography


U.S.E.P.A. (2003). National Priorities List Website:
http://www.epa.gov/superfund/sites/npl/index.htm


Table 3. System for rating severity of deformities in embryos and adults of snapping turtles and painted turtles at the JHNWR.

<table>
<thead>
<tr>
<th>Severity of Deformity</th>
<th>Types of Deformities</th>
</tr>
</thead>
</table>
| Minor: not likely to affect survival | • Misshapen Carapace or Plastron  
  • Deformed Tail  
  • Extra, missing or Misshapen Scutes  
  • Deformed or Missing Digits or 1 foot  
  • Lack of pigmentation  
  • Narrow body  
  • few or no scales |
| Moderate: may lower chances of survival but have seen adults with some of these conditions | • 3 or more of the above minor deformities  
  • skull smaller than normal  
  • 1 missing limb or eye  
  • Kyphosis or scoliosis  
  • One side of maxilla did not fuse with nasal process  
  • 2 deformed feet  
  • Developmental Asynchrony |
| Lethal: probability of survival slim, no adults seen with these conditions | • Dwarf  
  • Gastrotrichsis  
  • 2 or more limbs missing  
  • All brain abnormalities: exencephaly, hydrocephaly, missing lobes, etc.  
  • Missing or severely deformed jaw parts: maxillary processes not fused, maxillary process not fused with nasal process, absence of maxilla or mandible, malocclusions, etc.  
  • Missing plastron or carapace  
  • Ectocardia  
  • Anophthalmia  
  • Skull not fused  
  • Spine/notochord bent in 2 or more places so that movement is impaired |
Table 4. Number and percent of deformities of adult painted (*Chrysemys picta*) and snapping (*Chelydra serpentina*) turtles from JHNWR 2000-2003. Painted turtles had up to 4 deformities each.

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Normal (N)</th>
<th>Deformed (N)</th>
<th>Percent deformed</th>
<th>1 (N)</th>
<th>2 (N)</th>
<th>3 (N)</th>
<th>4 (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td><em>C. picta</em></td>
<td>153</td>
<td>14</td>
<td>9.2%</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>C. serpentina</em></td>
<td>101</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2001</td>
<td><em>C. picta</em></td>
<td>61</td>
<td>8</td>
<td>13.1%</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>C. serpentina</em></td>
<td>30</td>
<td>2</td>
<td>6.7%</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2002</td>
<td><em>C. picta</em></td>
<td>71</td>
<td>28</td>
<td>39.4%</td>
<td>11</td>
<td>10</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>C. serpentina</em></td>
<td>31</td>
<td>5</td>
<td>16.1%</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2003</td>
<td><em>C. picta</em></td>
<td>11</td>
<td>5</td>
<td>45.5%</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>C. serpentina</em></td>
<td>22</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Overall</td>
<td><em>C. picta</em></td>
<td>296</td>
<td>55</td>
<td>18.6%</td>
<td>29</td>
<td>16</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>C. serpentina</em></td>
<td>184</td>
<td>7</td>
<td>12.7%</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5. Incidence of abnormalities in *Chrysemys picta* and *Chelydra serpentina* of different age classes from the E.S. George Reserve in Ann Arbor, Michigan, 1975-2003.

<table>
<thead>
<tr>
<th>Species</th>
<th>Group</th>
<th>Individuals Examined (N)</th>
<th>Total Abnormalities 1 (N)</th>
<th>Abnormal Individuals (N)</th>
<th>Abnormal Marginal Scutes 2 (N)</th>
<th>Other Abnormal Scutes 3 (N)</th>
<th>Abnormal Spine (N)</th>
<th>Bacterial Granuloma (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. picta</em></td>
<td>Adult + Juvenile</td>
<td>4036</td>
<td>1324</td>
<td>577</td>
<td>597 (14.7%)</td>
<td>575 (14.2%)</td>
<td>34 (0.8%)</td>
<td>91 (2.0%)</td>
</tr>
<tr>
<td></td>
<td>Hatchling + Yearling</td>
<td>1432</td>
<td>563</td>
<td>299</td>
<td>213 (14.9%)</td>
<td>173 (12.1%)</td>
<td>5 (0.3%)</td>
<td>0</td>
</tr>
<tr>
<td><em>C. serpentina</em></td>
<td>Adult + Juvenile</td>
<td>865</td>
<td>72</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Some individuals had more than one abnormality
2 Extra or too few marginal scutes.
3 Inclusions or misshapen scutes
Figure 9. Percentage of each type of embryonic deformity out of all *C. serpentina* eggs with deformed embryos from JHNWR. Percentages were calculated from 4 years of data. 210 embryos and neonates out of 1460 eggs with visible embryos were deformed.
Figure 10. Deformities in snapping turtle (*Chelydra serpentina*) hatchlings. All at hatching stage 26 [Yntema, 1968]. A, B and D are hatchlings with lethal deformities, C is a hatchling with a minor deformity. A. “Corkscrew” tail; mandible and maxilla short; prefrontal bone grown backward; cyclops; brain protruding from skull; abnormal pigmentation; kyphosis. B. Bent tail; prefrontal bone grown backward; no eyes; brain protruding from skull; abnormal pigmentation; rear limbs deformed; cervical spine bent 90°. C. Normal (black) vs. abnormal (gray) pigmentation. D. Maxilla and premaxilla not fused, short; eyes slanted forward; right eye would not open wide; abnormal pigmentation; yolk sac still herniated.
Figure 11. Percentage of type of each embryonic deformity out of all C. picta eggs with deformed embryos from JHNWR. Percentages were calculated from 4 years of data. 84 embryos and neonates out of 155 eggs with visible embryos were deformed.
Figure 12. Deformities in painted turtles (*Chrysemys picta*). Staged according to [Mahmoud, 1984]. All turtles had lethal deformities. A. Stage 23 (hatching stage); dwarf. B. Stage 23; dwarf; plastron narrow so yolk opening wide; tail bent; body skewed along anteroventral axis. C. Stage 23; deformed carapace, plastron, rear limbs, head, jaws; no tail; no eyes. D. Detail of head of (C), maxilla and premaxilla not fused; mandible deformed; no eyes; brain covered by skin only. E. Stage 18; dwarf; clear area is brain with fluid buildup; all visible body parts deformed; no tail; ectocardia. F. Stage 18; dwarf; tail bent; no pigment; no carapace or plastron; organs herniated through back; brain protruding; most body parts deformed.
Figure 14. A. Severity of deformities in embryonic and hatchling snapping turtles (*Chelydra serpentina*) from the JHNWR 2000-2003. The four year mean shows that the majority of deformities were lethal. B. Severity of deformities in embryonic and hatchling painted turtles (*Chrysemys picta*) from the JHNWR, 2000-2003. The four year mean shows that the majority of deformities were lethal.
Figure 15. A. Percentage of each type of external deformity out of all adults captured, *C. serpentina*. B. Percentage of each type of external deformity out of all adults captured, *C. picta*. 
Figure 16. Examples of deformities seen in adult turtles. All deformities are minor.
A. *C. serpentina*. Scoliosis, scutes deformed as a result.  B. *C. picta*. 3 short toes with toenails, several extra protruding scales.  C. *C. picta*. One central toe; abnormal scales  D. *P. rubriventris* (hatchling). One extra vertebral scute.
Figure 17. Proportion of abnormalities for turtles from ESGR. A. All adult and juvenile painted turtles 1975-2003. B. Hatchling snapping turtles 2004.
Figure 18. Prevalence of minor, moderate and lethal deformities among all snapping turtle hatchlings examined at the JHNWR and ESGR.
CHAPTER 5. OIL EXPOSURE CAUSED DEFORMITIES IN EMBRYOS AND HATCHLINGS OF TURTLES IN THE JOHN HEINZ NATIONAL WILDLIFE REFUGE

Introduction

An underground pipeline leaked approximately 758,000 liters of Cabinda crude oil into a man-made lake (impoundment) in the John Heinz National Wildlife Refuge (JHNWR) in suburban Philadelphia in February 2000. Twenty turtles representing four species (the snapping turtle, *Chelydra serpentina*, the painted turtle, *Chrysemys picta*, the red-eared slider, *Trachemys scripta*, and the state-threatened red-bellied turtle, *Pseudemys rubriventris*) were pulled, covered with oil, from the spill area during the clean up and sent to a wildlife rehabilitation center for cleaning and veterinary care. Other individuals of the same species were enclosed within a fence that oil recovery workers erected around the spill site to prevent animals from entering the spill area. Those turtles lived in the enclosure for three months prior to the nesting season where they fed on oil-contaminated vegetation and prey and were exposed to contaminated sediment. Female turtles pass lipophilic contaminants to the yolk of their eggs (Bishop et al., 1991; Bishop et al., 1994; Bishop et al., 1996; Bishop et al., 1998; Pagano et al., 1999), and results from other studies in this laboratory indicate that JHNWR females transferred oil contaminants to their eggs (see Chapter 3).

Turtles in the JHNWR are already subject to contamination from multiple industrial and municipal sources that seep PCBs, PAHs, metals, volatile and semivolatile organic compounds into Darby Creek, which feeds the impoundment (U.S.E.P.A., 2003). Turtles at the refuge experience a high rate of deformity, ranging from minor abnormalities that do not affect survival to lethal malformations that prevent embryos
from completing development (see Steyermark and Spotila (2001) and Chapter 4 of this volume for a full description of background levels of deformities in turtles at JHNWR). It is possible that some of these deformities are caused by the background contamination at the refuge.

A small level of deformity occurs in natural turtle embryos, possibly due to fluctuating incubation temperatures, but not to the extent that occurs in turtles at this wildlife refuge. Several investigators have investigated the effect of incubation temperature in the laboratory with mixed results: Bishop et al. (1991) and Bobyn and Brooks (1994) did not find any correlation between incubation temperature and deformity while Packard et al. (1987) and Steyermark and Spotila (2001) did. Deformities in turtles also result from exposure to contaminants such as organochlorines and PCBs (Bishop, 1991; Bobyn and Brooks, 1994; Ashpole et al., 2001). Exposure to crude oil or its components caused morphological abnormalities in embryonic fish (Linden, 1976; Linden, 1978; Carls and Rice, 1988; Brown et al., 1996; Carls et al., 1999), birds (Hoffman, 1979; Gorsline and Holmes, 1982; Ellenton, 1982), and mammals (Feuston et al., 1997). The teratogenic effect of oil exposure depends not only on the dose, concentration, route of exposure, developmental timing of exposure, composition of the oil, uptake and release kinetics of oil components, but also on environmental factors at the time of exposure and alteration of components through metabolism (Neff et al., 1976; Sharp et al., 1979; Carls et al., 1999). Maternal effects can also influence deformity rate. The purpose of this research was to determine if maternal exposure to crude oil exacerbated existing developmental problems and increased the number and severity of deformities in turtles at JHNWR.
Materials and Methods

I captured turtles with hoop nets both inside the oil containment fence and at the opposite end of the 59-ha impoundment beginning in March 2000 and continuing each spring and summer through 2002. I also captured them by hand during the nesting seasons of 2000-2003. The fence and contaminated sediment were removed in 2001. Each turtle was marked with an individual alphabetic identification code similar to the numeric system established by Cagle (1939) by drilling or filing holes in the marginal scutes. I measured and weighed each turtle and examined it for deformities or signs of health problems before releasing it.

The eighteen surviving turtles initially exposed to the oil spill were transferred from the wildlife rehabilitation center to Drexel University in March of 2000. None of the females from that group produced eggs that year, nor were they recaptured in subsequent years. I collected eggs from other turtles in the field after they were laid or brought females to the laboratory for oxytocin induction (Ewert and Legler (1978); 19-21 IU/kg as per Steyermark, pers. comm.). I marked each egg with a unique alphanumeric code using a graphite pencil, weighed it, and placed it in 4% moisture (by mass, -100 to -170 kPa) sand in plastic boxes at 26.5 ± 0.5°C for incubation. I began egg incubation in a Rheem™ environmental chamber in 2000 but transferred eggs to a Precision™ brand incubator when the former malfunctioned and overheated to 40.0°C on June 14, 2000 and shut down. Moisture was determined gravimetrically and replaced three times per week. I determined egg fertility by external examination for the white spot that appears on the eggshell in living, fertile turtle eggs (Chan, 1989). I dissected eggs that did not appear to be fertile or that showed signs of embryonic death such as loss of turgidity, foul smell,
discoloration or arrested development (as determined by candling) and examined each embryo for deformities. In some cases I was unable to dissect eggs until 30 days after they were laid. I rated deformities as minor, moderate or lethal according to the system described in Chapter 4, Table 3. Embryos that survived were incubated full term, and hatchlings were weighed, measured and examined for deformities.

Data Analysis

Although four species of turtle were exposed to crude oil, only two species, the snapping turtle and the painted turtle, provided a large enough number of individuals and clutches to perform statistical analyses. Recapture rates of oil exposed turtles for both species were too low in 2001 and 2003 for statistical analysis; therefore I only present analyses for 2000 and 2002.

Percent deformity data was heteroscedastic, therefore I performed separate G tests to compare the ratio of deformed:normal individuals between groups of embryos from oil exposed and control females in 2000 and 2002. I also performed G tests to determine if there were significant differences in the occurrence of each type of deformity between oil exposed and control snapping turtles within 2000. I summed counts of individual embryos for all clutches for each G test performed in all analyses. Inspection of the distance between error bars of painted turtle and snapping turtle data in Figure 5.1 revealed that there was a pronounced difference in percent deformity of clutches between the two species. The standard error was particularly wide for oil exposed painted turtles in 2000 due to a small sample size and disparate deformity values, which made it less clear if the difference between species was significant during the year of the spill. I
therefore performed a G test to determine if the apparent difference between clutches of the two species was statistically significant that year.

Deformity severity data for snapping turtles was non-normal, therefore I used G tests to determine if there was a difference in the ratio of minor:moderate:lethal deformities between oil exposed and control snapping turtles in 2000 and 2002. I then performed a G test to determine if there was a difference between the aforementioned ratio in control turtles between 2000 and 2002. An initial G test indicated a difference, but not which group(s) differed from the others, so I performed multiple 2x2 G tests comparing two groups at a time to determine where the difference occurred. I did not have enough painted turtle clutches with embryos that survived long enough to exhibit visible deformities, so statistical analyses were not possible for this species.

Results

Crude oil exposure caused an increase in deformities in embryos and hatchlings. Percent deformity was higher for painted turtles than snapping turtles in both years for both control and oil exposed groups (Fig. 19). Graphical inspection of the data showed a clear difference between the two species except for the oil exposed group in 2000. A G-test confirmed that the difference between oil exposed painted turtles and snapping turtles was also significant in 2000 (G_{adj.}=10.03, \chi^2=3.84). The ratio of deformed:normal individuals for snapping turtles in 2000 was 50:338 (14.8%) for controls and 60:153 (39.2%) for oil exposed turtles, a difference that was significant (G_{adj.}=20.66, \chi^2=3.84). Graphical inspection of the 2002 data suggested that there was a greater incidence of deformities in embryos from oil exposed as compared to control female snapping turtles.
Because of the small sample size of clutches this apparent difference between control (33:249 or 13.3%) and oil exposed (15:73 or 20.5%) snapping turtles was not statistically significant. In both years painted turtle embryos from mothers exposed to oil appeared to have higher rates of deformities, but small sample sizes preempted statistical analysis of these data.

The patterns of prevalence of different types of deformities were similar in oil exposed and control embryos and hatchlings for both years (Fig. 20). Tail (Fig. 21 C, D, E and F) and shell (Fig. 21 F) abnormalities were the most common of all deformities for both exposure groups in both years, but in 2000 control turtles had a higher percentage of tail deformities ($G_{adj.}=6.94, \chi^2=3.84$), while in 2002 the percent of oil exposed turtles with tail deformities was more than twice that of control turtles ($G_{adj.}=10.94, \chi^2=3.84$). Oil exposed turtles had more jaw/facial (Fig 21 B, C and E) abnormalities than control turtles in both years as well ($G_{adj.}=4.37, \chi^2=3.84$). Although Figure 20 seems to suggest that there were more dwarf (Fig. 21 A, B) control turtles than oil exposed turtles in 2002, this difference was not significant ($G_{adj.}=0.68, \chi^2=3.84$). Turtles characterized as “small” always had a significant amount of yolk remaining at hatching (or death) due to their small size. Oil exposed turtles had more major organ abnormalities (usually consisting of enlarged livers or hearts) in 2002 than control turtles, but the latter had slightly more limb deformities than oil exposed both years.

There was an intriguing pattern of severity of deformities in snapping turtle embryos and hatchlings between years (Fig. 22). There was no difference in the ratio of minor:moderate:lethal categories between oiled and control turtles in either year ($G_{adj.2000}=1.00, G_{adj.2002}=1.46, \chi^2=5.99$). However, there was a significant difference in
the ratio of the three categories between years for control turtles \((G_{adj.}=14.13, \chi^2=5.99)\). Since oiled turtles showed the same pattern of occurrence of severity as control turtles, the ratio differed between years for the former as well. Contrary to what Fig. 23 would indicate, a G test indicated that the difference between years in the ratios of minor:moderate categories as well as moderate:lethal deformities did not reach significance at the 0.05 level, but would have at the 0.10 level. The ratio of minor:lethal deformities, however, was significantly different between years \((G_{adj.}=13.99, \chi^2=3.841)\). In 2000, lethal deformities appeared most common, although not significantly more so than any other severity category. In 2002, minor deformities predominated.

**Discussion**

While embryos from control turtles at JHNWR experienced a wide range of deformities ranging from inconsequential to lethal malformations that arrested embryonic development, embryos from oil exposed females had a greater incidence of deformities and a greater prevalence of craniofacial abnormalities. These jaw/facial deformities were most likely related to improper formation or closing of the neural tube (the embryonic precursor to the central nervous system). The neural tube becomes the brain and spinal cord, and neural crest cells, which arise from the neural tube, “migrate extensively to generate a bewildering number of differentiated cell types (Gilbert, 1997).” The neural crest cells differentiate into medulla cells, neurons, melanocytes (pigment-producing skin cells) and many skeletal and tissue components of the head. Damage to genes that control growth factors or other determinants of development of these cells often causes abnormalities. For example, mice with mutations in various *Pax-3* alleles (essential to
the formation of the mammalian neural tube) developed mutations ranging from pigment abnormalities to failure of the neural tube to close (Gilbert, 1998). Recent research by Qi et al. (2003) indicated that disruption of cell differentiation and proliferation by the mammalian notochord was pivotal to the formation of sacral vertebral defects and anorectal malformations, both of which occur in embryos from JHNWR.

The potential effect of crude oil components on these gene-level processes has not been investigated, but observational studies on oil-induced abnormalities confirm that exposure to petrochemicals does indeed lead to malformations like those seen in this study. Van Meter (2003) produced the same types of abnormalities seen in oil-exposed and control turtles in this study after applying either crude oil, benzo[a]pyrene or 7,12-dimethylbenz[a]anthracene to the eggshells of snapping turtle eggs from JHNWR and from two control sites. The author found that the JHNWR group of eggs showed the smallest magnitude of change in percent and severity of deformity of all three groups, possibly because the added PAHs did not add significantly to the effect of background contaminants already in the egg. Carls and Rice (1988) induced eye, brain, intestine and jaw deformities in pollock fish embryos by exposing fertilized eggs to the water soluble fraction (WSF) of Cook Inlet crude oil. Carls et al. (1999) found that exposure to the WSF of weathered Exxon Valdez crude oil (EVO) also caused jaw deformities, as well as pericardial edema, spinal and other skeletal defects. Effects were dose-dependent in both studies. Inland silverside fish embryos exposed to the water soluble fraction of a crude oil at different times during incubation showed the greatest susceptibility to severe developmental defects in the two- to four-cell stage (Middaugh et al., 2002). Killifish embryos exposed to the WSF of #2 fuel oil (a crude oil derivative) were shorter and had
larger yolk sacs than controls (Sharp et al., 1979). Exposure to EVO caused morphological defects in Pacific herring similar to those seen in the aforementioned studies that were more severe than in control larvae (Brown et al., 1996). The authors cited genetic damage as measured by anaphase aberration rate as the response most specific to crude oil when compared to other responses such as deformities, which could be caused by a wider variety of environmental stressors.

Tail and shell abnormalities occurred most often in both exposure groups. In addition to the possible contribution of abnormal notochord formation already mentioned, disruption of normal formation of the carapacial ridge (CR) may also lead to shell malformation (Gilbert et al., 2001). The CR is an embryonic structure unique to turtles that directs the transformation of ribs and dermal tissue into the familiar carapace and bridges of the shell. Bishop et al. (1991) also found that tail abnormalities were the most common type of deformity at their contaminated study sites in Ontario. The prevalence of tail deformities at polluted sites suggests that the development of the sacral and caudal vertebrae is extremely sensitive to environmental perturbation. The cause of the increased percent of oil exposed snapping turtles with tail deformities in 2002 compared to 2000 is not clear, but the higher incidence of such minor deformities compared to more lethal deformities suggests that oil exposure had little or no effect two years later.

The increased incidence of abnormally small embryos in clutches from oil exposed females corroborates findings from other studies. Hoffman (1979) saw a decrease in birth weight of mallards exposed to a mixture of aromatic constituents of crude oil, and Hoffman and Gay (1981) found that exposure to a single crude oil PAH (benzo[a]pyrene) caused stunted growth in the same species. The fibroblast growth
factors (FGF) are a family of proteins responsible for formation of numerous organ, skeletal and cutaneous systems. The primary cause of dwarfism is genetic mutations that prematurely activate FGF receptors (Gilbert, 1997). The increased incidence of growth-inhibited embryos in oil exposed clutches suggests that petrochemicals may cause such mutations, although the fact that control turtles had a higher incidence of true dwarfism in 2002 suggests that other contaminants may have this effect as well.

The small sample size of recaptured oil exposed females affected detection of statistical significance in analyses of differences between the categories of deformity severity. There was a difference in the ratio of minor:moderate and moderate:lethal deformities between years (Fig. 22). Results of analyses came near to but did not reach statistical significance. Multiplying my sample size by 1.05, however, produced a large enough sample size to reach significance at the 0.05 level. The ratios of minor:lethal deformities were so diametrically opposed between years, however, that a G test revealed a strongly significant difference.

Sediment sampling showed that the heavier components of oil did not extend more than 1 ha into the 59 ha impoundment, but several indicators point to the possibility that the water soluble fraction may have spread farther. The pattern of occurrence of severity of deformity, in which lethal deformities dominated in 2000 but minor deformities dominated in 2002 is one such indication. Embryonic death rates were also higher in both species in 2000 than 2002 (see Chapter 3). There was no difference in fertility between species either year, yet fertility was lower for both species the year of the spill (See Chapter 2).
There are several possible causes of differences in deformity rates between species (Chapter 4), such as differential efficiencies of mixed function oxidase systems. In brief, metabolization of xenobiotics often results in more toxic compounds (Hoffman, 1979). Petroleum products are no exception (Lee et al., 1986). Interspecies differences in sensitivity to contaminants have been documented in other species (Longwell, 1977; Sharp et al., 1979; Giesy et al., 1994). Giesy et al. (1994) attributed these differences to different metabolic processes as well as variation in diet between species of colonial water birds (see Chapter 4). In addition, Naf et al. (1992) found that chickens and eider ducks had similar capacities for PAH metabolization, yet eider embryos accumulated more PAHs in their gallbladders than chickens, a difference they attributed to the significantly higher lipid concentration in eider gallbladders.

Armstrong (1986) suggests that mutagenesis from environmental agents is not passed on to offspring frequently because most oocytes with induced mutations do not survive and affected embryos often die. Yet there is a very high incidence of deformity in turtles at the JHNWR. Longwell (1977) noted that chromosome abnormalities can lead to developmental arrest and “mutations that occur during the period of major organogenesis lead to developmental abnormalities.” Carls and Rice (1988) noted that mortality of pollock larvae after hatching was correlated with embryonic deformities and was dose dependent. Chromosome abnormality gives a partial explanation for the high incidence of early death in embryos from JHNWR (see chapter 3). The great number of surviving deformed turtles at JHNWR, however, indicates that not all mutations are lethal and that turtles can survive in environments that would be lethal to less tolerant species.
Summary

The data for snapping turtles the year of the spill suggest that exposure to crude oil contaminants passed from mother to egg increased the incidence and severity of deformities in snapping turtles at JHNWR. Although there was no difference in the types of deformities occurring in oil exposed turtle embryos from JHNWR compared to controls, the former had greater incidence of craniofacial, scale and pigment abnormalities than the latter. This suggests that exposure to oil may have affected proper formation of the neural tube or neural crest cells. Exposure to crude oil increased the number but not the severity of deformities seen in turtles at the refuge. There was no difference in the distribution of deformities among the three descriptive categories minor, moderate and lethal between oil exposed and control turtles. The trend toward lethal deformities the year of the spill was completely reversed two years later between exposure groups, which suggest that factors other than oil exposure were affecting the severity of deformities at the refuge. The high incidence of deformed embryos suggests that exposure to chemicals could be causing destructive, heritable genetic defects in turtles at JHNWR.
Bibliography


Figure 19. Percent deformity of embryos and hatchlings of painted turtles (C. picta) and snapping turtles (C. serpentina) at the JHNWR, 2000 and 2002. Numbers represent numbers of clutches examined. Error bars represent one standard error of the mean.
Figure 20. Types of deformities seen in embryos and hatchlings from oil exposed and control snapping turtles from JHNWR, 2000 and 2002.
Figure 21. Deformities in snapping turtle embryos and hatchlings from oil exposed females. A, B, C, and E were alive when egg was dissected after full incubation period, D emerged from the egg successfully. B and C were photographed after formalin preservation. A. Dwarf, hatching stage. B. Dwarf, no pigment, deformed jaw and frontal skull, hatching stage. C. Abnormal pigment, curled tail, deformed plastron, upper jaw not developed so eyes and skull deformed, brain exposed, hatching stage. D. Hatchling with curled tail. E. Undersized with facial, pigment and tail abnormalities, hatching stage. F. Hatchling with deformed tail and underdeveloped carapace (rear).
Figure 22. Percent of snapping turtle embryos/hatchlings from JHNWR that fell into each category of severity of deformity for each exposure group, 2000 and 2002. Numbers represent numbers of clutches examined. Error bars represent one standard error.
CHAPTER 6. CONCLUSIONS

I conducted four years of field research (2000-2003) at the John Heinz National Wildlife Refuge in Philadelphia, Pennsylvania to investigate the effects of a crude oil spill that occurred in February 2000. I examined the effects of maternal crude oil exposure on fertility, reproductive output as measured by relative clutch mass (RCM), embryonic death, and embryonic deformity rates and types. Snapping turtles and painted turtles were contained at the oil spill site within a fence from shortly after the spill until they were captured and removed one to two months later. I captured and marked females from the oil spill site as well as other sites in the refuge starting a month after the spill and continuing through nesting season. I incubated eggs from oil exposed and unexposed females in the laboratory and examined each egg for signs of fertility and embryonic death. I examined each undeveloped egg for signs of an embryo and each embryo for signs of deformity. I rated deformities as minor, moderate or lethal according to criteria that I developed (Table 3).

Crude oil exposure did not affect the fertility of snapping turtles or painted turtles. Very few studies published to date specifically address the effect of crude oil exposure on egg fertility in vertebrates. Most previous studies examined the effect of oil on pregnancy or on the number of active breeding pairs before and after a spill, and demonstrated a negative effect. Although I did not see an effect of oil on fertility in the year of the spill, fertility decreased for both oil-exposed and unexposed groups in 2002. Although environmental contamination is one possible cause of this decrease, other environmental and physiological factors could have played a role as well.
Crude oil exposure did not affect the reproductive output of snapping turtles or painted turtles as measured by relative clutch mass. Other studies have demonstrated a negative effect of maternal oil exposure on reproductive output as measured by clutch size or number of progeny produced. There was a sharp decrease in clutch size in two out of three oil exposed turtles two years after the spill. In both cases, the decrease was more than 30 eggs, which is most of a clutch.

Painted turtles had higher embryonic death rates than snapping turtles in all years. Embryonic death was higher in 2000 than 2002 for both species, probably due to a spike in incubator temperature in 2000 that lasted almost a full 24 hours. More embryos from female snapping turtles exposed to crude oil in 2000 died than those from unexposed mothers, but there was no difference in embryonic death rates between exposure groups in 2002. Previous studies of the effects of maternal crude oil exposure on embryos in vertebrates and invertebrates also showed increased embryonic death rates. Snapping turtle embryos died very early in development or just before hatching, regardless of oil exposure. Snapping turtle embryos from oil exposed females had a greater incidence of early embryonic death than those from unexposed females. Sample sizes were too small for painted turtles to make the same comparison. Hatching success was much lower for both species at the John Heinz National Wildlife Refuge than at other areas, a difference that could be due to background pollution.

Painted turtles also had higher rates of developmental deformities than snapping turtles. The deformities seen most frequently in unexposed painted turtle embryos were tail abnormalities or dwarfism, while tail and shell abnormalities were seen most frequently in unexposed snapping turtles. With the exception of 2002, the majority of
deformities in embryos of both species were lethal whether the mothers were exposed to oil or not. Maternal oil exposure caused an increase in jaw/facial and abnormalities, all of which were lethal and related to the formation of the neural tube. Other investigators reported deformities in turtle embryos from their polluted sites similar to those seen in oil exposed and unexposed embryos from the John Heinz National Wildlife Refuge. There was a greater prevalence of lethal deformities in unexposed hatchlings at the refuge than in those from a reference site in the E. S. George Reserve in Michigan.

This dissertation answered the question “how does oil affect the reproductive life history of a turtle?” Maternal oil exposure did not affect the adult stage through fertility or relative clutch mass, but it did affect the embryonic stages through increased embryonic death and increased prevalence of deformities. The question still remains: what physiological mechanism causes these changes in embryos when mothers are exposed to oil? Are the eggs damaged before fertilization, or do changes occur at the genetic level? Are painted turtles more susceptible to environmental contamination than snapping turtles, and if so, what mechanism is responsible? Understanding the physiological mechanisms for susceptibility to negative effects of pollution exposure will be important not only to conservation of threatened and endangered species in the face of increasing urbanization, but could shed light on differences in susceptibility in humans to toxic chemicals as well.
VITA

Barbara Allen Bell

Education:
2005    Ph.D., Drexel University, Biology, Ecology and Environmental Science
2000    M.S., Drexel University, Biology and Ecology
1996    B.S., College of William and Mary, Biology

Professional Experience:
2004-present    Research Associate, Eyak Environmental Science, LLC
2003-2004    Teaching Assistant, Research Assistant, Drexel University
2000-2003    Research Specialist II, Drexel University

Publications:

Presentations:


Professional Societies:
American Society of Ichthyologists and Herpetologists, Herpetologist’s League