

Food availability is expressed through physiological stress indicators in nestling white ibis: a food supplementation experiment

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Summary

1. Physiological responses to environmental stress such as adrenocortical hormones and cellular stress proteins have recently emerged as potentially powerful tools for investigating physiological effects of avian food limitation. However, little is known about the physiological stress responses of free-living nestling birds to environmental variation in food availability.

2. We experimentally tested how hydrologically mediated changes in food availability affect the physiological stress responses of juvenile white ibises *Eudocimus albus* in a fluctuating wetland. We provided supplementary food to free-living nestlings during 2 years with contrasting hydrologic and food availability conditions, and used plasma (PCORT) and faecal (FCORT) corticosterone and heat shock proteins (HSP60 and HSP70) from first-hatched (A-nestlings) and second-hatched (B-nestlings) to detect relatively short- to long-term responses to food limitation.

3. Nestling physiological stress responses were relatively low in all treatments during the year with optimal food availability, but PCORT, FCORT and HSP60 levels increased during the poor food year. FCORT and HSP60 responses were clearly due to nutritional condition as elevated concentrations were evident primarily in control nestlings. Significant year by hatch order interactions for both FCORT and HSP60 revealed that these increases were largely incurred by B-nestlings. FCORT and HSP60 responses were also well developed early in neonatal development and remained elevated for the duration of the experiment suggesting a chronic stress response. PCORT and HSP70 were less informative stress responses.

4. The nutritionally mediated increases in FCORT and HSP60 provide compelling evidence that white ibis nestlings can be physiologically affected by environmental food levels. FCORT and HSP60 are effective indicators of nutritional mediated stress for nestling white ibises and potentially for other species prone to capture or handling stress.

Key-words: corticosterone, Everglades wetlands, *Eudocimus albus*, feeding experiment, food limitation, heat shock proteins, wading birds

Introduction

Limited nutrients during avian postnatal development can increase offspring mortality through starvation, affect fitness related traits at adult-hood, and ultimately play a profound role in avian population dynamics (Martin 1987;

Newton 1998). Identifying those responses of neonatal birds that react predictably to variations in food supply is a critical first step for examining the underlying environmental drivers of food limitation, and ultimately for designing appropriate management strategies for recovering declining species. However, detecting food limitation is often problematic as the phenotypic and fitness consequences of nutritional deficit traditionally examined in food limitation studies (e.g. growth and fledging success) may vary unpredictably with resource availability (Williams & Croxall 1990), or may be masked by compensatory mechanisms with unknown fitness costs (Metcalf & Monaghan 2001).

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To further understand the role that food availability plays in avian ecology, ecologists are increasingly examining physiological functions as these have the potential to respond directly and predictably to environmental conditions, and may avoid some of the issues associated with traditional approaches (Kitaysky *et al.* 1999).

Prominent among these physiological responses is the vertebrate hypothalamic-pituitary-adrenal (HPA) stress response, which in birds results in increased circulating levels of the hormone corticosterone during episodes of environmental stress (Wingfield & Ramenofsky 1999). Corticosterone initiates the breakdown of stored energy through the catabolism of protein (Axelrod & Reisine 1984), and in juvenile birds can effect behavioural changes related to food acquisition such as increased begging rates and aggression (Ramos-Fernandez *et al.* 2000; Kitaysky, Wingfield & Piatt 2001; Kitaysky *et al.* 2003). These responses function to reduce the negative impacts of short-term nutritional stress (Wingfield, O'Reilly & Astheimer 1995). However, the ontogeny of the HPA response is highly variable among species: for some species the HPA axis is both well developed and responsive at, or relatively soon after, hatching (Kitaysky *et al.* 1999; Sockman & Schwabl 2001), while in others it develops slowly (Walker, Wingfield & Boesma 2005) or is delayed or uncoupled until maturity (Romero, Soma & Wingfield 1998; Sims & Holberton 2000; Love, Bird & Shutt 2003; Sears & Hatch 2008). Thus, corticosterone is potentially useful as a measure of neonatal nutritional condition, but its utility is likely species-specific and reliant on the development pattern of the HPA response. While a limited number of studies have examined HPA responses of free-living nestlings in relation to environmental quality (Blas *et al.* 2005), brood size manipulations (Lobato *et al.* 2008) and experimental food reduction (Saino *et al.* 2003), the role of natural variation in environmental food availability remains largely unresolved.

An alternative but less utilized group of physiological parameters for measuring nutritional condition are the stress proteins, a set of highly conserved intracellular polypeptides (Linguist 1986; Bierkens 2000) that function as molecular chaperones for proteins within cells (Tomás, Martínez & Merino 2004). During periods of increased stress the molecular chaperone role is amplified to minimize cell protein damage (Locke & Noble 1995; Råberg *et al.* 1998). Their delayed response relative to plasma corticosterone (Burel *et al.* 1992) suggests that they better indicate chronic long-term stress (Martínez-Padilla *et al.* 2004; Tomás, Martínez & Merino 2004) and may be independent of capture stress. Recent studies evaluating the effectiveness of stress proteins to examine physiological condition in avian ecology (Moreno *et al.* 2002; Martínez-Padilla *et al.* 2004) suggest that they can be a valuable metric of stress. However, our understanding of neonatal stress protein responses to food limitation is limited to that of studies manipulating brood size, an indirect measure of environmental food availability (e.g. Merino *et al.* 2006).

Here, we experimentally evaluate the influence of food availability on the nestling physiological stress responses of a



Fig. 1. White ibis adult provisioning nestlings in the Arthur R. Marshall Loxahatchee National Wildlife Refuge.

declining wading bird (Ciconiiform), the white ibis *Eudocimus albus* Linnaeus (Fig. 1), in a large, hydrologically pulsed freshwater wetland, the Florida Everglades. Food availability for wading birds in pulsed wetlands is primarily a function of the abundance, density, and timing of seasonal concentrations of aquatic prey (fish and macroinvertebrates), which in turn are driven by dynamic hydrologic processes such as water depth and water level recession rate (e.g. Kushlan 1981a; Gawlik 2002). Anthropogenic modifications to the hydrology of the Everglades that began in the 1940s (Ogden, Davis & Brandt 2003) have radically altered the timing, magnitude and predictability of prey concentration events. The white ibis is considered particularly sensitive to these fluctuations in food resources because it is a specialized tactile forager, reliant on a constant source of high quality foraging patches (Kushlan 1977a). Nesting effort in the Everglades declined by c. 87% from 1939–1940 to 1990–2000 (Crozier & Gawlik 2003).

We provided supplementary food to free-living white ibis nestlings during two breeding seasons with contrasting hydrologic conditions and concomitant high and low environmental food availability (e.g. Beerens 2008), and examined the effects of food availability on several physiological stress parameters that potentially reflect relatively short-term [plasma corticosterone (PCORT)], medium-term [faecal corticosterone metabolites (FCORT)], and long-term [heat shock protein 60 (HSP60), heat shock protein 70 (HSP70)] responses to food limitation. These measurements were part of a larger experiment where we also quantified the growth, behaviour and survival of nestlings (M. I. Cook, unpublished data). We expected white ibis nestlings to exhibit elevated

levels of corticosterone and stress proteins in response to limited food availability. Specifically, we predicted that each physiological stress response would increase with decreasing food consumption such that supplemented nestlings would show decreased stress levels compared to controls and, due to hatching asynchrony, first-hatched A-nestlings would be less stressed than smaller and competitively inferior younger B-nestlings (e.g. Mock & Parker 1998). We examined the stress levels at two ages to examine the maturation and persistence of the stress responses.

Materials and methods

STUDY SPECIES

The white ibis exhibits a flexible breeding period, colonial nesting behaviour, and bi-parental care. In the Everglades, breeding occurs primarily in the dry season from March to May when hydrologic conditions and food availability are relatively conducive to successful reproduction. Females lay a clutch of 2–4 eggs, which they incubate for *c.* 21 days and hatch asynchronously 1–2 days apart. Nestlings are semi-altricial, nidicolous until approximately age 20 days, and fledge at between 50 and 60 days (Kushlan & Bildstein 1992).

STUDY AREA AND PREY SAMPLING

The supplementary feeding experiment was conducted at a large wading bird colony (> 5000 white ibis nests) in the northern Everglades within the Arthur R. Marshall Loxahatchee National Wildlife Refuge (Lox), Palm Beach County, Florida (26°31'N, 80°17'W) during April to May 2006 and 2007. The location of the colony moved slightly between years (1.6 km) but since white ibis forage on average about 10 km from a colony (Beerens 2008) both sites encompassed the same general foraging habitat. The colony comprised *c.* 30–40 individual tree islands 0.04–0.13 ha in size, which allowed us to manipulate nests on one island and limit colony disturbance.

Estimates of prey biomass were obtained from Beerens (2008) and data specifically pertaining to Lox are published in a recent study on white ibis nest survival (see Herring *et al.* 2010). In short, radio-tagged adult ibises were located at foraging sites within Lox and then prey biomass at each site was estimated using 1-m² throw traps (Kushlan 1981b) within 24 h. Throw trapping is the standard procedure for sampling aquatic prey in the Everglades and provides an accurate means to assess both small fish and macroinvertebrate densities (Jordan, Coyne & Trexler 1997; Dorn, Urgelles & Trexler 2005). Biomass estimates were obtained for 49 sites in 2006 and 46 sites in 2007. Prey biomass data from Beerens (2008) were consistent with unpublished data by D. E. Gawlik from an ongoing study of prey availability across the entire Everglades landscape.

Hydrologic conditions and wading bird food availability within the Everglades can vary markedly among years (DeAngelis 1994). The northern Everglades during the 2006 dry season were considered close to optimal for wading bird nesting according to Gawlik (2002). Water levels were above average prior to the winter dry season (which promotes aquatic prey production), receded steadily throughout the season (which concentrates prey) and were unimpeded by major water level reversals (which re-disperses concentrated prey). This fostered relatively high dry-season prey biomass in Lox (mean 15.2 g m⁻² ± 0.80 SE, *n* = 49; Herring *et al.* 2010) and near record numbers of wading bird nests (Cook & Call 2006). This recession and

subsequent unusually dormant summer rainy season of 2006 produced abnormally low water levels during the 2006 rainy season and 2007 dry season. Similar to 2006, the 2007 dry season had few reversals and an uninterrupted recession (Cook & Herring 2007a); however, mean prey biomass in Lox was significantly reduced (5.57 g m⁻² ± 0.14 SE, *n* = 46; Herring *et al.* 2010). The low water levels of the preceding wet season appeared to have constrained the growth and reproduction of prey populations leading into the 2007 dry season. This limited food availability was evident in reduced adult white ibis physiological condition, reduced numbers of nests, lower nest survival, and slower nestling growth rates (Cook & Herring 2007b; Herring 2008; Herring *et al.* 2010).

FOOD SUPPLEMENTATION

Shortly after clutch completion, white ibis nests with two or three eggs were randomly selected and assigned to either a control or food supplemented group. Thirty-six nests were studied in 2006 (18 supplemented and 18 control) and 46 nests in 2007 (22 supplemented and 24 control). We checked nests daily beginning a few days prior to hatching to record hatch date and hatching order of nestlings (within ± 1 day). Date of first hatching was relatively synchronous among study nests, ranging from 17 April to 28 April in 2006 and 4 April to 16 April in 2007. Within each brood, siblings were referred to as either A-, B- or C-nestling, according to hatch order. On hatching, nestlings were initially marked on a toe with liquid paper to identify hatch order and later (age 5–8 days) with U.S. Geological Survey Service leg bands and unique colored leg bands for individual identification. Food supplementation began at age 6 days, (by which age chicks were able to fully thermoregulate and were not brooded by parents) and continued to age 20 days. All nestlings in supplemented nests were hand fed 10 g of freshly caught Everglades fish between the hours of 07.00–10.00 for five consecutive days per week. Fish were placed directly into the proventriculus, which took between 1 and 3 min to complete. The 10-g supplement represented a daily energy increase of *c.* 7% (age 15–20 days) to 42% (age 6 days; Kushlan 1977b). This quantity was expected to induce improved physiological condition without straining digestive capacity. Fish species included eastern mosquitofish *Gambusia holbrooki*, flagfish *Jordanella floridae*, golden topminnow *Fundulus chrysotus*, and sailfin molly *Poecilia latipinna*, which are common in the diet of white ibis nestlings in the Everglades (Kushlan 1979; Dorn, Herring & Gawlik 2008). Nestlings were then returned to the natal nest and observed for a further 2 min to ensure no regurgitation of the supplements. Control nestlings received no supplement but were handled to standardize any disturbance effect.

PHYSIOLOGICAL MARKERS

We sampled blood and faecal samples from one nestling per nest such that each nestling was sampled just once, at either 10 days or 20 days post-hatch (mean 11.6 days ± 0.4 SE and 21.9 days ± 1.0 SE; hereafter young and old). To ensure that the capacity for sibling competition was uniform among nests, we selected nests where two siblings were alive at the time of sampling. Blood and faecal samples were collected from 43 nestlings during 2006 (24 supplemented and 19 control) and 21 during 2007 (13 supplemented and 8 control). Sample sizes for hatch order (A, B, and C nestlings) were 31, 11, and 1 respectively, in 2006, and 12, 7, and 2 respectively, in 2007.

On sampling days, nestlings were removed from nests and taken to an adjacent shady site to minimize stress of sampled nestlings and

disturbance of local nests. Because blood samples could not be obtained in less than 3 min to achieve a baseline level of PCORT (Romero & Reed 2005), we standardized holding time (5 min) for nestlings before sampling, recognizing that standard capture-and-handling stress protocols may in fact underestimate the stress response due to haemodilution (Duffy 2008). We collected up to 1 mL of blood from the brachial or jugular veins using a 27.5-gauge needle. Blood samples were stored in heparinized Vacutainers and placed on ice. We then extracted *c.* 2 mL of faecal material from the cloaca of the nestling using a micropipette, stored it in a micro centrifuge tube and placed it on ice. In the laboratory, blood samples were centrifuged (15 min, 10 000 *g*) and plasma and red blood cells were separated. Plasma, red blood cells and faecal samples were frozen at -20°C until later analysis.

For analysis, PCORT samples were homogenized and then mixed with 95% ethanol and vortexed for 30 min. After centrifugation (15 min, 2500 *g*), we transferred the supernatant to a new vial, which was then evaporated under a stream of nitrogen gas. Faecal samples were homogenized, divided into two equal wet portions, and dried using a Labconco CentriVap Concentrator (Labconco, Kansas City, Missouri, USA). Dried samples (*c.* 0.25 g) were mixed with 5 mL of 95% ethanol and vortexed for 30 min. After centrifugation (15 min, 2500 *g*), we transferred the supernatant to a new vial, which was then evaporated under a stream of nitrogen gas. PCORT and FCORT metabolites were then resuspended in diluted extraction buffer and measured using the Correlate-EIATM Corticosterone Enzyme Immunoassay Kit (ELISA; Rothschild *et al.* 2008; Herring, Gawlik & Rumbold 2009) following manufacturer instructions (Assay Design, Inc., Ann Arbor, Michigan, USA). Inter- and intra-assay coefficients of variation for PCORT and FCORT internal standards were 6% and 9%, and 8% and 9%, respectively. ELISAs were validated for the measurement of both PCORT and FCORT metabolites using an assessment of parallelism and recovery of exogenous corticosterone (Rothschild *et al.* 2008). We also validated that FCORT levels did not change after freezing, as in the case of mammals (Khan *et al.* 2003), by freezing and measuring FCORT levels monthly for 6 months (Herring & Gawlik 2009).

Red blood cells were washed three times using phosphate buffered saline, centrifuged and the supernatant was removed after the final wash. Red blood cell supernatant was then mixed with 1 $\times$ extraction reagent and a protease inhibitor cocktail (Sigma, St. Louis, Missouri, USA), vortexed for 5 min and then sonicated for 1 min. Samples were again centrifuged (15 min, 2500 *g*) and the supernatant removed. We measured HSP60 (HSPD1) and HSP70 (HSP72/HSPA1A) in the

supernatant using ELISA kits specific to these stress proteins. Inter- and intra-assay coefficients of variation for HSP60 and HSP70 internal standards were 5% and 7%, and 6% and 7%, respectively. All samples were run in duplicate, and means of duplicates were used in all analyses. ELISA kits were validated using an assessment of parallelism and recovery of exogenous stress protein (Herring & Gawlik 2009; Herring, Gawlik & Rumbold 2009).

STATISTICAL ANALYSES

We constructed general linear models using program JMP 8.0 (Sall, Lehman & Creighton 2001) to examine how independent variables affected each physiological metric. Independent variables included year (2006 and 2007), treatment (control and food supplemented), hatch order (A- and B-nestling), age (young and old), and brood size. We omitted C-nestlings from analyses due to small samples size. The global model contained terms for all main effects and their two-way interactions. Interaction terms were dropped from the final model when not significant. The critical level of all statistical tests was 0.05. FCORT data were log transformed to meet assumptions of equal variance (Levene's test). All other data met the assumptions of equal variance and normal residuals. Results are presented as least squares means $\pm$ SE.

Results

Plasma corticosterone levels generally did not respond predictably to variation in food availability. PCORT was not affected by feeding treatment, hatch order or age, but did respond significantly to year, with the highest stress levels occurring during the year with poor food availability, 2007 (Table 1). Mean PCORT concentrations were $5.0 \text{ ng g}^{-1} \pm 1.0$ and $18.7 \text{ ng g}^{-1} \pm 4.3$ during 2006 and 2007 respectively.

Faecal corticosterone levels, by contrast, were far more responsive to nestling nutritional condition. FCORT levels were significantly higher in control than supplemented nestlings, and were also influenced by year, hatch order and nestling age as revealed by significant hatch order $\times$ year and hatch order $\times$ age interactions (Table 1, Fig. 2). These interactions show that B-nestlings had higher FCORT levels during 2007 than 2006, while levels in A-nestlings did not differ

Table 1. General linear model results of the influence of year, treatment, hatch order, collection date, brood size, and handling time on white ibis nestling physiological condition during 2006 and 2007 in the Florida Everglades. Non-significant interactions are omitted

Effect	PCORT			FCORT			HSP60			HSP70		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
Year	1,18	7.28	0.01	1,36	26.17	<0.01	1,26	36.9	<0.01	1,16	0.21	0.64
Treatment	1,18	0.02	0.88	1,36	5.19	0.02	1,26	9.95	<0.01	1,16	1.17	0.29
Hatch order	1,18	1.52	0.23	1,36	5.04	0.03	1,26	0.71	0.40	1,16	3.37	0.08
Age	1,18	0.18	0.66	1,36	12.76	<0.01	1,26	7.91	<0.01	1,16	0.38	0.54
Brood size	1,18	0.14	0.70	1,36	1.01	0.32	1,26	4.08	0.05	1,16	0.19	0.66
Handling time	1,18	0.03	0.85									
Hatch order $\times$ year				1,36	10.53	<0.01	1,26	18.04	<0.01			
Hatch order $\times$ age				1,36	10.53	<0.01	1,26	12.06	<0.01			

PCORT, plasma corticosterone; FCORT, faecal corticosterone; HSP60, heat shock protein 60; HSP70, heat shock protein 70.

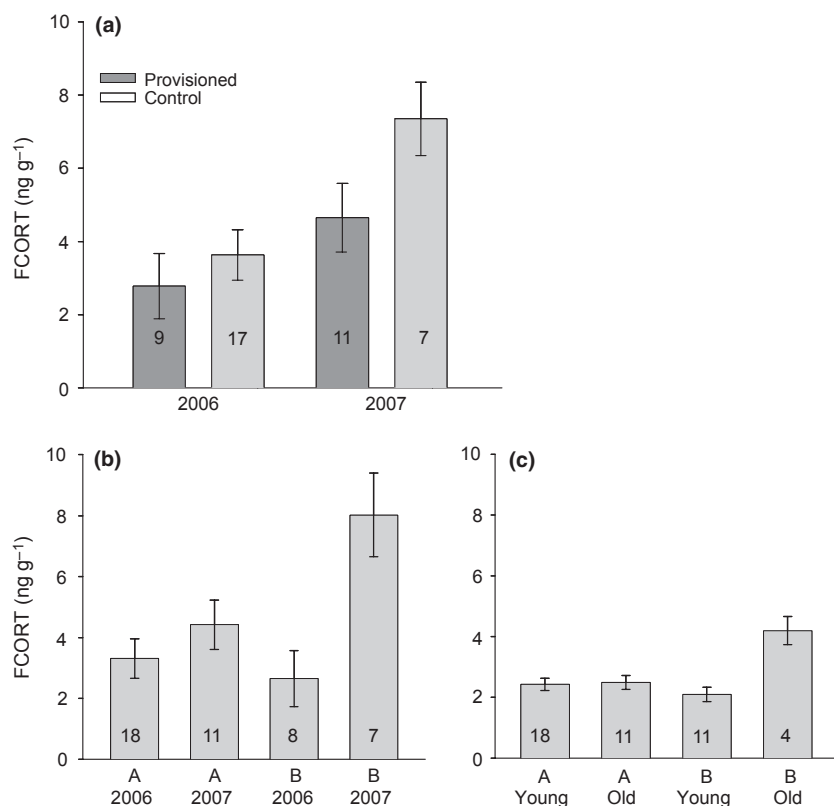


Fig. 2. Faecal corticosterone metabolite concentrations ($\pm$ SE) in white ibis nestlings (a) during 2006 and 2007 in food supplemented and control nestlings, (b) in A- and B-hatched nestlings by year, and (c) in A and B-hatched nestlings by age. Sample sizes are indicated in the bars.

by year (Fig. 2b); moreover, older B-nestlings had higher FCORT levels relative to younger B-nestlings, while A-nestling levels did not differ by age (Fig. 2c).

The responses of HSP60 were remarkably similar to those of FCORT. HSP60 levels were significantly higher in control than supplemented nestlings, and the importance of year,

hatch order and age were again evident from the significant hatch order $\times$ year and hatch order $\times$ age interactions (Table 1, Fig. 3). As with FCORT, HSP60 stress levels were similar between A- and B-nestlings in the good food year but B-nestlings suffered relatively higher levels than A-nestlings in the poor food year (Fig. 3b). Also, older B-nestlings had

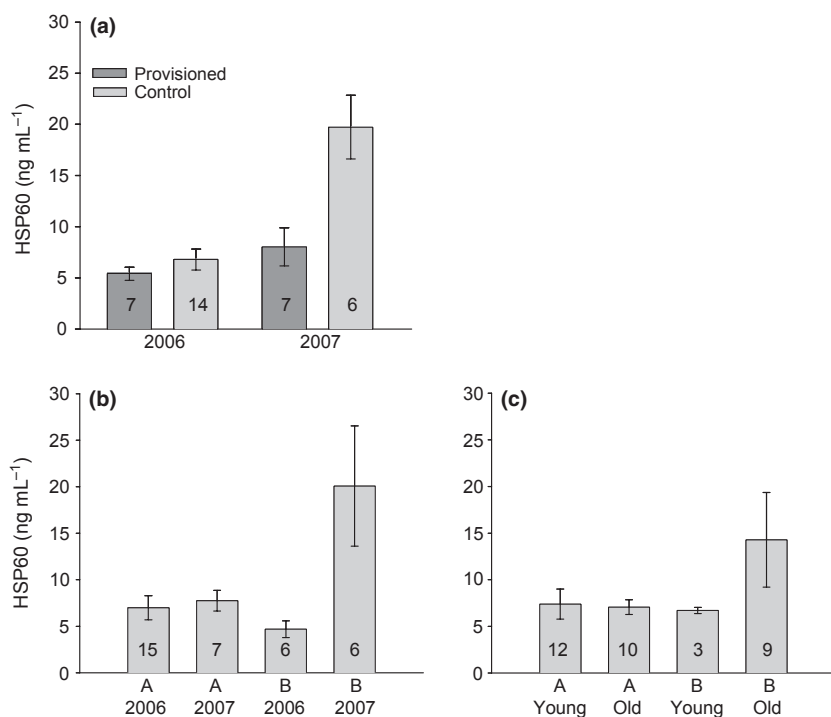


Fig. 3. Heat shock protein 60 concentrations ($\pm$ SE) in white ibis nestlings (a) during 2006 and 2007 in food supplemented and control nestlings, (b) in A- and B-hatched nestlings by year, and (c) in A and B-hatched nestlings by age. Sample sizes are indicated in the bars.

higher stress levels than younger B-nestlings, while A-nestlings did not differ by age (Fig. 3c).

There was no evidence of a response in HSP70 levels among year, treatment, or collection date, but higher HSP70 levels in B- relative to A-nestlings did approach significance (Table 1).

Discussion

The physiological stress responses of free-living nestling birds to environmental food availability are generally poorly understood. Recent studies have examined cellular stress proteins in response to parasitic infection, sibling competition and brood size (Merino *et al.* 1998, 2006; Martínez-Padilla *et al.* 2004), but direct responses to environmental food resources have not been investigated. The role of nutritional limitation on the HPA stress response has received comparatively more attention but studies to date were largely conducted under laboratory conditions and have been limited to few taxa (passerines: Romero, Soma & Wingfield 1998; Pravosudov & Kitaysky 2006; seabirds: Kitaysky *et al.* 2005; Sears & Hatch 2008; and raptors: Heath & Dufty 1998; Love, Bird & Shutt 2003). These laboratory dominated approaches have been useful for characterizing physiological responses to specific stressors, often under highly controlled conditions, but in general the ecological interpretations of the physiological responses were limited. A smaller number of studies have examined the HPA response in free-living nestling birds (e.g. Saino *et al.* 2003; Suorsa *et al.* 2003; Blas *et al.* 2005; Lobato *et al.* 2008), but the role of food limitation remains poorly resolved, and the potentially useful faecal metabolites of corticosterone (FCORT), which has methodological advantages over PCORT in that it may be less affected by handling stress, have received only minor consideration (but see Lobato *et al.* 2008).

PHYSIOLOGICAL STRESS RESPONSES TO FOOD LIMITATION

The current study is one of few to demonstrate elevated physiological stress levels in free-living nestling birds (see also Saino *et al.* 2003; Suorsa *et al.* 2003; Lobato *et al.* 2008), and

the first to do so by experimentally manipulating food supply in association with natural variation in food resources. Circulating and faecal concentrations of corticosterone and stress proteins were relatively low in all white ibis nestlings during 2006 suggesting that they did not exhibit symptoms of acute physiological stress during a breeding season when hydrologic conditions were considered optimal for reproduction (Gawlik 2002). By contrast, concentrations of PCORT, FCORT and HSP60 increased during 2007, a year of reduced food availability. These physiological stress responses were clearly due to nutritional condition, as elevated concentrations of FCORT and HSP60 were evident primarily in control nestlings. The responses of PCORT and HSP70 were generally muted and less informative but did increase as predicted in relation to year (PCORT) and potentially with respect to hatch order (HSP70).

Such nutritionally mediated increases in FCORT and HSP60 during 2007 provide compelling evidence that white ibis nestlings are physiologically affected by environmental food resources. Moreover, these food-related responses are potentially linked to nestling survival. This idea is supported by reproductive data associated with both the current experiment (M. I. Cook, unpublished data) and an independent study (Herring *et al.* 2010) wherein survival of unsupplemented nestlings to age 15–20 days was considerably lower in 2007 relative to 2006 (Table 2). Similar negative relationships between corticosterone and nestling survival have been noted for other species (e.g. Suorsa *et al.* 2003), and Morales *et al.* (2006) found that high stress protein levels can be associated with depressed humoral and cell-mediated immune responses, which in turn can be linked to post-fledging survival (e.g. Moreno *et al.* 2005). While relationships among white ibis nestling stress levels, survival and food availability need to be explored in greater detail, their apparent close associations in this study is consistent with the idea that the current Everglades ecosystem often limits reproduction through effects on food availability (Kushlan 1976; Frederick & Spalding 1994; Gawlik 2002).

Previous studies have linked food limitation to physiological stress responses of nestling birds, but did so indirectly using brood manipulation experiments with equivocal results. Contrary to the current study, experimental brood-size

Table 2. Evidence that white ibis reproductive output was compromised in the Everglades during 2007 relative to 2006

	2006			2007		
	Mean	Error‡	n	Mean	Error‡	n
i) General nesting data for the Everglades*						
Clutch size	2.5	0.07	73	2.2	0.04	263
Daily survival rate	0.933	0.838–1.0	91	0.47	0.414–0.555	242
ii) Breeding success for current experiment†						
Chicks per nest supplemented	1.47	0.18	19	1.75	0.14	16
Chicks per nest controls	1.79	0.15	19	0.83	0.15	23

*Herring *et al.* 2010.

†M. I. Cook, unpublished data.

‡Errors are SE except for Daily Survival Rate (95% CI).

increases failed to effect HSP60 responses in nestling blue tits *Cyanistes caeruleus* (Merino *et al.* 2006) or FCORT responses in nestling pied flycatchers *Ficedula hypoleuca* and blue tits (Lobato *et al.* 2008). Passerine species inhabiting temperate climates are short-lived, have a limited number of breeding opportunities, and according to life-history theory are considered more likely to increase investment in current reproduction than are longer-lived species such as the white ibis (e.g. Stearns 1992). It is possible, therefore, that parents of these short-lived species compensated for the brood-size manipulations by increasing brood food supply and offsetting the physiological stress of their nestlings. Provisioning behaviour was not quantified in these studies, but this idea was supported by elevated HSP60 levels in parents of the experimentally enlarged blue tits broods (Merino *et al.* 2006). Contrary to these ideas, however, both brood-size increases and food reduction manipulations induced elevated PCORT responses in nestlings barn swallows *Hirundo rustica* (Saino *et al.* 2003), while in the European tree creeper *Certhia familiaris* PCORT concentrations increased with declining habitat quality and food supply (Suorsa *et al.* 2003). These studies suggest that adults were unable or unwilling to compensate for a shortfall in brood food supply, perhaps because current reproductive effort is constrained in these short-lived species such that parents are already investing maximum effort in current reproduction (e.g. Nur 1984). A further understanding of nestling stress responses in altricial species may be elucidated with similar manipulative studies that simultaneously quantify parental efforts and environmental food resources.

Faecal corticosterone and HSP60 became elevated at a relatively early stage in white ibis development (between 10 and 20 days post-hatch) and FCORT levels were comparable to those of physiologically stressed adult ibises (Herring 2008), indicating that the HPA response matures relatively early during development in this species (fledging age is *c.* 60 days). These patterns generally contrast with those of other altricial and semi-altricial species, which do not tend to release adult-like quantities of corticosterone until close to fledging age (Sims & Holberton 2000; Love, Bird & Shutt 2003; Walker, Wingfield & Boesma 2005; but see Suorsa *et al.* 2003; Pravosudov & Kitaysky 2006), and are more akin to species with a precocial mode of development, which are characterized by full maturation of the HPA axis shortly after hatching (Holmes, Redondo & Cronshaw 1989). Short-term releases of corticosterone (hours to days) promote fitness in neonatal birds by triggering the release of stored energy (Axelrod & Reisine 1984) and by escalating food acquisition through behavioural changes such as increased begging rates (Ramos-Fernandez *et al.* 2000; Kitaysky, Wingfield & Piatt 2001). Hence, a well developed HPA complex might be advantageous for those non-precocial species that regularly experience short-term and unpredictable fluctuations in food supply during neonatal development. White ibis nesting habitat is generally characterized by random fluctuations in hydrologic conditions (DeAngelis 1994), which can result in short-term but potentially marked changes in food availability at any time

throughout the nesting period. Thus, the unusually early maturation of the white ibis HPA axis might function to moderate the effects of this short-term but temporally unpredictable environmental variability.

In addition to the strong stress responses observed during early development, our data also reveal that elevated FCORT and HSP60 levels persisted until at least age 20 days post-hatch. These prolonged elevations are representative of chronic stress, and reflect the limited food available to nestling birds throughout the study period during 2007. Unlike acute releases of stress hormones that aid immediate survival in developing vertebrates, prolonged elevations (days to weeks) can induce muscle atrophy, neuronal cell death and suppression of immune function (e.g. Sapolsky, Romero & Munck 2000; Kitaysky *et al.* 2003). Chronic elevations of corticosterone can thus be detrimental to fitness, and may indicate a disconnect between an individual's adaptations to its environment and its current ecological conditions. Accordingly, the chronic stress responses and reduced survival of unsupplemented white ibis nestlings (Table 2) suggest that they are unable to cope with relatively long-term periods of food shortage. While the white ibis is adapted to the natural stochasticity of foraging conditions inherent to fluctuating wetlands, the magnitude and frequency of that variability in the current Everglades may now be more extreme than during the pre-drainage period, and conditions such as those evident during 2007 may not be expected events during the nestling period.

White ibis stress responses were also related to hatching order. As predicted, HSP60, HSP70 and FCORT stress levels were on average higher in second-hatched B-nestlings than in A-nestlings. This is consistent with other avian studies demonstrating relatively higher concentrations of HSP60, HSP70 and PCORT in younger siblings (Kitaysky, Wingfield & Piatt 2001; Martínez-Padilla *et al.* 2004), and suggests that physiological responses to environmental perturbations are expressed foremost among younger members of the brood possibly as a result of sibling competition for parental provisions. The significant interactions of hatch order by year for both FCORT and HSP60 are in accordance with this idea, and reveal that the relative increase of stress levels in B-nestlings compared to A-nestlings was most marked during 2007 when competition for limited food was expected to be acute, while levels were generally equitable between nestlings in 2006 when food was plentiful (Table 1, Figs 2 and 3). The limited stress response by A-nestlings during 2007 suggests that they largely compensated for the reduction in food availability, probably by consuming a relatively larger proportion of total parental provisions.

UTILITY OF PHYSIOLOGICAL STRESS MARKERS FOR MEASURING ENVIRONMENTAL CONDITIONS

The unequivocal responses of FCORT and HSP60 to nutritional stress suggest that these physiological responses can be used as key biomarkers not only for the white ibis, an important indicator species of wetland restoration success for

the Comprehensive Everglades Restoration Plan (Frederick *et al.* 2009), but potentially for other avian species. A conspicuous result in this study was the detection of the HPA stress response as a medium-term faecal response rather than as the short-term plasma response evident in previous studies. Our ambiguous PCORT results were probably an artefact of capture- and handling-time and likely do not accurately reflect nestling nutritional condition. Sampling blood in less than 3 min is considered necessary for minimizing potential handling bias on PCORT levels (Romero & Reed 2005), but was not feasible in this study where nestlings were highly mobile and researcher disturbance was relatively high (mean time to blood sample $6.8 \text{ min} \pm 2.75 \text{ SD}$). FCORT is likely the more appropriate HPA biomarker for the white ibis and potentially for other species where capture and handling might bias the plasma response. Also noteworthy was the long-term elevated nature of this FCORT stress response. The close association among FCORT, food availability and nestling survival suggests that the duration of the FCORT response may be a particularly useful gauge of wetland environmental conditions for white ibis during nesting.

Of the stress proteins only HSP60 levels increased predictably in response to food limitation. Other studies also provide evidence that HSP60 and HSP70 do not respond similarly to a common stressor. Martínez-Padilla *et al.* (2004) observed a significant increase in HSP60 during the course of a breeding season in Eurasian kestrels *Falco tinnunculus*, while HSP70 remained relatively constant. Moreno *et al.* (2002) found a positive correlation between HSP60 levels and heterophil/lymphocyte ratios, but no relationship for HSP70 levels in pied flycatchers. Taken together, results from the current and previous studies suggest that HSP60 may be an appropriate physiological marker for understanding environmental food limitation in birds. Indeed, HSP60 may have greater general utility as a measure of environmental stress in neonatal birds than corticosterone and its metabolites. A primary limitation of employing PCORT and FCORT is the considerable variability in the ontogeny of the corticosterone response among altricial species, with few options available for detecting or understanding environmentally mediated stress in species with delayed development of the HPA. While little is known about developmental patterns of HSP60 in birds, the early development of this stress response in the current study suggests it may be a promising gauge of early neonatal stress for those species with delayed HPA development.

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