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INITIAL EVALUATION OF CYCLIC ADENOSINE MONOPHOSPHATE ENZYME IMMUNOASSAY FOR USE WITH CRANE SEMEN SAMPLES

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Abstract: The management of *ex situ* and *in situ* populations of many wildlife species requires detailed knowledge of a species' reproductive biology. For species such as cranes, where artificial insemination is a critical component of *ex situ* management strategies, understanding normal sperm function is especially important. Previous research has shown that captive cranes exhibit highly variable production and quality of semen samples produced by individual males and high levels of variation of cell concentration and motility across different species. Cyclic adenosine monophosphate (cyclic AMP) has been implicated in regulating sperm function, such as cell motility, and may affect an individual's ability to successfully fertilize. Here we demonstrate the feasibility of an enzyme-linked immunosorbent assay (ELISA) for measuring cyclic AMP produced by crane sperm to facilitate future research into its role in sperm function and fertilization.

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Key words: *Antigone vipio*, cyclic adenosine monophosphate, ELISA, *Grus americana*, *Grus canadensis*, *Grus monacha*, hooded crane, motility, sandhill crane, sperm, white-naped crane, whooping crane.

The management of *ex situ* and *in situ* populations of many wildlife species requires detailed knowledge of that species' reproductive biology. Historically, whooping crane (*Grus americana*) reintroduction programs have relied on captive pairs to produce chicks for release (Ellis and Gee 2001). Current reintroduction goals for the whooping crane are impeded by poor reproduction (Harrell and Bidwell 2016); specifically, low numbers of fertile eggs produced (Harrell and Bidwell 2016, Black and Swan 2019). But this issue with fertility is not limited to whooping cranes, and other crane species managed *ex situ* have an ongoing issue with low offspring production and variable levels of fertility (Gee 1983, Mirande et al 1996).

Previous research investigating possible causes of low egg fertility in captive whooping cranes has shown highly variable volume and quality of semen samples between individual males (Brown et al. 2017, Brown et al. 2018). High variation in seminal parameters and semen quality between different species of cranes has also been observed (Songsasen et al. 2019). The objective of this study was to assess the feasibility of using an enzyme-linked immunosorbent assay (ELISA) for easy measurement of a commonly studied nucleotide, cyclic adenosine monophosphate (cyclic AMP) in 4 different crane species: hooded crane (*Grus monacha*), white-naped crane (*Antigone vipio*), sandhill crane (*G. canadensis*), and whooping crane. It is our hope that this preliminary evaluation will facilitate future investigations into crane sperm function and fertilization ability and possibly improve artificial insemination and semen cryopreservation protocols.

Cyclic AMP is a critical nucleotide that acts as a second messenger and is active in cellular signaling pathways (Majumder et al. 1990), which activates a phosphorylation cascade and mediates cellular responses. The correlation between sperm function, sperm motility, and cyclic AMP production has been well established in mammals (Buffone et al. 2014), and decreased or defective cyclic AMP production has been linked to infertility (Xie et al. 2006). While this same relationship is less established in avian species, there is evidence of cyclic AMP-dependent pathways required for motility and acrosome reaction in chickens (Priyadarshana et al. 2018). Prior to ejaculation, avian sperm already possess full motility and a majority of their fertilizing capability (Etches 1996, Blesbois 2012), thus at ejaculation avian sperm actively produce cyclic AMP.

This study was approved by the Smithsonian National Zoological Park Animal Care and Use Committee (#17-14). Semen samples were collected

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Table 1: Summary of seminal parameters and cyclic AMP for crane semen samples. The non-diluted sample (see text) is included in the table and for statistical analyses.

Individual	Crane species	Sample volume	Concentration ($\times 10^6$ mL)	Motile sperm (%)	Normal sperm (%)	Total sperm ($\times 10^6$)	Total motile sperm ($\times 10^6$)	Total normal sperm ($\times 10^6$)	Cyclic AMP (pg/mL)
Male 1	Hooded[a]	20	2.44	61	45	48.8	29.8	21.9	71.0
Male 2	Whooping[a]	20	0.61	57	79	12.2	6.9	9.7	35.4
Male 3	White-naped	60	0.05	17	56	3.2	0.6	1.8	33.6
Male 4	Sandhill	70	0.13	63	31	9.0	5.7	2.7	25.2
Male 5	Whooping	73	0.53	31	70	38.3	11.9	26.8	32.4
Male 6	Whooping	85	0.16	19	71	13.9	2.7	9.9	46.0
Male 7	Whooping	60	0.05	5	36	2.7	0.1	0.9	18.0

[a] Males included in the recovery samples.

from 7 cranes across 4 different species housed at the International Crane Foundation, Baraboo, Wisconsin, where normal husbandry practices and established collection methodology were followed (Ellis et al. 1996). Individual males were selected for collection based on known semen concentrations, willingness to be collected, and because they were not currently being utilized for artificial insemination attempts. Samples were diluted 1:1 with crane semen extender to help stabilize cells (Blanco et al. 2012) then shipped overnight to Smithsonian Conservation Biology Institute, Front Royal, Virginia, in a styrofoam cooler containing frozen ice packs. A paper towel barrier was included between the sample and the ice-packs to prevent direct contact. Upon receipt, the following seminal quality metrics were obtained using previously validated methods: sample volume, sperm concentration, percent motile sperm, and percent morphologically normal sperm (Brown et al. 2018). Total sperm (sample volume × sperm concentration), total motile sperm (total sperm × percent motile sperm), and total normal sperm (total sperm × percent normal sperm) were also calculated (Table 1).

Cyclic AMP concentrations were assessed using Cyclic AMP direct ELISA kit (Arbor Assays, K019-H1) following the manufacturer's protocol. This assay has previously been used to quantify cyclic AMP concentrations in lysed human sperm (Allouche-Fitoussi et al. 2018, Martínez-León et al. 2019, Itzhakov et al. 2019). Briefly, cells were lysed using the sample diluent provided with the assay, which additionally stabilizes cyclic AMP by acidification. Cell lysis was confirmed via brightfield microscopy, then samples were centrifuged at 600 g for 15 minutes. Following centrifugation, supernatant was collected and stored at –80°C until further analysis.

As per protocol recommendation for cell lysates, we followed the acetylation assay protocol for improved assay sensitivity, thus an acetylation reagent was added to all assay standards and semen samples. All assay components and samples were prepared and assessed following assay protocol specifications. The assay plate was read using a FilterMax F5 microplate reader (Molecular Devices, San Jose, CA, USA) with the 450-nm filter, and cyclic AMP concentrations were calculated for the known assay standards and unknown semen sample using SoftMax Pro software (Molecular Devices, San Jose, CA, USA). As part of our preliminary validation of the cyclic AMP ELISA, we determined percent recovery based on cyclic AMP in 2 highly concentrated semen samples, 1 each from a hooded crane and a whooping crane. These recovery samples were split and serially diluted once, with the expectation that the cyclic AMP concentrations would reduce by ~50% upon dilution, and thus we calculated the average percentage of observed versus expected concentrations. Further dilution was not possible due to limited sample volume. This also allowed us to infer any matrix interference for this new sample type because with a high level of matrix interference in the assay we would observe low optical density values or expression of falsely depressed or falsely elevated sample measurements (Cygnus Technologies 2018). All other samples were run as test samples, without additional dilution, to determine the actual cyclic AMP concentration at neat total sperm concentrations (Table 1). For our recovery samples, we observed linearity following dilution and saw an average recovery

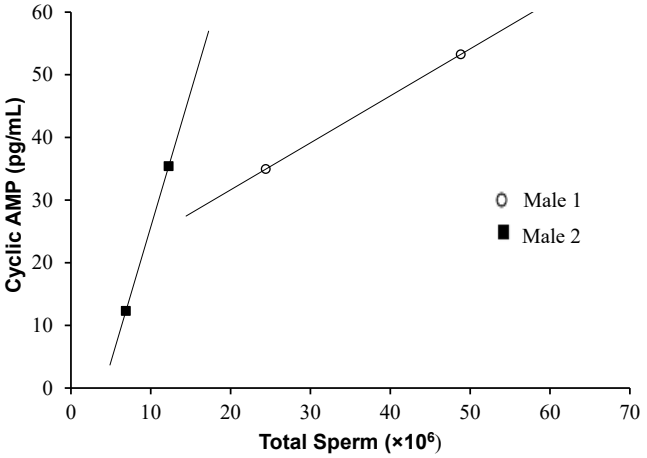

Figure 1. Linearity of semen samples collected from 1 hooded crane (Male 1) and 1 whooping crane (Male 2). Samples were serial diluted and analyzed via cyclic AMP ELISA. Samples showed 72.8% recovery.

of 72.8% from expected values (Fig. 1). Although recovery was lower than expected based on the serial dilution, as target percent recovery is typically between 80-120%, each male's concentrations declined at a similar rate; this probably reflects potential underestimation of concentrations rather than an overestimation. We also feel that the assay is likely measuring cyclic AMP within our samples rather than a matrix interference, as no decrease in concentration would have been observed or the decrease would have been more erratic and different between individuals had a matrix interference been observed.

Following this, we assessed the remaining test samples for potential relationship between cyclic AMP concentrations and total sperm, total motile sperm, and total normal sperm (Figs. 2 A, B, and C, respectively). Different species are denoted by differently shaped data points. We performed Pearson's correlation to assess linear relationships. Each variable displayed a positive correlation with the strongest relationship between cyclic AMP and total motile sperm (Fig. 2B; $R^2 = 0.75$, $P = 0.05$), followed by total sperm alone (Fig. 2A; $R^2 = 0.65$, $P = 0.08$) and then total normal sperm (Fig. 2C; $R^2 = 0.55$, $P = 0.19$). Additionally, we did not observe any patterns within or between species of crane; however, no true relationship can be discussed at this time as only 1 species included more than 1 male (Table 1). As in previous studies where variability occurred between males within a species (Brown et al. 2017, Brown et al. 2018), we observed

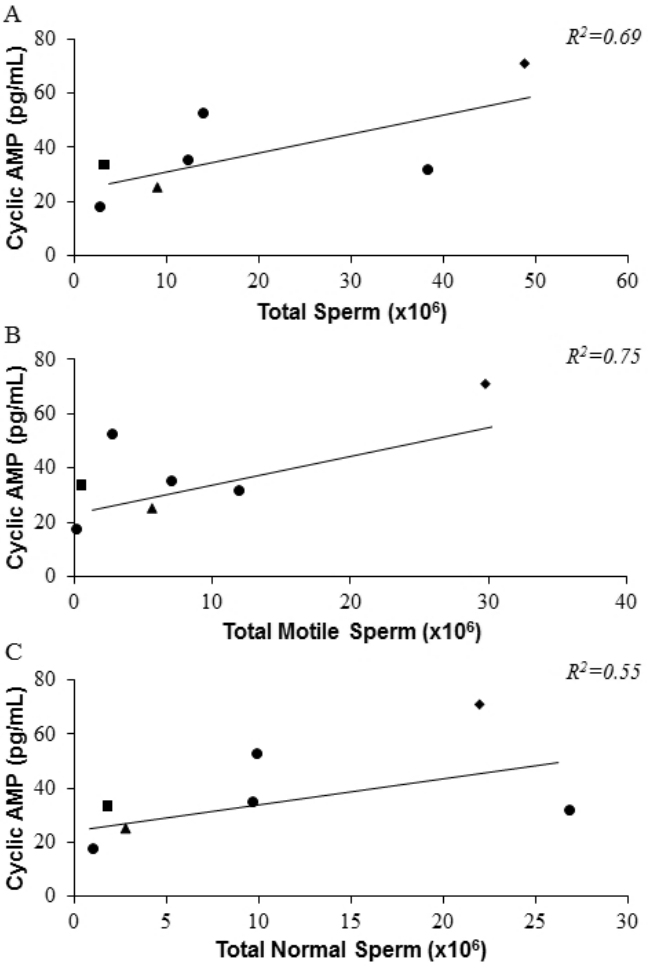

Figure 2. Relationship between cyclic AMP and A) total sperm, B) total motile sperm, and C) total normal sperm in crane semen samples. Individual species are denoted by differently shaped data points: hooded crane (diamond), whooping cranes (circles), white-naped crane (square), and sandhill crane (triangle). A positive relationship is observed in each comparison, the strongest relationship was between cyclic AMP and total motile sperm. There was no discernable pattern based on individual species but rather indicated variation between individual males within a species.

differences between individuals (Table 1), although each male was sampled only once and multiple sample testing is required to inform any such pattern or repeatability across semen collections.

Given the pilot results of this limited assay validation and sample testing, we feel that this direct detection ELISA is a promising candidate for measuring cyclic AMP activity in crane semen samples. While we cannot yet definitively discuss any patterns or influence on semen quality and fertility, cyclic AMP values were measurable and not attributed to matrix

interference. Additional validation is recommended to ensure accurate diagnostics and measurement of cyclic AMP; however, we were able to detect some difference based on number of cells present (total sperm) and motility of the cells (total motile sperm), although neither explained all variation in cyclic AMP concentrations. Further analysis is needed to explore the relationship between cyclic AMP, sperm function, and fertility in captively housed cranes and any possible mediation to restore fertility if a relationship does exist.

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