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The whooping crane (*Grus americana*) is listed as endangered under the IUCN Red List, the United States Endangered Species Act, and the Canadian Species at Risk Act (BirdLife International 2012, CWS and USFWS 2007). A major focus of recovery efforts for this endangered species is reintroduction to establish new populations (CWS and USFWS 2007). Captive populations are critical as a source of individuals for reintroduction efforts and also serve as insurance populations. Currently, there are a total of 157 whooping cranes held in captive breeding centers across North America, with the largest at the USGS Patuxent Wildlife Research Center (PWRC) in Laurel, Maryland. Birds produced in this facility are currently being released as part of efforts to establish the Eastern Migratory Population (EMP, Urbanek et al. 2005) and in an effort to establish a non-migratory population in Louisiana. In the past decade, PWRC has produced and released annually an average of 18 birds into the wild; however, reproductive performance of birds at this facility is lower than desired. PWRC had a 60% fertility rate for eggs laid from 2000 through 2010 (J. N. Chandler, personal communication, 2011). Furthermore, reproductive onset in this captive population appears to be delayed compared to wild populations. In wild populations, reproductive onset (production of sperm and eggs) normally occurs ~5 years of age in both males and females, ~2 years after initial pair formation occurs (Ellis et al., 1996), while some females in the EMP have laid eggs earlier than 5 years of age (Converse et al. 2011). However, PWRC females in some cases do not start to lay eggs until 7 years of age (Mirande et al. 1996). Currently, the PWRC population consists of a total of 74 whooping cranes, including 22 pairs. Six of these pairs (27%) are consistently infertile (i.e., no production of fertile eggs) and 3 other pairs (14%) have low fertility (30-45% fertility in eggs laid), which is variable from year to year. Six pairs (27%) are recently formed and have not produced eggs, and so have unknown fertility. This leaves only 7 pairs (33%) which contribute maximally to PWRC's chick production (J. N. Chandler, personal communication, 2011). Because of the challenges occurring within this captive colony, PWRC and Smithsonian National Zoo have initiated a joint research project to identify potential underlying causes of poor reproduction in captive whooping cranes.

One method critical to this research is non-invasive hormone monitoring, which has been used in a variety of studies focused on examining basic animal biology, health, and reproduction, as well as physiological responses of animals to captive management. Hormone metabolite concentrations can be sampled in a variety of materials including feces, urine, hair, feathers, and saliva (Brown 2008, Brown et al. 2001, Holt et al. 2003, Lobato et al. 2010, Moore et al. 1984, Wielebnowski et al. 2002). In the giant panda (*Ailuropoda melanoleuca*) hormone metabolites have been monitored in urine samples in order to understand the timing of estrus and ovulation, which aids in planning animal introductions and artificial inseminations (Moore et al. 1984). In the clouded leopard (*Neofelis nebulosa*) fecal hormone sampling has helped researchers understand relationships between aspects of enclosure design and location and stress responses (Wielebnowski et al. 2002).

Already used in a variety of wild mammal species in both *ex situ* and *in situ* studies, non-invasive hormone monitoring is also gradually being adapted to birds. Most avian hormone studies to date have

utilized blood sampling (Angelier and Chastel 2009, Angelier et al. 2009, Angelier et al. 2006, Bluhm et al. 1983), a process which has been shown to cause stress (Gratto-Trevor et al. 1991). Studies have validated the effectiveness and feasibility of non-invasive hormone monitoring in some bird species. Ludders et al. (2001) showed that serum corticosterone patterns were similar to those in fecal samples collected from the same bird in Florida sandhill cranes (*Grus canadensis pratensis*). Stanley et al. (2007) validated reproductive steroid hormone assays for both golden eagles (*Aquila chrysaetos*) and peregrine falcons (*Falco peregrinus*) housed in a captive setting. To date, non-invasive hormone monitoring has not been used to assess gonadal activity and little work has been done assessing adrenal activity and function in whooping cranes. Ongoing data collection at PWRC is one of the first efforts to use non-invasive hormone monitoring in an attempt to understand whooping crane reproductive biology.

The first critical step in this work was to establish a method to identify fecal samples from an individual bird within a breeding pair. Trials with different types of food dyes in varying amounts were unsuccessful. In the present study, we determined the feasibility of using chromic oxide (Cr_2O_3) and iron oxide (Fe_2O_3) as fecal markers. Both chromic oxide and iron oxide were obtained from Prince Agri Products, Inc. (Quincy, IL). These dyes have been used in nutritional studies in a variety of species, including chickens, ducks, cows, horses, and humans, especially in studies that involve more than 1 feeding trial or those aiming to assess the digestibility of a food item (Schurch et al. 1950). Both are non-biological, insoluble compounds which, when ingested, are not absorbed by the digestive system (Dansky and Hill 1952, Schurch et al. 1950). Instead, they pass directly through the digestive tract and subsequently color the animal's feces.

In our first trial, cranes housed individually in outdoor pens were given smelt (*Osmerus mordax mordax*) containing a capsule filled with 450 mg green chromic oxide (n = 5 birds) or yellow (n = 5), red (n = 4), orange (n = 3), or black (n = 3) iron oxide. The appearance of color in the feces was visually determined 8 hours later, with color intensity judged on a scale of 0 to 3, with 3 indicating intense color and 0 indicating no visible color. Visibility was determined in the field, where subsequent endocrine studies will take place, because it is important to know which color would be easiest to find where vegetation and other factors obscure sample visibility. Chromic oxide in green, and iron oxide in orange, red, and black (but not yellow) were visible in feces (green = 3; red = 2; black = 1.5; orange = 1; and yellow = 0).

In a second trial, we assessed the time required until chromic oxide could be observed post-feeding. Four whooping cranes were housed individually in indoor pens (Fig. 1) and fed smelt containing 230 mg of green chromic oxide. The pens were checked every 30 minutes until first appearance of the dye in the feces, and then every hour until the end of the day (8 hr post feeding). At the beginning of day 2 (24 hr post-feeding), the pens were cleared of all feces to ensure that any subsequent samples which showed a presence of chromic oxide were fresh samples. The marker first appeared on average (± SE) 1.5 ± 0.2 hours after feeding and remained detectable until 27.7 ± 0.2 hours for a total duration of 26.2 ± 0.2 hours. Therefore, use of chromic oxide allows for a flexible collection interval and increased chance of finding an individual's fecal samples. We observed no adverse consequences of feeding either substance, as fecal production (size, consistency, and overall number of fecals) appeared normal.

Finally, it was necessary to verify that chromic oxide and iron oxide would not interfere with hormone assay performance. Feces were collected daily at 0730 hours for 5 days from 3 male and 3 female whooping

Figure 1. Indoor pens where cranes were housed for trial 2. Small pens with wood shavings used as bedding allowed easy detection and identification of dyed samples.

crane adults, housed individually. On the afternoon of the second day (Day 2) each crane was given smelt containing a capsule filled with 230 mg of either green chromic oxide (females) or red iron oxide (males) so that the fecal samples collected on the morning of Day 3 were dyed. Samples were extracted with a modified dry shaking extraction using 70% ethanol (Brown 2008). Once extracted, all samples were assessed for corticosterone using a RIA kit (MP Biomedicals, Solon, OH; Fig. 2a). Female samples were also evaluated for progestagen metabolites using an enzyme immunoassay (EIA, monoclonal pregnane CL425; Fig. 2b), and male samples were also examined for testosterone using an EIA (polyclonal R156/7; Fig. 2c). Antibodies for protestagen and testosterone EIAs were obtained from C. Munro (University of California, Davis, CA). Hormone metabolite concentrations remained constant over the collection period (Fig. 2), providing no evidence that either colorant interfered with the evaluation of excreted hormones. The only individual that showed a significant difference between the Day 3 sample and the other collected samples, using a standard z score, was the corticosterone value for female crane number F2.

In summary, our findings indicate that both chromic oxide and iron oxide can be used as fecal markers for non-invasive hormone monitoring. This method will aid ongoing studies aimed at advancing the understanding of reproductive endocrinology and underlying causes of poor reproduction in captive whooping cranes. Studies are in progress to evaluate hormone metabolite concentrations and patterns in male and female whooping cranes during the breeding season. The method will be easily transferrable to a host of other avian species aiding in their conservation and captive management.

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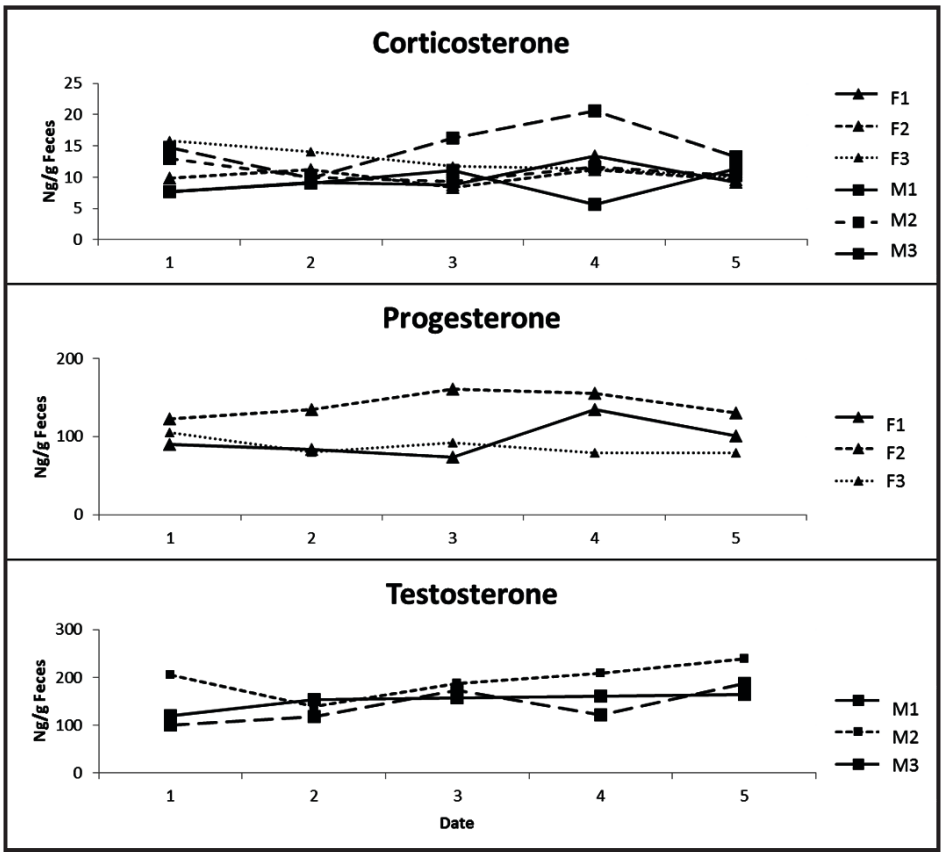

Figure 2. Metabolite concentrations assessed during a 5-day period in 6 adult whooping cranes. M indicates males (n = 3) and F designates females (n = 3). Sample collected on Day 3 contained fecal marker: iron oxide for males and chromic oxide for females.

oxide in her own poultry nutrition research and was instrumental to the idea to use this substance as a fecal marker for this species. We would also like to thank everyone on the USGS PWRC crane crew and vet staff for all of their help. N. Presley, S. Putman, and N. Parker of Smithsonian's Conservation Biology Institute Endocrine Lab were extremely helpful with mentorship and training on each of the hormone assays. Support for this project was provided by Morris Animal Foundation. All methods and animal use were approved by both the Smithsonian and USGS Patuxent Animal Care and Use Committees.

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