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Implementing artificial insemination as an effective tool for *ex situ* conservation of endangered avian species

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Abstract

Approximately 503 of the known species of birds are classified as 'endangered' or 'critical'. Captive propagation programs have proven useful in maintaining genetic diversity and restoring wild populations of certain species, including the Peregrine falcon, California condor and Whooping crane. Artificial insemination (AI) has the potential of solving problems inherent to reproductive management of small, closed populations of endangered birds, including dealing with demographic instability, physical and behavioral disabilities, sexual incompatibility, lack of synchrony, and need to maintain gene diversity. In this review, we address the necessary methods and factors that allow AI to be applied effectively to manage rare bird populations. It is clear that semen availability and quality are the greatest limiting factors to implementing consistently successful AI for birds. Behavioral sensitivity to animal handling and the ability to minimize stress in individual birds also are keys to success. Multiple, deep vaginal inseminations can improve fertility, particularly when semen quality is marginal. Laparoscopic methods of semen transfer also have produced fertile eggs. All of these practices leading to successful AI remain dependent on having adequate basic knowledge on female reproductive status, copulatory behavior, endocrine profiles and duration of fertility, especially as related to oviposition. The overall greatest challenge and highest priority is defining these normative traits, which are highly species-specific.

Keywords: Artificial insemination; Endangered species; Birds; Conservation; Sperm

1. State of the art

1.1. Brief historical perspective of avian AI

Artificial insemination (AI) was first successful in birds almost a century ago when Ivanov produced fertile chicken eggs using semen recovered from the ductus deferens [1]. The most widely used technique of intravaginal insemination was first reported by Quinn and Burrows in 1936 [1]. Since then, AI has evolved to become an important, yet common production method for the poultry industry. This assisted breeding technique now is integral to commercial turkey production, resulting in more than 300 million hatched turkey poults annually in the United States (USDA Statistical Service [2]). This same process also has been adapted successfully to produce chicks in more than 40 types of wild birds, including species of raptors, cranes, waterfowl, psittacines, and passerines [3]. Some efforts

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have been emblematic of potential. For example, Samour reported hatching more than 90 Peregrine falcon chicks during an 8-y interval using AI [4]. Furthermore, there is no evidence that reproductive success is compromised by artificial breeding, as fertility to AI was comparable to natural mating in the American kestrel [5].

1.2. Current use of AI in avian conservation programs

Most AI efforts in wild bird species have been research-oriented and/or for 'demonstration' purposes, rather than genetic management and conservation. However, there is a substantial need for consistently successful AI to assist in creating viable, self-sustaining populations. Of the known 9672 existing bird species, more than 5% are classified formally as 'vulnerable', 'endangered' or 'critically endangered' (i.e., almost 500 species are at serious risk for extinction) [6].

Although the highest priority always is securing species in nature, *ex situ* breeding has played an important role in recovering certain bird species. For example, nonprofit institutions produced and released 6000 captive-bred Peregrine falcons in 34 U.S. states from 1974 through 1997. That, combined with organized protection of nest sites and foraging habitats, resulted in one of the most successful species restorations programs ever [7]. Currently, other *ex situ* efforts are returning similarly spectacular species to nature (e.g., California condor, Mauritius kestrel, Whooping crane, American bald eagle [8]).

However, clearly there are too few recovery programs where *in situ* conservation and *ex situ* breeding/research efforts are successfully linked. Furthermore, there are even fewer examples where AI has had a measurable impact. Nonetheless, there are important programs that now stand as models to show how assisted breeding can contribute to species recovery (e.g., Peregrine falcon, [7]; Houbara bustard [9]). Most impressive have been efforts directed at the Whooping crane, where success can be attributed to intensive research into the biology of reproduction combined with unique field science to restore birds to nature. In 1941, the migratory population of Whooping crane was estimated at 15 birds. Fortunately, with collaboration involving the Patuxent Wildlife Center, the U.S. Fish & Wildlife Service, the Canadian Wildlife Service and the International Crane Foundation, there now are approximately 190 Whooping cranes in the Aransas-Wood Buffalo population, 75 in the Florida non-migratory flock, and ~120 adult birds in captivity. This population growth largely resulted from systematic studies to understand bird behavior, genetics, husbandry, chick-rearing, and medical requirements, as well as basic and applied reproductive biology [10]. Especially important has been the development of semen collection, evaluation, processing, and AI that collectively has contributed to effective species management [10,11].

1.3. Value of AI and introduction to its limitations for wild bird species

There are multiple reasons why assisted breeding is essential to propagation programs for rare bird species. Most are related to the inability of designated mates to achieve natural copulation, often because of an insufficient pair bond at the onset of egg laying. Even in the presence of a well-established pair bond, ineffective semen deposition may result from bouts of aggression, stress, lack of libido or confidence, attempts to mate with inanimate objects, or physical disabilities. Asynchrony within the pair also is common. For example, in our experience, older females lay early in the season when many males still are not producing ejaculates with a high concentration of mature spermatozoa. Poor animal management, nutritional deficiencies, and associated diseases are common contributors to poor seminal quality, unsuccessful copulation, or oviductal evertion (that results in decreased fertility). As a result of all of these challenges, captive rare bird populations generally have a preponderance of genetically under-represented (or non-represented) founders—that is, extremely valuable individuals with genomes that can be traced to wild counterparts. When such individual do not reproduce, the consequence is lost genetic heterozygosity and a decrease in fitness, including reduced breeding competency.

Fortunately, all of the problems noted above can be circumvented through the judicious and effective use of AI. Even when 80–85% of eggs are fertile, fertility can be increased by another 5–10% simply by adding AI to the propagation program [12]. Additionally, AI theoretically becomes even a more powerful tool, given that sperm can survive cryopreservation. The ability to retain post-thaw sperm viability offers substantial opportunities for sustaining genetic diversity by facilitating the shipment of viable sperm between geographically disparate populations or re-deriving genetic material from long-dead sperm donors.

Although there are enormous benefits from using fresh and thawed sperm via AI for rare wild bird species, there are important challenges to practical implementation to enhance conservation. The primary limitation appears to be the inability to consistently collect semen that is of sufficiently high quality to result in fertility post-AI. Unlike domestic poultry, semen quality in wild male donors generally is poor (i.e., low volume and sperm concentration), with ejaculates frequently contaminated with urine. Although there are few objective data for wild avian species, it is well established that stress is an important disrupting factor in implementing successful assisted breeding in the commercial poultry industry [13]. Outcomes have included delayed ovulation, premature laying of soft-shelled eggs, or interrupted oviposition [13,14]. Since these serious perturbations occur in common, domesticated stock, it is logical to expect similar, if not more severe disruptions in wild counterparts. In that regard, a majority of wild individuals in captivity demonstrate clear behavioral indices of nervousness and 'stress' during handling for semen collection or insemination. Such stress may contribute to the lack of consistency in collecting good quality semen samples from males in some captive populations. Thus, a high priority in successful programs has been to use socialized individuals, especially those imprinted on humans and preferably those from valuable founder stock. Of course, this often is not the case as founders generally are wild born, often disabled (the reason they are now held *ex situ*), but with extremely friable temperaments.

The other high priority is the need to increase our knowledge base of the fundamental reproductive and behavioral processes of each species. Reproductive mechanisms pertaining to seasonality, cyclicity, copulatory behaviors, and duration of fertility are as diverse as the number, morphology and geographic distribution of extant species. Having access to this fundamental information is the foundation of devising and implementing a successful AI program for wildlife species. The remainder of this manuscript highlights these 'limiting factors', as well as 'best known practices', from data published in the literature or from the authors' experiences. In the end, it will be necessary for those interested in implementing AI to a wild bird managed care program to address the following four questions:

1. Does the species truly experience severe challenges to natural reproduction that contributes to reproductive failure, poor genetic diversity, and underrepresented founders?
2. Are adequate numbers of birds (proven semen donors and healthy female conspecifics) available during the breeding season for basic and applied research, including AI attempts?
3. Is there adequate, at least preliminary scholarly and practical knowledge about the species (i.e., information on fundamental physiology, behavior, temperament, anatomy, and stress sensitivity) to justify the chance of ultimate success?
4. Are sufficient resources available in terms of scientific/husbandry expertise, time, and equipment?

All of these questions require a positive affirmation before beginning a serious initiative.

2. Methods and observations associated with bird AI

2.1. Semen collection

Unlike domesticated poultry where flocks are housed under environmental conditions aimed at maximizing egg and semen production for 30–60 wk, wild birds invariably experience a short (30–120 d) season of sexual activity. Consequences include restricted (1) time periods for research and (2) opportunities for semen collection, processing, storage, and use [15,16]. Seasonality and natural wild behaviors also require that birds be conditioned for handling and collection prior to sperm production onset [17].

Three semen collection methods have been used successfully in birds. The first, a 'cooperative' approach, was pioneered by falconers using sexually imprinted birds-of-prey [18]. Here, birds voluntarily copulate on special devices in response to a behavioral stimulus (e.g., adequate vocalization, food, and nest material transfer followed by a copulatory display). There is no animal handling, so stress and risk of trauma (to the bird or handler) are minimized. The advantage of this method is that the ejaculate generally is not severely contaminated by urine or feces. However, seminal volume varies significantly among individuals, and some birds perform copulatory behavior, but fail to ejaculate or produce few or no spermatozoa. Improved results have been produced using an artificial vagina in the Muscovy duck [19] or a 'dummy' female in the Houbara bustard [9]. A second method is electroejaculation that has been applied to ducks and geese [20], pigeons [21] and a variety of psittacines [22]. Although safe when conducted properly, anesthesia is required, and ejaculates frequently are contaminated with urine.

Consequently, the third method, abdominal massage [1], has clearly emerged as the most practical and often used procedure for recovering bird semen. In 1936, Burrows and Quinn first described this noninvasive method for collecting poultry semen [1]. The male is restrained, followed by gentle but rapid stroking of the back region from behind the wings towards the tail. Most males respond with phallic tumescence, at which time the handler gently squeezes the cloaca, expressing semen through the external papilla of the ductus deferens. Collecting semen in this fashion from small passerines is relatively easy [23]. During the reproductive season, most songbirds have a well-developed cloacal protuberance; a pea-like swelling on the dorsal cloacal lip where the semen is stored [24]. Small volumes of semen can be obtained by applying gentle, steady pressure to the protuberance. Because of a minute ejaculate volume, diluent is added immediately to prevent sperm dehydration. Adaptations in massage collection method have been made for waterfowl, ratites, guans, and tinamous, because these species have a penis-like copulatory appendage [25]. For birds producing limited ejaculate volume, the operator must evert the phallus early in the collection process, and a suction device often is used to avoid losing semen on the phallic surface [15]. Semen from larger-sized individuals (i.e., cranes, storks, eagles) is recovered with the bird in a standing position [26]. The male is cradled between an assistant's legs, with the bird's head and neck behind the handler and the animal's breast propped against the assistant's thigh. Safety for the bird and operator is critical [3]. During the breeding season, cranes can be aggressive, and a broom often is used as an object for the male to attack and to help corral the bird for restraint [17]. Clear, rigid plastic tubes, slightly larger than a bird's body, have been modified for parrots [3], largely eliminating serious biting. The bird is placed headfirst into a tube that is sufficiently long to ensure that the head remains completely protected. The bird's tail and cloaca are accessible from the contralateral end with the feet restrained with jesses through holes in the tube bottom. This positioning still allows the male to be adequately stroked. Birds are easily released through the tube front after collection.

2.2. Ejaculate quality and sperm characteristics

There is a remarkable variation in ejaculate volume and sperm concentration and quality among avian species, even those species of similar size [27,28]. For example, the American kestrel produces a diminutive ejaculate volume (10–15 μL), containing less than 0.03×10^6 sperm/mL (compared to the domestic turkey ejaculate that averages 300–400 μL, containing $8–12 \times 10^9$ sperm/mL) [12]. The method of collection and the frequency of natural mating influence ejaculate volume [17]. Although semen quantity is low early and late in the breeding season, limited volume also can occur during peak breeding season due to frequent copulations [16]. Larger semen volumes have been associated with males in a pair experiencing compromised fertility, in contrast to highly fecund pairs where the male is ejaculating a smaller volume [29]. Collection attempts earlier in the morning also can improve semen yields and, therefore, is standard protocol for domestic turkey stud farms, as well as for some wild species (e.g., Piping guan [30]).

Interspecies differences in sperm morphology, mitochondria numbers, metabolism, motility, and duration of storage in the female also are important. These variations, in turn, markedly influence strategies for AI and liquid semen storage/cryopreservation. Sperm morphology varies from a simple sauropsid form (e.g., domestic poultry) to a complex helical type with an exterior ribbon-like membrane and long flagellum (e.g., passerine) to a rounded, flattened shape (e.g., American kestrel) [31]. Avian sperm size (head-to-flagellum tip length) ranges from 30 to 300 μm and is unrelated to bird mass [31]. In fresh semen samples, sperm pleomorphisms are uncommon, although one study of Houbara bustards revealed that as many as 64% of sperm had large nuclei, perhaps due to aberrant spermatogenesis [32]. The midpiece appears to be the most variable component of avian sperm [33], especially in numbers of mitochondria that provide the energy for cellular motility. Although similar in ultrastructural appearance, Japanese quail spermatozoa contain more than 1400 mitochondria, compared to only 20–30 for the turkey [34]. Fowl and turkey spermatozoa have similar morphology and mitochondrial numbers, yet the former are capable of anerobic glycolysis, whereas the latter depend on aerobic oxidation [35]. This suggests that there may be variations in energy requirements for sperm motility or survival within the female reproductive tract.

2.3. Female reproductive features of interest

The avian reproductive tract is inherently complex (reviewed in [39]), and this review focuses strictly on those aspects having the potential of influencing AI success. Birds commonly present only a single, functional ovary and oviduct on the left side of the

body. As a result, the AI procedure needs to be directed to the left side of the cloaca. The oviduct is comprised of five morphologically distinguishable regions: infundibulum, magnum, isthmus, shellgland, and vagina. Successful AI requires an operator who is highly familiar with the specific anatomical features of the species of interest, including having the skill to locate the vaginal orifice. Both the ovary and oviduct size and weight increase markedly as the reproductive season approaches, largely as a result of increased steroidogenesis [36]. Simultaneously, there are enhanced size, vascularisation, and mucus production in the vagina, development of the brood patch (an area on the chest and abdomen comprised of feather-free skin where the subcutaneous tissue is modified for improved egg surface contact-incubation), abdominal distension, and increased flexibility and distance between the pubic bones, all being collective indicators of an ideal time to AI. It must be emphasized that the oviduct is susceptible to disease. Insemination must be performed carefully. If not, one can expose the vagina to infection, trauma, or stress that, in turn, can cause (1) egg yolk peritonitis (i.e., yolk entering the abdominal cavity [37]) or hyperperistalsis that can elicit premature laying of soft-shelled eggs [38].

All domestic and wild birds examined to date have at the anterior end of the vagina, a uterovaginal junction (UVJ) and specialized tubular invaginations of the surface epithelium known as sperm storage tubules (SST) [39]. This important adaptation no doubt improves overall reproductive fitness by ensuring the presence of sperm, potentially important in the case of a wild female losing its mate, thereby allowing a 're-clutch'. In an *ex situ* breeding program, the SST serves as a sperm reservoir to facilitate fertilization between inseminations. Although the length of sperm storage is unknown for most species, fertile eggs have been produced as long as 45 d after AI in the domestic turkey [40]. Typically 6–10-d intervals between successive inseminations are used in commercial poultry industry programs to ensure fertility. The SST also has been observed microscopically in the folds of the mucosa of the American kestrel [41], Peregrine falcon, European eagle owl and Marsh harrier [42]. Although the physicochemical mechanism for sperm storage and release has not been well studied for birds, the presence of the SST offers a unique advantage for avian AI programs by extending the window of opportunity for fertilization and reducing the number of required inseminations. This is a fascinating and rich area for future research.

2.4. Artificial insemination technique and equipment

2.4.1. Insemination in unconditioned (non-cooperative) birds

Captive breeding programs often are comprised of wild-caught birds that have become physically disabled. Although some of these animals can be conditioned to voluntarily accept AI after several breeding seasons, most cannot. There are three steps for artificially inseminating these type birds. The first involves female restraint that must be done quickly and preferably in the bird's home cage to avoid transport-related stress. The handler approaches the female from the rear and secures each leg and corresponding side wings, each side with one hand. This allows the female to be positioned in a natural mating position. The bird's head is covered immediately with a hood or towel to reduce fear and prevent biting. Alternatively, in larger and long-neck species (i.e., cranes or vultures), the head is directed backwards from the handler. Rarely is it recommended that a female be permitted to stand on or grab a perch during the procedure, as this allows the bird incentive and purchase to move excessively, thereby complicating the insemination. Risk of self-injury also can be reduced by securing talons into special leader sacs. There are several exceptions to these recommendations. For example, when full oviductal evertion is required in large species (e.g., eagles), then there can be an advantage to allowing feet on the ground or talons affixed to a perch. In such cases, the female can better contract muscles that, in turn, facilitate oviductal protrusion. Additionally, if a vaginal speculum is to be used in larger-sized birds and evertion is not required, then we recommend holding the female suspended head down on a 90° vertical angle, with the body tilted slightly to the right. A small portable table can be used to support the bird's chest to increase animal comfort. The primary advantage to this approach is to avoid inseminate contamination. Excreted urine can inhibit locating the vagina, with urine flow being especially voluminous when a female is near oviposition onset or in an egg laying period. Vertical positioning helps retain urine in the urodeum, avoiding it streaming into the oviduct and damaging sperm or, more seriously, causing tract infections (Fig. 1).

The second step to AI is determining the optimal insemination site, of which there are three possibilities: cloacal, intravaginal, or intramagnal insemination. Cloacal insemination is substantially easier, faster, and less stressful compared to the other two approaches. However, fertility is approximately four times higher

Fig. 1. Female positioning for AI includes restraining the same side wing and leg together, while the bird is maintained in the headdown, vertical position, with a slight tilt to the right. This is often crucial during non-cooperative AI in larger species so urine is less likely to flow from the urodeum obscuring the location of the entrance to the oviduct.

when sperm are deposited within the vagina compared to the cloaca (using the same number of viable sperm; Blanco, unpublished observations). Nevertheless, this compromised fertility sometimes can be circumvented by boosting both the number of inseminated sperm (usually to more than 15×10^6 total motile cells) and insemination frequency to three or more times per week. This strategy has allowed cloacal AI to result in 80% fertility in crane species [11,17,43].

Certainly, an intravaginal approach is used most often in birds. There are two ways to identify the oviduct, either by simple visualization or by manually everting the oviduct at the cloaca. The latter is advantageous in providing substantial space for depositing large seminal volumes, while concurrently helping prevent tract peristalsis that can eject the transferred semen. The oviduct is everted by applying a stimulating massage (similar to that described earlier for semen collection), followed by digitally manipulating the paracloacal area. This procedure is most applicable to passerines, but can be stressful to most other wild birds and, thus, is rarely used. In larger birds (e.g., Golden or Imperial eagle), eversion generally is difficult if there is a hard-shelled egg mass in the uterus. In contrast, the oviduct of small birds (e.g., quail or Red partridge) actually is easier to evert manually in the presence of a uterine egg mass which exerts some facilitative pressure.

It is noteworthy that females often react positively to the initial massage, raising the tail, spreading tail feathers, lowering abdominal feathers, and exposing the cloaca. However, resistance to final eversion occurs when the paracloacal area is touched. This can require forced manipulation, with success varying by species and individual. For this process, one attempts to stabilize the cloaca by firmly retracting the skin to

the left and dorsal aspects, while gently applying rigid and continuous pressure to the lower abdomen to expose the oviduct. For larger birds (e.g., eagles), this procedure requires two people, one restraining the individual and exposing the oviduct, whereas the other performs the AI. An alternative approach involves using a vaginal speculum (sized according to the target species) that is introduced superficially while applying pressure to the paracloacal region. This technique generally is far simpler, faster, and less stressful than manual eversion, especially for species such as the Peregrine falcon and eagles. Blades of the speculum are introduced into the cloaca being directed to the left to more easily identify the vaginal orifice that can be visually observed with assistance of an adjacent cold light source (Fig. 2). Over-insertion of the speculum can actually allow the blades to enter the oviduct, thereby causing disorientation and contamination. Finally, a palpation method also has been described for locating the oviduct in cranes and ostriches [12]. Rather than a speculum, this technique involves palpating the vagina with a sterile probe (with attached insemination cannula) or finger.

The third approach for eventually achieving successful AI is via an intramagnal route. Our laboratory has demonstrated the feasibility of this minimally invasive technique [28]. Originally, anesthesia was required, but more recent refinements have allowed inserting an endoscope through the tract and into the magnum of many individuals (e.g., mostly various eagle species) without chemical anesthesia or sedation. The female is placed in ventro-dorsal recumbency, the oviduct observed with the aid of a vaginal speculum, and then a catheter (of appropriate species-specific diameter) introduced. The length of insertion into the tract is determined by earlier assessments of post-mortem birds or as a result of contrast medium studies. These lengths are species-specific and ensure that sperm eventually are delivered to the correct tract location. This is the primary advantage, which substantially reduces the total number of sperm required for delivery. The limitation to the intramagnal approach is that it requires substantial operator knowledge and skill; otherwise, the procedure can be overly protracted and stressful. It also circumvents the intense sperm competition within the vagina and permits unfit sperm otherwise expelled by the vagina to populate the upper part of the oviduct [34]. Consequently, duration of fertility is generally no more than 24–48 h depending on species (Blanco, unpublished observations). This by-pass approach also limits the ability of the female to 'select' spermatozoa, which may be important. For example, the lack of selection in poultry has been determined to increase embryonic mortality from a normal 9 to 57% [45]. Of course, there also is the potential of greater contamination risk to the proximal aspects of the reproductive tract, requiring extreme care, sterilized catheters and washed spermatozoa.

The final step to AI involves actual semen deposition into the tract. When everted or using a speculum, the protruded, swollen oviduct is 'volcanic' in appearance. The preferred insemination device is a plastic intravenous catheter that is available in a wide variety of diameters and lengths (i.e., Nipro®, Terumo®). 'French' catheters with a blunted tip are ideal, having the advantage of minimal residual volume post-insemination. Glass insemination devices are avoided, and commercial catheters produced for the poultry industry have little applicability to wild birds and are not recommended. The catheter is inserted to a species-specific depth, based on multiple factors (see below). Once the insemination device is in place, semen is delivered with the aid of a 1 mL (sterile) nontoxic plastic syringe with an air embolus adjacent to the plunger to ensure expelling fluid, all with a coordinated withdrawal of the catheter as semen is injected. The female can be released immediately into its enclosure.

2.4.2. *Insemination in conditioned birds*

Properly trained, imprinted females may establish a pair bond with the caretaker that stimulates courtship displays and vocalization that, in turn, elicit copulatory postures and oviductal exposure. The enormous advantage of this unusual response is that no restraint is required, and no stress occurs. Often, simply applying light hand pressure on the bird's lower back causes a copulatory reflex (depending on species). Usually the operator can use one hand to direct the tail feathers slightly cranially while using the other hand to insert the insemination device straight into the everted oviduct followed by semen injection and simultaneous catheter withdrawal. The primary limitation is the significant time invested initially to train each bird to accept the procedure (usually twice daily for several weeks or even months). However, once a pair bond is established between animal and handler, the bird will allow this insemination approach for the remainder of its reproductive lifespan. An additional benefit is that this technique has been shown to stimulate ovipositioning and overall fertile egg production in several raptor species (i.e., Golden eagle and Peregrine falcon; Blanco, unpublished observations) (Fig. 3). Thus, properly managed, imprinted females start laying

Fig. 2. An alternative approach to oviductal evertion involves using a vaginal speculum, a technique generally that is simpler, faster, and less stressful. Speculum blades are introduced into the cloaca, and directed to the left, to more easily identify the vaginal orifice that can be visually observed with the assistance of an adjacent cold light source.

earlier, produce more total eggs, and experience overall higher fertility compared to untrained or unconditioned counterparts. Excellent descriptions on the training of conditioned, imprinted birds, especially raptors, cranes, storks, and waterfowl, are available [46–48].

2.5. Management and selection of AI candidates

We have found that certain procedures maximize fertility. First, it is prudent to consider fasting the female 24 h in advance of a scheduled AI, largely to minimize the presence of accumulated urates and fecal debris in the urodeum. Second, it is important to capture and handle the bird in a physical area away from the nest to avoid accidental egg breakage. Third, inseminations should be conducted in birds that have had the opportunity to naturally mate or express a copulatory display, as both activities facilitate sperm transport post-AI. Finally, all females (regardless of type) are rewarded with a favorite food immediately after sperm deposition.

Although candidates for AI include both unconditioned and conditioned birds, clearly the best targets for developing a new program or increasing the odds of offspring production are imprinted individuals. Any bird that is substantially stressed is prone to premature, interrupted, or stopped ovipositioning, egg breakage, and sperm rejection. Imprinted individuals are least susceptible to such perturbations, especially if managed appropriately to maintain the pair bond with the caretaker/handler. This relationship is enhanced by person-to-animal vocalizations or physical displays, providing more food or nesting material, and massaging the bird's lower back and paracloacal area. The protocol is substantially different for the unconditioned, non-imprinted counterpart. Here, most of the focus is on intensive and meticulous monitoring of breeding behavior and, more recently, noninvasive assessments of longitudinal endocrine metabolites extracted from feces [49]. Whereas imprinted females routinely tolerate multiple inseminations, AI number in unconditioned birds is minimized, relying as much as possible

Fig. 3. First Golden eagle produced using intramagnal insemination. Artificial insemination (AI) has the potential of solving problems inherent to reproductive management of small, closed populations of endangered birds, including dealing with demographic instability, physical and behavioral disabilities, sexual incompatibility, lack of synchrony, and need to maintain gene diversity.

on experience or empirical data on minimal numbers of sperm required and estimates of survival in the SST (see above).

2.6. Artificial insemination timing

In general, larger-size avian species (e.g., eagles and cranes) are inseminated twice per week during the 2 wk prior to onset of egg laying, and then after each oviposition. In contrast, smaller counterparts (e.g., Booted eagle or Eleonoras falcon) are inseminated three times during the week before oviposition begins. Of course, optimal AI protocols are species-dependent and related to specific biological norms. The three most critical factors are: most practical insemination method (see above, Section 2.4); required minimum sperm number (see below, Section 2.8); and duration of female fertility for the species of interest. For larger species laying only one or two eggs per breeding season, it is advisable to start inseminations 7–14 d in advance of the first oviposition. For example, optimal fertility is achieved in cranes inseminated 10–14 d before the first egg is laid [3]. Some birds (i.e., Mississippi sandhill crane) easily tolerate twice per week inseminations for two or three consecutive weeks in advance, and a few hours after each oviposition [17]. Two or three inseminations every other day, or every 3 d, also has produced excellent results in pheasants [50]. Japanese quail often are inseminated every other day [51], whereas good fertility occurs in turkeys by inseminating only once every 3 wk [40]. For species or individuals prone to stress, it is prudent to inseminate only once on the day before the first oviposition, thereby 'sacrificing' the first egg to secure fertility in the second and subsequent eggs. Otherwise, such sensitive females may refuse to lay, or oviposition is delayed in the face of multiple restraints. However, once laying begins, egg production rarely is interrupted by insemination, regardless of bird type.

In general, the smaller the body mass, the shorter the duration of fertility. Additionally, it appears that sperm retention in the SST is improved (at least in the turkey) by inseminating before the first egg is laid [52]. Recent endocrine monitoring data from our laboratory also

suggest that there are hormonal profile traits during a few days prior to the first oviposition that also signal a milieu highly accommodating to sperm, a period when the female readily accepts copulation at a higher frequency. Studies in the turkey have demonstrated that delivering sperm immediately before egg production onset maximizes sperm filling the SST, thereby promoting fertility throughout the laying interval [53].

It also is clear that AI should be performed as soon as possible after each oviposition. However, the oviductal expulsion peristalsis associated with laying continues for at least 30 min, which compromises the ascendant passive transport of the deposited sperm. Therefore, AI should occur coincident with the antiperistalsis associated with retraction of the uterus. In contrast, AI immediately prior to egg release has been related to decreased fertility, perhaps because fewer sperm reach the SST or more are being expelled during egg laying [54]. The actual practicality of inseminating soon after each egg is laid depends on the minimum time required between AI and egg laying that, in turn, is species-specific. For example, this interval is 60 h in the Peregrine falcon [48]. Thus, for such females that lay an egg every other day, a given insemination will fertilize the third egg in a sequence, but not the one produced on the next day after AI. Insemination efficiency overall also may be influenced by time within the breeding season. The suggestion that there is incomplete filling of the SST early during egg production followed by ineffective storage later in the season has been refuted by Robinson et al. [55]. Likewise, there has been at least one report suggesting that Golden eagle eggs laid later in sequence are more fertile than earlier counterparts [47]. Finally, it has been asserted that older females probably require more (and more frequent) inseminations as aging adversely influences SST integrity and/or function [39].

2.7. Depth of insemination

Although much more effective than cloacal insemination, vaginal AI remains highly inefficient. For example, in the Bengalese finch, 95–99% of deposited semen leaks almost immediately from the vagina and is lost [23]. Therefore, deep intravaginal AI is the best approach for achieving high fertility, as long as depth of insertion does not completely by-pass the uterovaginal SST. Lengths of insertion compatible with the intramagnal AI technique (see Section 2.4.1) should be restricted to special circumstances where only a few spermatozoa are available, and insemination is planned for a known time of ovulation.

Depth of insemination depends largely on species size and the presence/absence of fully formed eggs. For example, in the case of the latter, length of insertion in the Bonelli's or Golden eagle decreases from 35 to 25 mm. At times prior to onset of egg laying, the level of female receptivity and endocrine status significantly influence depth of cannula insertion. Additionally, if the oviduct has not been everted, the cannula requires deeper penetration to compensate for the absence of oviductal protrusion.

2.8. Semen volume, dilution, and in vitro storage for AI

Inseminate volumes vary substantially and, as expected, vary according to species size and ejaculate quality. Inseminate volumes range from 5 to 10 μL in passerines [44], 33 to 150 μL in medium-sizes species (i.e., falcons, monals, trapogans) [48,50], 100 to 200 μL in cranes [26] and 600 to 1400 μL in ostriches [44]. The smaller the female's size, obviously the lesser the ability to accommodate a larger inseminate volume. Regardless, the preference always is to AI with a limited volume containing a high sperm concentration, thereby reducing opportunities for backflow and semen loss. A large ejaculate volume does not necessarily translate into more sperm recovered (bird semen samples can be very dilute with low sperm concentrations). Therefore, it may be necessary to inseminate a greater than desirable volume to ensure adequate numbers of deposited sperm. Optimal fertility also is secured with fresh, undiluted semen, with dilution only recommended for highly viscous ejaculates containing high sperm concentrations such as observed in passerines, or when semen is not used immediately for insemination. For these species/circumstances, dilution should be one part semen to one part diluent. In the absence of a species-specific diluent, Beltsville Poultry Semen Extender (BPSE) or Lake's diluent (maximally, a 1:2 ratio) can be used. However, it is important to minimize dilution to allow retaining valuable sperm proteins and viscosity that actually may enhance post-AI fertility [56].

Little is known about the species-specific minimal number of sperm required to maximize fertility. Generally, an inseminate contains all the viable sperm available from a given male for that particular day. Understanding the sensitivity of fertilization in birds to sperm number for assisted (as well as natural) breeding is a high priority for future research. No doubt results are influenced by species, age, sperm quality (concentration, motility, structural integrity), time to oviposition, and

type of insemination approach used. More than 16×10^6 sperm twice per week produces excellent fertility in cranes [17], whereas 8×10^6 cells usually are sufficient in falcons. Generally, AI of wild birds requires about 20×10^6 spermatozoa to achieve fertility.

It is beyond the scope of this paper to address issues associated with short- or long-term storage of avian spermatozoa. Detailed reviews are available [12,44]. It is noteworthy that fertility with refrigerated or thawed sperm in the Golden eagle has mimicked the success achieved using fresh semen (Blanco, unpublished observations). However, inevitably there is loss in sperm integrity, motility, and probably function from the cryo-related stress, requiring that final inseminated sperm number be increased to account for this loss.

3. Disease control and AI

Birds present special challenges in disease control. Avian anatomy predisposes a semen sample to fecal contamination and, thereby, an array of problem-causing bacteria, the most common being *E. coli* [57]. The semen diluent may be another common source of contamination, especially for *E. coli* and pseudomonas [58]. These agents can trigger significant sperm mortality in raw or diluted ejaculate and, when used for AI, may cause both systemic disease and infertility [58]. This problem is commonly addressed by adding antibiotic to diluent (i.e., penicillin and streptomycin) although these drugs may adversely impact sperm viability [59], an issue deserving more research attention.

Other pathogens also are worthy of note. For example, an ureaplasma organism related to malformed sperm and infertility has been detected in the semen of some turkey strains [14]. It also is known that pasterellosis and salmonellosis can induce histopathological changes in the avian testis leading to decreased sperm production and increased abnormalities. Although it is unclear if these diseases can be transmitted via AI, horizontal transmission has been demonstrated in virgin hens inseminated with salmonella-contaminated semen [13]. Vertical transmission of this pathogen also has been reported, with either route having the potential of causing irreversible infertility [13].

Candida albicans, a yeast, has been isolated from the papilla of infected birds-of-prey and geese (Blanco, unpublished observations). The result is an abnormal swelling of the male copulatory organ that can contribute to both discomfort to the male during collection and contamination after semen collection. *Mycoplasma meleagridis* has been found in turkey semen and is known to be spread by AI [14]. The parasite hexamita also has been reported to cause low fertility in the turkey [60].

Latent herpesvirus infection has been reported to produce malformed spermatozoa [61]. Marek's disease virus, equine encephalitis virus, Highlands J virus, chicken anemia virus and pox virus now are recognized as being transmittable in birds via semen by natural mating [62–64]. The possible transmission of avian leukosis virus still is being debated [66]. This pathogen is absent in the ejaculate after intratesticular inoculation [64], but other findings have revealed presence in semen [65] and have suggested male-mediated venereal transmission [67].

Collectively, results to-date strongly emphasize the need to minimize ejaculate contamination by focusing on sanitary semen collection and processing (including using appropriate, yet prudent doses of broad spectrum antibiotic and anti-fungals) as well as protecting birds from pathogens. This includes maintaining thorough pathogen monitoring protocols and even strict isolation/quarantine practices for breeder populations. Once infectious situations arise, rapid mitigation is mandatory, although it is challenging to alleviate certain viruses (i.e., West Nile virus) from infected birds. Studies are in progress in our laboratories evaluating the feasibility and efficacy of sperm washing and 'purification'. Nonetheless, the most sensible and cost-effective strategy is to implement and maintain a rigorous prophylactic program [68].

4. Practical challenges and future priorities

In summarizing the challenges and needs, it is important to begin by simply encouraging that more research be directed at birds. Most investigations in the reproductive sciences have been mammal-centric, even though there are almost two-fold more bird than mammal species on the planet. Due to electrocution, poisonings, gun wounds, and other human-related causes, there is a constant influx of wild birds entering *ex situ* collections. Few of these animals can ever be returned to nature and even a more trivial proportion is used in research. These genetically valuable founders are an incredible resource for basic and applied research and for implementing new, or supporting existing, genetic management programs. A related priority is building more facilities and recruiting scientists who can learn from these invaluable specimens and populations.

Certainly the highest priority for implementing a successful AI program for any bird is having substantial fundamental knowledge regarding the target species, beginning with information on natural history and basic

behavior. In the context of wild individuals now living in captivity, it is essential that these animals be placed in an appropriate *ex situ* environment that minimizes stress and provides appropriate daily needs. Even then, such founders may require years of conditioning before showing behavioral interest in reproduction or producing viable sperm or laying eggs. Thus, a first order priority is ensuring that the captive facility is minimally adequate and preferably 'enriched' to hopefully provoke reproductive behavior. As early studies have demonstrated, noninvasive hormonal metabolite assessments (via urine or feces) offer enormous opportunities to understand reproductive status short-term (e.g., during the breeding season) or longitudinally (e.g., circannually) or, interestingly, across genders, between individuals, or within a population. Furthermore, because it is possible to measure adrenal hormones [49], there are many opportunities to examine how altering captive conditions can minimize stress (e.g., impact of spatial enrichment or reduced/eliminated human contact).

It is noteworthy that AI is to be considered for genetically valuable birds that are reproductively sound, but fail to naturally mate in an appropriate captive environment. Throughout this paper, we have identified the primary challenges related to the ability to collect high quality, uncontaminated, semen samples. There is a need to determine if this problem is technical (i.e., ideal collection methods remain to be discovered), physiological (i.e., reproduction in wild birds placed in captivity is compromised by stress and/or other inadequate environmental factors, or both). We also find the differences among individuals in a given species intriguing (e.g., variations in sperm concentration and incidence of pleiomorphisms). We suspect that understanding this variation could be improved by more studies of wild-caught founders, especially comparing individuals that have just entered captivity versus those living *ex situ* for protracted intervals. Additionally, it would be useful to examine male reproductive fitness in the face of altered husbandry and other management tactics. Regardless, it is clear that there is significant species-specificity, and yet we are encouraged because intensive research in some birds has demonstrated the ability to obtain, high quality ejaculates for research and assisted breeding.

Only a few studies by pioneers in the field have dealt with the more detailed characteristics of bird reproductive physiology. There is a need for systematic studies of seminal traits, natural copulatory patterns, duration of fertility, and even basic anatomical features like the presence or absence of a seminal glomus. We are fascinated with the operational mechanisms of the SST. Indeed, how do these structures facilitate sperm viability (both structure and function) and then appropriately distribute these sperm over time? Is there something to be learned that has relevance to long-term sperm storage or transport in other species, including humans?

Finally, in the context of actually conducting an AI, much more information is needed on best practices for semen processing, including washing to both concentrate sperm and minimize pathogen contamination of the female tract. Even deposition of the semen requires substantial examination. Priorities include determining how to reduce (or eliminate) the passive efflux of semen from the vagina post-AI, which is related in part to better understand the importance of insemination depth within the tract. Of course, it is clear that there is a need for more studies on AI timing (number and duration between inseminates) and minimal numbers of sperm to maximize fertility. The latter topic could be facilitated by developing *in vitro* fertilization assays for birds, an approach that would offer exciting opportunities to study sperm–egg interaction in this taxon. Lastly, although the topic of semen cryopreservation was beyond the scope of this paper, the ability to produce offspring routinely from thawed sperm would significantly advance endangered species conservation. There already are a few encouraging examples (e.g., cranes or Golden eagles) [17,42] which can serve as models for the taxon as a whole. Given the number of rare birds coming into captivity and that few will ever naturally breed, then a priority is improving sperm cryopreservation science so this important genetic diversity can be preserved for AI use now or in the future.

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