Environmental Science & Technology

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Article

Early Breeding Failure in Birds Due to Environmental Toxins: A Potentially Powerful but Hidden Effect of Contamination

Jabi Zabala, Joel C. Trexler, Nilmini Jayasena, and Peter Frederick*

Cite This: *Environ. Sci. Technol.* 2020, 54, 13786−13796

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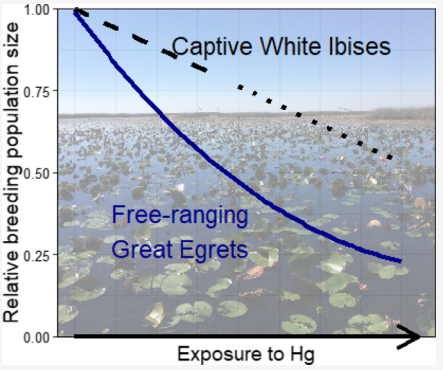
ABSTRACT: Toxin emissions and legacies are major global issues affecting many species through, among other effects, endocrine disruption and reproductive impairment. Assessment of toxin risk to wildlife focuses mostly on offspring-related metrics, while the lack of breeding initiation or early breeding failure has received less attention. We tested whether exposure to methyl mercury (MeHg) results in early breeding failure and reduced number of breeding birds using observational and experimental data. We used 21 years of numbers of breeding pairs of colonially breeding wild Great Egrets (*Ardea alba*) in response to annual and geographical variation upon exposure to environmental MeHg. After controlling for food availability, we found a strong negative association between MeHg exposure and the number of breeding Great Egrets. We report reductions of >50% in breeding numbers under exposure levels otherwise associated with <20% reduction in post-egg-laying breeding success. Experimental exposure of White Ibises (*Eudocimus albus*) to MeHg also caused early breeding failure and a ∼20% reduction in breeding numbers at environmentally relevant exposures. The demographic consequences of reductions in breeding pairs are additive to known and typically studied impairments in postlaying reproductive success. Net demographic effects of exposure to endocrine disruptors may often be strongly underestimated if early breeding failure is not measured.



INTRODUCTION

Environmental toxins are now widely distributed in the world through human activity.[1−3] The effects of sublethal exposure to persistent organic pollutants, pesticides, and heavy metals include, among others, endocrine disruption and reproductive impairment.[4−8] Experimental and field studies aimed at detecting and quantifying the possible effects of exposure to toxins in wildlife often analyze breeding impairment through variation in breeding output using clutch or brood size, offspring production, offspring survival, or other endpoints related to breeding productivity.[3,9−12] However, toxins, particularly endocrine disruptors, can influence breeding propensity or induce breeding failure in early stages of the breeding cycle before traditional endpoints are detectable. Therefore, net reproductive impairment associated with toxins, particularly, with endocrine disruptors, may be underestimated. Mercury (Hg) is a toxic metal whose concentrations increased worldwide as a consequence of human activities and which bioaccumulates and biomagnifies, particularly within aquatic food webs.[3,12] Depressed reproductive success is the most widely investigated and reported consequence of Hg exposure,[13] though studies have focused primarily on the success of breeding once initiated, and little attention has been devoted to effects on propensity to breed or early failure.[12−14] In most animals, the success of embryos once formed and their survival to later stages until the independence of offspring are only partial components of reproduction, and it is possible that reproductive impairment associated with toxins may also include the inability to initiate breeding or failure in early stages prior to egg laying or embryo formation. Contaminants, in general, and heavy metals, in particular, can disrupt the endocrine system affecting the courtship behavior, breeding propensity, and parental behavior of vertebrates.[3,7,8,15,16] Nonbreeding and failure in early breeding phases may be difficult to detect because breeding is typically documented from a specific telltale point (nest initiation, egg laying, denning, parturition, etc.). However, without knowledge of effects on breeding propensity, the effects of contaminants may be systematically underestimated.

Experimental evidence suggests that birds exposed to dietary concentrations of the methylated form of mercury (MeHg) in the upper end of values likely from their prey (1.2 ppm wwt of MeHg in diet) were less likely to initiate nests or lay eggs.[14] White Ibis (*Eudocimus albus*) exposed to MeHg concentrations in the middle of the range experienced in the wild (0.05, 0.1, and 0.3 ppm wwt MeHg in diet) resulted in an increase in the

Received: June 25, 2020
Revised: September 11, 2020
Accepted: October 1, 2020
Published: October 26, 2020

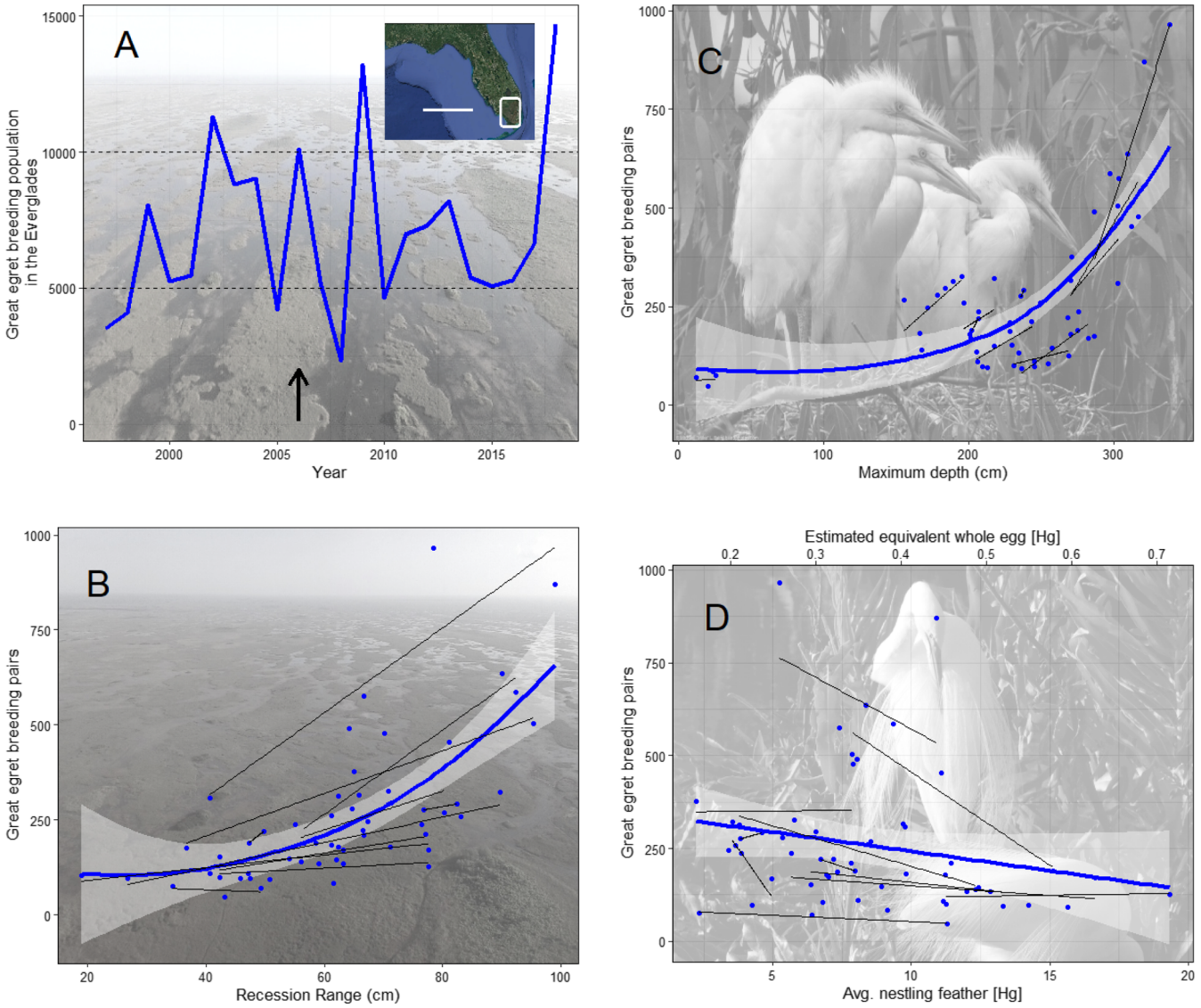


Figure 1. (A) Temporal variation in numbers of breeding pairs of Great Egrets in the Greater Everglades area (blue line, Everglades National Park and Water Conservation Areas). The black arrow indicates the separation point of the data used to develop the model (2006−2018) and those used to validate it (1994−2005). The inset shows the location of the study site in the Florida peninsula (USA), and the white line indicates a scale of 300 km. (B) Association between numbers of breeding pairs and surface water recession range. The thick blue line shows smoothed model-predicted values (blue dots) with standard error shaded. Thin black lines are linear regression fits over predicted values for individual colonies included in the study. (C) Association between numbers of breeding pairs and the averaged maximum water depth around the colony at the start of the breeding season. Thick blue line shows smoothed model-predicted values (blue dots) with standard error shaded. Thin black lines are linear regression fits over predicted values for individual colonies. (D) Association between numbers of breeding pairs of Great Egrets and average [Hg] in feathers from nestlings raised in the colony. Thick blue line shows smoothed model-predicted values (blue dots) with standard error shaded for numbers of Great Egrets breeding pairs. Thin black lines are linear regressions fitted over predicted values for individual colonies (blue points) included in the study. Background images in (A,B) show tree islands in the Everglades at two different water levels (note that it is not the same area); in (C) nestlings of Great Egrets, our study species, in the age range of those we sampled for feathers to estimate exposure to Hg in each colony during the breeding season; and in (D) breeding adults of Great Egret.

frequency of homosexual, nonproductive pairs,[17] which was linked to endocrine disruption.[7] In field settings, studies of long-lived seabirds show that high contaminant levels (including Hg) in one year may negatively influence the breeding probability in the same or the following year.[8,18−20] However, the available information on the influence of toxicant exposure on the number of breeding pairs remains limited, particularly, in natural settings, and it is unclear what the net effect size or shape of the response curves might be. This is not surprising as (1) in field studies, it is difficult to account for interactive stressors like food availability, predation, or disease, (2) field studies often do not have estimates of total breeding population size, and (3) multiannual monitoring data may be required to ensure a representative range of contaminant exposures and interactive stressors.

Here, we use observational and experimental approaches to understand the influence of exposure to MeHg on the numbers of breeding pairs of two wading bird species. We hypothesized that increased exposure to environmental MeHg would cause increased incidence of early breeding failure in birds and, therefore, reduce the effective breeding population size, approximated as the number of breeding pairs. Early breeding

https://dx.doi.org/10.1021/acs.est.0c04098
Environ. Sci. Technol. 2020, 54, 13786−13796

failure encompasses both failure at an early stage (courtship, nest building, or egg laying) and failure to initiate breeding behavior. We defined breeding pairs as those that lay at least one egg and, therefore, would typically be considered in studies of reproductive impairment. To test this hypothesis, we first analyzed 25 years of systematic counts of Great Egret (*Ardea alba*) breeding pairs in colonies of the Florida Everglades (Figure 1A) and investigated their association with temporal and geographic variation in food availability and exposure to MeHg. We predicted that increased exposure to MeHg would be associated with reduced numbers of breeding pairs, once the variation in food availability and the possible effects of extreme weather conditions were controlled. Second, we investigated the causation using an independent dataset from experimentally dosed captive White Ibises.[7,17] We predicted that (1) breeding attempts in dosed White Ibises would more likely fail in early stages (before laying eggs), in consequence, (2) the breeding population size of dosed groups would be proportionally smaller than that in the control group; and (3) reduction in the number of breeding pairs would be associated with the experimental dose of MeHg.

METHODS

Observational Study: Study Area, Species Monitoring, and Hg Sample Collection. We annually monitored the number of breeding pairs of Great Egrets, egret food availability, and exposure to Hg in breeding colonies in Miami-Dade, Broward, and Monroe County, Florida, USA. Great Egrets are piscivorous and the number of breeding pairs is known to vary annually with local food availability.[21] Great Egrets breed in colonies on tree islands widely spaced (2−15 km) within the extensive graminoid Everglades wetlands (9200 km^2). In this ecosystem, individuals in different colonies are exposed to geographically and temporally variable Hg through food,[22,23] driven by variation in the rainfall, hydroperiod (time inundated), and water recession rate.[21,24−26]

We used monthly (January to July), systematic, 100% coverage, aerial, and ground surveys to detect and quantify breeding pairs in breeding colonies each year between 1994 and 2019.[27−29] We counted nests in pictures taken of these colonies from the aircraft to produce accurate counts of nests.[30] Evidence of breeding pairs included incubating adults, adults sitting next to nestlings, or nestlings in nests. We did not count roosting adults, empty nests, or courting pairs as breeding. As Great Egret breeding in colonies tends to be relatively synchronous, we used the highest monthly count of nesting pairs during each nesting season as the response variable and as the best proxy of breeding population size in that colony and year.

Each year, we selected 5−6 colonies to monitor Hg exposure. Colony selection was based on the location to ensure a geographically representative sample of breeding and Hg conditions within the Everglades.[22] Although we aimed to sample the same colonies annually, this was not always possible as Great Egrets did not use all colonies each year, and occasionally some colonies were not accessible to sample feathers due to water conditions. Thus, this study included 17 different colonies. We aimed to sample at least 10 randomly selected nests in each colony[31] in which nestlings were 20−28 days of age, estimated from the culmen length or hatching dates. In each nest, we collected up to 10 scapular feathers from the second largest chick by pulling fully grown feathers (feather, feather shaft, and pulp) and stored feathers in sealed paper envelopes in a dry place until analysis. To estimate Hg exposure in each colony and year, we used [Hg] in nestling feathers from the same colony and year (referred to as feather [Hg], hereafter). Individual nestling feather [Hg] showed a normal distribution around the colony-averaged value (Figure S1), and because arithmetic and geometric means were closely related (linear relationship of arithmetic average to geometric average, $\beta = 1.015 \pm 0.007$, $P < 0.001$, $R^2 = 0.99$, $N = 130$), we used the arithmetic mean of observed [Hg] values. Although we have found that the accuracy of colony-averaged [Hg] is compromised if estimates are based on five or less nests,[31] we also included colonies that had four or more samples to allow the inclusion of colonies with low-nestling success and environmentally or toxicologically adverse conditions. We assessed the possible effects of this cutoff point on results by rerunning analyses excluding colonies whose [Hg] was estimated using less than six samples.

Observational Study: Food Availability. The diet of breeding Great Egrets in the Everglades is composed almost entirely of fish (>95% by biomass),[32] which are obtained by hunting in shallow water (5−25 cm). Fish become available to wading birds because of a combination of fish biomass and the concentrating effect of shrinking pools of water through drying during the dry season.[24,33] To describe food availability for Great Egrets nesting in each colony and year, we used three parameters: fish biomass (g/m^2; a measure of food abundance), the average maximum depth around colonies at the start of the breeding season (a measure of the area flooded and available for fish production), and water recession range (a measure of food vulnerability).

We approximated food abundance using the average fish biomass at different sampling points within a 20 km range around the colony, a distance that encompassed >95% of the foraging flights of breeding Great Egrets in the Everglades.[34] Starting in 2005, we conducted an annual systemwide description of small fish communities focusing on species typically consumed by wading birds. Our fish biomass sampling design included primary sampling units (PSUs) distributed across the Greater Everglades area to provide spatial information on wading bird prey availability and its variability. Within each PSU, we estimated the fish biomass using standardized replicated 1-m^2 throw traps (see further details in the Supporting Information). We used wet-season fish biomass estimates that were a predictor of fish biomass during the egret breeding season.[24] While hydroperiod affects the fish biomass,[35] the total area covered with water can expand and contract by thousands of Ha per year, thereby influencing the total fish breeding habitat. To account for the depth and the wetted area, we included the maximum water depth around colonies at the start of the breeding season (highest water level during the first three months of the year) using established gages (see below).

To model the hydropattern, we downloaded daily average water-depth values from January 1994 to December 2018 from 128 gaging stations within the study area (https://sofia.usgs.gov/eden/). To approximate the maximum water depth around colonies at the start of the breeding season, we used the maximum water depth at each gage in the first 3 months of the year. Great Egret prey become more vulnerable in shallow water.[33] As water dries out during the breeding season, fish get concentrated in the remaining shallow ponds where wading birds prey on them.[24] To approximate food vulnerability, we documented the water recession range (highest water level

https://dx.doi.org/10.1021/acs.est.0c04098
Environ. Sci. Technol. 2020, 54, 13786−13796

during the first 3 months of the year minus the lowest water level achieved before the seasonal reversal of the recession [cm]). We estimated the maximum water depth at the start of the breeding season and the recession range in each year and at each gaging station, and averaged these over all stations within 20 km of the focal colony in each year.

Heavy rain can result in abandonment of some nests.[36] While some of the effect of such storms on nesting occurs through food availability (as above), there may also be direct effects of heavy rainfall on nest structures and nest contents. To control these possible influences, we included the number of days with heavy rain (>0.95 quantile of total daily rain in all the stations between January and May 2002−2018) in the nearest gaging station to each colony in March and April. We focused on March and April because the highest numbers of breeding Great Egret pairs typically occur in these months. To control for the possible influence of extreme temperatures, we added the average temperature in March to the models. We obtained temperature records from the nearest NOAA station to the study area. (Big Cypress: https://www.ncdc.noaa.gov/IPS/cd/cd.html?_page=0&jsessionid=4275AA08591C28326ADFFD00F380B040&state=FL&_target1=Next+%3E.)

We obtained fish biomass from >1300 PSU-years (118.18 ± 33.09 PSUs per year [range: 21−138, N = 11]) and hydrological information using >500,000 measures of daily average water depth from 128 gaging stations in the 11 years. To characterize the annual food availability in each colony, we used fish biomass data from an average of 24.24 ± 7.2 (7−39, N = 57) PSU sampling stations and water depth data from an average of 23.96 ± 6.3 (13−35, N = 57) gaging stations per colony and year. Hg was not correlated with any of the other predictors (Pearson correlation coefficient <0.3 in every case, Figure S2). The strongest correlation was between the fish biomass and the maximum depth at the start of the breeding seasons (Pearson correlation coefficient = 0.64). To avoid issues arising from collinearity among these two covariates, we split models into two blocks: one included fish biomass as a covariate and the other the maximum depth at the start of the breeding season.

Observational Study: Model Specifications. We assessed the association between feather [Hg] and annual breeding pairs using generalized linear mixed models (GLMMs) with a log link function and a negative binomial error distribution. We found evidence of overdispersion in nest counts (variance to mean ratio = 278.2) and, therefore, favored negative binomial models over Poisson error distributions, as the former includes two separate parameters to estimate the mean and the variation. We only used data from 2006 to 2018, the years in which we had data on fish biomass. We later used data from 1998 to 2006 to evaluate the model developed using 2006−2018 data.

We modeled breeding pairs as a function of feather [Hg], average fish biomass, averaged range of water recession around the colony, average maximum water depth around the colony at the start of the breeding season, number of heavy rain events in the colony during March−April, and average temperature in March. As the fish biomass and the maximum depth at the start of the breeding season were strongly correlated, we ran two sets of models, one including fish biomass and the other covariates (but not the maximum depth) and the other assessing the maximum depth at the start of the breeding seasons instead of fish biomass. To account for the differences in colony island characteristics, as well as the possible among-colony differences in predation risk and habitat quality, we added colony as a random factor in every model. To control for temporal pseudocorrelation of breeding population size (i.e., breeding population size in year$_y$ being correlated with the breeding population in year $_{y-1}$ or showing structured temporal trends), we included year as a predictor covariate in the models, and we inspected model residuals for temporal autocorrelation at different time lags. We also ran a generalized least squares (GLS) version of our best model and compared it in terms of the corrected Akaike information criterion (AICc) with the same GLS plus an autocorrelation structure.[37]

Because breeders could redistribute from the breeding grounds outside the study area, accounting for part of the inter-annual variation in the total numbers of breeders in the area (Figure 1A), we ran a parallel group of models that included year as a random factor crossed with colony. Therefore, we ran four blocks of models: two models including fish biomass and year as a covariate or random factor and another two with maximum depth at the start of the breeding season instead of fish biomass and year as a covariate or random. In every model, we scaled predictor covariates by centering them and then dividing by their standard deviation. In each set of models, we ran six competing models, including (1) a null model, as reference, that had no predictor covariates but included the random structure of other models in the set, (2) a base model with additive effects of covariates, (3) another model adding the quadratic term of feather [Hg] to assess nonlinear responses, and (4−6) three linear response models assessing the possible interactions between feather [Hg] and recession range, recession range and fish biomass (or maximum depth), and feather [Hg] and fish biomass (or maximum depth). We used AICc[38] to select among competing models. Next, we inspected the best performing model and reran it dropping nonsignificant covariates in a stepwise manner until we achieved no further improvement in terms of AICc. From the best model, we calculated the semipartial coefficient of determination (R_{part}^2), the proportion of observed variation explained exclusively by covariates and their interactions. We used commonality analysis[39−41] to deconstruct the R^2 of a set of predictors into unique and common, or shared, effects.

To compare the results of observational and experimental approaches, we used the best model in each case to predict the effects of egret or ibis exposure to Hg at a gradual increase of 1 mg/kg dw nestling feather [Hg], while environmental covariates retained values observed in the field.

Observational Study: Model Validation. We used two approaches to validate the results of the best model obtained above. First, we used data collected before 2006 which had not been not included in previous analyses to assess the predictive performance of the best model. We used 35 colony-year observations from the 1994−2005 period for which we had: counts of nesting pairs; estimates of feather [Hg] based on feathers from four or more nests; information on maximum water depth and recession range; and the specific colony was included at least once in the development of the best model. We did not use fish biomass because that variable was not included in the best model (see results). We compared the deviation of predicted population sizes from the observed ones in 1994−2005 using: (1) a null model without fixed predictors but the same random structure as our best model (i.e., a model only with random effects) and (2) our best model. We compared the proportional difference between the observed

https://dx.doi.org/10.1021/acs.est.0c04098
Environ. Sci. Technol. 2020, 54, 13786−13796

Table 1. Output of the Best Model of the Effect of Feather Hg and Food Availability on Breeding Pairs of Great Egrets in the Everglades[a]

	variable						
	fixed	random	Var	est	SE	*z* val.	*p*
N = 57		colony	0.12		0.35		
	intercept			5.30	0.13	40.35	<0.001
R_{marg}^2 = 0.70	rec. range			0.31	0.09	3.47	<0.001
R_{cond}^2 = 0.99	max. depth			0.32	0.12	2.74	0.006
	feather [Hg]			−0.26	0.09	−2.88	0.004

[a]The variation (var), beta estimate (est), standard error (SE), Z statistic (z. Val), and associated p value of random and fixed factors retained in the best model are reported. Rec. range stands for recession range, the difference in water depth between the highest water level at the start of the year and the lowest water level recorded during the breeding season. Max depth stands for the maximum water depth around the colony at the start of the breeding season and feather [Hg] indicates the average [Hg] in feathers of nestlings from the colony. We also indicate the marginal (R_{marg}^2) and conditional coefficients of determination (R_{cond}^2).

values and those predicted by each model as an indicator of model performance.

Second, historic data show a steep increase in breeding pairs in the Everglades during the late 1990's, and one possible mechanism is through a gross reduction in Hg availability during that period. Systematic counts of nesting numbers of Great Egret breeding pairs before 1998 (mean 2928 ± 1217 pairs per year; N = 11; 1986−1997) and after (mean 7189 ± 3116 pairs per year; N = 21; 1998−2018) showed an average 2.46-fold increase. We used our best model with values for recession ranges and colony random effect estimates from 2006 to 2018 but substituted observed feather [Hg] values with the average of the three highest observed values of feather [Hg] during 1994−1997 to predict the number of breeding pairs under previously high Hg exposure. We then compared those values with observed counts of breeding pairs during 1990−1998.

Experimental Confirmation. To examine the effect of Hg exposure on nesting responses experimentally, we reanalyzed data on the number of nests of captive White Ibises exposed to different concentrations of MeHg through food.[7,17] Briefly, White Ibis nestlings captured in 2005 in breeding colonies in the Everglades were randomly distributed into groups of 20 of each sex in four Hg exposure treatment groups. Birds were housed outdoors in a circular aviary divided into four quadrants by nets. Each year from 2006 to 2008, groups were randomly moved among quadrants to minimize the location effects. Each quadrant had 6 perch modules, 48 nest platforms, and *ad libitum* nesting material. Exposure to MeHg started when nestlings were 90 days of age and continued until the end of the experiment. We offered birds *ad libitum* pelletized food infused with Hg doses using a corn-oil vehicle. The groups were control (corn-oil vehicle alone), low-, medium-, and high-exposure diets (0.05, 0.1, and 0.3 ppm wwt MeHg in food, respectively). In 2008, we collected adult blood (N = 41; 11 control, with 6 females and 5 males; and 10 each for the other three groups, with 5 females and 5 males each) and nestling feathers (N = 57; 18 control, 13 low-dose, 15 medium-dose, and 11 high-dose groups) to compare [Hg] in a way directly comparable to that in the field study (see below). We stored feathers of each individual in labeled paper envelopes and conserved them in a cool and dry place until analysis.

White Ibis nesting activity was monitored daily during each of the three breeding seasons. We defined as nesting attempts every occasion in which two individuals established a bond through courtship and started building a nest or occupied an existing one, even if they were homosexual pairs.[17] We defined breeding pairs as those nesting attempts that produced at least one egg, even if the egg did not survive. To evaluate Hg effects on the probability of pairs producing at least one egg, we ran a model assessing the differences in nesting attempts resulting in breeding pairs between control and all dosed individuals, regardless of the dose. We also fit a model of linear relationships between the probability of a breeding attempt resulting in a breeding pair and the average nestling feather [Hg] for their Hg dose group. In both models, we accounted for the age of the breeder and the order of the breeding attempt within the season. In these two analyses, we used a generalized linear model (GLM) with a logit link function and a binomial error distribution.

To allow comparison of the effect of Hg on breeding pairs with the observational study, we expressed the maximum number of synchronous breeding pairs in treatment groups as a proportion of maximum number of synchronous breeding pairs in the control group. To analyze the dose−proportional reduction in nesting pairs, we used a GLM with the identity link and a Gaussian distribution of error in which the proportion of breeding pairs with reference to the control group was the response variable and the average [Hg] in nestling feathers the sole predictor. We used model results to predict the effects of exposure to Hg at doses in the observational study that were higher than those actually used in the experimental approach.

Hg Determination. We determined [Hg] using two different analytical procedures. From 1994 to 2013, Great Egret feather samples and White Ibis blood samples were analyzed by the Chemistry Section of the Florida Department of Environmental Protection (see the Supporting Information). From 2014 onward and for the White Ibis feathers collected in 2008, we used a Direct Mercury Analyzer (Milestone DMA 80). In each analysis run of the DMA 80, we included at least two blanks, four samples of standard reference materials (DORM-2, DORM-4, DOLT-5, and TORT-21 [National Research Council, Canada]), and 15−25% of sample duplicates. All the duplicate concentrations were within 10% of the original sample. Recovery rates for reference materials in the DMA 80 ranged between 88.3 and 102.4%. Further details on methods and quality control are provided in the Supporting Information. To facilitate the interpretation and comparison with other studies, we approximated the Great Egret nestling feather [Hg] to total egg [Hg], a commonly used measure of Hg exposure with well-studied dose−response relationships[12] (details are provided in

https://dx.doi.org/10.1021/acs.est.0c04098
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the Supporting Information), and added it to relevant plots. Based on previous research, we assumed that virtually all Hg in feathers was MeHg,[42,43] yet we report results as total Hg (THg) on a dry weight (dw) basis and refer to all concentrations as [Hg], which we assume to represent [MeHg] in feathers.

Data Analysis Software. We used R 3.5.1[44] to perform analyses. For GLMMs, we used the packages "lme4 1.1−19"[45] and "PiecewiseSEM 1.2.1"[46] to calculate R^2 of GLMMs, the package "r2glmm" to calculate semipartial R^2,[47] and AICcmodavg 2.1−4[48] for AIC*c* values. We produced plots using "ggplot 2 3.1.0".[49] We report descriptive statistics as mean ±1 standard deviation with the range in parenthesis, unless otherwise stated.

RESULTS

Observational Study. Annual numbers of Great Egret breeding pairs were positively associated with the recession range and maximum water depth at the start of the breeding season (Table 1, Figure 1B,C), while feather [Hg] was negatively associated with breeding pairs (Table 1, Figure 1D), in accordance with our predictions. These three covariates explained 70% of the observed variation in breeding numbers (R_{Marg}^2 = 0.70), and when considering the conditional R^2 with colony as a random factor, our best model explained virtually all the observed variation in the breeding population size (R_{cond}^2 = 0.99; Table 1). Commonality analysis showed that the proportion of observed variation explained in exclusivity by each covariate was similar: 0.18 for the recession range (95% CI = 0.03−0.39), 0.18 (95% CI = 0.03−0.39) for the maximum depth, and 0.15 (95% CI = 0.02−0.36) for feather [Hg]. Model selection using fish biomass instead of maximum depth resulted in a model retaining only the recession range and feather [Hg] (Table S1) with very similar β estimates to these in the model with maximum depth instead of fish biomass (Table S2). This model without maximum water depth had a smaller marginal R^2 (0.41; Table S2) and its AICc was 2.55 units higher. Therefore, and bearing in mind that these two models can be considered nested, we disregarded the second model.[38,50] In both sets of models, the estimated intercept variance for year as a random factor did not differ from zero (estimated Var. $<21 \times 10^{-10}$) and estimates of coefficients for the fixed factors were virtually identical to those of the model with only colony as the random factor (Table S2). We did not find support for the influence of redistribution of breeders from or to other areas in the interannual variation in breeding pairs, as adding year as a crossed random factor resulted in a worse model in terms of AICc compared to the same model with only colony as a random factor (ΔAICc > 2.2 in both cases). Therefore, the results we report refer to the best model of the set with the maximum depth and year as covariates.

We found no evidence of interactions among covariates (Table S1) or effects of heavy rainfall (β = −0.10 ± 0.10; P = 0.323) or temperature (β = −0.04 ± 0.08; P = 0.623). We found no strong temporal pseudocorrelation or a clear temporal structure in residuals of the best model (Figure S3), suggesting that the breeding population size was not influenced by the breeding population size in previous years and the lack of temporal patterns in the population size. The GLS model with an autocorrelation structure performed worse (ΔAICc = 2.5) and its autocorrelation estimate was 0 (-6^{-18}). The residuals of the best model showed a reasonable fit too (Figure S4). The inclusion of colonies characterized for [Hg] using feather samples from 4 to 5 nests (N = 10) did not seem to affect the results—discarding these colonies resulted in the same best-model structure, similar estimates of coefficients of determination, and similar slope estimates (Table S3). When the recession range and maximum depth were fixed to their median value observed in data (63.2 and 234.9 cm), respectively, our results predicted a steep decrease in breeding pairs with increasing Hg exposure at the low end of the concentration range, while the slope of the decline decreased at high Hg levels, probably because the relative population size was already very small (Figure 2).

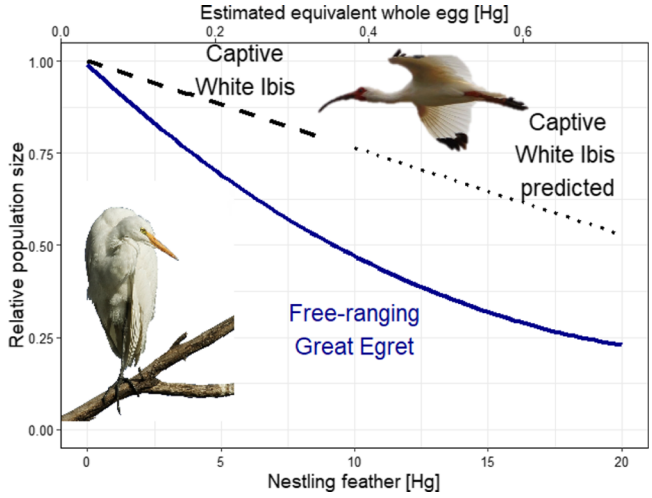

Figure 2. Relative change in the breeding population size with increasing [Hg] in nestling feathers predicted from models derived from observational (Great Egret: blue line) and experimental data (White Ibis, black broken line). For Great Egret predictions, we kept the recession range and maximum depth values at the median observed and changed only the Hg exposure. The dotted line shows the estimated reduction of ibis breeding population in Hg exposures beyond the range of exposure values experienced in the experiment. We estimated the whole egg [Hg] equivalent to nestling feather [Hg] data using Great Egret regression values to enhance the comparability of results using formulas described in the text.

Model Validation. Our best model performed well when fit to data from 1994 to 2005. Our model reduced the distance between the predicted and observed values by 36.5% (from a deviation of 77.2 ± 110.6% in the null model to 49.0 ± 49.5%). The mean bias for model-predicted breeding populations (considering whether models over- or under-predicted the number of breeding pairs) was 17.8% (±67.8) compared to 53.3% (±124.3) in the null model. This suggests that our model not only reduced the departure of predicted values from field observations but also centered the error, correcting biases in the null model.

Finally, using the average of the three highest observed values of feather [Hg] during 1994−1997 (27.1 THg μg/g dw) as an approximation to high Hg exposure values typical of the early 1990s, our best model predicted a reduction in breeding pairs of 62.7% (±22.5) compared to the observed numbers in the field after 1998. That reduction encompasses and resembles the observed average 59.3% fewer breeding pairs in the early 1990s compared to those in post-1998.

Experimental Exposure to Hg: Breeding Responses. Captive White Ibises often made more than one breeding

https://dx.doi.org/10.1021/acs.est.0c04098
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attempt within the same breeding season. White Ibises in the control group made 107 breeding attempts, 112 in the low-dose group, 125 in the medium-dose group, and 105 in the high-dose group. Of 898 individual breeding attempts (two individuals in each breeding attempt) recorded, 46.1% (N = 414) were first breeding attempts, 32.4% (N = 291) were second breeding attempts, 15.6% (N = 140) were third, 5.2% (N = 47) were fourth, and 0.7% (N = 6) were fifth attempts. No significant difference existed in the number of breeding attempts per individual among treatment groups (χ^2 = 9.82; df = 12; P = 0.632; N = 898). The average clutch size was 2.5 ± 1.6 eggs (range: 1−10; note possible egg dumping in large clutches). Pairs in treated groups laid significantly smaller clutches compared to those in the control group (β = −0.393 ± 0.16; t val = −2.40; P = 0.017, N = 449), but this decrease in size was not linearly associated with the increase in exposure to Hg (β = −0.019 ± 0.02; t val = −0.87; P = 0.387, N = 449). The breeding season, measured as the net span of egg-laying days, lasted 70 days in 2006, 130 days in 2007, and 198 days in 2008.

Adult blood and nestling feather [Hg] of experimentally dosed captive White Ibises were strongly correlated. Bootstrap analyses showed a strong correlation between blood [Hg] from breeding adults and nestling feather [Hg] among dose groups. Nestling feather [Hg] was −0.119 (±0.023) × 0.439 (±0.007) adult blood [Hg] ($P < 10^{-17}$ in every case; R^2 = 0.917 [±0.019; 95% CI = 0.886−0.950]).

Effect of Experimental Hg Exposure on the Probability of Reaching the Egg-Laying Stage. We found experimental evidence of a negative effect of Hg on captive White Ibis egg-laying, confirming our prediction. Breeding attempts (i.e. pairs building a nest or occupying an already existing one) of exposed individuals in all dosed groups combined were 14% less likely to result in eggs laid than those in the control group. Breeding attempts of dosed individuals regardless of dose were significatively less likely to result in laying at least one egg (β = −1.937 ± 0.55; z val = −3.55; P < 0.001, N = 449; R_{part}^2 = 0.06 [range 0.025−0.108]; further details on the results of this model are given in the Supporting Information).

The model assessing a linear relationship between the Hg dose level and the probability of laying at least one egg also revealed a significant negative relationship (β = −0.10 ± 0.04; z val = −2.23; P = 0.025, N = 449; R_{part}^2 = 0.011 [range 0.000−0.038]). The linear model predicted average probabilities of a breeding attempt progressing to egg-laying as 0.88 (±0.09) for control pairs, 0.86 (±0.09) for the low-dose group, 0.85 (±0.11) for the medium-dose group, and 0.80 (±0.14) for individuals in the high-dose group (further details of the results of this model are given in the Supporting Information).

Experimental Exposure to Hg and Relative Breeding Population Size. The maximum number of breeding White Ibis pairs in any season was significantly smaller in dosed groups compared to the control group (up to 22% reduction), as predicted from our hypothesis. The maximum number of breeding pairs in low-, medium-, and high-dose groups compared to the control was 90% (±12%; range: 76−100%), 87% (±9%; range: 78−95%), and 78% (±2%; range: 77−80%), respectively. We also found a significant negative association between average [Hg] in White Ibis nestling feathers from each treatment group and the maximum number of breeding pairs observable during the breeding season relative to control group (β = −0.023 ± 0.007; z val = −3.50; P = 0.006, N = 12; R_{part}^2 = 0.551 [range 0.179−0.840], Figure 2). These results confirmed our prediction that increased exposure to MeHg would increase the proportional reduction in the number of breeding pairs. The slopes of reduction in the relative population size in ibis experimental conditions and in the egret observational study were similar, yet they were slightly steeper in the egret observational data (Figure 2). The relative breeding population size of ibises predicted at the high end of the range of Hg in the field study resulted in 47% reduction (Figure 2). Reductions predicted from experimental data were considerably smaller (up to 30% less) than those from the model based on observational data.

DISCUSSION

We found a significant negative association between numbers of breeding pairs and the average [Hg] in nestling feathers in Great Egret colonies when effects of food availability were accounted for. On average, an increase of 1 μg/g dw THg in nestling feathers was associated with 4% incremental reduction in breeding population size. This effect of Hg exposure on breeding numbers was also experimentally confirmed in captive dosed White Ibises, although the reduction in breeding population in the latter was smaller (2.4% reduction for each increase of 1 μg/g dw THg in nestling feathers). The number of breeding pairs in wild egret colonies more than halved in association with feather [Hg] within the upper end of the observed range of feather [Hg] values in this study (19.3 THg μg/g dw; Figures 1D and 2). The magnitude of reduction in breeding pairs is particularly worrisome as all the estimated equivalents of whole egg [Hg] we observed were considered below MeHg toxicity reference values associated with a 20% reduction in post-egg-laying reproductive success.[12] Furthermore, the negative association we report is additive to other Hg sublethal effects because within the range of [Hg] values observed, we also found negative associations of Hg with later reproductive endpoints.[51] Thus, a 50% reduction in breeding pairs could easily be added to a 10−20% reduction in nest success at the upper range of Hg exposure observed, resulting in a net 55−60% reduction in successful nests. The White Ibises experimentally dosed with Hg also showed a reduction in the likelihood of breeding attempts resulting in egg-laying. This resulted in a reduction of about 20% in the numbers of breeding pairs compared to the control group within the range of experimental doses and a predicted decrease of 47% within the upper end of exposure values observed in the field (Figure 2).

Our results are in line with previous reports of capture−recapture studies showing reduced breeding propensity associated with pollutant concentrations,[8,18−20] and with anecdotal field observations suggesting a lower occupancy of nest boxes in polluted areas.[52,53] Unlike those studies, we were able to control the possible influence of several sources of ecological variation (food availability, rainfall, weather, and colony) on the number of breeding pairs, disentangle it from association with [Hg], and estimate the influence of both ecological and exposure variables on local breeding population sizes. A recent study experimentally investigated the effects of Hg exposure on the nesting probability of zebra finches (*Taeniopygia guttata*).[14] The authors found that adult finches exposed to dietary Hg in doses that resulted in whole egg [Hg] higher than those in our study (3.35 ± 0.21 ppm compared to the estimated <0.7 ppm in this study) were less likely to start nests and spent less time constructing them. The probability of

https://dx.doi.org/10.1021/acs.est.0c04098
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dosed zebra finches laying eggs in that study was 0.64 while that of control finches was 0.78.[14] Thus, dosed finch pairs were 18% ([1−0.64/0.78] × 100) less likely to produce eggs than control pairs. As with our captive White Ibis study, there is strong experimental evidence of the negative effect of Hg on breeding propensity and breeding numbers in experimental settings, but both experimental studies demonstrated considerably smaller effects than those we report from our observational study of free-ranging birds (experimental studies: 18−20% reduction; free ranging: 50% reduction).

The difference in the effect size between our field and experimental studies (∼30%) is considerable, yet perhaps to be expected. Although this could be due to species-specific sensitivity to Hg, White Ibis embryonic survival was more sensitive than that of Great Egrets to Hg exposure,[54] an effect opposite to our results. As Hg is the only likely toxin of concern in the Everglades,[55] interactions with other contaminants do not seem to be a likely effect.[55] Neither predation[56] nor disease[55] has a large influence on breeding outcomes in this population.

The tissue sampled also does not appear to elicit an explanation for differences in the effect size in experimental and observational studies. A potential drawback of using nestling feathers to approximate exposure to Hg of adult breeders is that only successful nests can be sampled for this tissue. If nests fail early as a result of higher exposure to Hg, this can result in underestimated exposure to Hg.[51] The strong correlation between adult blood and nestling feather [Hg] in the White Ibis data suggests that nestling feathers accurately indicate exposure to Hg in adults if exposure is not variable during the breeding season. The same appears to be true in Great Egrets. Colony-averaged nestling feather and egg albumen [Hg] in Great Egrets in the Everglades showed a good correlation (r = 0.703)[23] within the range of 5.26−19.34 mg/g dw THg in nestling feathers. As albumen [Hg] is derived directly from adult tissues, that relationship suggested nestling feathers to be a reasonable proxy for adult exposure early in the breeding season. That range also virtually encompasses the values of nestling feather [Hg] used to develop the model presented here (2.24−19.34 mg/g dw THg). Experimental research showed that [Hg] in scapular nestling feathers integrates exposure over the early nestling period and is not susceptible to short-term variation in [Hg] in food.[42] Therefore, within that range of [Hg] values included in the analyses, any putative bias arising from sampling nestling feathers does not appears to be strong enough to affect the correlation, although a partial mismatch between exposure to Hg during the early breeding season and exposure to Hg during chick-rearing stages might add noise to the models and reduce the strength of the association reported here.

We believe that there are two potentially overlapping causes that probably account for a relevant part of the difference in the effect size of Hg exposure between observational and experimental studies. First, similar Great Egret and White Ibis nestling feather [Hg] might reflect different Hg exposures. Growing feathers effectively capture Hg in blood.[42,57] While both species have similar masses and sizes and eat similar foods, the developmental period of Great Egrets lasts longer than that of White Ibises (Table S4). Therefore, these species seem to be similar physiological models, but there are some ecological differences, particularly, in the speed of reproduction. In consequence, the same exposure to Hg through food could result in different nestling feather [Hg] values, hindering the comparison of effects between species. The other likely cause is a result of lack of stressors and/or the lack of interindividual variance in exposure to them, in the experimental study. Typically, experiments conducted under controlled conditions can provide evidence for causal mechanisms, but their low environmental realism results in difficult translation to the field.[58−60] In the aviary-kept ibises, interannual and interindividual variability in exposure to variation in food supply, thermal stress, predation, and other natural stressors were purposefully absent, homogenized or reduced in the experimental aviary conditions. White Ibises in the aviary had *ad libitum* food, laid eggs in up to five events per season, had clutch sizes of up to 10 eggs, and breeding seasons that lasted up to 198 days. None of these conditions or reproductive outcomes match what typically happens in wild colonies.[61] On the other hand, White Ibises were probably subjected to captivity stress that could potentially influence the dose−response relationship.

We also found a positive association between the maximum depth at the start of the breeding season and the recession range with numbers of Great Egret breeding pairs. High water conditions at the start of the breeding season and strong recession, resulting in a large reduction of the flooded area and formation of many shallow pools, were associated with larger numbers of breeding pairs. The association between a large recession and several parameters of reproductive success of wading birds in the Everglades has been reported before.[21,24,33] The lack of association between fish biomass and breeding numbers in this study was unexpected. However, fish biomass was strongly and positively correlated with water depth at the start of the breeding season (Pearson correlation coefficient = 0.64; Figure S2) which, in turn, was positively associated with numbers of breeding pairs. Long periods of inundation, related to high water at the start of the breeding season, increase the time for fish growth and reproduction and, consequently, standing stocks.[35] Considering the flat topography of the Everglades and the association between fish biomass and water depth at the start of the season, water depth could be a better proxy for total fish abundance in the system than actual fish biomass at sampling points, as it is closely related to the extent of flooded area and therefore the amount of available fish habitat.

Our results collectively suggest a strong influence of exposure to Hg on numbers of pairs attempting to breed in natural settings. This influence could be widespread, and particularly, acute in populations in extreme environments already facing other natural stressors.[9,62] The reduction in breeding numbers we report is complementary to other known breeding impairments resulting from Hg exposure (e.g., nest success, chick survival) and is of special concern because the endpoint (reduced propensity to initiate breeding and breeding failures prior to egg-laying) is difficult to observe in wild populations. Endpoints assessed in studies of Hg effects typically range from egg fertility to fledgling production[12,13] and, therefore, are entirely separate from any effect previous to egg-laying. Our results suggest that estimates of breeding impairment in wild populations associated with Hg may be systematically underestimated in the literature and, consequently, in management and conservation decisions. The range of estimates of nesting reduction we report suggest that underestimates of effects could be in the range of 10−45% in captive White Ibises and up to 70% in wild Great Egrets, within the range of [Hg] values we observed in the field. As

https://dx.doi.org/10.1021/acs.est.0c04098
Environ. Sci. Technol. 2020, 54, 13786−13796

much of the effect we saw with Hg on the breeding propensity of ibises was a result of endocrine disruption,[7] these conclusions may have application to a large class of toxicants worldwide.[4−6,8] We urge researchers to seek observational and experimental validation and extension of our results, and managers to consider the possible influence of pollutants on reduced propensity to breed in vertebrate species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c04098.

Methods for sampling fish biomass, Hg determination, and transformation of Great Egret nestling feather [Hg] into whole egg [Hg];results of the effect of experimental Hg exposure on the probability of reaching the egg-laying stage; results of model selection for each block of Great Egret models; output of the best Great Egret model of each block; output of the best model for Great Egrets using only colonies with exposure to Hg based on >5 nestling feathers; comparison of breeding and life-history traits of Great Egret and White Ibis; deviation of individual Great Egret feather [Hg] values from their colony averaged values; correlation among predictive covariates in observational models; temporal pseudo-correlation in the residuals of the best model for Great Egret breeding number pairs; and residuals of the best model for Great Egret numbers of breeding pairs (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Peter Frederick − *Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, Florida 32611, United States*; Email: pfred@ufl.edu

Authors

Jabi Zabala − *Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, Florida 32611, United States*; orcid.org/0000-0002-1425-1594

Joel C. Trexler − *Department of Biological Sciences, Florida International University, North Miami, Florida 33181, United States*

Nilmini Jayasena − *Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya 20400, Sri Lanka*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.0c04098

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the U.S. Army Corps of Engineers (W912HZ-12-02-0007) and by grants of equipment from the University of Florida Institute of Food and Agricultural Sciences. Egret monitoring was performed under the University of Florida IACUC permit 201708650. Fish sampling was supported by Cooperative Agreements W912HZ-11-2-0048 and W912HZ-16-2-0008 between Florida International University (FIU) and the U.S. Army Corps of Engineers and Contract 4600001083 between FIU and the South Florida Water Management District. Fish monitoring was performed under FIU IACUC permits including IACUC-08-004, -09-029, -10-026, 12-020, -13-060, and -16-033. Nestling Ibises were collected from the field under Florida Fish and Wildlife Conservation Commission permit WX03527, which also allowed for their maintenance in captivity, and with a modification, re-release into the wild. Ibises were held in captivity under Institutional Animal Care and Use Committee permit D424-2006. Background images in the graphical abstract, Figure 1A,B and D were taken by J.Z. The background egret image on Figure 1C was cropped from the picture nest with three chicks at the Morro Bay Heron Rookery, taken by M. Baird licensed under CC BY 2.0. The image of the Florida peninsula used in the inset of Figure 1A was modified from Google Earth (Data SIO, NOAA, US Navy, NGA, GEBCO, Image Landsat/Copernicus). The Great egret picture in Figure 2 was taken by J. A. Gonzalez-Oreja and used with his permission, while the White Ibis image in the same figure was extracted from a picture downloaded from www.all-free-photos.com licensed under CC BY-SA 2.5. We are grateful to four anonymous reviewers whose comments greatly improved the manuscript.

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https://dx.doi.org/10.1021/acs.est.0c04098
Environ. Sci. Technol. 2020, 54, 13786−13796