



Timing and magnitude of net methylmercury effects on waterbird reproductive output are dependent on food availability

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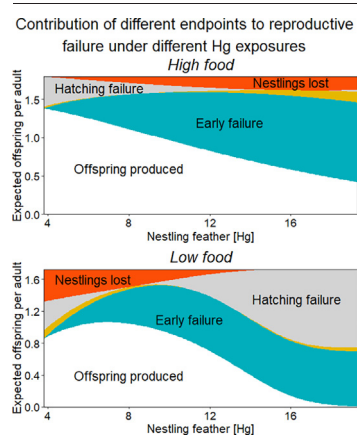
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HIGHLIGHTS

- Using 4 endpoints we estimated timing and net reproductive effects of MeHg in birds
- Effects vary considerably among endpoints and total effect was twice the predicted.
- Timing and magnitude of failure varied strongly with food availability in great egrets.
- No single endpoint best captures reproductive impairment under all conditions.
- Net effects of Hg exposure may often be underestimated for wild animal populations.

GRAPHICAL ABSTRACT



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ABSTRACT

Mercury (Hg) is a globally distributed pollutant. Its sub-lethal effects on reproduction of birds have been used as indicators of contamination and of potential demographic effects. However, studies typically used single endpoints that might not be representative of entire reproductive cycle. To estimate timing and net cumulative effects of Hg exposure under field conditions, we used observational data over 11 years from >1200 nests of great egrets breeding under temporally and spatially varying food availability and Hg exposures in the Florida Everglades. We collected measures of fish biomass and availability (>100 locations annually) and used four avian reproductive endpoints that represented the entire breeding cycle. We calculated net reproductive loss by adding estimated Hg effects on failures prior to egg laying, clutch size, hatching success and nestling survival in response to food availability and Hg exposure. To validate and assess results of the observational egret study, we ran the same analyses with data of captive breeding white ibises experimentally exposed to Hg with *ad libitum* food over 3 years. We found large (>50 %) reductions in great egret offspring with high Hg exposure (18 µg/g dw THg nestling feather, ~0.7 µg/g ww whole egg THg) and high food availability, and even larger reductions (up to 100 %) with high Hg exposure and low food. Timing and the relative contribution of different endpoints to overall reproductive failure varied with food availability. Failures prior to egg laying were relevant at all food availabilities and proportionally most important during high food availability (~70 % of total losses). Under high food, post-hatching failures increased moderately with increasing exposure (~10 % of total losses),

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and under low food, hatching failures became dominant (~50 % of total losses). Patterns of failure of captive white ibis fed *ad libitum* resembled those of great egrets under high food availability but differed in total magnitude. We suggest that, a) net reproductive effects of Hg in free-ranging animals are probably much higher than generally reported in studies using single endpoints, b) Hg effect sizes vary considerably among different endpoints and c) food availability is a strong driver of timing and net effects of Hg exposure.

1. Introduction

Industrial and agricultural development has resulted in the release in the environment of considerable quantities of heavy metals, biocides and other chemicals, many of which may have long half-lives (Krabbenhoft and Sunderland, 2013; Nriagu, 1996). While the effects of many of these contaminants is often well understood in controlled lab settings, their impact on free-ranging animal populations that are chronically exposed and encountering multiple stressors remains poorly understood. Important impediments to our understanding include the difficulty of accurately measuring exposure and consequences to free ranging wildlife (Zabala et al., 2020a), the simultaneous exposure to several chemicals at variable concentrations (Goutte et al., 2015; Tartu et al., 2014), and the influence of natural stressors that might mask or interact with contaminant effects (Relyea and Mills, 2001; Sih et al., 2004; Zabala et al., 2021).

Mercury (Hg) has been released in large quantities through industrial activities, and dispersed atmospherically across the globe (Krabbenhoft and Sunderland, 2013; Travnikov et al., 2017). Mercury can be methylated and converted into the highly toxic methyl-mercury (MeHg; de Oliveira Ribeiro et al., 2002; Rice et al., 2014). MeHg biomagnifies in food-webs, particularly in aquatic ones and top predators in aquatic ecosystems, such as piscivorous birds, are therefore often used as model or study species (Fuchsman et al., 2017; Wolfe et al., 1998). Hg exposure in vertebrates affects multiple organs and physiological processes, and reproduction is often a target endpoint because it integrates the effects of degradation on many of these systems. While the consensus from lab studies and some field studies is that Hg has negative effects on reproduction, some studies of free-ranging birds have reported contradictory findings, such as birds with higher Hg in blood being in better physiological condition (Herring et al., 2009), effects being detectable only under certain weather conditions (Hallinger and Cristol, 2011) or in certain age-groups (Brasso and Cristol, 2008). This may have to do with interactions of Hg effects with temporally or spatially varying stressors such as food, crowding or predation pressures. For example, Hg effects in wild animals may be modulated strongly by food availability, presumably though effects on nutrition and body condition (Zabala et al., 2021). Further, interactions with environmental stressors may also affect the relative sensitivity of various endpoints to Hg (Zabala et al., 2021). This is of particular importance because most studies of effects on avian reproduction use only one endpoint (e.g., hatching success or clutch size) even though effects at different parts of the reproductive cycle are cumulative and interactive. Therefore, although sub-lethal reproductive effects of exposure to Hg have been widely documented on particular reproductive endpoints (Brasso and Cristol, 2008; Fuchsman et al., 2017; Jackson et al., 2011; Whitney and Cristol, 2017; Wolfe et al., 1998; Zabala et al., 2019a), effects on the entire reproductive cycle have not been measured and it remains unclear what the total net effect of Hg may be, especially for free ranging wild populations.

In the geographically extensive Everglades wetlands of southern Florida, Hg bioavailability varies both spatially and temporally depending on antecedent water conditions, geographically varying deposition and methylation rates, and composition and size of the prey community (Liu et al., 2015; Loftus, 2000). In previous work, we have shown that exposure to Hg and variation in food availability influenced the annual number of breeding great egret (*Ardea alba*) pairs in the Everglades (Zabala et al., 2020a) and that food availability modulates Hg effects on some reproductive endpoints of that species (Zabala et al., 2021). However, there has not been a synthesis that examines cumulative effects using endpoints from throughout the breeding cycle to estimate a net effect of Hg on great egret reproduction.

Here, we used two data sets to model cumulative losses during the whole breeding cycle, from early failure prior to egg laying to loss of nestlings, and to assess net losses associated with environmental and experimental exposures to MeHg. To the best of our knowledge, this is the first study to take this approach. While some experimental studies reported failure and losses at different stages of the breeding cycle (Chin et al., 2017), none has approximated cumulative losses. To accomplish that, we used two unique data sets: observational data of great egrets breeding over a large wetland ecosystem and experimental data from white ibises (*Eudocimus albus*) dosed with MeHg in a controlled aviary setting.

For the observational study we used a unique data set of >20 years of observational data on great egret breeding success, exposure to Hg and spatial and temporal variation in food availability (Fig. 1), to 1) estimate magnitude of cumulative effects of Hg throughout the breeding cycle by adding effects expected at consecutive endpoints; 2) determine timing of effects; and 3) assess whether effects and timing vary with food availability. To do that, we used particular models for each endpoint assessed. As no model for clutch-size was available for our data, we first developed a model to predict the effects of Hg on clutch size. We then coupled the clutch size model with previous results on the effect of Hg on nesting probability, hatching success and post-hatching survival, to estimate the net reproductive effect of exposure to Hg. To assess whether effects and timing vary with food availability, we considered low, medium and high food availability scenarios based on quantiles of observed values, to assess variation in reproductive reduction and in affected endpoints.

We compared the results of the observational field study with experimental data from white ibises. We used results of a 3 year long Hg dosing experiment (Jayasena et al., 2011) using captive white ibises under *ad libitum* food availability and compared them to our high food availability egret results. This allowed us to directly compare the observational results of naturally exposed great egrets under favorable food conditions with results of an ecologically similar species experimentally dosed on *ad libitum* diets. Thus, the white ibis dataset provided experimental validation for a part of the observational result. Based on prior results and toxicity reference values available in the literature (Fuchsman et al., 2017; Zabala et al., 2021), we predicted reproductive loss for both species to be <20 % within the observed Hg exposures. We also predicted that in field conditions low food availability would exacerbate reproductive impairment associated with Hg, while high food availability would be associated with much lower levels of impairment. We hypothesized that timing and degree of reproductive losses associated with Hg would vary with food availability and it would manifest most often as hatching failures under low food availability scenarios, and nestling mortality at high food availability.

2. Material and methods

2.1. Observational study of great egrets

We used observational data on great egrets breeding in the Everglades of Miami-Dade, Broward and Monroe counties, Florida, USA. Great egrets nest communally each year on tree islands widely spaced (2–15 km) within the Everglades herbaceous marsh. Annual numbers of nests initiated vary in response to temporally and geographically variable food availability and exposure to Hg (Beerens et al., 2011; Frederick et al., 2002; Zabala et al., 2019b). Great egrets are piscivorous (Powell, 1983) and fish availability in the Everglades is influenced by annually variable fish biomass, and the degree of seasonal drying (November through April) that forces fish to concentrate in remaining shallow pools, where they become vulnerable and

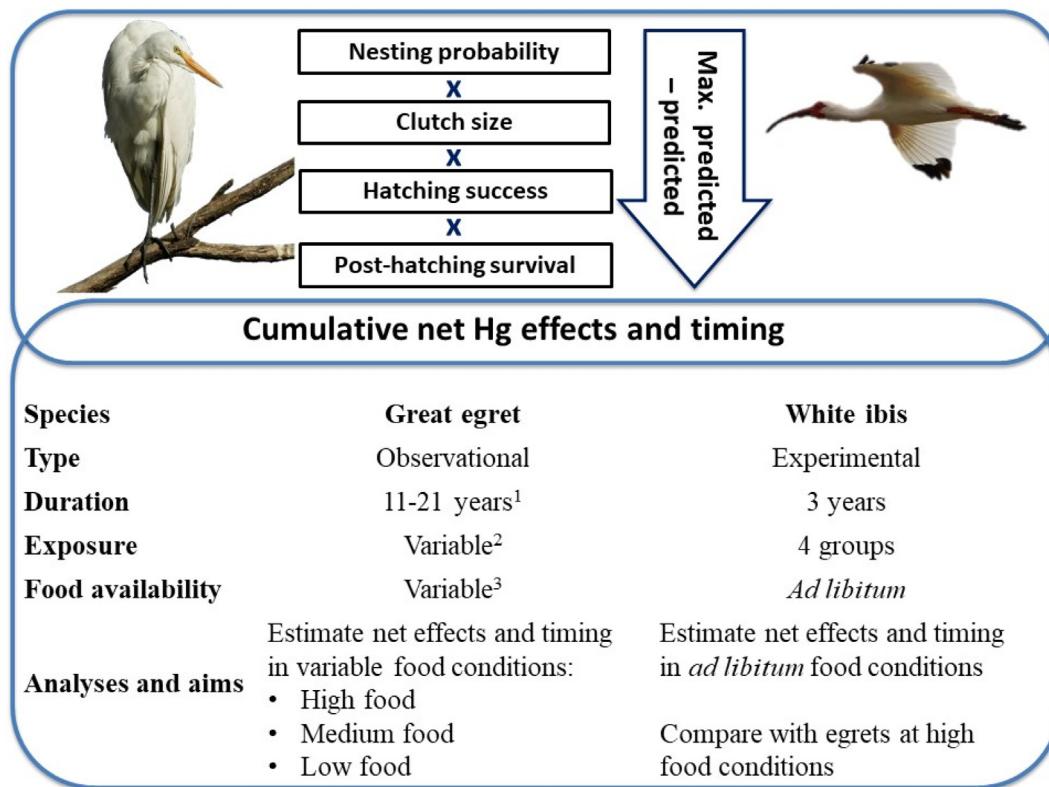


Fig. 1. Conceptual flowchart of the analytical approach. In the upper block we show the four endpoints assessed. For each endpoint we predicted expected output under different Hg exposures and, in the great egret data set, food availability conditions. Then we approximated failure at each endpoint as the difference between the predicted value for that Hg and food conditions and the maximum value predicted for that endpoint at any exposure and same food conditions. Next, we added failure at all four endpoints to estimate cumulative net failure associated with Hg and its timing. We also provide basic detail on characteristics, aims, conditions and duration of studies with each species. ¹ Duration in great egret is variable because one endpoint (nesting probability) is based on 21 years of data while the other three are based on 11 years of monitoring. ² Exposure in great egrets was environmental and varied among years and breeding colonies, we used average nestling feather [Hg] to approximate Hg exposure at each colony. ³ Food availability for great egrets varied among colonies and years, we measured it using fish biomass and water level data within 20 km of the colony using annual fish biomass sampling in >100 points and daily water levels measured in >100 gages in the Everglades. Inset images show a great egret (left) and a white ibis (right), the model species of this study.

available to birds (Beerens et al., 2015; Botson et al., 2016). As the dry season proceeds, typically November–May, water recedes over a 5-month period, creating a sequence of temporary, shallow pools that can offer a continuous supply of food to breeding birds. Seasonal rainfall beginning in May or June interrupts the water recession with rising water levels, making fish harder to capture (Gawlik, 2002), usually leading to the cessation of nesting.

Starting in 1998, we annually monitored breeding success of great egrets in selected colonies of the Everglades. We marked a sample of active nests along transects within breeding colonies, and monitored eggs and chicks weekly. Further detail on field data collection is provided elsewhere (Zabala et al., 2021). We divided the nesting cycle into four metrics representing serially consecutive steps leading to fledgling production (Table 1; Fig. 1): (1) nesting probability, defined here as the probability that a bird

will attempt breeding (lay at least one egg): (2) clutch size, defined as the maximum number of eggs observed simultaneously in the same nest; (3) hatching success, the probability of a laid egg resulting in a hatchling; and (4) post-hatching survival, the probability of a hatchling surviving to an age of 21 days.

2.2. Modelling great egret failures at each endpoint

For understanding Hg effects in great egrets, we partially relied on previously published models for all metrics except for clutch size (described below, Table 1), and then looked at net effects of Hg by combining the results of all models over the reproductive cycle. We modeled nesting probability using a published model (Zabala et al., 2020a) for the same study population. That study correlated numbers of nesting pairs in breeding

Table 1

Endpoints considered, from earliest to latest in the breeding cycle, the definition of each we used and the source from which we took the model for that endpoint and each study species. Published models were developed for the same great egret population and using the same data we use in this paper. “In this paper” indicates that models were developed for this study and their results are reported in Table 2.

Endpoint	Definition	Model
Nesting probability	Birds will attempt to nest and lay at least 1 egg.	Approximated by a breeding index using negative binomial GLMM for observational data (great egrets) and binomial GLMM for experimental data (white ibis; both published in Zabala et al., 2020a)
Clutch size	Maximum number of eggs simultaneously observed in the nest	Gaussian GLMM in this paper for both species (Table 2).
Hatching success	Probability of each egg in the clutch resulting in a hatchling	Binomial GLMM. In this paper for white ibis (Table 2), published elsewhere for great egrets (Zabala et al., 2021)
Post-hatching survival	Probability of a hatchling surviving to fledgling (age 21 days for great egrets, 14 days for white ibis)	Binomial GLMM. In this paper for white ibis (Table 2), published elsewhere for great egrets (Zabala et al., 2021)

colonies with exposure to Hg (measured as annual average [Hg] in feathers of nestlings from the colony; Zabala et al., 2021), recession range (the difference between water depth at the start of the breeding season –highest water level in January–February– and the minimum depth registered before the June–July reversal), and maximum water depth at the start of the breeding season, a proxy for extent of fish breeding habitat (Zabala et al., 2020a). Although we call it probability, we acknowledge that this is more a breeding index than actual individual nesting probability. Yet, we assumed that, in the long term and in the absence of strong temporal trends in population size, the variation in relative number of nesting-pairs predicted under different Hg exposures approximated the variation in nesting probability of individuals. For instance, when predicted breeding-population size dropped from 1.0 to 0.5, we assumed that individual nesting probability halved accordingly.

To model hatching probability and post-hatching survival (Table 1) we also used published models for the study population (Zabala et al., 2021). Those models predicted success of individual nests using colony-wide averages of food availability and Hg exposure. In them, food availability was modeled using two components: recession range and fish biomass (Zabala et al., 2021). We then constructed a model of clutch size effects, using Hg exposure, while accounting for variation in food availability. Based on previous results (McDonald and White, 2010; Zabala et al., 2021), we modeled clutch size using Generalized Linear Mixed Models (GLMMs) with a Gaussian error and an identity link function. To control for background differences among colonies or years, we added year and colony as crossed random factors. We first ran a general model with annual Hg, the quadratic form of annual Hg, fish biomass, recession range, and interactions between food availability components, Hg and quadratic Hg. We optimized that general model using backwards stepwise regression (Zuur et al., 2009). In each step, we deleted the covariate with the highest *P* value, and compared the performance of the reduced model using AICc. We stopped the optimization when no further reduction in AICc was observed.

Clutch size included data from 1290 clutches gathered over 11 years (2006–2018) in 13 different colonies and in 43 different colony-year combinations. We kept the modelling approach used for other endpoints. Therefore, to account for variation of food availability we included local fish biomass, as a proxy measure of food abundance, in the models. We also included water level recession range, as a proxy for the magnitude of the concentration effect resulting from the drying of shallow pools where fish became vulnerable. To measure recession range, we used daily average depths recorded by >100 water gauges distributed across the study area (Everglades Depth Estimation Network EDEN; <https://sofia.usgs.gov/eden/>). To estimate food density, we used data from >100 annual fish biomass sampling points distributed throughout the study area (Dorn et al., 2005; Jordan et al., 1997). We estimated colony-specific values for both recession range and fish biomass using water level gages and fish sampling points within 20 km of the breeding colony. We used a 20 km radius because >95 % of foraging flights of great egrets in the Everglades were within 20 km of the colony (Bancroft et al., 1994). We have successfully used both metrics in previous studies, including those in which we developed the models used here that can be consulted for more details on methods (Zabala et al., 2021, 2020a). To estimate Hg exposure, we used average [Hg] in nestling feathers from the same colony and year. For colonies with less than six independent feather samples, we weighted the data based on feather sample size (Zabala et al., 2019a). Average nestling feather Hg values observed during the study period ranged between 2.24 and 19.335 THg $\mu\text{g/g}$ dw, which are approximately equivalent to 0.06–1.31 THg $\mu\text{g/g}$ ww of thin albumen and 0.18–0.72 THg $\mu\text{g/g}$ ww whole egg (Zabala et al., 2020a).

2.3. Experimental white ibis reference-study

We used white ibis reproductive data collected over four breeding seasons (2006–2008) from birds captured as fledglings in the Everglades in 2005 and raised to adulthood in a large aviary in four different dosage groups (Jayasena et al., 2011). Dosage groups consisted of 20 birds of each

sex housed in an outdoor aviary divided into four equal sections by hanging net walls. Each year, treatment groups were randomly reassigned to other sections (but same dose) to minimize possible location effects. Each section was provided with 48 nest platforms and *ad libitum* food and nesting material. We fed birds pelletized food infused with MeHg using a corn-oil vehicle. Observers were blind to dose group, which consisted of only corn-oil for the control group and 0.05, 0.1, and 0.3 ppm wet weight MeHg for low, medium and high dose groups (Frederick and Jayasena, 2011; Jayasena et al., 2011). These levels of exposure were the control, low, medium and high dose groups respectively, resulting in breeding adult blood THg concentrations of 0.079 ± 0.015 ; 0.732 ± 0.092 ; 1.599 ± 0.319 ; and 3.946 ± 0.678 ppm ww; and in nestling feather THg values of 0.319 ± 0.068 ; 1.676 ± 0.270 ; 3.711 ± 0.559 ; and 8.867 ± 0.903 ppm dw. White ibis adult blood and nestling feather [Hg] showed a strong correlation when compared among groups ($r^2 = 0.92$; Zabala et al., 2020a).

Individual white ibises experimentally exposed to Hg nested up to five times within a season, and breeding seasons lasted up to 198 days. The ability to re-nest so often, and to have such a long breeding season was not typical of wild birds, and probably was an artifact of *ad libitum* food in captivity (Zabala et al., 2020a). In any case, re-nesting at least once does occur in natural conditions, and is important to breeding output (Heath et al., 2020). Thus, in our models we included the possibility of only the first two nesting attempts per season: a first attempt and a re-nesting. We split the raw data in two blocks: one included only first attempts and the other re-nesting attempts. Some nesting attempts recorded were brief (<10 days) and then the pair split and moved to other nests. Some of these attempts might have been exploratory. To accommodate that, we defined here as first attempt all these attempts that were the first attempt for both individuals in the pair, and those that were the first attempt for one of them and the second for the other ($N = 215$; in 19 of them one of the birds was in its second attempt). The block with re-nesting attempts included all attempts that did not fall into this former group. White ibises switched pairs between breeding attempts and pairs could include a bird in its second attempt and another in their fourth. We modeled white ibis nesting probability, clutch size, hatching probability and post-hatching survival of first and re-nesting attempts separately. Previous analyses showed that presence of a juvenile-plumaged individual in the pair influenced their breeding success (Frederick and Jayasena, 2011; Zabala et al., 2020a). Thus, here we modeled white ibis endpoints as a function of exposure to Hg and the presence of juvenile-plumaged individuals in the pair. We used GLMMs to model endpoints. For clutch size, we used the identity link and a Gaussian error distribution and assessed linear and log responses. For nesting probability, hatching probability and post-hatching survival, we used the logit link and a binomial error distribution. For hatching-success and post-hatching survival, we used a proportional model in which each nesting attempt was considered as a series of binomial trials and each egg or nestling an independent attempt (Zuur et al., 2009). For instance, hatching success of a nest with three eggs resulting in two nestlings was considered as three independent binomial trials, two of which were successful and the third resulted in a failure.

2.4. Estimating breeding success under different Hg exposures

We predicted expected reproductive success at each of the four steps of the reproductive cycle (above; Fig. 1) under varying Hg exposure by substituting observed Hg values with increasing Hg exposure values. In the great egret observational study, to predict the expected output of endpoints, we used the range of Hg exposure (measured as averaged nestling feather [Hg]) observed in the field (3.8 to 19.36 $\mu\text{g/g}$ THg dry weight) in stepped increases of 0.1 $\mu\text{g/g}$ THg dw. To account for the influence of variation in food availability on great egrets (Zabala et al., 2021), we considered three scenarios: (1) a median food availability scenario in which fish biomass, recession range, and water depth at the start of the season were set at median observed values (4.67 g m^{-2} ; 62.34 cm and 234.89 cm respectively); (2) a high food availability scenario with values set at the 0.75 quantile of observed values (6.54 g m^{-2} ; 77.21 cm and

271.27 cm respectively); and (3) a low food availability scenario set at 0.25 quantiles of observed values (3.89 g m⁻²; 56.56 cm and 201.67 cm respectively). All other parameters in the model, including random factors, were kept at their original values. To see whether our models predicted significantly different trends for the same endpoint between low and high food availability conditions in great egrets, we compared the performance of two models. In a first model, we used predicted values as response and exposure to Hg and food conditions as additive covariates. In a second model we added the interaction between exposure to Hg and food conditions to the first model and inspected its *P* value and the change in AICc with regards to the first model (Burnham and Anderson, 2002). In the case of white ibises, we accounted for probability of re-nesting. There was no significant difference in the probability of re-nesting between successful or failed first nesting attempts (respectively 75.9 and 68.8 % of them re-nested; *P* = 0.288; *N* = 427), or between treatments compared to control (*P*_{Low} = 0.803; *P*_{Med} = 0.749; *P*_{High} = 0.301; *N* = 427) thus, we used the average re-nesting probability of 0.70.

Using predicted output at each step, we estimated the expected number of fledglings produced by an individual bird by multiplying the expected success at each step. Thus- expected number of fledglings = predicted probability of nesting attempt to reach egg-laying × predicted clutch size × predicted hatching success × predicted probability of post-hatching survival. We caution that we used model predictions despite the fact that in some cases white ibis endpoints did not significantly associate with Hg (see results). To model the influence of white ibis re-nesting, we separately estimated the expected number of fledglings in first nesting and re-nesting attempts. Next, we multiplied the latter by re-nesting probability (0.70) and added the result to the numbers of fledglings expected from the first nesting attempt.

2.5. Determining the contribution of partial failures to net fledgling losses

To determine how failures or reduced success at different stages contributed to the total reduction in fledglings produced, we first estimated the highest number of fledglings expectable per individual. To do that we multiplied the highest value predicted for each endpoint regardless of what Hg exposure was used to predict it. That value was constant for each scenario (one for each of the three great egret food availability scenarios and one for white ibis first attempts and another for re-nesting white ibises). Next, we calculated the difference between that value and the number of fledglings predicted at each Hg exposure. Then, we estimated the proportional reduction of success at each endpoint compared to the highest value predicted for that endpoint. Finally, we multiplied the difference in fledglings expected by the proportional reduction of each endpoint divided by the sum of the proportional reduction of all the four endpoints at that Hg exposure. Thus, we calculated the contribution of failure at each endpoint to reduction in fledglings produced while also accounting for the effects of failure at previous or later endpoints.

2.6. Analysis software

We performed all analyses using R 3.5.1 (R Team, 2016). For GLMMs we used the package lme4 1.1–19 (Bates et al., 2015). We used AICcmodavg 2.1–4 (Mazerolle, 2017) to calculate AICc values. We produced plots of ggplot2 3.1.0 (Wickham, 2009) packages.

3. Results

3.1. Modelling great egret failures at endpoints: clutch size and mercury

The optimized model for predicting great egret clutch size retained recession range and interactions between recession range, fish biomass and both Hg and quadratic Hg (Table 2). The optimized model performed 17.397 AICc lower than the most general model and 25.537 units lower than the null model. When food availability was low, our top model predicted for great egrets a slightly smaller clutch size compared to high food availability (2.6 and 2.72 respectively). However, our best model

predicted no change in clutch size with increasing Hg exposure under low food availability conditions, but predicted clutch size decreases with increasing Hg exposures under high food availability conditions (Fig. S1).

3.2. Modelling failures in the experimental white ibis study

For white ibis, probability of reaching the egg laying stage in the first breeding attempt was negatively associated with exposure to Hg (Table 2). Clutch size in Hg-exposed pairs was significantly smaller than in control pairs ($\beta = -0.561 \pm 0.260$; *P* = 0.032; *N* = 215), but the linear and log associations were not significant. Estimates for Hg effects on hatching success and post-hatching survival were negative but none reached statistical significance (Table 2). Analysis of endpoints with re-nesting data yielded similar results. In every endpoint, with the exception of hatching success, Hg had a negative effect, yet none reached statistical significance (Table 2). We used these estimates and models to predict change in endpoints under increasing Hg exposure, but lack of statistically supported associations with Hg needs to be kept in mind and results should be regarded as indicative. We discuss possible causes and consequences of the lack of significant associations below.

3.3. Net cumulative effects of Hg in numbers of offspring produced

3.3.1. Observational great egret study

Model predictions for great egrets resulted in strong reductions (>50 % in expected offspring) with increasing exposure to Hg under every food availability scenario (Fig. 2A). Differences in expected offspring produced under contrasting food conditions (i.e., high versus low food availability) were very small in the low-mid and mid ranges of observed Hg exposures, but increased towards the high end. The marked difference in reduction in expected offspring between low and high food at high Hg exposure became more apparent when the difference was expressed as a proportion of offspring expected at high food availabilities (Fig. 2B). The absolute reduction was similar at both ends of Hg exposure, around 0.5 fewer fledglings per nest were expected when food was low. However, the difference in fledglings expected at high and low food availability conditions expressed as a proportion of the number of fledglings expected at high Hg exposures was quite large (Fig. 2B). Expected number of offspring of great egrets breeding at the high end of observed Hg exposures and experiencing low food conditions was >50 % less than those expected by egrets breeding at the same Hg exposure but under high food availability conditions. This supported, at least partially, our hypothesis that Hg-induced reductions would be smaller under high food availability. However, predicted reductions in offspring produced were non-trivial in every case. Reduction in number of fledglings expected at the high end of Hg exposure were in every case >50 %, which greatly exceeded our 20 % assumption based on published toxicity reference values.

3.3.2. Experimental white ibis study

In experimentally exposed ibises, our results predicted a reduction of around 10 % in fledglings produced under the range of Hg doses provided, and predicted reductions in fledglings of 50 % at higher modeled exposures (Fig. 2C). Again, as in the great egret case, predicted reductions in number of fledglings produced under high Hg exposure values (18 µg/g dw THg nestling feather, ~0.7 µg/g ww whole egg THg) were > 40 %, much larger than our initial prediction of 20 % reduction based on reference values.

3.4. Timing of failure and influence of food availability

3.4.1. Observational great egret study

In great egrets, variation in food availability also resulted in differences in which endpoints were most strongly affected. Predicted shapes for all four endpoints differed significantly among high and low food availability scenarios, and the model with the interaction between Hg and food availability clearly outperformed the model with only additive effects of Hg and food availability (*P* of interaction <0.001 and Δ AICc > 113.36 in all

Table 2

Output of the best model for great egret clutch size and white ibis models. In the great egret model “Rec Range” stands for recession range (difference between maximum and minimum water depth during the breeding season), “Fish” indicates average fish biomass, and “Hg” and “Hg²” average [Hg] in nestling feathers from the colony and its quadratic form, respectively. Random shows the random factors included. In the white ibis models, “juvenile” indicates if any of the birds in the breeding pair was a juvenile and “Hg” the average Hg in nestling feathers in the dosage group. For every model we indicate the estimate of the slope or intercept change of the variable (Est), its standard error (SE), the value of the statistic used (“Stat.”; t statistic for clutch size analyses and z statistic for all the others), and the associated P-value. We also indicate sample size (N) for each model. In white ibis models we provided separate results for first breeding attempts and re-nesting attempts.

	Variable	Random	Variance	Est	SE	Stat.	P		
Great egret		Year	0.0442		0.210				
		Colony	0.0002		0.015				
Clutch-size	Intercept			2.57	0.07	37.90	>0.001		
	Rec Range			0.10	0.03	3.78	0.002		
	Fish * Hg			0.07	0.03	2.15	0.033		
	Rec Range * Hg			-0.09	0.02	-3.79	0.000		
	Rec Range * Fish * Hg			0.50	0.14	3.69	0.001		
	Rec Range * Fish * Hg ²			-0.49	0.14	-3.42	0.002		
		N = 1288							
White ibis		First attempt				Re-nest			
	Variable	Est	SE	Stat.	P	Est	SE	Stat.	P
Nesting probability	Intercept	2.066	0.313	6.59	<0.001	2.961	0.431	6.87	<0.001
	Juvenile	-1.069	0.362	-2.96	0.003	-2.756	0.697	-3.96	<0.001
	Hg	-0.104	0.051	-2.01	0.044	-0.066	0.080	-0.83	0.409
		N = 215				N = 234			
Clutch size	Intercept	2.952	0.184	16.08	<0.001	2.726	0.132	20.72	<0.001
	Juvenile	-1.451	0.261	-5.57	<0.001	-1.622	0.424	-3.83	<0.001
	Hg	-0.011	0.035	-0.30	0.765	-0.034	0.028	-1.22	0.226
		N = 215				N = 234			
Clutch size	Intercept	3.010	0.147	20.43	<0.001	2.682	0.102	26.22	<0.001
	Juvenile	-1.278	0.092	-1.38	<0.001	-1.606	0.423	-3.79	0.158
	Log (Hg)	-1.460	0.259	-5.62	0.168	-0.103	0.073	-1.42	<0.001
		N = 215				N = 234			
Hatching-success	Intercept	0.208	0.133	1.56	0.119	-0.286	0.124	-2.30	0.021
	Juvenile	-0.271	0.244	-1.11	0.267	-0.182	0.652	-0.28	0.780
	Hg	-0.004	0.026	-0.16	0.871	0.031	0.027	1.17	0.243
		N = 168				N = 214			
Post-hatching survival	Intercept	-1.568	0.236	-6.66	<0.001	-1.185	0.222	-5.34	<0.001
	Juvenile	-0.097	0.475	-0.20	0.838	2.371	1.165	2.04	0.042
	Hg	-0.002	0.047	-0.05	0.958	-0.033	0.048	-0.68	0.495
		N = 130				N = 136			

four endpoints). Thus, at the same Hg exposure level, failures happened at different stages of the breeding cycle depending on food availability (Fig. 3). This difference was most noticeable in the mid to high observed levels of Hg exposure. At median food conditions and mid-high Hg exposures, reproductive impairment was mostly expressed through early nest failures and decreased hatching success (Fig. 3 A). Post-hatching nest survival was a secondary source of nesting failure at mid exposures and became an irrelevant source at higher Hg exposures. In contrast, in high food availability conditions, most of the reduction in fledglings produced was attributable to nest failures in early stages. Reduced fledging survival also became more important at higher Hg exposures, as did, to a lower extent, the influence of reduced clutch size (Fig. 3. B). Finally, when food availability was low, virtually all failures at mid-high Hg exposures were early failures or hatching failures, with hatching failures becoming dominant as exposure to Hg increased (Fig. 3 C). Thus, results supported our hypothesis that timing of reproductive losses associated with Hg would vary with food availability. They also supported the prediction that losses manifest most often as hatching failures under low food availability scenarios, although early failures were also very relevant (Fig. 3C). At high food availability failures were mostly early failures and deaths of nestlings, which is somewhat contrary to our prediction that these sources of failure would be of lower relevance (Fig. 3B).

3.4.2. Experimental white ibis study

Our estimates of the timing of nest failure for white ibises experimentally dosed under *ad libitum* food conditions, strongly resembled those of great egrets breeding under high food availability conditions. Most of the reduction was attributable to early failures with post-hatching failures and reduced clutch size becoming more important at higher Hg exposures

(Fig. 3 D). Hatching failure, as in great egrets under high food availability conditions, was most relevant at low Hg exposures, becoming less important as Hg increased.

4. Discussion

In the observational approach, our results predicted strong reductions in numbers of great egret offspring produced with increasing Hg exposure under all food availability conditions. In scenarios of low food availability indicative of nutritional stress, decreases in numbers of offspring produced were larger in absolute values (<0.5 fledglings produced per nest attempt) and particularly evident when expressed as proportional decreases compared to the same Hg exposure under high food availability conditions (up to 60 % more). Furthermore, reductions in reproductive output associated with Hg exposure took place at different nest stages depending on food availability. When food availability was low, most Hg-related losses happened early in breeding, particularly as hatching failures, and virtually no losses were predicted after hatching. On the other hand, when food availability was high, reduced clutch size and reduced fledgling survival also became important sources of Hg-related losses. This pattern of sources of nest failures relative to the nest cycle under high food availability was reinforced by experimental results of the study of *ad libitum* fed captive white ibises, whose nest failure pattern resembled that of wild great egrets under high food availability conditions. Thus, results of the ibis experiment suggest that patterns described here are not a particular characteristic of the studied great egret population but a general feature of birds exposed to MeHg.

Contrary to our predictions, our models suggested large absolute values of reproductive impairment due to Hg, even when food availability was

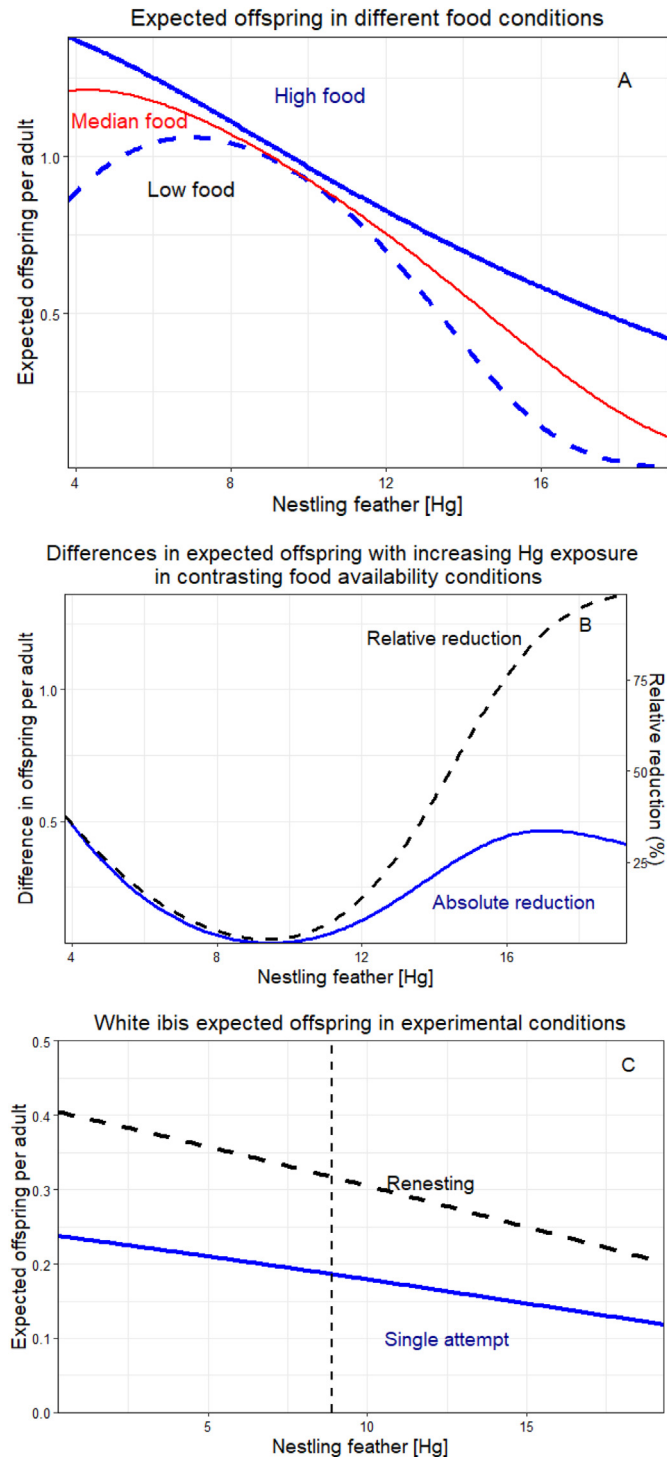


Fig. 2. A) Changes in expected reproductive output (fledglings per individual) of great egrets with increasing exposure to Hg and different food availability conditions (high, median and low food availabilities defined as food availability parameters at their 0.75, 0.5 and 0.25 quantiles respectively). B) Absolute reduction in offspring expected (offspring expected in high minus offspring expected in low food availability at the same Hg exposure) and percentage of difference ($100 - [(\text{expected offspring in low food conditions} \times 100) / \text{expected offspring in high food conditions}]$) from the observation great egret study C) Expected offspring of captive white ibis experimentally exposed to Hg in relation to exposure. We show offspring expected considering a single (first) attempt per breeding season, and re-nesting after failed and successful attempts (see text). Hg in nestling feathers indicates THg $\mu\text{g/g dw}$.

high. Under any food conditions, our predicted reductions in great egret fledglings at the higher Hg exposure levels exceeded dose-dependent predictions from other avian studies (Fuchsman et al., 2017) by at least 20 %. This may be due to a difference in methods used to estimate reproductive impairment. Many of the studies used to create toxicology reference values include only one or two endpoints and by omission assume no effects at other stages particularly for early breeding failure. For example, in the white ibis experiment most of the failures were in the earliest stages, and might not have been accounted for in the studies that were used to create dose-response curves. Indeed, if we excluded early failures, our predicted decreases in fledglings produced by great egrets (Fig. 3B, D) fit in most cases within the predicted range of reductions expected from using toxicity reference values (Fuchsman et al., 2017).

The absolute differences in the reduction of numbers of fledglings produced between high and low food availability scenarios was large (<0.5 fledglings), and the proportional differences between offspring expected in high/low food conditions was huge (25–100 % less; Fig. 2B). Reproductive losses also took place at different endpoints (Fig. 3). For both, observational and experimental data sets, early failures accounted for nontrivial amounts of predicted reduction in total fledglings under mid-high Hg exposures in all food availability conditions. While early failure in egrets may itself be largely insensitive to food availability (Zabala et al., 2020a), when expressed as a proportion of total failure, it may be indirectly related to food conditions. For instance, under low food availability and high Hg exposure conditions, the contribution of early failures to total reproductive losses were diminished simply because hatching success took on an overwhelming role.

Post-hatching losses of egret offspring under high Hg and low food availability were negligible, in large part because most of the losses had already occurred early in the breeding cycle. Conversely, early failures were comparatively low under high food and Hg, and those were the only conditions under which clutch size reduction showed any importance (Fig. 3). In general, in low food availability scenarios, reproductive losses took place early in the breeding cycle. The combination of nutritive stress and Hg toxicity might make parents more prone to abandon, or might induce aberrant nest attendance (Evers et al., 2008) leaving the nest and exposing eggs to cold or heat for long periods, killing the embryos. These results highlight that we currently lack a solid ecological, biological or physiological explanation for this commonly reported pattern of pre-hatching failure in response to Hg exposure.

Food availability appears to strongly influence the effect size, and the timing of Hg related reductions in reproductive output. If food availability is variable among studies, it could strongly affect the importance of each endpoint. In consequence, these results strongly suggest that comparisons of Hg effects across studies or situations based on a single or a few reproductive endpoints should be carefully regarded. Another consequence of these findings is that, at least for great egrets, there is no single best endpoint to assess Hg in the field. Thus, studies not accounting for the whole breeding cycle could easily underestimate the importance of Hg to reproduction, or its relative importance at any endpoint, depending on food availability, and perhaps other stressors (Brasso and Cristol, 2008; Hallinger and Cristol, 2011; Zabala et al., 2021). A particular feature of the great egret results is the change in reproductive success predicted at low Hg exposures, compared to that at medium exposures, especially the initial increase predicted in low food availability conditions. In field conditions, virtually all body Hg of nestlings comes from Hg in food (Spalding et al., 2000). Thus, low feather [Hg] can indicate intake of food with low Hg or intake of low biomass of food that contains average Hg levels. In consequence, reduced reproductive success expected at low Hg exposure and low food availability probably reflects negative effects of malnourishment rather than hormetic effects of Hg (Heinz et al., 2010). We also note that failure at different stages might be compensatory rather than additive. For instance, fledgling losses associated with Hg under high food availability might have been abandoned as eggs, or breeding failed at even earlier stages, if food availability had been lower. Many of the reproductive endpoints discussed (clutch size, hatch success, nestling survival) can be

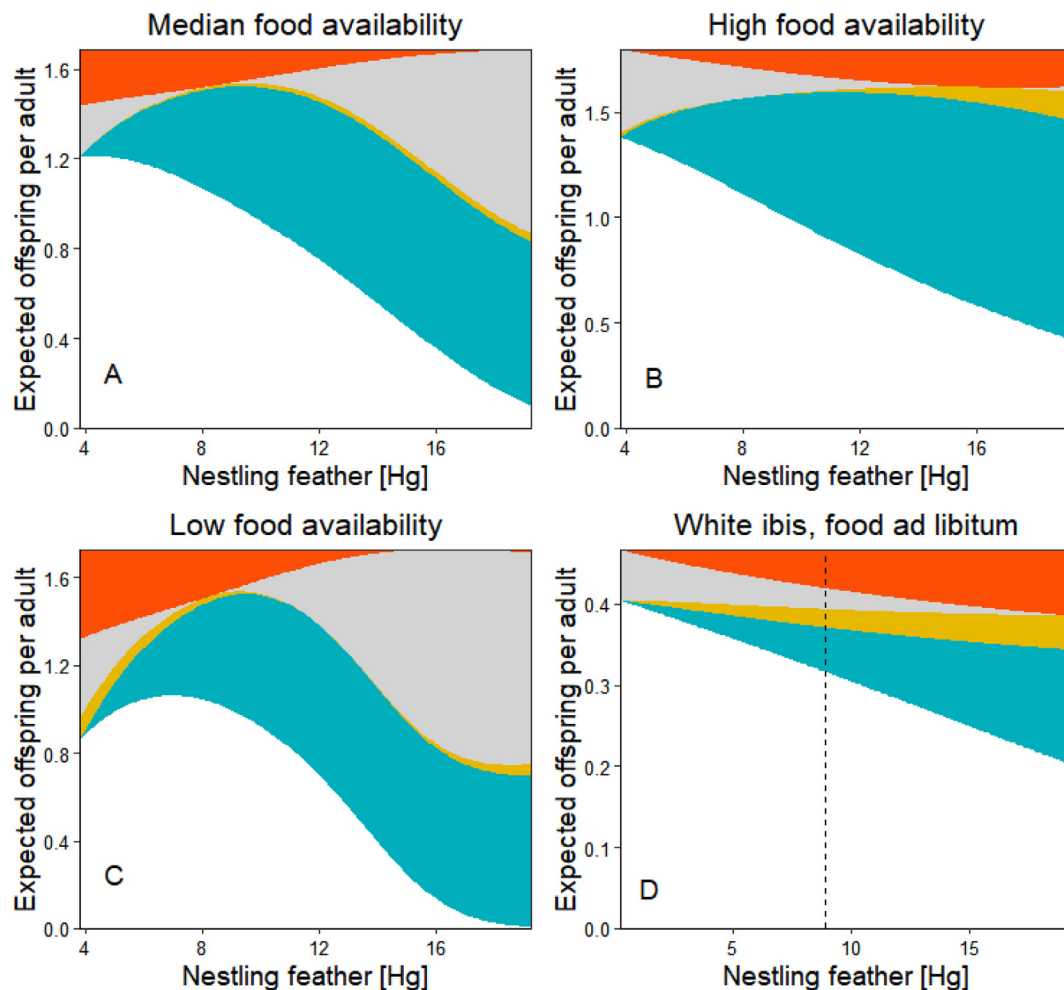


Fig. 3. Expected offspring of great egrets at different food availability conditions (A, B and C; observational study) and white ibis (D, experimental study) at different Hg exposures and timing of failures. In all plots (starting from bottom to top) white areas indicate successfully fledged offspring; blue areas potential offspring lost as early failures; yellow areas offspring lost due to clutch size reduction; gray areas hatching failures, and red areas, at the top of the plot, post-hatching failures –chicks that successfully hatched but died before reaching the fledgling state. The curved shape at low dose of the egret plots are putatively a consequence of the association between food intake and Hg in food in field conditions and the models might partially confound low exposure with starvation (see text). Note that the scale varies among plots depending on the maximum number of offspring expected in each scenario. The dashed vertical line in the white ibis plot indicates maximum experimental dose and predictions beyond those exposures are predications for unobserved conditions. Hg in nestling feathers indicates THg $\mu\text{g/g dw}$.

integrated into a single measure of fledglings produced per nesting attempt, and so capture the reproductive cycle in a single measure. However, the ability to identify nesting attempts prior to egg laying is generally difficult unless one has a lot of prior data and modeled results or is somehow capable of estimating number of potentially breeding pairs (known numbers of breeding territories or similar). Our study highlights the importance of this stage to reproductive failure, and suggests a need for better methods for measuring this effect.

There are several limitations to our models and results, particularly to the estimated distribution of failures in different scenarios. First, the plot of white ibis experimental exposure (Fig. 3.D) is in several cases based on models that were not statistically significant. In our opinion, that lack of significance does not represent a lack of effect but weak effects and trends that are difficult to detect because of relatively low sample size (Johnson, 1999), and the fact that any effect of food is masked by *ad libitum* feeding. While sample size and variability in food availability was constrained for the ibis results, the great egret models predicted strong responses of endpoints to Hg in median or low food conditions but flat responses to the same exposure to Hg in high food availability (Zabala et al., 2021). This suggests that the weak-non significant trends in white ibis exposed to moderate Hg levels with *ad libitum* food may have been real rather than artifactual.

Another limitation is that our great egret models are based on Hg in nestling feathers. If early failures affect a subset of pairs with higher Hg exposure, those nests would never be expected to produce chicks old enough to sample feathers from, and the use of nestling feathers could therefore underestimate exposure to Hg (Zabala et al., 2019a). Further, the association between Hg in nestling feathers and early endpoints is weaker than with later endpoints (Zabala et al., 2021). Nonetheless, in the studied colonies and within a range of nestling feather [Hg] of 5.26–19.34 mg/g dw , very similar to the used in this paper, average [Hg] in nestling feathers and egg albumen showed a good correlation ($r^2 = 0.703$; Zabala et al., 2019b).

Observational studies have reported several other natural stressors that seemed to mediate Hg effects, such as temperature (Hallinger and Cristol, 2011) or age-related effects (Brasso and Cristol, 2008). Apart from its influence in recession range, weather during the breeding season, namely temperature and high wind conditions and heavy rainfall during storms, did not seem to affect the probability of egrets reaching the egg laying stage in the study population during the study period (Zabala et al., 2020a). We could not estimate age of adult great egrets breeding in Everglades colonies, but in captive ibises, age was known, and was a significant factor. Thus, it is likely that those and other environmental stressors unaccounted for are adding noise to our estimates and reducing their accuracy. Thus, our absolute estimates of endpoint effects are likely to be

somewhat inaccurate, but unbiased, suggesting the direction and relative magnitude of effects can be trusted. Another limitation of our models is that we lack a framework to interpret variation in food availability. Although our fish biomass estimates are sound and extensive, we cannot ascertain what they mean for great egrets in terms of individual nutritional status and we have no way of translating them to other systems. Similarly, the variation in recession range captures a particular dynamic of Everglades food availability that is difficult to apply to other systems directly. Many species rely on pulsed resources for breeding (Chevallier et al., 2020; Frederick and Ogden, 2001; Ostfeld and Keesing, 2000; Schmidt and Ostfeld, 2003; Zabala et al., 2020b), but the nature, dynamics and amplitude of these pulsed cycles are not comparable. In any case, we emphasize the internal comparability of our results and value of reported trends and variations in relative importance of failure between endpoints in different food availability conditions, rather than the exact estimates of the proportions of failures.

The results of this study suggest that the pattern and timing of reproductive impairment is strongly driven by an interaction between contamination and food availability, and potentially other natural stressors, that affects multiple endpoints of the reproductive cycle differentially. Our results show that failures associated with exposure to MeHg take place during the whole breeding cycle but they are not equally distributed along it. Therefore, studies measuring a single or a few endpoints are likely to result in strong underestimated of overall impairment. This is particularly concerning in observational studies since net effects and the relative importance of different endpoints can change depending on environmental conditions, which may vary temporally and spatially. In our great egret case, the same MeHg exposure can be associated with high hatching failures under low food availability while in high food conditions hatching failures would be small but nestlings losses relevant. Therefore, results of observational studies of contaminant associated effects using only one, or a few reproductive endpoints, may produce highly variable results and often may result in misleading impressions of when failure takes place or what are the most sensitive endpoints.

We found strong net Hg effects on reproductive success that at least doubled effects predicted from reference values (Fuchsman et al., 2017). That was probably because studies used to create toxicity reference values often assessed a single or a few endpoints, rather than cumulative effects over the whole breeding cycle, and many studies might have failed to detect effects or underestimated effects because they did not assess some of the most relevant endpoints. Therefore, current reference values probably underestimate Hg effects on reproduction and Hg risk in the field. While the exact mechanisms underlying this problem may change with species and ecosystem, we suggest it is likely that incomplete measurement of effects throughout the reproductive cycle, and a lack of understanding of other stressors may consistently result in underestimates of contaminant effects in free ranging animal populations.

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CRediT authorship contribution statement

Jabi Zabala: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Joel C. Trexler:** Data curation, Methodology, Funding acquisition, Project administration, Validation, Resources, Writing – review & editing. **Nilmini Jayasena:** Methodology, Investigation, Validation, Writing – review & editing. **Peter Frederick:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Validation, Resources, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

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

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