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Reproductive physiology of the female Magellanic penguin (*Spheniscus magellanicus*): Insights from the study of a zoological colony

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ABSTRACT

Eight captive female Magellanic penguins (*Spheniscus magellanicus*) were monitored over a 10 week period, commencing at 5 weeks prior to egg lay (EL), to increase our understanding of the species' reproductive biology. Females in cordoned nest sites underwent cloacal artificial insemination (AI) every 4–7 days with different semen donors for each insemination. The EL interval was 97.9 ± 3.6 h (range: 84–108 h) and paternity analyses revealed that conceptive inseminations occurred from 11.5 to 4.5 days before oviposition. A biphasic pattern of estradiol, testosterone, progesterone and the biochemical analytes triglyceride, iron, calcium and phosphorus occurred in relation to EL, with values increasing ($P < 0.05$) to maximal concentrations during the three weeks preceding oviposition, then decreasing ($P < 0.05$) rapidly after oviposition completion. In comparison with post-lay (baseline) values, concentrations of estradiol and testosterone relative to the first oviposition were elevated at Week-5, and those of triglyceride, a yolk formation index, as well as iron, calcium and phosphorus, became elevated at Week-4 ($P < 0.05$). Collective data indicate an estimated total egg formation interval of 29 days, with oviducal transit of the ovulated ovum occurring over the majority of the ~4 day EL interval. These findings indicate that egg formation is prolonged with folliculogenesis initiated at 5 weeks or more prior to oviposition. Consequently, the period of folliculogenesis and egg formation is estimated to overlap with the final ~3 weeks that wild females spend at sea prior to returning to land for breeding.

1. Introduction

The avian order *Sphenisciformes* (penguins), which includes one family and six genera, is considered the most threatened group of seabird along with species of the order Procellariiformes (petrels and albatrosses; Croxall et al., 2012). Eleven of the 18 penguin species are currently listed as threatened, with degradation of terrestrial habitats, pollution and fisheries activities representing primary causes of population decline (IUCN, 2013; Trathan et al., 2015). Four species exist in the genus *Spheniscus* and all of those are classified as threatened with extinction (vulnerable or endangered categories) except the Magellanic penguin (*Spheniscus magellanicus*), which was up-listed to near-threatened status in 2000 in response to declining population trends (IUCN, 2013).

The wild Magellanic penguin population comprises approximately 2.5 million individuals distributed over its primary range on the coast of South America including Argentina, southern Chile and the Falkland Islands. At one of the species' largest breeding colonies (Punta Tombo, Argentina) there has been a 20% decrease in numbers of breeding penguins since 1987, with petroleum pollution reported to be one of the factors associated with this decrease (Boersma, 2008; Boersma and Rebstock, 2014). Other significant threats to the species include fisheries by catch and disease (Trathan et al., 2015), and reproductive success and future population survival may also be negatively influenced by climate patterns that are predicted to occur more frequently in the future over the species breeding ranges. Specifically, these include an increase in the intensity and frequency of storms, which have already been shown to decimate Magellanic penguin chick numbers during vulnerable developmental stages (Boersma and Rebstock, 2014).

Information on migratory patterns of Magellanic penguin populations in southern Argentina has revealed crucial information for conservation efforts of this species (Stokes et al., 2014). An understanding of a species' reproductive biology is another necessary component of species management strategies, for both wild and captive populations. The reproductive endocrinology of the Magellanic penguin encompassing plasma production of sex steroids and

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LH in relation to oviposition has been described from studies on wild males and females (Fowler et al., 1994). However, since females of this pelagic species spend on average only 11 days on land prior to oviposition (interval computed from Table 2 in Yorio and Boersma, 1994), little is known about the physiology of the initial phase of the breeding cycle and the timing of gonadal recrudescence, which is presumed to occur while Magellanic penguins are at sea (Fowler et al., 1994).

In seasonally reproductive avian species the most important cue that mediates gonadotrophin production and ensuing ovarian activity and breeding is the photoperiod (reviewed by Williams, 2012). Non-photic factors such as air temperature, food availability, body condition and presence of a mate are also important (yet poorly understood) cues for some avian species (e.g., Jacobs and Wingfield, 2000; Dunn, 2004; Williams, 2012), and for marine species such as the penguin, average sea surface temperatures (which are linked to food availability and ensuing body condition) have been linked to the timing of breeding onset (as inferred by laying dates) and reproductive success (Boersma, 1978; Cannell et al., 2012; Hinke et al., 2012). With regard to the temporal characteristics of egg production, dye ingestion and yolk staining studies are the most accurate means for examining the rate and duration of yolk formation and this procedure, using laid eggs, has been performed in numerous avian species including three penguin species, the Fiordland crested penguin (*Eudyptes pachyrhynchus*), the formerly known white-flippered penguin (*Eudyptula albosignata*; Grau, 1984) and the Adélie penguin (*Pygoscelis adeliae*; Astheimer and Grau, 1985). Such penguin studies have shown that initiation of yolk formation for the first laid egg (A-egg) starts several days before that for the second egg (B-egg), and that the interval of rapid yolk deposition spans ~14–16 days (Astheimer and Grau, 1990), meaning that A-egg yolk formation is presumed to occur almost entirely while Adélie penguin females are migrating to breeding grounds (Astheimer and Grau, 1985). Useful blood biomarkers of yolk production that have been described in birds include the egg yolk precursors vitellogenin (measured as vitellogenic zinc) and low density lipoproteins (measured as total triglyceride; reviewed by Williams, 2012). One or both of these yolk precursors have been examined in the emperor, macaroni and rockhopper penguin (Groscolas, 1982; Crossin et al., 2010, 2012a) but as with other penguin studies, females were only sampled after arrival on land for breeding when precursor concentrations were already elevated, so the duration of vitellogenesis and the accompanying dynamics of yolk production remain to be determined for those species and for the majority of those in the order *Sphenisciformes*.

Determination of the temporal characteristics of egg yolk production in the penguin might also be aided by examining the timing of elevation of other metabolites required for egg formation, including minerals such as calcium and iron which have long been known to be elevated in laying females in response to increased estrogen concentrations (Morgan, 1975; Bacon et al., 1980). Prebreeding plasma biochemistries have been reported for the macaroni penguin, however, the first blood sample for such research was collected no earlier than Day-14 from oviposition (coincident when females returned to land for breeding; Ghebremeskel et al., 1991; Crossin et al., 2010). To the authors' knowledge there exists no information on blood biochemistry profiles during the entire reproductive cycle of the Magellanic penguin or in any penguin species. There is also a scarcity of information on the species' precise timing of peak endocrine activity in relation to oviposition. An understanding of the temporal relationships of reproductive hormones and biochemical analytes relative to oviposition in conceptive females would not only be helpful for characterizing normal reproduction in the species for future health monitoring, but may also facilitate the optimal timing of artificial insemination (AI) procedures, which in turn have a potential role in maximizing reproductive rates and maintaining the genetic diversity of penguin species in conjunction with natural breeding efforts (O'Brien et al., 1999).

The overall goal of this research was to therefore characterize normal reproductive processes in the Magellanic penguin toward an increased understanding of female reproductive biology, particularly egg formation. Specific objectives were to: (i) characterize profiles of plasma hormone and biochemical parameters during the species' breeding cycle to identify biomarkers of egg production and to examine health status; (ii) determine the interval between mating and oviposition using mixed-male AI trials and paternity analysis of resultant fertile eggs and; (iii) develop a working model for the species' timeline relative to oviposition of steroidogenesis, egg formation, female sperm storage and fertilization using plasma endocrine and biochemistry data from conceptive inseminations.

2. Materials and methods

Blood collection and AI procedures were performed on restrained animals. Semen samples were collected using routine husbandry training and were obtained from unrestrained animals. All procedures described herein were reviewed and approved by the SeaWorld Parks and Entertainment Incorporated Institutional Animal Care and Use Committee, and were performed in accordance with the US Animal Welfare Act.

2.1. Animals

Eight adult female Magellanic penguins all of proven fertility were used in the research during the 2013 ($n = 4$) and 2014 ($n = 4$) breeding seasons for characterization of plasma hormone and biochemistry profiles (Table 1). Females, all captive-born, were aged 6.8–22.8 years, weighed 4.0–5.3 kg, and were all of proven fertility. Semen was collected from seven Magellanic penguins at SeaWorld San Diego, aged 4.8–18.8 years. Five of the seven males were of proven fertility; the two non-proven males, aged 4.8 years, had not yet paired with a female at the time of the study. Penguins were maintained in an outdoor natural habitat at SeaWorld San Diego in a colony of ~30 animals and were given a standardized fish diet that was supplemented with vitamins and minerals. Additional vitamins and minerals including calcium carbonate (162 mg per day) were also given starting from 2 weeks prior to expected egg lay (based on the date of first egg lay for the colony from the previous year) until completion of oviposition.

Females were maintained in a cordoned nest site separate from their paired mate during the presumptive copulatory period (Weeks-3 to -1 from egg lay). Transparent fencing material integrated into natural vegetation around the nest burrow was used to permit visual, auditory and tactile contact but prevented copulation between pairs. Nest-sites were cordoned until the completion of lay (2013) or until the first egg was laid (2014), after which time fencing material was removed. Throughout the entire study, females were offered food and monitored for health/general body condition daily as per standard husbandry practices, and were weighed weekly at the same time at blood collection.

2.2. Plasma hormone and biochemistry analysis

Blood sampling was initiated during the first week of March in the 2013 breeding season, and during the last week of February in the 2014 breeding season (Table 1). Blood samples were collected every 4–7 days until oviposition (coincident with AI procedures), then every 10–14 days until Week 5 from the first oviposition.