EFFECTS OF CHRONIC AND LOW METHYL MERCURY EXPOSURE IN JUVENILE WHITE IBISES (*Eudocimus albus*)

By

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To my parents, for encouraging me to explore
ACKNOWLEDGMENTS

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Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

EFFECTS OF CHRONIC AND LOW METHYLMERCURY EXPOSURE IN JUVENILE
WHITE IBISES (*Eudocimus albus*)

By

Evan M. Adams

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Chair: Peter Frederick
Major: Wildlife Ecology and Conservation

Methylmercury is a global contaminant with reported neurological, endocrine disrupting, and teratogenic effects that has been a problem specifically in the Everglades ecosystem in South Florida. I used a free-flight aviary to hold White Ibises (*Eudocimus albus*) captive and allow the controlled delivery of dietary methylmercury to four exposure groups—control, 0.05, 0.1, and 0.3 mg/kg per day in diet, wet weight. My research has focused upon the sublethal effects of chronic, environmentally relevant exposure on the behavior and endocrine development in juvenile White Ibises. In this study I examine the method we used to capture 220 nestling White Ibises from two colonies in Florida and in the process monitored a colony-wide starvation event. During my week long collection period in the Everglades I report a significant decrease in the proportion of females caught by my methods and report on differences in size-corrected mass.

In Chapter 2, I design an experiment testing the foraging efficiency of juvenile White Ibises in a group setting. Using stereotyped foraging arenas with varying quantities of structural complexity, I allowed all exposure groups to a set amount of time to forage upon a pre-defined number of small fish. I hypothesized that increasing methylmercury exposure and increasing structural complexity would negatively affect a young ibises foraging efficiency and that increasing methylmercury would decrease this ability to improve foraging efficiency with time.
I found that while structural complexity had a strong negative effect upon group foraging efficiency, methylmercury had a weak (though statistically significant) effect that is non-linear with respect to exposure. The low and medium groups were the best foragers, while the high and control groups were the worst overall. Methylmercury did not affect the improvement of foraging efficiency with time.

In Chapter 3, I investigated the changes in fecal estradiol, testosterone, and corticosterone metabolites over time with respect to methylmercury exposure group in juvenile White Ibises. I collected over 350 samples non-invasively from individual birds over 7 months and developed tests for each hormone of interest for this species. I hypothesized that increasing methylmercury would impact estradiol, testosterone, and corticosterone in dose-dependent fashion with respect to time and that sex would affect each hormone. I found that estradiol and testosterone did not change in any biologically explicable manner with respect to dose, and that sex was not important in determining steroid hormone levels. With corticosterone, I found that a significant non-linear relationship with methylmercury exposure. While this result was not predicted, a biological explanation of hormesis may explain these data.

In conclusion, I found no linear relationships between endpoint—behavior or endocrine function—and methylmercury exposure. White Ibises may not be sensitive to methylmercury exposure at either 1) these dose ranges or 2) while juveniles. Future research during the breeding season will test these new hypotheses.
CHAPTER 1
SEX-RELATED MORTALITY DUE TO A STARVATION EVENT IN WHITE IBIS
(Eudocimus albus) IN THE FLORIDA EVERGLADES

Introduction

Sexually size dimorphic (SSD) avian species have often been found to exhibit sex
dependent pre-fledgling mortality during periods of food limitation (Hipkiss et al. 2002). This
may be because larger sized nestlings have higher mortality due to greater nutritional need
(Clutton-Brock 1985; Arroyo 2002; Laaksonen et al. 2004; Bortolotti 1986; Anderson et al.
1993; Kalmbach et al. 2005), but there are also numerous examples in which both the smaller sex
has higher mortality as nestlings or juveniles (Nager et al. 2000; Torres and Drummond 1999;
Teather and Weatherhead 1989; Røskaft and Slagsvold 1985). This suggests that a different
mechanism of mortality must be acting upon these nestlings. Possible mechanisms include, large
siblings dominating smaller ones (Oddie 2000), larger offspring being better able to withstand
starvation or extreme temperature resilience (Greenberg 1979) and sex specific, size unrelated
mortality (Müller et al. 2005) are potential mechanisms. Covariates linked to sex-related
mortality that are independent of size and sex are brood size (Raberg et al. 2005), prey size
(Anderson et al. 1993), prey availability (McDonald et al. 2005), and hatching asynchrony
(Gonzalez-Solis et al. 2005; Ostreiher 1997); these factors influence the dynamics of sibling
competition in nests and may determine whether a species or population exhibits significant sex-
related mortality.

Here, we report on differential sex related mortality in young of the sexually dimorphic
White Ibis (Eudocimus albus). Adult males are larger than females in most measurements of
body size by roughly 15% (Babbitt and Frederick 2007; Kushlan 1977). A variety of selective
costs and benefits to adults have been proposed for sustaining the sexual dimorphism (Bildstein
Breeding propensity and breeding success are largely limited by prey availability in White Ibises (Kushlan 1974; Kushlan and Bildstein 1992; Frederick 2002). In south Florida, USA, patterns of surface water recession is typically associated with highly available prey (fishes and crustaceans), while rising surface waters are typically associated with poor availability and abandonment of nesting (Kushlan 1974; Frederick and Spalding 1994; Frederick and Collopy 1989; Gawlik 2002). In many years, nesting may be wholly or partly abandoned in response to unseasonable rainfall and associated water level increases (Frederick 2001). Incubation and hatching of eggs in this species is asynchronous, and a size differential is typical for nestmates up to the approximate midpoint of the 40-50 d nestling stage (Kushlan 1974). A sex related size differential is also typical of nestlings, ranging from very little at hatching to nearly adult ratios at fledging.

In this paper, we document changes in secondary sex ratio and body condition in nestling White Ibises during a widespread nest abandonment event resulting from a food shortage.

**Methods**

As part of a study of captive ibis behavior and reproduction we collected young ibises from 1) in the Alley North colony in Water Conservation Area 3 (WCA3) in the Florida Everglades (Broward Co. Florida, N 26° 11.179, W -80° 31.431) and 2) a colony in White Springs, Hamilton County, FL (30°19.900 N, -82°45.367 W). Alley North is one of the largest multi-species wading bird colonies in North America and is composed of a 4-km long willow tree island/cattail complex in the northeast corner of WCA3. In an attempt to collect only the first-hatched chick (i.e., the largest nestling) of each brood we banded 300 nestlings before they could
leave the nest (about 10 days old). The bands were individually numbered plastic spirals (National Band and Tag Co., Lexington, KY, USA).

Some of the banded birds were re-captured at 25–32 d on 14, 17, 19, and 21 April 2005 when they had become vagile and existed in large, somewhat mobile crèches (De Santo et al. 1990). We collected birds using a combination of herding techniques to concentrate them, followed by capture by hand or with hand nets. All 21 birds collected on 14 April 2005 were banded, first-hatched nestlings marked previously (above). After this date we were able to discover no other banded birds, and so we collected any birds available to our techniques. All birds were transported overnight to a free-flight aviary. The following morning prior to any feeding all birds were weighed, had both the culmen and tarsus measured, and blood samples (about 50 μL) were drawn from the brachial vein. We used blood for DNA sexing (Fridolfsson and Hellegren 1999) at Avian Biotech (Tallahassee, FL).

The White Springs colony was on an island within a dredge-spoil lake. Here, we banded chicks between ca. 2 and 7 d of age and identified the first-hatched chicks by relative size and development. We sexed 60 first-hatched chicks by blood DNA, and returned a week later (ca. 10 d of age) to collect chicks.

We used an ANOVA to examine size and mass corrected size (culmen length/total mass) by collection date and sex. The binomial test (expected value = 0.5) was used to test a departure from random for each collection period. All analyses were conducted using JMP IN v. 5.1.

Results

Prior to our first collection date there was evidence of colony-wide nest abandonment at the Alley North colony. Dead chicks were a common sight beginning in early April and continuing through the collection dates. Circling vultures were observed during the first pre-collection foray into the colony where we banded first-hatched chicks. However, adults were
still attending many nests. The abandonments temporally co-occurred with sharp increases in water level due to heavy rains in middle and late March and again in early April (Cook and Call 2005). Between the middle of March and April 21, local water levels increased by 22.9 cm.

Over the course of the week-long collections of ibises at the Alley North colony, the ratio of male to female chicks captured increased dramatically (Table 1-1). We found marginally significant or significant departures from a 50:50 male/female ratio on both the third and fourth collection dates (P= 0.0571 and 0.0427, respectively, binomial test) while birds collected from the first and second collection did not significantly differ from 0.5 (binomial test, P=0.662 and P=0.37, respectively). The sex ratio of first-hatched birds from the White Springs, FL colony, did not differ significantly from 0.5 (P=0.84, n=59, binomial test). The first collection in Alley North and the collection at White Springs only included first-hatched nestlings and we found no evidence of sex ratio bias in the samples of 21 and 69 birds, respectively.

Body condition—as quantified by the ratio of weight (g) to tarsus length (cm)—changed during the course of the one-week collections and was different between the sexes (Fig. 1-1). We found a significant effect of collection date and sex on mass/tarsus ratio (ANCOVA, P<0.001 for both tests), but found no significant interaction (P= 0.6639). Both sexes increased in body condition index over time. While males had significantly higher condition indices than females, the rate of condition increase was virtually identical between the sexes.

Discussion

Our data suggest that there were sex-related differences in mortality and body condition in 25-32 d White Ibis nestlings during a widespread food shortage event that this was not a result of sex bias in the hatch order. Water level had been increasing for about a month previous to our collection and such increases are associated with nest abandonment and limited prey availability in White Ibises (Russell et al. 2002; Frederick and Collopy 1989; Bildstein et al. 1990; Frederick
1987a; Gawlik 2002). We found no evidence of predation or disease at the colony; in fact, most dead ibis chicks were untouched by predators or scavengers. Although disease seems unlikely because no adult birds were found dead, it is sometimes difficult to determine if disease or parasites are causing nest abandonments and colony failure (Frederick and Spalding 1994) and some SSD birds have been found to have sex-related differences in immunity (Fargello et al. 2003). However, the repeated examples of co-occurrence of food shortage and abandonment under very similar hydrological conditions (Frederick and Collopy 1989; Frederick and Spalding 1994) lend strong support to interpretation of food shortage as a causative factor.

We do not believe that the differences in sex ratio of captive birds were due to our capture techniques. The technique did not change over the collection period and was not size-specific in any apparent way. We also would not expect a size-biased collection technique to result in a changing capture sex ratio. The data indicate a linear pattern between sex ratio and collection only after the first (biased) collection, with the later three collection dates showing progressively larger departures from parity. Despite the statistical difficulties with hypothesis testing in sex ratio studies due to small sample size when the expected values of a ratio is 0.5 (Ewen et al. 2002) our data showed biologically and statistically significant trends toward a male bias.

We found that male body condition was higher than female condition on each collection date, though rate of increase in body condition over time was not different between the sexes. Size-corrected mass increased for both sexes over the sampling period which implies that these birds were not starving; however, our technique only sampled the live birds, not the many dead. An increase in size-corrected mass has been correlated with increased survival and reproduction in non-SSD avian species (Magrath 1991; Krementz et al. 1989). While this implies that male
ibises were in better condition than females, an alternative hypothesis would be that male body size is allometrically scaling with mass. The adaptive significance of allometric scaling between sexes in SSD species has been well-researched and allometric scaling is generally correlated with body size in adult (Fairbairn 1997) and nestling (Teather and Weatherhead 1994) birds. In breeding adults and in juvenile ibises, males have higher size-corrected mass than females (Kushlan 1977a, Kushlan and Bildstein 1992, Kushlan 1977b, unpublished data). The apparent lack of difference in rate of increase in body condition between males and females that we found is consistent with the allometric scaling hypothesis. We do not know when sex-related differences in size-corrected mass would begin to manifest in White Ibises, and therefore allometric scaling remains a plausible explanation for males being larger.

We suggest three possible mechanisms by which females might obtain less food than males when food is scarce. First, male nestlings may out-compete their female counterparts for food given by parents. Older and larger ibis nestlings typically are fed first by parents (Bildstein 1992, Rudegeair 1975); if food is limiting then the smaller sex may get few or no regurgitated boluses. There is some evidence to suggest that among birds there is higher mortality in the smaller sex when prey items are small (Anderson et al. 1993) and the size of the brood is small (Raberg et al. 2005). Using relative definitions supplied by these studies, White Ibises deliver small prey and have small brood sizes. Males may also be better equipped to deal with long intervals between feedings. Poor foraging is associated with longer feeding intervals (Bildstein et al. 1990; Rudegair 1975; Frederick 2001), and a larger animal may also be better able to survive longer periods between food deliveries. This explanation is not necessarily mutually exclusive of the food dominance explanation (above). Finally, parent birds could be investing more food in male offspring during times of food limitation. It is not clear that there would be a
benefit to doing so, but the decision would be presumably be affected by the likelihood of male
survival in relation to nutrition, and by the breeding sex ratio likely to be in place at the time that
the young birds become mature. The evidence presented here suggests that females were dying
during the food shortage at a higher rate than males, probably because they were receiving less
food than males. The mechanism by which this occurred is unknown, but there seems to be good
circumstantial evidence that males would be in a better position to compete for food and to
survive long interfeeding intervals because of their size.
Table 1-1. The number of male and female nestlings captured for each collection date. The proportion of males captured is displayed parenthetically after the column totals.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Collection date</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>10</td>
<td>21</td>
<td>41</td>
<td>31</td>
<td>103</td>
</tr>
<tr>
<td>Females</td>
<td>11</td>
<td>18</td>
<td>27</td>
<td>18</td>
<td>74</td>
</tr>
<tr>
<td>Total</td>
<td>21 (0.476)</td>
<td>39 (0.538)</td>
<td>68 (0.603)</td>
<td>49 (0.633)</td>
<td>177 (0.582)</td>
</tr>
</tbody>
</table>
Figure 1-1. The increase in mean mass corrected for tarsus length over collection date split by sex. N=21, 39, 68, and 49 in ascending order of collection dates. Error bars indicate the standard error of the mean for each data point.
CHAPTER 2
EFFECTS OF METHYLMERCURY AND SPATIAL COMPLEXITY ON FORAGING BEHAVIOR AND EFFICIENCY IN JUVENILE WHITE IBISES (Eudocimus albus)

Introduction

Methylmercury is a neurotoxin, endocrine disrupting chemical (EDC), and teratogen in which exposure has been connected to changes in behavior in humans and wildlife (Scheuhammer et al. 2007). The most common acute, neurological effects are a loss of motor skills, coordination and reduction in motivation (Wolfe 1998; Scheuhammer 1987). In captive mallards (Anas platyrhynchos) (Heinz 1979) 3 ppm (wet weight in diet) methylmercury caused changes in the duckling flight response, brain lesions and demyelination of neurons (Heinz and Locke 1976) and 5 ppm (wet weight in diet) methylmercury caused decreases in weight and appetite in Great Egrets (Ardea alba) (Spalding et al. 2000a) along with changes in hematology, neurology and histology (Spalding et al. 2000b). Nocera and Taylor (1998) found methylmercury exposure to be correlated with behavioral changes in young Common Loons (Gavia immer). Bouton et al. (1999) found that at a much lower dose, (0.5 mg/kg ww in diet) juvenile Great Egrets showed decreased activity, altered thermoregulatory behavior, and decreased motivation to hunt. Although there appeared to be no effect of mercury dose upon foraging efficacy, any potential effects seemed confounded by individual foraging strategies.

There are direct and indirect links between methylmercury exposure and learning; first, methylmercury has been suggested to alter thyroid hormones in vertebrates (Facemire et al. 1995), which has been correlated to changes in testosterone, estradiol, and perhaps progesterone in the White Ibis (Eudocimus albus) (Heath 2002). Estrogens, androgens, thyroid hormones and glucocorticoids have important roles in brain development; steroid hormones are involved in critical to early development (Schlinger 1997) and song learning in passerines (Gahr 2004) and hippocampal (i.e. spatial) learning, while thyroid (Schantz and Widholm 2001; Zala and Penn
hormones help regulated nervous system development, and impact learning and memory. Prenatal exposure to methylmercury in humans can lead to learning disabilities later in the life of the child (Grandjean et al. 1997). Therefore, it seems reasonable to expect low level methylmercury exposure to have an impact on learning in other vertebrates. However, the level of mercury necessary to induce this kind of effect is unknown.

Behaviors under high levels of hormonal control are at risk of changing in response to endocrine disrupting chemicals (EDCs) like methylmercury and such changes may have a large impact on important ecological and demographic parameters (Vos et al. 2000). Avian behaviors linked to changes in the hypothalamus-pituitary-adrenal axis and its associated hormones may also impact foraging rates and suppression of breeding behavior (Wingfield et al. 1998). Steroid hormones influence a variety of physiological parameters (i.e., metabolism), and behaviors (i.e., territoriality, foraging rates, courtship, incubation) (Ketterson and Nolan 1992). A wide variety of behaviors are tightly linked with endocrine function and may therefore be susceptible to EDCs, including courtship and breeding behaviors, altered social and dominance behaviors, and learning (Zala and Penn 2004).

We report here on an experimental examination of the effects of low, chronic doses of methylmercury on the ability of naive juvenile White Ibises to forage in differing levels of habitat complexity. White Ibises (hereafter, ibises) are a tactile-foraging aquatic bird that forages in flocks on crabs, crayfish, insects and small fish in a variety of aquatic habitats (Kushlan 1974). Foraging efficiency is directly linked with conditions that produce high availability of prey (Frederick and Collopy 1989; Gawlik 2002), and this parameter has high
biological relevance because food intake can be related to reproductive success (Frederick and Collopy 1989; Frederick and Spalding 1994; Frederick 2001).

We hypothesized that the learning of novel foraging behaviors may be more difficult for birds exposed to methylmercury at environmentally realistic levels. In this experiment we attempted to test the specific prediction that mercury would impair the ability of ibises to forage in the context of increasing difficulty—in this case, structural complexity in the foraging arena. We tested the assumptions that 1) increasing structural complexity would decrease capture rates, and 2) foraging efficiency would increase over the weeks of the experiment. We used foraging efficiency (prey depleted in a given time) and motivation (numbers of birds attempting to forage over time) of groups of foraging ibises as response variables to four different exposure levels of mercury.

Methods

We used 168 captive White Ibises in a large, free-flight aviary at the US Department of Agriculture Wildlife Research Center in Gainesville, FL, USA to conduct this experiment. These birds were collected as nestlings at 10–35 d of age from breeding colonies in the northern Everglades (Broward Co., FL, N 26° 11.179, W -80° 31.431) and from White Springs, FL, USA (Hamilton Co, FL, 30°19.900 N, -82°45.367 W) (Ch. I). Young birds were randomly assigned to one of four dietary exposure groups receiving 0, 0.05, 0.1, or 0.3 mg/kg methylmercury (wet weight in diet) after 90 d of age. Exposure groups were housed in the same circular, open-air aviary (cf. 1200 m²) separated into quadrants by interior net walls. These levels of exposure mimic the range that might be encountered in the Everglades (Frederick et al. 2002; Loftus 1999).

This experiment was run from 11 Oct to 17 Nov 2005. During each daily bout, all treatment groups were simultaneously presented with 200 live fathead minnow juveniles.
(Pimephales promelas) in 2.4 x 3.7 m rectangular foraging pools between 0800 and 0900 h. All groups were given access to the fish for 15 min. The foraging arenas were all similar, and had continuously varying water depths of 2–15 cm as a result of a sloping floor. Water depth was standardized for each experimental run each morning because of the possibility of prey availability differing with water depth (as mentioned above) and we placed varying levels of physical structure (see below). All cages were deprived of food starting at sundown the night before each bout, and food was restored ad libitum after each bout. All bouts were video recorded and reviewed later to determine how many birds participated at standard times in each bout.

The experiment was run for six weeks, and during each week each cage experienced four bouts in a total of four different levels of structural complexity. No more or less than one bout was experienced on any day by any treatment group, and on any one day no two cages would be assigned the same structural complexity level. Daily and weekly order of habitat complexity given to each treatment was pseudorandom. We used four levels of structural complexity: 1) Open pools, 2) pools with panels of agricultural fencing (mesh size ranging from 13–38 cm) supported approximately 3 cm off the bottom of the pool and occupying the entire surface of the pool), 3) the same as #2 but we attached six pieces of 1 m by 1 m shade cloth and about 30 artificial plant leaves—evenly spaced—to the panels, and 4) Same as #2 but with 16 pieces of shade cloth and about 60 artificial plant leaves/fronds. All shade cloth and plastic plants provided a partial visual and physical barrier, but were flexible and did not make any area of the arena inaccessible to the foraging ibises.

After birds had foraged for 15 min, two researchers entered the cages and placed large pieces of shade cloth over the foraging arena to ensure that no more foraging could take place.
Once foraging was stopped in all cages we drained the foraging arenas through a seine net, and counted the remaining fish.

The video recordings were used to obtain an accurate measure of the number of birds in each treatment group that were foraging at standardized times. Each video was analyzed by stopping the tape 30, 60, 240, 420, 600, 780 s into the experiment and the number of ibises in (not around or on the edges of) the wading pool were counted. We estimated the total number of bird-minutes foraged during the entire bout by using rectangles bounded by the total time foraged and the number of birds to estimate the area under the curve. This approximation was a consistent underestimate of the true area under the curve.

We used a repeated measures general linear model for analysis. The response was proportion of fish remaining and the dependent variables were day, week, methylmercury exposure group, and structural complexity. We modeled the proportion of fish remaining adding 1 to the number of fish remaining and the number given (to eliminate zeroes) and transforming these data with an arcsine square root. This transformation was found to produce a set of residuals with an approximately normal distribution. We also modeled group motivation with the same factors to test our assumption of equal motivation. We used ANOVAs to compare numbers of birds foraging across exposure groups while controlling for the effects of structural complexity. We selected models from a base \textit{a priori} set using backward selection. The linear models were analyzed using SAS v. 7.2 (PROC MIXED) and all other statistics were analyzed using JMP IN v. 5.1. Alpha was equal to 0.05.

**Results**

Time, structural complexity, and exposure group were all determined to be important variables \textit{a priori}, and were all included as main effects in our best model (Table 2-1). However, while structural complexity showed a significant (and quadratic) relationship with time,
methylmercury exposure did not. The resulting model fit well and all these terms were considered to be biologically important and plausible. The repeated measures aspect of our analysis (date) explained a small amount of the variance; this implies that our Latin square experimental design was effective in removing the potential bias of day effect. While there was an effect of mercury dose group on foraging, it was small by comparison with effects of structural complexity and time.

The hypothesis that structural complexity would decrease foraging efficiency was supported by this experiment (Fig. 2-1). Foraging efficiency decreased with increasing vegetation/structure (Table 2-2), and this effect varied with time. When compared to the highest group of habitat complexity the lowest two levels showed significantly faster learning (the time*complexity effect) but no significant non-linear change in their improvement (the time*time*complexity effect). While the medium complexity level did not differ from the high in linear improvement, there was a significant non-linear effect and had significantly less fish remaining independent of time. Thus, the hypothesis that feeding efficiency would increase over time was supported and the degree of improvement was related to degree of structural complexity.

Although we predicted that methylmercury exposure would decrease foraging efficiency, result in a change in foraging efficiency over time, or both, in a dose dependent fashion we did not find any interaction of treatment group with time. The medium dose group has the most efficient foragers, the low dose group has the second most efficient and the control and high dose being almost equally less efficient than the other two groups (Fig. 2-2). Statistically, the control and high dose were similarly worse than the low and medium groups (Table 2-2). Contrary to our predictions, this relationship was highly nonlinear.
One assumption of the experiment was that foraging efficiency during the bout would not decline as a result of decreased motivation. The number of birds foraging during the course of the bouts was first analyzed as a response variable by using the same model and dependent variables. This model showed no significant effects. When the model was stripped down to the main effects, complexity was found to be significant (P<0.0001) while time and exposure group were not. In summary, our attempt to control motivational differences between exposure groups via temporary food restriction appears to have been successful.

**Discussion**

One of our objectives was to establish a foraging environment that offered enough challenge to motivated foraging groups that it would gradually decrease foraging efficiency. This was achieved, as evidenced by the inhibitory effect of increasing structural complexity. We also wanted to establish an environment in which the birds might learn how to improve foraging efficiency over time and thus test whether learning might be affected by treatment. We also achieved this goal, since we found an effect of time on foraging efficiency at all levels of foraging difficulty. Although we found a significant effect of methylmercury exposure in this experimental context, methylmercury did not have the expected linearly increasing effect; the middle two exposure groups were more efficient foragers than the control and high dose groups. More to the point, our strongest prediction was that the high and control treatment groups should provide the greatest contrast in mercury effects, yet they were not significantly different. Controlling for group motivation also proved successful and differences between exposure groups are not due to differences in participation.

Over time, all treatment groups improved in their foraging efficiency, with no interaction with dietary methylmercury exposure. Our results do indicate that all cages learned at an exponential rate (the time*time term in the model) when challenged by higher habitat complexity.
and that the degree of improvement in foraging efficiency depended on the difficulty of the task (the time*complexity and the time*time*complexity terms). The prediction that methylmercury (at the dose rates given) would inhibit learning seemed to have little supporting evidence. However, this finding does not rule out the possibility that learning may be affected at higher dose rates or with different behaviors.

There are two potential explanations for the nonlinear effect of mercury seen in this experiment: hormesis and/or confounding effects. Hormesis suggests that certain toxicants stimulate an animal in apparently positive ways at low doses, yet cause negative effects at higher doses (Noel et al. 2006; Calabrese 2005). This pattern has been found increasingly in studies of endocrine effects, and especially with behavioral endpoints (Clotfelter et al. 2004). The amount of methylmercury with which these birds were exposed could be considered quite low by comparison with the existing literature on effects (Wolfe 1998), and it is possible that we approached a threshold for hormesis.

There are two potential factors in the experimental setup that could confound effects of methylmercury exposure: individual composition of groups, and location. Due to differences in social structure of groups between cages and asymmetrical natural events (like the differential effect of light or shade on different cage locations) we may be placing certain cages at a foraging advantage over the others. While we cannot find any ready mechanisms that suggest such a location effect, we also cannot rule such an effect out, since we had only a single replicate for each group.

Increasing structural complexity made it more difficult for ibises to forage. Our measurement of motivation indicates that higher structural complexity encourage more birds to forage longer; despite such differences there were still large differences in foraging efficiency
between complexity groups. While availability of prey to visual and tactile aquatic birds is known to be affected by prey density, hydroperiod, temperature, dissolved oxygen and water depth (Maccarone and Brzorad 2005; Smith et al. 2003; Gawlik 2002; Frederick 2001) the effect of vegetation and obstructions has received relatively little attention (Bancroft et al. 2002; Surdick 1998). Although the ibises showed improvement in the more challenging habitat structure, they never achieved the levels of efficiency seen in the low complexity environment. Although such an effect has been suggested by habitat selection studies of wading birds (Stolen 2006; Surdick 1998; Bancroft et al. 1994), we believe this is the first experimental demonstration of an experimentally measured effect of structural complexity for foraging by long-legged wading birds. It therefore seems likely that vegetation density and type is an important determinant of foraging success and habitat selection for tactile-foraging waders.
Table 2-1. Overall effect of factors in our best model. Significance was determined using an F-test based on Kenward-Rogers degrees of freedom estimation.

<table>
<thead>
<tr>
<th>Effect</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
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</tr>
<tr>
<td>Exposure Group</td>
<td>0.0038</td>
</tr>
<tr>
<td>Time*Time</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Structural Complexity</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Structural Complexity*Time</td>
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</tr>
<tr>
<td>Structural Complexity<em>Time</em>Time</td>
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</tr>
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Table 2-2. Parameter estimates from the best model (PROC MIXED). Note that Beta values are relative to the control group in the case of methylmercury exposure and the high group in the habitat complexity.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Parameter estimate (β)</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td>Time</td>
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<td>&lt;.0001</td>
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<tr>
<td>Time*Time</td>
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<td>0.008699</td>
<td>0.0002</td>
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<tr>
<td>Methylmercury Exposure</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
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<td>0.03069</td>
<td>0.0145</td>
</tr>
<tr>
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<td>0.03069</td>
<td>0.0036</td>
</tr>
<tr>
<td>High</td>
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<td>0.03069</td>
<td>0.8756</td>
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<tr>
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</tr>
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<td></td>
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<tr>
<td>High</td>
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<td>0.001</td>
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<td>&lt;.0001</td>
</tr>
<tr>
<td>Habitat Complexity * Time</td>
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<td>High</td>
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</tr>
<tr>
<td>Control</td>
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Figure 2-1. Mean proportion of fish remaining for each week for each methylmercury exposure group for A) complexity level 1 (control), B) complexity level 2 (low), C) complexity level 3 (medium), D) complexity level 4 (high).
Figure 2-2. Mean proportion of prey remaining grouped by methylmercury exposure group and structural complexity over all weeks. Error bars represent standard error of the mean.
CHAPTER 3
SUBLETHAL EFFECTS OF METHYLMERCURY ON TESTOSTERONE, ESTRADIOL, AND CORTICOSTERONE FECAL METABOLITES IN CAPTIVE JUVENILE WHITE IBISES (Eudocimus albus)

Introduction

Methylmercury is a globally distributed contaminant that has a wide range of endocrine disrupting, neurological, and developmental effects in animals (Wolfe et al. 1998) with significant differences between avian species (Heinz et al. 2006). Within an environmentally relevant exposure range however, endocrine effects have not been well studied, especially in birds. Increased levels of methylmercury in feather samples over the past thirty years are also indirectly correlated with a decrease in number of breeding White Ibises (Eudocimus albus) in the Florida Everglades, where mercury contamination has been high (Heath and Frederick 2005). Heath and Frederick (2005) showed that methylmercury in breeding White Ibises was correlated with a decrease in estradiol in females and an increase in testosterone in males. In Common loons (Gavia immer) Evers et al. (2003) demonstrated a positive association between blood mercury concentrations and blood corticosterone levels and Nocera and Taylor (1998) correlated mercury exposure with changes in chick behaviors. In contrast, Thaxton et al. (1982) showed a decrease in corticosterone with increasing methylmercury in chickens (Gallus domesticus) and Heath and Frederick (2005) found no relationship between mercury exposure and corticosterone in breeding White Ibises. Methylmercury exposure is correlated to changes in endocrine function; however such changes are sensitive to species, dose range, and season.

The mechanism by which mercury exposure affects endocrine function in birds is also unknown. Methylmercury may impact the endocrine system of birds in ovo, during the rapid growth stage as nestlings, as non-breeding juveniles. Alternatively, there may be no developmental aspect to the effect, and methylmercury exposure as an adult may be the primary
route by which adult breeding is affected. Currently, evidence for methylmercury as an
endocrine disrupting chemical (EDC) in birds is solely correlative; a causal relationship between
exposure and endocrine function needs to be demonstrated.

The case for causal relationships between various EDCs and birds is strong; effects in birds
have been commonly found using the steroid hormones estradiol (MacLellan et al. 1996; Giesy
et al. 2003) and testosterone (Colborn et al. 1993; Giesy et al. 2003) along with corticosterone
(Baos et al. 2006; Thaxton et al. 1982), a hormone that is part of the hypothalamus-pituitary-
adrenal stress axis. Estradiol is the sex determining hormone in early development (Bigsby et al.
1999) and is correlated with breeding cycle as adults (Wingfield and Farner 1978; Maguet et al.
1994). Testosterone has been linked to territoriality (Beleskey et al. 1990) and mating behaviors
(Wingfield 1984; Brunstrom et al. 1999). Corticosterone plays an important role in coping with
environmental stressors (Harvey et al. 1984; Kitaysky et al. 1999; Marra and Holberton 1998;
Holberton et al. 1999).

Currently, we lack causal evidence in support of the connection between changes in steroid
and glucocorticoid hormones and methylmercury exposure, however, most studies have not been
adequately designed to answer such a question and we believe this has yet to be fully explored.
As a result we lack strong evidence connecting endocrine changes to reproductive failure in birds
(Bosveld and van den Berg 2002). Field studies are primarily correlative when causality is
needed for decision-making, contaminant exposure may be correlated to confounded ecological
variables (Meyer et al. 1998), and multiple contaminants—with unknown synergies—may be
present at field sites (Tyler et al. 1998). Captive studies may be difficult to generalize to a free-
living population of interest by selecting ecologically irrelevant endpoints (Zala and Penn 2002;
Chapman 2002), species selection (Ottinger et al. 2005), and differential endpoint sensitivities
(Clotfelter et al. 2004). Complicating issues further is our lack of knowledge of endocrine disruption in juvenile birds and the effect this may have on adults; in mammals, EDCs can delay the onset of juvenile sexual maturation (Marty et al. 1999). Such relationship may exist in EDC-exposed juvenile birds, however, no studies have looked at this experimentally.

The available literature therefore suggests that methylmercury exposure in birds could be altering expression of hormones that are important for development and reproduction. If these effects exist, they may have the potential to result in population change, by affecting survival and fecundity rates. Here, we report on a study of the effects of mercury on endocrine function in a developing juvenile aquatic bird, the White Ibis, using a controlled experimental approach. We studied the White Ibis because there is considerable information on its breeding ecology (Kushlan 1974; Kushlan and Bildstein 1992; Bildstein 1994; Frederick 1987a; Frederick 1987b), because there are demonstrated effects of methylmercury on endocrine function in adult birds (Heath et al. 2003), and because the species continues to be exposed to methylmercury in the wild (Frederick and Heath 2005). We measured estradiol, testosterone, and corticosterone metabolites in fecal samples, and used an environmentally relevant range of experimental mercury exposures. We hypothesized that increasing exposure to methylmercury would significantly alter fecal estradiol, testosterone, and corticosterone concentrations in a dose-dependent fashion.

**Methods**

**Study Site**

We used an experimental approach by measuring endocrine responses of captive White Ibises that were exposed to differing levels of methylmercury through their diet. In April 2005, wild-caught White Ibis nestlings were raised in four dose groups in a large free-flight aviary. The aviary was circular (21 m radius, 10 m tall) and divided into quadrants with net walls. The
interior of the aviary contained numerous perches, artificial nest cups and a feeding/loafing pool. We fed all birds in each enclosure on a different diet containing methylmercury: control, 0.05 ppm MeHg, 0.1 ppm MeHg, and 0.3 ppm MeHg (all values in diet, wet weight). The flooring was impermeable PVC sheeting that drained towards a common central drain. White Ibises were collected as 25–32 d old nestlings from the northern Everglades (Water Conservation Area 3, Broward Co. Florida, N 26° 11.179, W -80° 31.431) and from a colony near White Springs, Hamilton Co., Florida (N 30° 19.900, W -82° 45.367). The Alley North birds were collected on 14, 17, 19, and 21 April 2005 and transported overnight to Gainesville, FL (see also Chapter 1). We sexed, weighed, measured, banded and removed approximately 4 scapular feathers for mercury analysis from all nestlings before randomly distributing them to enclosure/exposure groups.

Methylmercury was administered via diet beginning at 90 d of age by dissolving MeHgCL into corn oil and spraying the mixture onto Flamingo diet pelletized feed (Mazuri Company, Brentwood, MO, USA) while the mass was being rotated in a cement mixer in 11.3 kg batches. Each dose group had a complete set of glassware and mixing devices (including cement mixers) dedicated solely to that dose regime. Stock solution concentrations of methylmercury were fine tuned by direct measurements of mercury content of food prior to the onset of feeding to ibises. Dose regime was also verified by determining mercury concentrations of scapular feathers (Frederick et al. 2002) in January of 2006 and 2007 using standard cold vapor techniques (Figure 3-1).

**Fecal Hormone Sampling Technique, Storage and Extraction**

Steroid and corticosteroid hormones are most commonly measured in the blood plasma of animals. However, a number of recent studies (Creel 2001, Palme et al. 2005, Wasser and Hunt 2005, Hinson and Raven 2006) have demonstrated that useful hormone levels can also be
obtained from fecal samples. The latter sampling method is noninvasive, thus avoiding the need to correct for short term stress response. In addition, hormone levels in feces represent an integration of fluctuating hormone levels over the gut passage time, which allows for a more reliable measure of baseline hormone levels (Tyler et al. 1998). Potential difficulties with the method include validating the relationship between blood hormones and fecal metabolites, and developing effective extraction techniques (Goymann 2005).

We collected fecal samples on clean black plastic sheeting placed below perching structures, and identified feces of individual ibises by observing excretion of individually banded birds directly. The location, time, and band number of each excretion was recorded. On collection days, we collected feces for two 1-hr periods separated by 1 hr (typically between 1100-1200 h and 1300-1400 h). During collection bouts we removed samples from the plastic every 10 min. Two observers usually watched during each collection bout, and at each 10-min interval both observers approached the plastic sheets at the same time and collected fecal samples that were visually marked. Unidentified samples were crossed-out to avoid confusion. If unidentified samples were in very close proximity (e.g., within 0.5 m) to the sample of interest, the sample was considered contaminated and was not collected. We estimated throughput time (time from ingestion to excretion) at 2-3 h using food marked with colored plastic beads.

There were 4 collection periods during this study. The first was early June 2005 immediately before dosing began (when birds were ca. 90 d old), the second was late June immediately after the initiation of dosing (ca. 110 d old), the third was late July 2005 (ca. 140 d old), and the fourth was in late December 2005 and early January 2006 (ca. 290 d old).
Individual fecal samples were collected in 2 mL plastic cryotubes and placed in an ice-filled cooler for no more than 3 hours until they could be stored at -20°C temperature; all samples were analyzed for hormone concentrations in May 2006. Fecal samples were individually lyophilized and the dried and stable samples were then weighed and placed into glass extraction vials. Samples that contained more than 0.05 g of sample were homogenized and subsampled while samples that contained 0.05 g or less were used in their entirety.

Using a combination of techniques (Hunt and Wasser 2003; Wasser and Hunt 2005) we used ethanol diluted by deionized water to extract hormone metabolites from the sample. We added the ethanol to a measured amount of fecal hormone in a capped glass culture tube, and used a multi-tube vortexer to shake the mixture for 30 min, cycling the vortexer on for 1 min and off for the next. Culture tubes were then spun in a refrigerated ultra-centrifuge at 3000 rpm for 20 min. The resulting ethanol supernatant was decanted into clean culture tubes and placed in -80°C freezer for storage. We compared the extraction efficiency of 80% (20% deionized water), 90% (10% deionized water), and 100% ethanol solutions by adding a known amount of radio-labeled hormone to standardized desiccated fecal matrix then performing the extraction procedure. We used 80% ethanol because its extraction efficiency was highest when considering all hormone tests (Table 3-1). All results were adjusted for mean extraction efficiency (estradiol 77%, testosterone 64%, corticosterone, 90%).

Radioimmunoassay

We tested each sample extract for estradiol metabolites using Estradiol 125I Coat-a-Count RIA kits (Diagnostic Products, Los Angeles, CA, USA). We used the 3 h, room temperate incubation for all samples. The manufacturer’s protocol was used with 100 µL of extract used initially and dilutions used when needed. This kit has shown high accuracy and dependability in previous avian fecal hormone studies (Wasser and Hunt 2005). The manufacturer-reported
antibody cross-reactivities were 10% for estrone, 4.4% for δ-Equilenin, 1.8% for Estrone-β-δ-
glucuronide and less than 1% for all other tested steroids.

We tested for testosterone metabolites using Testosterone $^{125}$I double-antibody RIA kits
(MP Biomedicals, Solon, OH, USA). The manufacturer’s protocol was used with 50 μL of
extract used initially and dilutions used when needed. This kit has been validated for fecal
metabolites previously in birds (Wasser and Hunt 2005) and other vertebrates (Hunt and Wasser
2003). The manufacturer reported cross-reactivities were 3.4% for 5α-dihydrotestosterone, 2.2%
for 5α-androstane-3β, 17β-diol, 2% for 11-oxotestosterone, and less than 1% for all other tested
steroids.

We tested for corticosterone metabolites using Corticosterone $^{125}$I double-antibody RIA
kits (MP biomedicals, Solon, OH, USA). The manufacturer’s protocol was used with 50 μL of
extract used initially and dilutions used when needed. This and similar kits have been validated
in several avian fecal glucorticoid studies (Wasser and Hunt 2005; Ludders et al. 2001). The
manufacturer-reported cross-reactivities were 34% for desoxycorticosterone, 10% for
testosterone, 5% for cortisol, 3% for aldosterone, 2% for progesterone, 1% for androstenedione,
1% 5α-dihydrotestosterone, and less than 1% for all other tested steroids and glucocorticoids.

All kits were validated by running a set of internal standards into standard hormone extract
and hormone-stripped extract using the manufacturer’s standard curve. In order to determine
whether the extraction matrix would interfere with the accuracy of the assay we tested for
differences between all curves for all kits and found none to be significantly different (ANCOVA
all P’s > 0.22). Thus, we find our assay to be internally valid for each hormone.

**Statistical Analysis**

We looked for differences in hormone concentration due to treatment using a repeated
measures ANCOVA. Sampling was not uniform across sampling periods (i.e. certain individuals
were not represented in some sampling periods) so traditional repeated measures methods could not be used. After averaging individual hormone concentrations for each collection for immunoreactive estradiol (E) and testosterone (T) and for each day for corticosterone (CORT)) we took the natural log of the hormone concentration to normalize them. Because we wished to correlate hormone values from the same individuals at different collection times, we used a compound symmetry structure to the covariance matrix to link individuals (nested within treatment) over time (SAS v. 9.1 PROC MIXED). We also used Kenward-Rogers calculated degrees of freedom—a technique that has been shown to minimize Type I errors in repeated measures studies with gaps in the data due to sampling inequity (Padilla and Algina 2004). Using treatment group, time (either collection period or collection day as above), and sex as main effects, we developed a set of 21 biologically relevant a priori models based on our predictions (see Introduction). We included all possible combinations of the three terms up to the most complicated model that included a time*treatment*sex term. We also included a time*time interaction that allowed for non-linear changes in hormone concentration over time but we did not allow this term to interact with other main effects. We included one term a posteriori: a categorical grouping that compared the control group against all experimental groups. Model selection was based upon AICc (Burnham and Anderson 2002) and models were ranked by AIC weight. The AIC method is invalid when used with data generated via restricted maximum likelihood methods (REML) that are default in PROC MIXED, and we used standard maximum likelihood methods instead.

Finally, in an effort to examine the possible effects due to individual variation in methylmercury exposure within groups (Fig. 3-1) we regrouped each individual as being either high or low methylmercury exposure based upon the median feather mercury quantity for each
exposure group. We then removed the *a priori* treatment factor (control, low, medium and high) from the model and replaced it with the new (control, low-low, high-low, low-medium, high-medium, low-high, and high-high) in the best model as previously selected by AIC. The new parameter estimates are then indicators of difference from each new group and the control. Alpha was set at 0.05.

**Results**

The model that best explained variation in E included treatment, sex, and time as main effects with time*time and treatment*time interactions (Table 3-2). This model was substantially better than all remaining models, with a difference in AICc greater than two for the next closest model. Fecal E metabolites showed an overall downward trend with time (Fig. 3-2), and showed significant upward curvature via the time*time term (Table 3-4). Although the effect of treatment was significant (F test, P<.0022), it was also nonlinear with respect to exposure group. The medium dose group was the only group significantly different than the control group, and high, low and control groups were not different. This pattern was also found for the treatment*time interaction (F test, P<.0136) (Table 3-4). The medium group had less E than all other groups, but relatively greater increase over time. Our *a posteriori* test of non-linear dose-response relationship (control compared to all experimental groups) also ranked poorly relative to our best model (ΔAICc= 4.8). Finally, although female ibises tended to have less E than males, the difference was not significant and there was no overall effect of sex on E (Table 3-4).

Fecal T metabolites tended to decrease over time and showed few differences between treatment groups (Fig. 3-3). Two models received similar support from the model selection process. The top model included time, time*time, exposure group. The second-ranked model (ΔAICc = 1) included time as a main effect and a time*time interaction (Table 3-2). However,
the top-ranked model is an *a posteriori test* of control versus all experimental groups combined; we chose to analyze this model to test our prediction of methylmercury exposure with the caveat that any effect is unpredicted. Looking at the results of the top model in greater depth, T increased over time (F-test, P=0.0015) with a significant (F-test, P<0.001) negative parameter estimate for the time*time interaction allows for concave curvature (and an overall decrease) of T with increasing time (Table 3-4). The *a posteriori* treatment term did not show significant differences between groups. Females tended to have less T than males, though the effect was non-significant (e.g., P=0.6484).

Three models seemed to explain fecal CORT metabolite concentrations well (i.e., ΔAICc< 2) (Table 3-2). The third model, however, was marginal compared to the first two, so we will limit the discussion to the two top models. Further, the two top models had similar structure—the only difference was the inclusion of the sex effect—so our discussion will focus upon the more complicated model to test our hypothesis on the importance of sex. There was no obvious trend in CORT over time (Fig. 3-4). The model shows an insignificant upward trend and a significant negative time*time interaction suggesting concave curvature (Table 3-5). Treatment group had a significant, nonlinear effect on CORT (F-test, P=0.0148). While no individual exposure groups differed significantly from the control, both the low and high groups were significantly different from each other. Females had lower levels of CORT than males, although the effect of sex was not significant (Table 3-5).

In our analysis of within-group variance in mercury exposure showed differences depending on hormone endpoint and exposure group (Table 3-6). Variance in methylmercury effect appears to increase with increasing methylmercury exposure; the low group is relatively consistent in direction and magnitude of effect. The medium and high group parameter estimates
show larger differences between there subgroups than the low groups, particularly in the CORT endpoint.

**Discussion**

Our predictions about the relationship between methylmercury and the hormones studied were not generally supported by our findings. The most consistently violated prediction was that responses would be linearly related to methylmercury exposure. While we found effects of experimentally administered methylmercury on hormones, all were non-linear. Methylmercury exposure significantly altered E, although this result appeared to be driven by large differences between the medium exposure group and all other groups. T showed no significant dose-response relationship with all *a priori* tests of methylmercury exposure, although the *a posteriori* test comparing the control and all other groups showed an apparent decrease in T with mercury exposure. CORT varied significantly with methylmercury exposure, but the lowest mean concentrations were found in the low exposure group and the highest in the high exposure group with control and medium groups between the two. Our prediction that levels of steroid and glucocorticoid hormones would be affected by sex were not supported by any of the tests. Finally, our analysis of variation in intragroup methylmercury exposure did not aid in explaining these nonlinear patterns as they did not suggest that higher intragroup exposure yielded a response of greater magnitude; however, these data do suggest there are differences within these groups and the high intragroup variance may be a product of exposure variance.

It is possible that the levels of mercury exposure were simply not high enough to have an effect, and/or that effects of mercury were overwhelmed by random or confounding effects. For example, the dose-dependent patterns identified in the E, T and CORT data sets may have been confounded with location effect, since each treatment was represented by only one replicate. We attempted to control for cage effect though physical and experimental design; the cages were
physically identical within a circular aviary that carried equal edge effects. We also chose response variables that were measured by individual bird and used statistical techniques (like repeated measures) that take use the individual as the base unit—albeit nested within exposure group. Cage effect might also have derived from a cohort effect, whereby the makeup of individuals in the group affected the endocrine expression within that group.

There appears to be no biologically significant explanation for the medium dose group having more E than other groups; we therefore suggest that at the dose levels we use, methylmercury has little effect on fecal E metabolites in juvenile ibises in the non-breeding season. This conclusion is reinforced somewhat by the fact that there was no effect of sex on E levels. Thus in first-year ibises, it may be that E has simply not been expressed to the degree that it is in breeding adults, at which point there is a marked difference (Heath and Frederick 2005). Thus the capacity for expressing E in juveniles may not have been tested during the juvenile period, and so may not be a very good indicator of potential EDC effects.

With T, the only near-significant effect occurred when all experimental groups were \textit{a posteriori} combined and compared with the control—\textit{all a priori} models that included the effect of (uncombined) methylmercury had little support from AIC. The pattern from this last analysis suggested that the control group may have, on average, higher T levels than all others during the non-breeding season. However, as with E, the lack of sexual differences in testosterone indicates that expression of T is not very active during the juvenile period, and therefore may not be a very useful indicator of endocrine dysfunction.

Thus while steroid hormones may be important to sexual differentiation \textit{in ovo} and to the timing and onset of breeding, there is little information on endocrine expression in developing juvenile birds (though see Schlinger 1997 for information on hormones and song learning).
The significant effect of time in all models shows that T and E are changing, however, the lack of sexual differences imply that these changes are not related to reproduction or differential sexual development. While we have found patterns that suggest an effect of methylmercury exposure on E and T levels in developing ibises, the patterns are not supportive of a linear dose response relationship, and the most likely explanation appears to be that these endpoints may be insensitive to methylmercury exposure.

Effects of methylmercury on fecal corticosterone metabolites are more explicable as non-linear responses have been reported in this endpoint. In the American Kestrel (*Falco sparverius*), Love et al. (2003) found that plasma CORT levels varied similar to an inverted parabola when compared to liver EDC load. This apparently hormetric pattern may be due to the multiple roles that CORT plays in the physiological function of birds. Short-term increases in CORT can be caused by environmental stressors like heat, handling or capture, cohabitating with conspecifics, and food consumption (Siegel 1980). In wild migrating birds high baseline CORT suppresses the acute CORT increase, at least in response to handling stress (Holberton et al. 1996). It is possible that high, chronic levels of methylmercury simulate high levels physiological stress or disease, resulting in an increase in baseline CORT. The lower exposure levels could be causing a decrease in baseline CORT by either mechanistically altering hormonogenesis or by providing low enough levels of physiological stress to downregulate the production of CORT. Further research is needed to evaluate these as explanations for the pattern we have reported.

Potential (non-exclusive) hypotheses explaining these findings are (1) that methylmercury does not alter the steroid hormones at these dose levels, and (2) that methylmercury does not alter steroid hormones in juvenile ibises. We have no direct evidence
for supporting or rejecting either hypothesis, although the earlier work of Heath (2005) suggests strongly that there is an effect of mercury on expression of these hormones in wild breeding adults. Since the dose levels in this study spanned the range of exposures estimated in the wild birds studied by Heath (2003), we believe the mercury effect seen in adults may simply not be manifested in young birds. It is also possible that there is something about the captive situation other than methylmercury exposure (lack of typical stressors, social environment, etc) that has blocked an effect of methylmercury on steroid hormones.

In conclusion, we found immunoreactive fecal estradiol, testosterone, and corticosterone levels were changing over time in captive juvenile White Ibises. While estradiol and testosterone showed an effect of dose, only corticosterone changed in manner that seemed to be explicable on biological principles. We suggest future studies of endocrine effects of methylmercury might profitably focus on endocrine expression during the breeding season, since the results with juveniles were inconclusive.
Table 3-1. The average extraction efficiencies for each hormone by percent ethanol used for extraction.

<table>
<thead>
<tr>
<th>Percent ethanol</th>
<th>Estradiol</th>
<th>Testosterone</th>
<th>Corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>77%</td>
<td>64%</td>
<td>90%</td>
</tr>
<tr>
<td>90</td>
<td>72%</td>
<td>62%</td>
<td>98%</td>
</tr>
<tr>
<td>100</td>
<td>38%</td>
<td>80%</td>
<td>102%</td>
</tr>
</tbody>
</table>

Table 3-2. Ranking of various models for all hormone metabolites by Akaike weight. Only models with a weight greater than 0.01 were included.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Model</th>
<th>AICc</th>
<th>ΔAICc</th>
<th>Akaike weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>time sex trt time<em>time time</em>trt</td>
<td>260.8</td>
<td>0</td>
<td>0.4697</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time sex</em>trt*time</td>
<td>262.9</td>
<td>2.1</td>
<td>0.1643</td>
</tr>
<tr>
<td></td>
<td>time time*time trt</td>
<td>264.2</td>
<td>3.4</td>
<td>0.0858</td>
</tr>
<tr>
<td></td>
<td>time time*trt</td>
<td>264.3</td>
<td>3.5</td>
<td>0.0816</td>
</tr>
<tr>
<td></td>
<td>time sex trt time*trt</td>
<td>265</td>
<td>4.2</td>
<td>0.0575</td>
</tr>
<tr>
<td></td>
<td>time time*sex</td>
<td>265.4</td>
<td>4.6</td>
<td>0.0471</td>
</tr>
<tr>
<td></td>
<td>time time*control</td>
<td>265.6</td>
<td>4.8</td>
<td>0.0426</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>trt time</em>time<em>trt</em>trt</td>
<td>267</td>
<td>6.2</td>
<td>0.0212</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time sex</em>trt</td>
<td>267.2</td>
<td>6.4</td>
<td>0.0191</td>
</tr>
<tr>
<td>Testosterone</td>
<td>time control time*time</td>
<td>158.7</td>
<td>0</td>
<td>0.4663</td>
</tr>
<tr>
<td></td>
<td>time time*time</td>
<td>159.7</td>
<td>1</td>
<td>0.2828</td>
</tr>
<tr>
<td></td>
<td>time sex time*time</td>
<td>161.7</td>
<td>3</td>
<td>0.1040</td>
</tr>
<tr>
<td></td>
<td>time trt time*time</td>
<td>161.9</td>
<td>3.2</td>
<td>0.0941</td>
</tr>
<tr>
<td></td>
<td>time sex trt time*time</td>
<td>163.9</td>
<td>5.2</td>
<td>0.0346</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time sex</em>trt</td>
<td>166</td>
<td>7.3</td>
<td>0.0121</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>time sex trt time*time</td>
<td>380.8</td>
<td>0</td>
<td>0.2350</td>
</tr>
<tr>
<td></td>
<td>time time*time</td>
<td>380.8</td>
<td>0</td>
<td>0.2350</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time time</em>trt</td>
<td>382.6</td>
<td>1.8</td>
<td>0.0955</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time</em>trt*time</td>
<td>382.9</td>
<td>2.1</td>
<td>0.0822</td>
</tr>
<tr>
<td></td>
<td>time time*time</td>
<td>384.1</td>
<td>3.3</td>
<td>0.0451</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time</em>trt<em>trt</em>time</td>
<td>384.8</td>
<td>4</td>
<td>0.0318</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time</em>trt*trt</td>
<td>384.9</td>
<td>4.1</td>
<td>0.0303</td>
</tr>
<tr>
<td></td>
<td>time time<em>trt time</em>trt</td>
<td>384.9</td>
<td>4.1</td>
<td>0.0303</td>
</tr>
<tr>
<td></td>
<td>time time<em>trt time</em>trt<em>trt</em>trt</td>
<td>386.2</td>
<td>5.4</td>
<td>0.0158</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time</em>trt*trt</td>
<td>386.9</td>
<td>6.1</td>
<td>0.0111</td>
</tr>
<tr>
<td></td>
<td>time sex trt time<em>time</em>trt<em>trt</em>trt</td>
<td>387.3</td>
<td>6.5</td>
<td>0.0091</td>
</tr>
</tbody>
</table>
Table 3-3. Estradiol parameter estimates from our best model selected by AIC with their respective standard error of the estimate. P-value is determined using an F-test. Note that treatment and sex effects are categorical and the control and male groups, respectively, act as a reference and all others are estimated relative to that group.

<table>
<thead>
<tr>
<th>Model parameter</th>
<th>Parameter estimate ($\beta$)</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>-0.6394</td>
<td>0.1379</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Time*Time</td>
<td>0.052</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>-0.05325</td>
<td>0.0521</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Control</td>
<td>-0.08078</td>
<td>0.1253</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>-0.4526</td>
<td>0.1318</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>-0.09285</td>
<td>0.129</td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>Control</td>
<td>0.004207</td>
<td>0.0294</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>0.1002</td>
<td>0.0348</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>0.03288</td>
<td>0.031</td>
</tr>
</tbody>
</table>

Table 3-4. Testosterone parameter estimates from our best model selected by AIC with their respective standard error of the estimate. P-value is determined using an F-test. Note that treatment and sex effects are categorical and the control and male groups, respectively, act as a reference and all others are estimated relative to that group.

<table>
<thead>
<tr>
<th>Model parameter</th>
<th>Parameter estimate ($\beta$)</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.1547</td>
<td>0.0481</td>
<td>0.0015</td>
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<tr>
<td>Time*Time</td>
<td>-0.02085</td>
<td>0.005</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>Control</td>
<td>-0.09756</td>
<td>0.0542</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3-5. Corticosterone parameter estimates from our best model selected by AIC with their respective standard error of the estimate. P-value is determined using an F-test. Note that treatment and sex effects are categorical and the control and male groups, respectively, act as a reference and all others are estimated relative to that group.

<table>
<thead>
<tr>
<th>Model parameter</th>
<th>Parameter estimate ($\beta$)</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.002567</td>
<td>0.002</td>
<td>0.195</td>
</tr>
<tr>
<td>Time*Time</td>
<td>-0.000002</td>
<td>9E-06</td>
<td>0.0351</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>-0.1065</td>
<td>0.0732</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Control</td>
<td>-0.17</td>
<td>0.0978</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>-0.0174</td>
<td>0.1006</td>
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<tr>
<td></td>
<td>Medium</td>
<td>0.14</td>
<td>0.0969</td>
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</tbody>
</table>

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Table 3-6. Parameter estimates, standard errors and P-values (t-test) for each treatment group subdivided by median feather mercury quantities (Fig. 1) for each hormone. The treatment grouping was added to our best model selected by AIC. The parameter estimate is for the highest order term that includes treatment; estradiol is treatment*time, testosterone is treatment, and corticosterone is treatment. All parameter estimates are relative to the control group.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Treatment Group</th>
<th>Parameter Estimate ($\beta$)</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low-Low</td>
<td>0.0139</td>
<td>0.0349</td>
<td>0.6917</td>
</tr>
<tr>
<td></td>
<td>High-Low</td>
<td>-0.0160</td>
<td>0.0353</td>
<td>0.6498</td>
</tr>
<tr>
<td></td>
<td>Low-Medium</td>
<td>0.1402</td>
<td>0.0505</td>
<td>0.0060</td>
</tr>
<tr>
<td></td>
<td>High-Medium</td>
<td>0.0771</td>
<td>0.0399</td>
<td>0.0548</td>
</tr>
<tr>
<td></td>
<td>Low-High</td>
<td>0.0010</td>
<td>0.0394</td>
<td>0.9795</td>
</tr>
<tr>
<td></td>
<td>High-High</td>
<td>0.0578</td>
<td>0.0354</td>
<td>0.1046</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low-Low</td>
<td>-0.1524</td>
<td>0.0794</td>
<td>0.0575</td>
</tr>
<tr>
<td></td>
<td>High-Low</td>
<td>-0.1067</td>
<td>0.0760</td>
<td>0.1628</td>
</tr>
<tr>
<td></td>
<td>Low-Medium</td>
<td>-0.0907</td>
<td>0.0815</td>
<td>0.2677</td>
</tr>
<tr>
<td></td>
<td>High-Medium</td>
<td>-0.1531</td>
<td>0.0828</td>
<td>0.0662</td>
</tr>
<tr>
<td></td>
<td>Low-High</td>
<td>-0.1143</td>
<td>0.0805</td>
<td>0.1584</td>
</tr>
<tr>
<td></td>
<td>High-High</td>
<td>-0.0268</td>
<td>0.0751</td>
<td>0.7223</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low-Low</td>
<td>-0.1745</td>
<td>0.1177</td>
<td>0.1416</td>
</tr>
<tr>
<td></td>
<td>High-Low</td>
<td>-0.1880</td>
<td>0.1168</td>
<td>0.1108</td>
</tr>
<tr>
<td></td>
<td>Low-Medium</td>
<td>0.1096</td>
<td>0.1229</td>
<td>0.3745</td>
</tr>
<tr>
<td></td>
<td>High-Medium</td>
<td>-0.1078</td>
<td>0.1249</td>
<td>0.3897</td>
</tr>
<tr>
<td></td>
<td>Low-High</td>
<td>0.2258</td>
<td>0.1201</td>
<td>0.0631</td>
</tr>
<tr>
<td></td>
<td>High-High</td>
<td>0.0456</td>
<td>0.1126</td>
<td>0.6865</td>
</tr>
</tbody>
</table>
Figure 3-1. Feather mercury levels for individual birds in each exposure group. Data are presented in box plots with outliers represented as dots, the gray box showing where 50% of the data lie, the line in box is mean.
Figure 3-2. Mean estradiol concentration by treatment group for each collection period. Error bars represent the standard error of the calculated mean and do not consider intracollection dependency issues as our statistical models do.
Figure 3-3. Mean testosterone concentration by treatment group for each collection period. Error bars represent the standard error of the calculated mean for each collection period and do not consider intracollection dependency issues as our statistical models do.
Figure 3-4. Mean corticosterone concentration by treatment group for each collection period. Error bars represent the standard error of the calculated mean for each collection period and do not consider intracollection dependency issues as our statistical models do.
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BIOGRAPHICAL SKETCH

Evan Adams was born and raised in Richland, WA, where he decided to delve into science at an early age with his investigation on how to protect strawberry plants from herbivory. Soon after, he began working for the local National Laboratory in particle physics. He earned an honors Bachelor of Arts in biology from Whitman College in Walla Walla, WA. There he discovered his true love for tropical avian ecology. In August 2007, he received his master’s degree in wildlife ecology and conservation from the University of Florida, focusing on avian ecotoxicology. He plans to start his doctorate at the University of Maine, studying the ecology of neotropical migratory birds.