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Proc. R. Soc. B 2011 **278**, 1851-1857 first published online 1 December 2010
doi: 10.1098/rspb.2010.2189

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Proc. R. Soc. B (2011) **278**, 1851–1857
doi:10.1098/rspb.2010.2189
Published online 1 December 2010

Altered pairing behaviour and reproductive success in white ibises exposed to environmentally relevant concentrations of methylmercury

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Methylmercury (MeHg) is the most biologically available and toxic form of mercury, and can act as a powerful teratogen, neurotoxin and endocrine disruptor in vertebrates. However, mechanisms of endocrine impairment and net effects on demography of biota are poorly understood. Here, we report that experimental exposure of an aquatic bird over 3 years to environmentally relevant dietary MeHg concentrations (0.05–0.3 ppm wet weight) resulted in dose-related increases in male–male pairing behaviour (to 55% of males), and decreases in egg productivity (to 30%). Dosed males showed decreased rates of key courtship behaviours, and were approached less by courting females in comparison to control males. Within dosed groups, homosexual males showed a similar reduction when compared with dosed heterosexual males. We found an average 35 per cent decrease in fledgling production in high-dose birds over the study duration. These results are of interest because (i) MeHg exposure is experimentally tied to demographically important reproductive deficits, (ii) these effects were found at low, chronic exposure levels commonly experienced by wildlife, and (iii) effects on reproductive behaviour and sexual preference mediated by endocrine disruption represent a novel and probably under-reported mechanism by which contaminants may influence wild populations of birds.

Keywords: methylmercury; sexual behaviour; ecotoxicology

1. INTRODUCTION

Humans and wildlife are increasingly exposed to contaminants of anthropogenic origin, yet causal mechanisms relating exposure levels to effects on reproduction or population structure are established for only a few chemicals and species [1]. Methylmercury (MeHg) is the most biologically active form of mercury (Hg) and is a globally distributed contaminant [2]. Exposure to MeHg in vertebrates results in neurotoxicity [3,4], embryotoxicity [2,5], impaired physiological function [6,7], endocrine disruption [8,9] and altered reproductive behaviour. Species in upper trophic levels in aquatic environments are generally considered to be at high risk of exposure owing to high bioaccumulative potential in these habitats [2,10], and predaceous aquatic birds have often been used as models of MeHg effects. Although there are large differences in susceptibility among species [11], studies of aquatic birds suggest that MeHg exposure at environmentally relevant levels can alter breeding propensity and reproductive success [8,12–14], depression of egg-laying and hatching success [5], increased incidence of developmental abnormalities [2–4] and altered parental and chick behaviour [15,16]. However, there may also be apparent hormetic effects in some species at some exposure levels [17]. Although MeHg is documented to disrupt endocrine function in vertebrates, the mechanisms and net effects on reproduction are poorly understood [9].

In this study, we measured effects of MeHg exposure on courtship, pairing behaviour, breeding propensity and breeding success in large groups of captive white ibises (*Eudocimus albus*) in an experimental setting. MeHg-exposed ibises have shown altered testosterone and oestradiol levels in the field [8] and laboratory [18], and breeding population size is inversely correlated with annual MeHg exposure in south Florida, USA [8]. Here, we report that chronic exposure at dietary MeHg levels commonly encountered by wild birds resulted in altered courtship behaviour in males, high levels of male–male pairing and reduced reproductive success in pairs that did raise young.

2. MATERIAL AND METHODS

(a) *Aviary set-up and dietary MeHg exposure*

Nestling white ibises were collected from breeding colonies in south Florida in April 2005, and randomly assigned to one of four treatment groups (20 of each sex per group). The birds were kept outdoors in a 1200 m^2 circular, free-flight aviary divided into four quadrants by net walls. The circular design minimized location effects, and ensured similar drainage, exposure to disturbance and lighting. Each treatment group was provided with six perch modules with 48 nest platforms in a similar spatial configuration.

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2010.2189 or via http://rspb.royalsocietypublishing.org.

Received 11 October 2010
Accepted 29 October 2010

We provided an ad libitum supply of twigs (*Quercus* spp.) and fresh cattail leaves (*Typha* sp.) as nesting material. Nesting in the wild is typically in very dense aggregations (inter-nest distances $\bar{x} = 0.69$ m, $n = 30$, s.d. $= 0.276$ m, [19]), and the inter-nest distances and breeding space we provided were larger than these measures. We recorded greater than 10 per cent unoccupied breeding spaces in each cage during all breeding seasons over the experimental period.

Exposure to disease agents was probably similar since the common net walls allowed free passage of mosquito vectors, surface water and direct bill to bill contact by individuals. Each group of birds was moved to a new cage location in a randomized order in October of each year (electronic supplementary material, figure A1). As ibises are colonial nesters, it was necessary to keep them in dense groups to stimulate breeding. This meant that individuals or pairs within a group-treatment were not truly independent of one another, though we have regarded them as such for statistical analyses and acknowledge that pseudoreplication [20] is a potential source of undesired bias in this experiment. All birds were genetically sexed (Avian Biotech International, Tallahassee, FL, USA), and wore individually identifiable leg-bands.

MeHg exposure was started when birds were 90 days old and continued through 2008 [18]. Prior to this age, young birds were still being fed a nestling diet in which it was difficult to introduce MeHg. Young birds in the wild are typically dependent upon parental feedings until they leave the nest area at approximately 50 days of age, and by 90 days have well-developed flight abilities and nearly adult proportions. While gonads, size and proportions clearly distinguish sexes at this age, full expression of sex steroids probably does not occur until after the first year of life [21]. Dietary MeHg exposure rates used in this study spanned the range found in prey of ibises in the Everglades during the mid-1990s [22,23]. Low (L), medium (M) and high (H) treatment groups corresponded to 0.05, 0.1 and 0.3 ppm wet weight (ww) MeHg in diet, respectively. MeHg was sprayed onto pelletized feed in a mixer using a corn oil vehicle (Flamingo and custom Ibis diets, Mazuri Company, Brentwood, MO, USA). Control (C) birds received pellets sprayed with the corn oil vehicle alone. MeHg concentration in pelletized food was measured as total Hg (THg) from every batch of food. Identity of dose levels was blinded to all observers, handlers, feeders and animal care staff throughout the duration of the study.

Scapular feather samples were analysed for THg levels each January using cold vapour atomic absorption spectroscopy by the Florida Department of Environmental Protection (Tallahassee, FL, USA) using a modified version of the USEPA method 245.6 (Standard Operating Procedure number HG-006-3.14). We also measured THg in blood from a random sample of 10 birds per treatment (five from each sex) in 2008, using direct Hg analysis.

(b) *Behavioural observations during the breeding season*

On each day during the breeding season, we noted the identity of all individuals displaying, nest-building, incubating, brooding or chick-rearing, and the contents of each nest. Courtship behaviour was observed intensively in 2008 from the start of courtship in each nest through cessation of nest-building. Each group (= treatment cage) was observed for 40 min within 2 h of sunset when courtship rates are typically at maxima [24]; two groups were observed each day by one observer each. Order of group observation within any 4 day period was randomized. Observations were begun after a habituation period of 5 min, from a point 8 m outside each cage edge using 16–48 × 65 mm telescopes. We recorded numbers of head bobs, pair bows and aggressive acts of individual displaying males, as well as identity of birds approaching each displaying male. Head bobbing is a courtship display performed early in the display period [25]. Females approach displaying males and if accepted by the male, the pair will bow together. Aggressive acts were defined as pecking/jabbing oriented towards approaching birds. Only data from the first nesting attempt of each displaying male were used in analyses of courtship behaviour. For each male, we averaged rates of courtship behaviours recorded during the 14 days prior to nest-initiation. The resulting database was from 4960 min of observation on 31 days.

(c) *Statistical methods*

R statistical software v. 2.10.0 [26] was used for all analyses. Results with a p-value of greater than 0.05 were considered significant unless otherwise stated. We used Fisher's exact tests to examine differences among groups in numbers of nests that did not contain eggs (='unproductive nests'), numbers of homosexually nesting birds and proportion of males accepting courtship approaches by males. We used analyses of variance (ANOVA) to test for differences in nest-initiation dates of first nesting attempts by MeHg-treatment group (=treatment) and type of pair bond (male–male or male–female).

We used χ^2-tests to examine differences in total numbers of heterosexual and homosexual pair days (no. pairs × numbers of days each pair active) by treatment for each year. Generalized linear models (GLMs; binomial distributions with logit links) were used to test whether pairing type of the previous breeding season affected whether birds switched partners in the subsequent season.

GLMs with Poisson or negative binomial errors (when data were over-dispersed) were used to model each behaviour by treatment or pairing type. Effects of MeHg treatment were only examined in males that subsequently paired heterosexually. Effects of pairing type were examined only within dosed groups. Quantile–quantile plots, residual plots and the Shapiro–Wilk statistic were examined to determine whether statistical assumptions were met. Fisher's exact tests were used to determine whether the number of males that accepted advances by males differed according to pair type.

Fisher's exact test was used to compare numbers of males or females that failed to produce hatched eggs in at least one of their first two nesting attempts in 2007 and 2008. More than two nesting attempts per season are very rare in the wild [24]. GLMs (with quasi-Poisson distributions) were used to test for treatment effects on the total number of fledglings and the total number of successful breeding attempts per individual female over all 3 years.

3. RESULTS

(a) *Hg levels in feathers and blood*

Mean THg levels in scapular feather samples of ibises showed clear treatment effects in all 3 years, varying between 0.47 and 51.3 ppm fw (table 1). Mean blood

Table 1. Total mercury concentrations in feather and blood samples of white ibises exposed to different levels of dietary methylmercury. All concentrations are on fresh-weight (fw) basis; s.d., standard deviation.

total mercury ($mg\ kg^{-1}$ fw)	year	control		low		medium		high	
		mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
feathers	2006	0.74	0.25	7.15	2.60	15.24	8.65	23.86	8.77
	2007	0.47	0.11	8.20	1.53	14.13	5.92	51.32	12.33
	2008	0.62	0.21	4.31	1.28	17.96	9.15	35.04	16.94
blood	2008	0.07	0.01	0.73	0.09	1.60	0.32	3.95	0.68

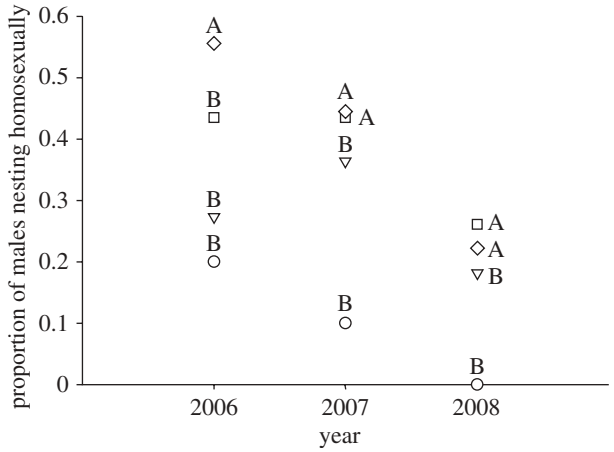

Figure 1. Proportions of males nesting homosexually by treatment in each breeding season. Circles, control; inverted triangles, low; squares, medium; diamonds, high.

Hg showed a similar dose-dependent response, varying from 0.07 (control) to 3.95 ppm ww.

(b) *Unproductive nesting and male–male pairing*

All three dosed groups had significantly more unproductive nests than the control, both when all nesting attempts in all years were combined (Fisher's exact tests, L: $p = 0.002$; M: $p = 0.001$; H: $p = 0.001$), and when only first nesting attempts were considered (Fisher's exact tests, L: $p = 0.004$; M: $p = 0.004$; H: $p = 0.003$). The loss of productivity owing to lack of egg-laying when compared with controls over all 3 years was 13.2 per cent, 14.6 per cent and 13.5 per cent for L, M and H, respectively. The percentage of total unproductive nests that was owing to homosexual pairing were 73.7 per cent, 91.3 per cent and 77.8 per cent for L, M and H, respectively. Degree and persistence of homosexual pairing increased with MeHg exposure. There were significantly higher proportions of homosexual males in H than C in all 3 years and significantly more in M than C in 2007 and 2008 (figure 1). Homosexual pairs in any group initiated nest-building significantly earlier than heterosexual pairs (ANOVA, $p < 0.05$; electronic supplementary material, table A1). A significantly larger proportion of the total pair days in the breeding season was occupied by unproductive male–male pairs in each of the dosed groups than in the control group in all years (χ^2-tests, all p-values < 0.0001). Homosexual males were significantly less likely to switch partners from 1 year to the next compared with heterosexual males, irrespective of treatment group (GLMs; 2006–2007: $p = 0.0024$; 2007–2008: $p = 0.0033$). The sex ratio was not significantly different from parity in any treatment-year combination (χ^2-tests, all p-values $\gg 0.05$).

(c) *Courtship behaviour*

Dosed males showed significant dose-related reductions in key courtship behaviours (head bobbing and pair bowing) compared with C males (GLMs, $p \leq 0.01$; figure 2*a*,*c*). H males were significantly less likely than C males to be approached by females (GLM, $p = 0.04$; figure 2*e*). There were no significant differences in the rate of aggression between treatment groups (GLM, $p > 0.05$).

Within dosed groups, homosexual males head bobbed and pair bowed significantly less often than heterosexual males (GLMs, $p \leq 0.001$; figure 2*b*,*d*). Dosed homosexual males were less aggressive (GLM, $p = 0.008$) and less likely to be approached by females (GLM, $p = 0.001$; figure 2*f*) than dosed heterosexual males. Dosed homosexual males were also more likely to be approached during courtship by males than dosed heterosexual males (GLM, $p = 0.0006$). However, courtship displays of displaying and approaching homosexual males were male-typical behaviours and neither displayed a female-typical behavioural role. Within dosed groups, a significantly lower proportion of homosexual males acted aggressively towards males who approached them during courtship than did heterosexual males (Fisher's exact test, $p = 0.0076$, see also electronic supplementary material, tables A2 and A3).

(d) *Production of nestlings*

Nestling production by dosed heterosexual males was significantly lower than by C heterosexual males in 2007 and 2008 (Fisher's exact tests; 2007: C versus L: $p = 0006$; C versus M: $p = 0.002$; C versus H: $p = 0.04$; 2008: C versus L: $p = 0.049$; C versus M: $p = 0.022$; C versus H: $p = 0.017$). Nestling production by dosed females was significantly lower than C females in L and H in 2007 (Fisher's exact tests; C versus L: $p = 0.003$; C versus M: $p = 0.49$; C versus H: $p = 0.049$); and in H females in 2008 (Fisher's exact tests; C versus L and M: $p = 1$; C versus H: $p = 0.009$).

There were no significant differences between numbers of dosed and C females fledging at least one young in 2007 or 2008. Nor were there significant differences in total numbers fledged per female over the entire period (GLM, $p > 0.05$; electronic supplementary material, table A4). While these comparisons were not significant, H females fledged 34.8 per cent fewer young per female than C (GLM, $p = 0.085$; electronic

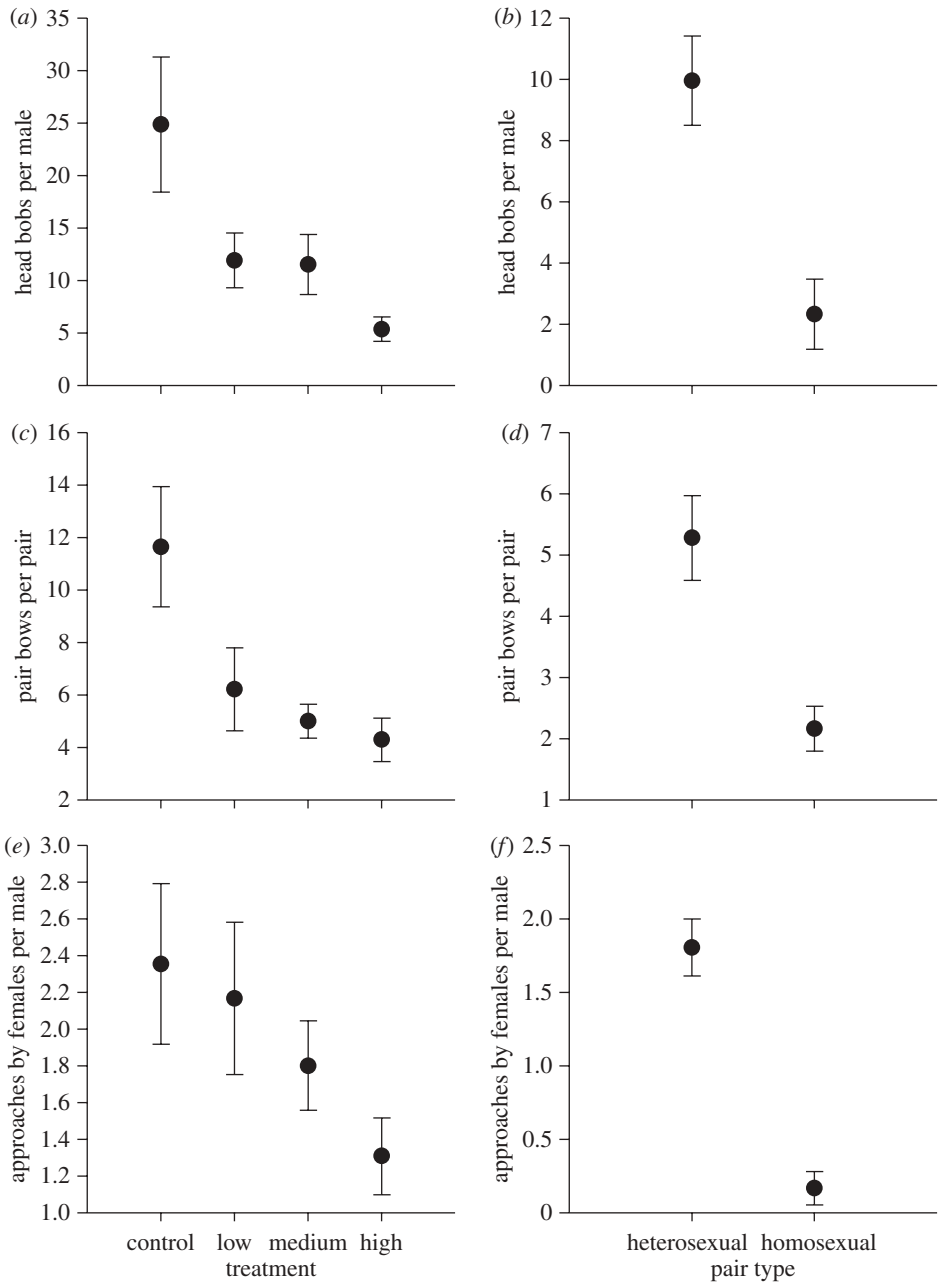

Figure 2. Average rates of courtship behaviours per male per 40 min observation sessions; error bars show ± one standard error from the mean. (*a*) Average head bobbing rate in heterosexual males by dose group. Differences were significant between control and each dosed group (GLM, $p \leq 0.01$). (*b*) Average head bobbing rate by pairing type. Rates were significantly different between pairing types (GLM, $p \leq 0.01$) (*c*) Average pair bowing rate in heterosexual pairs by treatment group. Rates were significantly different between control and each dosed group (GLM, $p < 0.001$). (*d*) Average pair bowing rate by pairing type. Rates were significantly different between pairing types (GLM, $p \leq 0.01$). (*e*) Average number of approaches per heterosexual male from females by treatment group. Significant differences were found between control and high dose groups (GLM, $p = 0.0425$). (*f*) Average number of approaches from females per dosed male, by pairing type. Rates were significantly different between pairing types (GLM, $p = 0.001$).

supplementary material, table A4) and L females fledged 33.5 per cent fewer young per female than C females (GLM, $p = 0.10$).

4. DISCUSSION

Male–male pairing contributed a large proportion of the reproductive deficits documented in this study, yet to our knowledge, this mechanism has not been reported as an effect of MeHg exposure or of other contaminants. In experimental studies of reproductive effects of MeHg to date, mates were pre-assigned, and mate choice, therefore, was not a measured endpoint [15,27]. Male–male pairing behaviour has been reported extensively in many species in both natural and captive settings (e.g. [28,29]) but is most commonly associated with strongly skewed sex ratios or mating opportunities [28,30]. In this study, numbers of potential mates were robust in any group, and sex ratio was not significantly different from parity. Further, homosexual pairing occurred early in the season, when many unpaired females were available. In a wild colony of white ibises with

minimal exposure to MeHg, there were no male–male pairings observed in 134 white ibis pairs studied over 15 580 pair hours of observation during four breeding seasons [31]. The incidence of a few homosexual males in the control group may have been an effect of captivity and/or social environment [32]. However, our results indicate that this effect is significantly exacerbated by MeHg exposure and there is no obvious reason why alteration of the same key behavioural pathways would be unaffected by MeHg exposure in wild populations.

The mechanism linking MeHg exposure to male–male pairing is unknown, but may be mediated through behavioural and endocrine processes. Reduced male display rates were probably an important reason why female approaches were markedly reduced towards dosed and especially homosexual males during courtship. Reduced display rates may have been part of a general reduction of activity associated with MeHg exposure [33], or an effect of impaired learning [34]. Sexual display behaviour in birds is also strongly influenced by circulating steroid hormone levels [30], and in this study, MeHg exposure was associated with a de-masculinized pattern of oestradiol and testosterone expression in males, especially during courtship [18].

Avian display behaviour and sexual preference appear to be controlled through decoupled and independent processes [30]. Altered sexual preference appears to have contributed to ibis male–male pairing, since males paired readily with one another early in the breeding season when females were available, and often approached one another during courtship. Sexual preference in birds is influenced by organizational changes in brain and receptor function, determined at some point during the developmental process [30,32,35]. The ibises in this study were not exposed to MeHg until 90 days of age, suggesting that if neuroendocrine organization is altered, it may be occurring fairly late in the developmental sequence.

A number of contaminants with xenobiotic activity have been shown to affect sexual behaviour, sex ratios, development of secondary sexual characteristics and altered profiles of sex hormones in a variety of animals [36–44]. Same-sex pairing is much less often reported, and is rarely reported as a consequence of contaminant exposure in birds. Exposure of California gulls (*Larus occidentalis*) to organochlorines was associated with a strongly female-biased sex ratio and related high incidence of female–female pairs [45,46]. Our study is somewhat different in that pairing patterns appeared to be altered directly by action of the contaminant, and the pattern was generated in part by altered sexual display behaviour rather than a change in sex ratio. The ibis example, therefore, suggests a novel mechanism by which contaminants may affect reproduction.

In this study, reproductive output was decreased by Hg administration both through homosexual behaviour (average 13–15% over 3 years) and as a result of reduced numbers of fledglings raised by dosed heterosexual pairs (33–35%). While the latter result was not significant ($p = 0.10$ and $p = 0.085$ for low- and high-dose groups, respectively), the two sources are clearly additive, and a worst-case scenario suggested by our results could therefore involve up to 50 per cent reduction in fledglings owing to MeHg exposure at 0.01–0.3 ppm ww in diet. These estimates may be conservative. If male–male pairing occurred in the wild with an assumed sex ratio of one, it would remove homosexual birds from productive breeding and induce a shortage of partners for females, particularly early in the breeding season. In the aviary situation, up to four breeding attempts were possible each season and nearly all females eventually were able to breed each year. In the wild, only one or two breeding attempts are possible, and the effect of homosexual breeding would therefore be considerably magnified compared with the captive situation.

The exposure levels we used in this study span the exposure rates reported for several avian studies, suggesting that our findings are relevant to many free-ranging bird populations [14,22,47]. MeHg exposure may therefore routinely lead to altered demographic patterns in wild bird populations. MeHg-induced reproductive deficits in birds have until now been attributed to altered parental behaviour or embryonic death. Our results demonstrate that a sizeable proportion of net reproductive deficits can result from effects of MeHg exposure on sexual behaviour and/or sexual preference of adults.

We thank T. Atkeson, D. Axelrad, G. Heinz and M. Avery for fruitful discussions leading to this work. This research was supported by grants from Florida Department of Environmental Protection, US Fish and Wildlife Service and US Geological Survey.

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