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Research article

Sex identification and sex ratio of the American flamingo *Phoenicopterus ruber* using molecular techniques: importance for management in zoological collections

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Abstract

In recent years, zoos have acquired an important role in biodiversity conservation, especially for birds which are the most representative group in zoos. Proper management and conservation of species within zoos requires knowledge of sex composition within collections, to form successful breeding groups. However, many bird species are monomorphic, making it difficult to differentiate males from females. This study employed a fragment-specific PCR technique using two primers, 2550F and 2718R exclusively for the CHD-W and CHD-Z introns of the CHD gene, to identify males and females in two collections of American flamingo *Phoenicopterus ruber* in Mexican zoos. In one zoo, all the adults acquired by the zoo were sampled (n=14); in the other, adults acquired by the zoo (n=92) and individuals hatched in the zoo (n=52) were sampled. Sex was identified with 95.6–100% effectiveness. The sex ratio for adult individuals acquired by both collections was about 1:1, while it was 1.89:1 in favour of males for individuals hatched in the zoo. The high quality of maternal conditions may overproduce sons (Trivers-Willard hypothesis) in flamingos hatched in this zoo. Despite its importance for zoo management and conservation programmes, this study is one of the first to use a molecular technique to evaluate sex ratio in captive American flamingo. The male-skewed sex ratio in one of the collections could have a negative impact on the long-term survival of this population. In conclusion, monitoring sex ratio in flamingos is important to improve management practices in zoos.

Introduction

Modern zoos have evolved to become conservation and management centres through captive breeding, scientific research and conservation activities, among others (Cuarón 2005). Zoos play a crucial role in biodiversity conservation by supporting breeding programmes of threatened species (Scott 2012). The role of zoos has become fundamental for birds, considering that bird extinction rates are expected to increase considerably in the 21st century (Pimm et al. 2006). Birds are a well-represented ex-situ group in zoos, with 23.1% of extant bird species held in zoos, in comparison with 17.9% for mammals and 11% for amphibians (reviewed in Biega et al. 2019). These captive populations must be managed appropriately to increase breeding rates, becoming sustainable populations that can provide individuals for reintroduction, restocking and translocations (Azevedo et al. 2010). A sustainable population is defined as a group of individuals that have the necessary resources to avoid the need for supplementation, meaning that the group has controlled birth and death rates and there are no inbreeding or sex ratio skews, among other factors (Lees and Wilcken 2009). However, maintaining a sustainable ex-situ

population for zoos can be challenging due to factors such as small population size, low reproductive success and skewed sex ratios (Mooney et al. 2023).

Flamingos are a very popular bird species in captivity; however, the species is underrepresented in zoo science literature. Studies on captive populations are necessary to increase knowledge and, in particular, reproductive success (Sandri et al. 2018). Different factors need to be controlled, such as flock size, diet, environment and sex ratio, among others, to optimise the reproductive success of flamingos in captive conditions (Mooney et al. 2023). Although sex ratios can vary among species due to adaptive processes (West and Sheldon 2002), studies have shown the importance of an even sex ratio in flamingos to increase ex-situ breeding success (Mooney et al. 2023). However, many captive flamingo collections are not sexed, which increases the risk of unbalanced sex ratios, with consequences such as low breeding success in the case of a male-skewed sex ratio (Mooney et al. 2023). Besides reproductive success, a deviation from adequate sex ratios in small captive populations can affect space use efficiency and genetic structure (Mooney et al. 2023; Tanaka et al. 2019). Therefore, identifying sex in birds is relevant to maintain an even sex ratio in wild flocks, and even more so in captive populations (Childress et al. 2005; Herring et al. 2010; Mooney et al. 2023). Reliable sexing methods are essential for sexually monomorphic species, given that sex identification based upon phenotypic characteristics is unfeasible. Sex determination is particularly relevant in birds since at least 50% of all bird species are sexually monomorphic (Dawson et al. 2016; Santamaria et al. 2010). Species belonging to the Phoenicopteridae family, including the American flamingo *Phoenicopterus ruber* (Linnaeus, 1758; Phoenicopteriformes, Phoenicopteridae) are sexually monomorphic (Phillips and McDermott 2012).

The principal techniques used to identify sex in birds include behaviour (Jodice et al. 2000), cloacal examination (Bazzano et al. 2012), endoscopy (Lumeij et al. 1985), hormonal assessment (Bercovitz and Sarver 1988), karyotype analysis (Garcelon et al. 1985) and morphometric measures (e.g. Gandini et al. 2009; Herring et al. 2010; Koczur et al. 2015). For flamingos, the most common techniques are morphometric measures (Childress et al. 2005; Montalti et al. 2012; Phillips and McDermott 2012; Richter and Bourne 1990; Richter et al. 1991) and gonad laparoscopy (Richter and Bourne 1990; Richter et al. 1991). However, these techniques are ineffective to sex young birds and not reliable in the field (Liza et al. 2008). The increase in the number of captive species programmes makes it necessary to use sex identification techniques in monomorphic species that do not compromise the integrity of the individuals (Garcelon et al. 1985).

Molecular techniques such as polymerase chain reaction (PCR) have revolutionised the identification of sex in bird species due to their high accuracy, speed and lower stress for the animal compared with invasive techniques that could affect an individual's health or biological stability (Gandini et al. 2009; Matta Camacho et al. 2009). Santamaria et al. (2010) used two intron sequences of the chromodomain-helicase-DNA-binding protein 1 (CHD1) gene to sex birds. The identification of bird sex consists of distinguishing, after PCR amplification, the heterogametic female with two bands and the homogametic male with only one band (Fridolfsson and Ellegren 1999; Santamaria et al. 2010; Thanou et al. 2013).

Molecular techniques have been used to successfully identify the sex of flamingo species such as the greater flamingo *Phoenicopterus roseus* (Balkiz et al. 2007; Bertault et al. 1999; Boucheker et al. 2020), the Chilean flamingo *Phoenicopterus chilensis* (Tomasulo et al. 2002) and the lesser flamingo *Phoenicopterus minor* (Childress et al. 2005). They were also tested successfully on a tiny sample of five individual American flamingos *Phoenicopterus ruber* (Fridolfsson and Ellegren 1999). In this study, molecular techniques were used to identify the sex of American flamingo individuals from two zoo collections, using modified steps from Fridolfsson and Ellegren's (1999) protocol. Additionally, the number of base pairs (bp) of the CHD-Z and CHD-W genes is determined. The data collected enabled determination of the sex ratio of each collection of adults acquired by the zoos, as well as that of zoo-hatched individuals, which is important for the long-term management of flamingos in captivity.

Methods

Sampling

Blood was sampled from individual American flamingos in two zoos in southern Mexico: Payo Obispo Zoo in Chetumal, Quintana Roo (14 individuals making up the collection) in October 2017 and Xcaret Park in Playa del Carmen, Quintana Roo (145 samples; 53 chicks hatched in the zoo and 92 adults acquired by the zoo) in February 2019. Flamingos of Xcaret Park come from donations, transfers from other collections or are hatched in the park, while in Payo Obispo Zoo, all individuals have been donated or transferred. Blood samples of 0.5 ml to 1 ml were obtained from each individual by puncturing the brachial vein with a 23-gauge (0.6 mm) needle (Zaccara et al. 2008). In Payo Obispo Zoo, two blood samples were collected per flamingo to test the effectiveness of blood conservation on filter paper (1 cm^2) compared with the classic conservation technique in vials. The paper samples were dried and stored in labelled glassine bags, while the vial samples were preserved in 96% ethanol. All samples were refrigerated at 4°C. Because blood preservation on filter paper was effective, that technique was used for the blood samples from Xcaret Park, using filter paper or Whatman FTA cards. Samples were dried and stored in labelled glassine bags at room temperature for one month until all samples had been collected, at which point all were stored at 4°C.

DNA extraction and PCR amplification

DNA was extracted using the salt protocol (Aljanabi and Martinez 1997) with some modifications that consisted mainly of working in cold conditions (using cooler plates) during the whole process. DNA was extracted using a small piece of filter paper placed directly inside the lysis buffer, after which the extraction process was carried out on a regular basis. DNA quality was confirmed using horizontal gel electrophoresis in 1% agarose gels using TAE buffer (1X) (90 volts for 1 hour). DNA was visualised using a solution of GelRed (BIOTUM; 20 µl) and blue/orange loading dye (PROMEGA; 500 µl) as a post-staining method under UV light and digitised using an Imaging System (PhotoDoc-it UVP®). Finally, DNA was conserved at −20°C.

Different molecular weights between CHD-Z and CHD-W genes were observed by using the following genetic primers: 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') (Fridolfsson and Ellegren 1999). DNA was amplified using PCR with a final volume of 15 µl using the following components: 1 µl of DNA, 0.2 µl of GoTaq Flexi DNA Polymerase (PROMEGA), 0.3 µl of each dNTP (dNTP mix, PROMEGA), 1.5 µl of 5X Green Buffer (PROMEGA), 0.5 µl of each genetic primer (INTEGRATED DNA TECHNOLOGIES) and 1.8 µl of MgCl2 (PROMEGA), adjusting the volume with ultra-pure water. Amplifications were conducted in a T100 Thermal Cycler (BIO-RADTM) using modified steps from Fridolfsson and Ellegren (1999). This technique consists of preheating for 2 min at 94°C followed by the first series of 10 cycles beginning with annealing at 94°C for 30 s, a step extension protocol starting at 60°C for 30 s and decreasing at 1°C per cycle until reaching 50°C terminating with an extension at 72°C for 45 s. After this first phase, the traditional run was performed, consisting of 25 cycles with the following

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conditions: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 45 s and a final extension at 72°C for 5 min. PCR products were separated on 3.4% high-resolution agarose gel (UltraPureTM Agarose-1000, INVITROGEN) running in a buffer TAE (1X) at 70 volts for 2 hours and visualised with the same method as extracted DNA.

Data processing

The agarose gels from each zoo were analysed and the number of females (two bands: CHD-W and CHD-Z) and males (one band: CHD-Z) were identified. BioVision Software (Vilber, US) was used to determine band size. Results were compared with the sex of American flamingos from Xcaret Park that had been previously commercially sexed by molecular technique, but without knowledge of the genetic primers used, to validate the effectiveness of the molecular method. Finally, the sex ratio (here defined as males/females) of the offspring collection in Xcaret Park was determined for each year with offspring (2009, 2012, 2014, 2015, 2017 and 2018; a single female was born in 2012 and 2014, so that no sex ratio could be determined) and overall (total of females and males for the six years of data). Then, to evaluate deviation from the balanced sex ratio (value of 1), a G-test (maximum likelihood ratio) was applied for each year with an estimated sex ratio (2009, 2015, 2017, 2018) and overall years grouped together.

Results

All samples were amplified correctly, except one sample from Xcaret Park. The reading of agarose gel (Figure 1) allowed identification of the sex of all individuals processed using the two genetic primers (2550F/2718R). For males, a single band (CHD-Z) of approximately 665 bp appeared. In contrast, for females two bands (CHD-W and CHD-Z) were observed with 461 and 665 bp, respectively.

The American flamingo collection in Payo Obispo Zoo has the same number of adult males and females, leading to a sex ratio of 1:1 (Table 1). In contrast, there are 47 male adults and 45 female adults in the collection of individuals acquired by Xcaret Park, leading to a sex ratio of 1.04:1. Of the 114 individuals previously sexed at Xcaret Park, five were assigned to a different sex. Thus, a high similarity is observed between results of the current study and those obtained previously by Xcaret, leading to a very high correspondence of 95.6% of the fragment-specific PCR technique using 2550F/2718T primers. A total of 53 chicks were hatched in Xcaret Park over six different years (from 2009 to 2018), 34 of them males and 18 females (one did not amplify), leading to a sex ratio of 1.89:1 with a significant departure from a balanced sex ratio (G=5.00, df=1, P=0.025) in favour of males (Figure 2). Two years (2012, 2014) had too few chicks to evaluate the sex ratio (only one female born each year), three years presented sex ratios from 0.86:1 to 3.5:1 without significant departure from a balanced sex ratio (2009: G=2.94, df=1, P=0.086; 2015: G=0.40, df=1, P=0.530; 2017: G=0.08, df=1, P=0.780) and chicks hatched in 2018 present a highly male-biased sex ratio (G=8.73, df=1, P=0.003; Figure 2).

Discussion

The role and objectives of zoos have evolved to define today's modern zoos; one objective is the conservation of species, in addition to education, research and recreation (Rose 2018; Scott 2012). An important factor in the success of breeding programmes is knowledge of the sex of individuals, which is difficult to determine in monomorphic species such as *Phoenicopterus* (Phillips and McDermott 2012) without the use of molecular techniques (Fridolfsson and Ellegren 1999). This study presents the effectiveness of a fragment-specific PCR technique (CHD genes) for sexing American flamingos. Evaluating the sex ratio of captive-born individuals can contribute to the long-term management and survival of a captive population.

Effectiveness of molecular sex determination in American flamingo

Molecular sexing is widely used in bird species (Çakmak et al. 2017; Dawson et al. 2016), including some *Phoenicopterus* species (Balkiz et al. 2007). The current study shows that molecular sexing is a cheap, quick and efficient method to determine the sex of American flamingos with almost 100% success (100% for Payo Obispo Zoo and 98.6% for Xcaret Park). Particularly, the use of paper filter tips to collect blood samples (Hagadorn et al. 2016) is quicker, easier and cheaper than the traditional capillary tubes with heparin or vacutainers with EDTA (e.g. Çakmak et al. 2017; Ravindran et al. 2019; Thanou et al. 2013).

The technique employed here allowed high efficiency of the

Table 1. Sex identification of the American flamingo *Phoenicopterus ruber* in two Mexican zoological Parks (Xcaret and Payo Obispo). Information from Xcaret Park before this molecular work was performed.

		Female	Male	Without information
This work: Payo Obispo		7	7	0
This work: Xcaret	Adults acquired	45	47	0
	Individuals hatched at the zoo	18	34	1
Total Xcaret for this work		63	81	1
Xcaret previous information		57	57	31

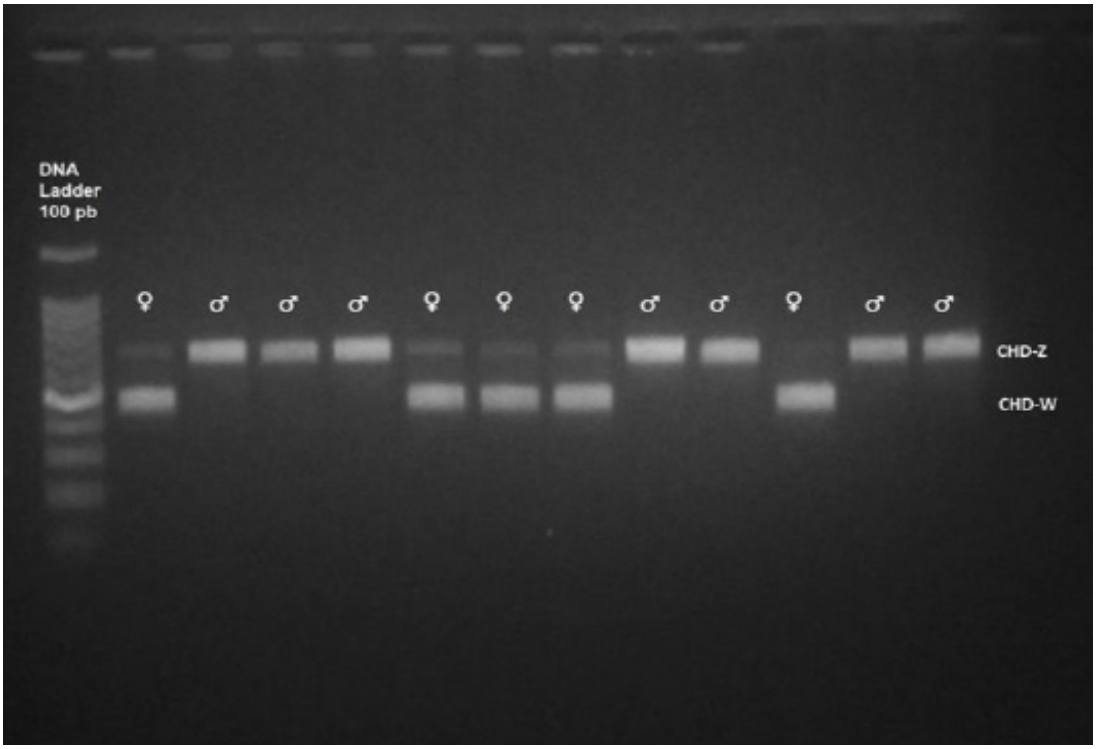

Figure 1. Sex determination of *Phoenicopterus ruber* using 2550F and 2718R primers. CHD-Z fragment at 665 pb and CHD-W fragment at 461 pb, reference based on 100 pb DNA Ladder.

genetic primers (2550F/2718R) for *Phoenicopterus ruber*, as shown for other avian species (Dawson et al. 2001; Sulandart and Zein 2012; Thanou et al. 2013; Vucicevic et al. 2013). However, for some species no amplification products are found (Çakmak et al. 2017; Zhang et al. 2013) or a single DNA product is produced for both sexes in others (Balkiz et al. 2007; Dubiec and Zagalska-

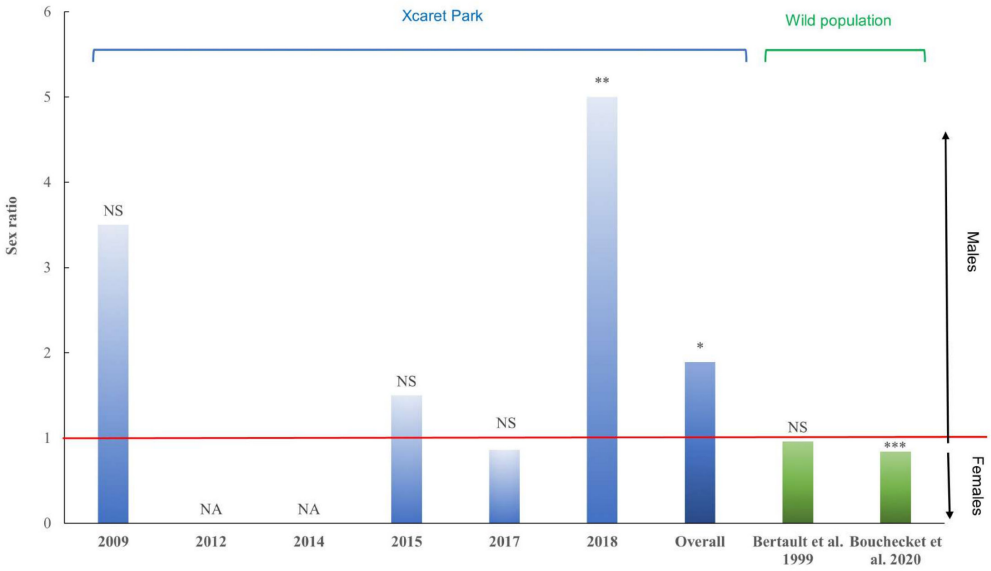

Figure 2. Sex ratio of American flamingo *Phoenicopterus ruber* chicks hatched in Xcaret Park compared with sex ratio of chicks in wild populations. Results of statistical test (G-test) for departure from balanced sex ratio: * P<0.05, ** P<0.01, *** P<0.001, NS not significant, NA does not apply. Red line represents sex ratio of 1:1.

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Neubauer 2006; Santamaria et al. 2010). A comparative study testing three different sets of genetic primers highlighted the importance of testing other primers when one fails (Çakmak et al. 2017). When comparing results with previous Xcaret Park results, there are five discrepancies in adult individuals. The effectiveness of the set of primers 2550F/2718R used in this study is not questioned. Nevertheless, when a set of primers does not produce results or has a low success rate, it is recommended to test other sets of genetic primers such as CHD1F/CHD1R, which have shown higher efficiency in some bird sex determination, while P2/P8 primers usually have a low success rate, particularly in agarose gel resolution (Çakmak et al. 2017). Some human error may have occurred in determining sex in Xcaret Park or in the laboratory. The size of the amplified bands (CHD-W and CHD-Z) produced in this study is 461 pb and 665 pb, respectively. These sizes of genetic fragments are slightly above those previously reported for other bird species (Fridolfsson and Ellegren 1999) though size difference between both fragments (here, 204 pb) lies within the reported range (150–250 bp; Dawson et al. 2001; Fridolfsson and Ellegren 1999; Thanou et al. 2013). To the authors' knowledge, there are no studies for the American flamingo using this set of primers that present the fragment size. Balkiz et al. (2007) studied greater flamingo *Phoenicopterus roseus* using the same set of primers, but did not report the size of genetic fragments.

Using sex ratios for the management of captive American flamingo

The success of a breeding flamingo flock in captivity involves, among other factors, a balanced proportion of both sexes (Phillips and McDermott 2012). The results, which indicate an adult sex ratio of 1:1 or close to it in both zoos for the birds they acquired, suggest that both institutions have adequate sex ratios for successful reproduction. Nevertheless, although Xcaret Park has successfully reproduced its population (52 chicks in 6 years), the Payo Obispo Zoo has not. Successful breeding in captivity depends on other important factors such as demographic viability control (birth/death, sex classes, population size; Che-Castaldo et al. 2018), suitability of the environment (Pickering et al. 1992) and flock size (King 2008; Pickering et al. 1992). King (2008) considers flock size to be the most important factor for optimising breeding. Pickering et al. (1992) showed that larger flocks of captive flamingos breed more frequently and have higher success in rearing chicks than small flocks. For the Caribbean flamingo, they suggested that the minimum flock size to ensure breeding is around 20 birds, although they observed a small flock of 14 birds successfully rearing a chick. The IUCN Flamingo Specialist Group suggests a flock of more than 40 birds for breeding (King 2008). The low number of individuals (n=14) in the Payo Obispo Zoo could explain the lack of successful reproduction, even when flamingos exhibited courtship behaviours (Rivera, personal communication, 21 October 2021). Of course, other factors must be controlled to optimise breeding success (King 2008; Pickering et al. 1992) and as suggested by King (2008), a cumulative effect of different factors is required to reach some level of success.

Although the sex ratios of the adult individuals acquired by both zoos were balanced, the sex ratio of the individuals hatched in Xcaret Park was strongly biased in favour of males. When the sex is genetically determined, as in birds, it is expected that the sex of offspring shows a binomial distribution with a probability of 0.5. However, this expected distribution is not always observed (Bertault et al. 2000) and biased sex ratios are relatively common in wild and captive populations (Ewen et al. 2001). Many studies have found a biased offspring sex ratio favouring males in birds (e.g. Clotfelter 1996; Whittingham and Dunn 2000), while others have found a bias favouring females (e.g. Cordero et al. 2001; Ewen et al. 2001). Different hypotheses or processes have been proposed to explain sex allocation. These include local resource enhancement (sex ratio bias favouring the helping sex), local resource competition (wherein parents invest more in the dispersing sex), steroid-mediated sex ratio adjustment (wherein a higher level of maternal testosterone can produce more sons), sex-by-environment effect or the Trivers-Willard hypothesis (wherein maternal condition has a stronger effect on the fitness of sons than daughters, leading females in good condition to overproduce sons whereas females in poor condition will preferentially produce daughters) and seasonal variation in nestling sex ratio (Bowers et al. 2015; Ewen et al. 2001; Louder et al. 2020; Merkling et al. 2018; Navara 2018; Szász et al. 2012; Trivers and Willard 1973). Studies on the primary reasons for biased sex ratio in flamingos are scarce. Bertault et al. (2000) identified a seasonal bias in sex ratio of chicks in a wild population of *Phoenicopterus roseus*, with more males produced at the beginning of the nesting season and more females later. They suggested this bias might result from a sex-by-environment effect or from the faster maturation of male-producing follicles as an extended Trivers-Willard hypothesis interpretation. Other studies on wild flamingos (Bertault et al. 1999; Boucheker et al. 2020) did not discuss the slight female-biased sex ratio observed. None of these studies accounted for factors that could influence the sex ratio observed at a particular moment, such as predation, food quality and availability, among others. In Xcaret Park, the flamingo collection is in a controlled environment where food is not limiting, suggesting that the Trivers-Willard hypothesis could explain the male-biased sex ratio observed. The bias observed in the sex of flamingos hatched in Xcaret Park could have negative consequences for the flamingo collection in the future. In fact, previous studies (Mooney et al. 2023) have shown that an excess of males can provoke more unrest in the colony and more egg breakage, leading to lower reproductive success. Additionally, studies have shown that a male-skewed sex ratio could affect population management (Faust and Thompson 2000; Tanaka et al. 2019). For example, this may lead to the need for more space to maintain a sustainable population, unstable age structure and a decrease in reproduction (Faust and Thompson 2000). This sex bias favouring males has been a recurring pattern in Xcaret Park over the years, and if it continues it could seriously jeopardise the population's long-term survival.

In zoos, sex ratio can be influenced by the management strategy manifested in factors such as diet composition (considered fundamental for reproductive success in flamingos; Sandri et al. 2018), food disposition, group size and structure, stress levels and age of the parents, among other factors (Glatson 1997; King 2008; Tanaka et al. 2019). A number of conditions need to be met to ensure breeding success (King 2008). For the populations in this study, the effect of food supply and quality should be tested (Kilner 1998) and the important factors identified by King (2008) for breeding flamingos in captivity carefully analysed to understand the causes of the current sex bias in the American flamingos hatching at Xcaret Park.

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