That was then, this is now – Over two decades of progress in rhinoceros reproductive science and technology

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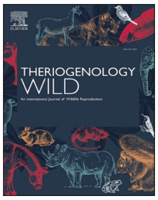

That was then, this is now – Over two decades of progress in rhinoceros reproductive science and technology

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ABSTRACT

Despite dipping to perilously low numbers over the past century, all five rhinoceros (rhino) species still survive in the wild with four also in managed breeding programs. These managed populations have been essential for advancing rhino reproductive science and technology. Despite a plethora of challenges and the incremental nature of sound science, researchers have made significant progress over the past quarter century in broadening our knowledge of rhino reproduction, developing new technologies, and expanding the scope of existing research tools. When we compare the state of this scientific field a quarter century ago to where it stands today, there is much to celebrate. For example, at the turn of the century, the Sumatran rhino breeding program had failed to produce a single calf, the first rhino artificial insemination (AI) procedures had just been described, but no pregnancies had been documented, and *in vitro* fertilization (IVF) had not succeeded in any rhino species. As we reach the end of 2023, 8 Sumatran rhino calves have been born, a total of 17 white and greater one-horned rhino calves have been produced by AI, and 51 white rhino IVF embryos have developed into blastocysts. Furthermore, several theories based on the evidence available at that time have been disproven as additional scientific data have deepened our knowledge and understanding. However, many unanswered questions still exist, and reproductive technologies require refinement, development, or application to additional rhino species, so plenty of challenges remain on the landscape for future generations of rhino reproductive scientists to conquer.

Introduction

The world's rhinoceros (rhino) numbers have fluctuated wildly over the past century, with all five species (representing four genera) reaching population lows that taunted extinction, but today the same five species still exist, with just three critically endangered [1]. However, due to a substantial increase in poaching since 2008, total rhino numbers in recent decades peaked in 2012 and have since declined despite some continued growth in black rhinos (*Diceros bicornis*) and impressive increases in greater one-horned (GOH) rhinos (*Rhinoceros unicornis*) [2]. Furthermore, two critically endangered species (Sumatran rhinos (*Dicerorhinus sumatrensis)* and Javan rhinos (*Rhinoceros sondaicus*)) number fewer than 100 individuals, a precarious state of existence. Finally, despite the survival of the four extant genera, several sub-species or geographical populations are now extinct including the Malaysian Sumatran rhino (*Dicerorhinus sumatrensis harrissoni)* [3] and western black rhino (*Diceros bicornis longipes*) [4], or functionally extinct like the northern white rhino (*Ceratotherium simum cottoni*) [5]. Given these losses, the historical volatility of wild rhino populations, and current, serious threats from poaching, political apathy, and competition for natural resources in the face of the burgeoning human population, the value of managed breeding programs is self-evident. A quarter century ago, there were breeding programs for four of the five rhino species. They have endured while playing an essential role in advancing the state of rhino reproductive science and technology. In turn, these scientific advances may prove pivotal in helping to save one or more of these species from extinction.

By its very nature, sound science progresses deliberately, incrementally, and maddeningly slowly for conservation scientists working with endangered species facing limited timelines. To explain the somewhat sluggish progress in reproductive science compared to that for humans and domestic species, researchers point to several factors including comparatively few individuals available for study, insufficient labs and programs focused on wildlife, the paucity of financial resources, regulatory hurdles, challenges in data collection, and the science-conservation disconnect [6,7]. All true, and yet in the face of these daunting obstacles, a small cohort of passionate, dedicated rhino researchers have persevered, making remarkable progress. Instead of

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belaboring what could have been accomplished if the above-mentioned obstacles had not existed, we celebrate achievements in rhino reproductive science and technology that transpired over the past two decades. To do so, reference is drawn from a similar presentation created for an International Symposium organized by the Companion Animals, Non-Domestic and Endangered Species (CANDES) founder, Dr. Naida Loskutoff, in January 2001 [8]. Comparing the state of the science then, to where we are now has been rewarding, and I hope many wildlife scientists toiling in the trenches today will find it similarly uplifting.

Although the collective team of rhino reproductive scientists has certainly done its part, to be fair, we have to acknowledge that many favorable factors facilitated this progress. First, though not as popular as the elephant or giant panda, rhinos have received significant scientific attention compared to many other wildlife species. Second, rhinos are fairly tractable, and operant conditioning training opens doors to research initially presumed unattainable. With rhinos voluntarily participating in scientific procedures, data collection can be more intense, accurately timed, and repeated at greater frequency compared to activities requiring sedation or anesthesia. As important, research methodologies are more palatable among animal care staff when animals participate by choice. Third, because rhinos are large, charismatic animals, zoo management personnel are more willing to allow staff the time/resources necessary to participate in rhino reproductive research and the sometimes labor-intensive efforts required. Furthermore, populations of three rhino species are sufficient in our zoos for generating statistically meaningful data. Finally, to a limited extent, the horse serves as a useful domestic research model [9].

All of these favorable factors, the eager participation of animal care staff, and resolute rhino scientists have coalesced over the past quarter century to advance rhino reproductive research. In return, the scientific discoveries, technologies, and their applications have been notable, ultimately resulting in tangible by-products that just may help tip the scale in favor of long-term survival for this taxon.

Advancing the tools of the trade

Inherent to reproductive sciences and especially ART are the development and advancement of technologies that generate or provide access to new/improved data and make feasible procedures once deemed beyond reach. Evidence of such progress with rhinos is exemplified by the expanded use of existing technologies and successful deployment of several newly developed techniques over the past quarter century.

Hormone monitoring - year 2000

Initiated in the early 1980 s, noninvasive hormone metabolite monitoring in feces or urine was one of the first and most powerful tools employed in the study of rhino reproductive physiology. Progesterone, testosterone, and corticoid metabolite data on black, white, and GOH rhinos already were abundant by 2001 [10–21] and had just been reported for Sumatran rhinos [22], but estrogen metabolite monitoring received mixed reviews and only proved biologically relevant if measured in GOH rhino samples [10,15,21]. Minimally invasive salivary hormone monitoring had also yielded promising progesterone data for diagnosing pregnancy in black rhinos [23]. Rhino serum hormone (progesterone and/or LH) evaluations had been conducted in isolated case studies of black rhinos and Sumatran rhinos [19,22] but were considered largely impractical due to challenges associated with serial blood collection.

Hormone monitoring – year 2024

The popularity and value of noninvasive hormone monitoring have not ebbed, but the breadth of research it supports extends beyond basic rhino reproductive physiology to include impacts of or associations with other factors including rhino behavior [24,25] management [26], dehorning [27], body condition [24], microbiome [28], seasonality [29–31] and adrenal activity [25,32,33]. Additionally, with operant conditioning training now considered essential to routine animal husbandry, and rhinos proving to be cooperative for blood collection, new opportunities for serum hormone studies beckon. Protein hormones, often degraded in fecal samples, now can be targeted in serum, and their relationships to reproductive function elucidated by strategically timed sampling strategies rather than the opportunistic single-point samples of the past collected from anesthetized rhinos [34]. Additionally, hormones like estrogen which are present in low systemic concentrations may reveal biologically informative patterns despite historic failure to transform into meaningful fecal metabolite data. There is also the advantage of improved inter-species antibody cross-reactivity when hormones are in native form rather than modified metabolites excreted in feces. Already, studies of anti-Müllerian hormone and prolactin have demonstrated that serum diversifies our tools for assessing reproductive function in rhinos and that we are no longer restricted to steroid hormone evaluations [35,36]. Finally, new multiplex technologies may allow concurrent evaluations of numerous serum reproductive biomarkers that extend beyond hormones [37], reducing labor and sparing valuable serum while yielding exponentially more physiological data.

Ultrasonography – year 2000

The feasibility of employing transrectal ultrasonography to examine the female rhino reproductive tract and diagnose pregnancy was first reported in 1991 [38]. In the following decade, the technology was used to characterize the reproductive anatomy of male and female Sumatran rhinos which revealed reproductive pathologies [39] and to monitor glandular changes during electroejaculation in male rhinos [40]. By 2001, it was becoming evident that rhinos could be trained through operant conditioning to voluntarily accept daily rectal ultrasound exams. Initial reports in a white rhino [20], GOH rhino [41], Sumatran rhino [22] and a couple of black rhinos [42] proved that the approach was both feasible and extremely informative across rhino species for monitoring ovarian activity, diagnosing pregnancy and documenting early embryonic death (EED). Additionally, an ultrasound-guided artificial insemination (AI) procedure for black and white rhinos had just been described [43].

Ultrasonography – year 2024

Serial rectal ultrasound exams are proving to be the second most powerful tool reproductive scientists can employ for studying basic rhino reproductive physiology [20,22,42,44], assessing female rhino fertility [39,45], performing ART [43,46–55] and assisting managed breeding programs [56–58]. In some cases, information gained from serial ultrasound exams has contradicted conclusions previously based solely on noninvasive hormone metabolite data. For example, a rise in progesterone is not always indicative of ovulation since anovulation often leads to luteinized follicle formation and some level of progesterone production [20,22,42,44,54,59]. Serial ultrasound exams have been essential in discovering a relatively high prevalence of EED (within the first ~3 months, and usually the first four weeks of gestation) in all rhino species maintained in different zoos, climates, countries and/or sanctuaries following natural breeding or AI [19,20,22,42,51,55,56,60] and for documenting fetal viability and growth during pregnancies carried naturally or supplemented with altrenogest which appears to help overcome EED in many (but not all) cases with no negative side effects [51,57,60,61]. Additional uses of ultrasonography have included examination of male reproductive tracts [39,40,62,63] monitoring tumor growth during hormonal down-regulation [64], testing efficacy of hormonal treatments for inducing follicular growth and/or ovulation [46,51,53,54,59], confirming intra-luminal semen deposition during transcervical AI [48,51] and guiding ovum pick-up [50]. Finally, the original use of rectal ultrasound exams for identifying reproductive tract pathologies remains relevant as tumors and cysts have been identified in more rhinos of all species, and the exact etiology of this phenomena has not been elucidated. There is clearly a strong association between the development of pathology and the extended non-pregnant state [45,65,66], but not all old, nulliparous female rhinos develop it, and there have been cases in which the severity of pathology worsened rapidly within 1–3 years of giving birth, suggesting pregnancy is not a cure. Furthermore, it no longer can be classified as a captive rhino condition because several female Sumatran rhinos have had severe reproductive pathology upon capture [67], likely due to years of isolation from males in natural habitats.

Sperm collection and cryopreservation – year 2000

Semen collection attempts had been conducted somewhat sporadically in black, white and GOH rhinos with mixed results since 1979 [68] using multiple methodologies including manual massage [69], post-mortem sperm rescue [70] and electroejaculation [40,68]. Sumatran rhino semen had been collected only post-coitum from a female following mating [70]. Scant rhino sperm cryopreservation attempts had been performed, but the limited post-thaw data suggested some sperm of all four species could survive the procedure [68,70,71].

Sperm collection and cryopreservation – year 2024

The science of semen collection and cryopreservation advanced rapidly in the early 21st century for three primary reasons, the first technical, the second out of necessity for the development of ART, and the third a fortuitous transition in veterinary anesthetic preferences. The technical advancement came in the form of custom-built rectal probes that better fit the anatomy of the rhino and provided more targeted prostate stimulation [62,72]. The caudal stimulation administered by these probes increased ejaculatory success and volume but did not entirely alleviate the problem of urine contamination. However, collecting in fractions typically ensured some portions of the sample contained negligible urine concentrations [60,63,72]. A strong desire to develop AI for the taxa also heightened efforts to improve semen collection, processing, and cryopreservation technologies, and because rhino sperm proved tolerant of varied environmental conditions and a variety of media and cryoprotectants, progress has been rapid and impressive [63,72–79]. It did not take long before cryopreserved rhino sperm proved functionally competent in producing pregnancies following AI in both GOH rhinos [51,80] and white rhinos [47] which also spurred interest and progress in sperm sex sorting technologies [81–83]. Throughout this time, many zoo/wildlife veterinarians started incorporating detomidine/medetomidine into their rhino anesthetic protocols [84]. As alpha-2-agonists, these anesthetics induce smooth muscle relaxation allowing semen to pool in the urethra while the rhino is anesthetized, thereby facilitating its collection by electrostimulation or even simple urethral catheterization [85]. How much the success of rhino semen collection can be attributed to new probes, improved expertise, or changes in anesthesia is unknown, but the end result is a win for rhino reproductive science. Regardless, variable sample quality [60,62,63,72,82], high seminal viscosity [78,81], and/or urine contamination [72,82] continue to plague ongoing efforts despite some progress in overcoming these challenges [78].

Endoscopy – year 2000

Reproductive endoscopy was largely ignored until the end of the 20th century due to early anatomical reports that the lengthy, tortuous rhino cervix contained multiple dense tissue folds and blind pockets, presumably rendering impossible the passage of a scope or catheter [86]. However, in 2000, the value of vaginal endoscopy in the development of an ultrasound-guided AI technique was reported [43].

Endoscopy – year 2024

The surprising ability to pass a standard flexible endoscope through a rhino's convoluted cervix was first discovered during GOH rhino AI trials [51]. With enough insufflation and experience in deciphering the passage through the folds while directing the scope appropriately, a sizeable (8.7 mm) colonoscope could be advanced through the entire GOH cervix for intra-uterine semen deposition. Although no calves were produced from the endoscopic AI procedures, there were conceptions followed by EED [51], and discovering it was possible led to other important reproductive endoscopic uses. For example, the intact Sumatran rhino hymen can be difficult for a male rhino to penetrate. In one case, endoscopy allowed visualization of the unusual hymen consisting of a vertical septum with bilateral tissue pouches just past the vestibular-vaginal junction (Fig. 1**A**), and identification of the tiny (3.5 mm) opening associated with the thinnest tissue of one pouch (Fig. 1**B**) that was targeted for manual tearing to access the cervical os (Fig. 1**C**). This procedure caused only minimal bleeding, was performed without any sedation/anesthesia, and did not appear to cause the rhino discomfort. Similar procedures were later conducted during standing sedation in white rhinos in which vertical vaginal septa also were reported but in slightly different intravaginal locations [87]. In another case, with initial manual assistance, a Storz 7.9 mm Telepak videoendoscope was passed through the cervix of a standing, sedated Sumatran rhino to visualize and biopsy her fibroid, and to closely examine the entire uterus (Z. Arsan, B. Bryant, S. Citino, M. Ferawati, J. O'Brien, and T. Roth, unpublished). In conclusion, we now realize that endoscopy is a viable tool for vaginal, cervical, and uterine reproductive assessments and procedures in both large and small rhino species.

ART – year 2000

The potential and some realized benefits of wildlife ART have been described in detail in several other reviews, some written decades ago [88,89] and others more recent [6,7,90,91], and will not be repeated here for fear of simply preaching to the choir. In short, rhinos stand to gain the same benefits from ART as other imperiled wildlife species assuming procedures eventually achieve consistent, reasonable levels of success, are broadly applicable with relative ease and minimum risk, and generally provide an adequate return on investment. From its meager beginnings, rhino ART has progressed rapidly (Table 1). In 2000, the first few white and black rhino transcervical AI procedures had just been reported [43], but a pregnancy had not yet been achieved. IVF had been attempted only opportunistically with oocytes collected post-mortem, and no embryos formed [92], and there had been no attempts at embryo collection or transfer in any rhino species.

ART – year 2024

Transcervical rhino AI has been conducted close to 200 hundred times across all four managed rhino species using similar methods and instrumentation following hormonal treatment or during natural estrus, and often in conjunction with an ovulation-inducing hormone treatment [47,48,51,55,80]. Multiple white and GOH rhino calves have been produced, but no successful pregnancies have yet been reported in black rhinos or Sumatran rhinos. Minimally invasive, trans-rectal OPU procedures have now been developed for rhinos, and oocytes have been recovered in three species [49,50,93–95]. The availability of fresh, partially in vivo matured oocytes facilitated progress with IVF, and the first four-cell rhino embryo was reported in 2009 [50]. In parallel, improvements to IVM for oocytes recovered post-mortem led to a single two-cell embryo [96], but the early developmental arrest of these first few cleaving embryos was not encouraging, and parthenogenesis could not be ruled out. However, a substantial scientific breakthrough in rhino IVF transpired several years later resulting in the first rhino blastocysts produced following OPU, IVM and ICSI [49]. The same study reported

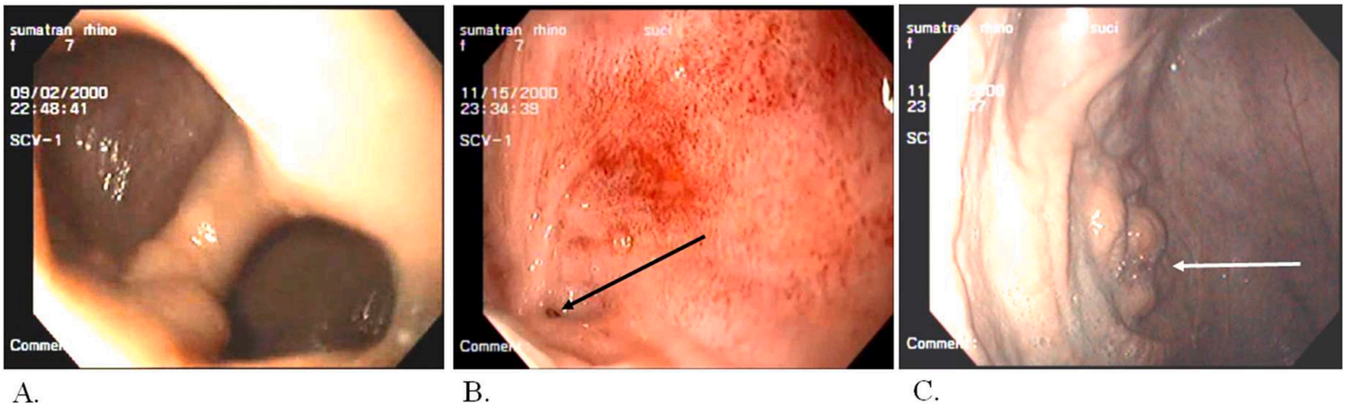

Fig. 1. Sumatran rhino hymen and vagina visualized through a flexible endoscope (8.7 mm). A) View just caudal to the vestibular-vaginal junction forced open by insufflation to reveal the vertical septum and bilateral pouches of the intact hymen. B) Hymen opening (black arrow) in one pouch next to the vertical septum. **C**) Cervical os (white arrow) viewed from within the vagina after the hymen was manually torn open. (Note: The date recorded on the video footage was not accurate. The procedure was performed in December 2011.).

Table 1

The current state of rhino assisted reproduction technologies. An “X” means the procedure has been conducted successfully and not just attempted.

Species	Ovulation induction	Treatment	AI	Semen type	AI calves	OPU	IVF	IVF Method	Blastocysts	Sperm sorting[a]
White rhino	X	hCG[46], GnRH analogue[b] [46,48,54]	X	Fresh[48], chilled[55], frozen[47,55]	10	X[49,50, 95]	X	ICSI[49]	51[95]	X[81,82]
Black rhino	X	GnRH analogue[59]				X[50]	X	Natural[50, 96]	0	X[81,83]
GOH rhino	X	GnRH analogue[51,52], hCG[108]	X	fresh, chilled[52], frozen [51]	7					X[83]
Sumatran rhino	X	GnRH analogue[53,59]				X[93]				

Acronyms: GOH = greater one-horned; AI = artificial insemination; OPU = ovum pick up; IVF = *in vitro* fertilization; ICSI = intracytoplasmic sperm injection

[a] The sperm sorting methodology used in all reports is fluorescent-activated cell sorting.

[b] GnRH analogues may include SucroMate, Suprelorin, Histrelin, Cystorelin, etc. See specific references for details.

the establishment of white rhino stem cells, and these embryo/cell culture efforts have continued with the target beneficiary being the northern white rhino [95]. Similar IVF success in other rhino species has yet to be realized.

White rhino reproductive discoveries and scientific achievements of the last quarter century

It is peculiar that the most abundant rhino species both *in situ* and *ex situ* with the greatest longevity has the worst reproductive record *ex situ*, but that seems to be the case with white rhinos. Historically, no more than half of female white rhinos reproduced under managed care, and far fewer of the captive-born females [16,97,98]. Therefore, they have been subject to research that extends across several scientific disciplines including behavior, nutrition, and physiology in efforts to understand the underlying cause(s) of reproductive failure in an otherwise healthy and easily managed rhino species.

Reproductive cycle irregularities

Early noninvasive hormone metabolite monitoring of female white rhinos produced puzzling results with some females exhibiting ~35 day cycles, others ~70 cycles and still others a mix of the two [10,16–18]. Because at that time pregnancies had been diagnosed only following 35-day cycles, the evidence suggested 35-day cycles were fertile and 70-day cycles infertile [18,20]. Today, we know that both cycle types can be fertile as pregnancies have been established following 70-day cycles by natural breeding [98] and AI [55]. This is good news for the white rhino population and important for animal staff who previously thought their rhino with a 70-day cycle was infertile. The 70-day cycle is characterized by an extended luteal phase and has been documented repeatedly in rhinos that are not breeding so it is not the result of EED as once suggested based on early observations [20]. To this day, the physiological drivers of the extended luteal phase cycle in this species remain unknown.

The same early endocrine studies revealed that almost half of the females monitored had static baseline progesterone concentrations with no evidence of reproductive activity (i.e., “flatliners”) [16,17]. The finding led some to conclude that these white rhinos were experiencing asymmetric reproductive aging [65,66] but more recent evidence refutes the theory of ovarian oocyte exhaustion. With increased integration of serial ultrasound examinations in rhino reproductive monitoring, we know that many of these flatliner rhinos are exhibiting ovarian activity in one of two ways, either they develop preovulatory follicles that fail to ovulate or luteinize and therefore do not produce appreciable progesterone, or they produce only small follicles that never reach pre-ovulatory size [54,59,65]. In either case, hormonal treatments appear to overcome these conditions leading to pre-ovulatory follicular development and/or ovulation and even pregnancies in some cases [46,48,54,55], all of which prove ovarian function is still possible, at least in a subset of individuals. The former condition simply requires a well-timed GnRH injection, and the latter responds to long-acting progestin downregulation that triggers new follicular growth, followed by GnRH to catalyze ovulation [46,54,59]. A more natural method of addressing acyclicity is to simply change a female’s social structure which can trigger improved reproductive cyclicity and successful breeding without any hormonal treatment [98]. Additionally, there are some data suggesting that white rhinos are sensitive to dietary phytoestrogens [99], and limiting their intake may have beneficial impacts on F1 reproduction [100,101]. No single finding deserves all of the credit for improved reproductive success in this species, but the good news is that the synergism of this new knowledge and improved population management have increased reproductive success in North America to 62% of females ≥ 10 years old, 20% higher than historical numbers (J. Capiro, personal communication). Furthermore, captive-bred females, once blamed for much of this species' poor reproduction [33,97,99], are succeeding similarly (58% of females ≥ 10 years old) indicating that the perceived F1 reproductive problem of the past has dissipated.

Artificial insemination

Since an AI procedure was originally developed in white rhinos [43], and white rhinos are most abundant in zoos, it is not surprising that it first succeeded in this species [48]. This ART breakthrough gave hope to similar ongoing efforts in other rhino species. Although the first two AI pregnancies were produced after insemination with entire freshly collected ejaculates, shortly thereafter, a calf was born following insemination with 500 x 10^6 motile, frozen-thawed sperm [47]. In total, eight calves have been produced by AI in several European countries (L. Versteege, personal communication). White rhino AI also has been successful in the U.S. where two calves have been produced after insemination with over 500 x 10^6 cryopreserved or chilled (overnight) sperm samples [55]. On both continents, the AI procedures included treating females with an ovulation-inducing formulation of GnRH to ensure timely ovulation of dominant follicles identified by ultrasound prior to AI. Of the 10 white rhino calves produced by AI, one (the first) was stillborn, nine are still alive, and six are sexually mature, but none have reproduced. However, the lack of reproduction is not likely to be physical but simply management related with several of the bulls in bachelor groups and females remaining with dams instead of moving to new social groups where reproduction is more likely (L. Versteege, personal communication; [98]).

Because white rhinos are best managed in herds, a surplus of bulls can develop in managed populations, and the ability to produce a female skew in the sex ratio is desirable. Fortunately, sperm sex-sorting technology, specifically fluorescent activated cell sorting (FACS), progressed rapidly in this species [81,82] resulting in samples of adequate post-thaw quality for AI trials by 2015 [82]. Though no female calf produced with an x-bearing enhanced sperm fraction has been born, it may happen soon. Meanwhile, new technologies are being developed that could be less costly, more efficient, and field-friendly compared to FACS; however, they are in their infancy and efficacy currently is unproven in rhinos [102].

IVF

Exceptional IVF progress has been achieved in white rhinos driven primarily by the desperate state of the northern subspecies that is functionally extinct with just two living females left on the planet [103]. The minimally invasive OPU procedure that allows the acquisition of fresh, healthy oocytes at later stages of maturation [50] together with involvement from world expert equine IVF specialists at Avantea Biotechnologies have resulted in 51 blastocysts produced by ICSI using a mixture of southern and northern white rhino gametes [49,95]. Efforts by the team have yielded 22 northern white rhino blastocysts that are cryopreserved and awaiting transfer [95]. Although embryo transfers (ET) have been initiated in southern white rhino surrogates with the goal of establishing a successful protocol for northern white rhinos, no calves have been produced after 15 ET attempts [95]. Similar white rhino OPU procedures have been conducted by other researchers/labs with oocytes successfully recovered, but to-date, embryo production has not been reported from these additional efforts [94]. Southern white rhino embryonic stem cell lines and primordial germ cell-like cells of both southern and northern white rhinos also have been produced and cryopreserved for future *in vitro* gamete production efforts [49,104].

Black rhino reproductive discoveries and scientific achievements of the last quarter century

The status of managed black rhinos a quarter century ago largely remains the same today in terms of reproductive needs. Of all the rhino species, black rhinos are the most prolific in human care with the fewest reproductive challenges if they are managed correctly and allowed to breed regularly. There have been similar reports of irregular cyclicity exhibited by a significant percentage of female black rhinos in both U.S. and European populations [17,24], but animal managers find breeding success sufficient for population needs (L. Smith and L. Versteege, personal communications), although scientists caution that there is the potential for genetic skew to occur in some populations [105].

Reproductive cycle irregularities

Though white rhino reproductive cycles have garnered the most attention over the past two decades, irregularities with black rhino reproductive cycles have not gone unnoticed and have been reported since fecal hormone metabolite monitoring began [17]. Unlike the two primary cycle types exhibited by white rhinos, the variation in black rhino cycle length is more random occurring intermittently throughout the year in many females [32]. Although many physiological and behavioral factors have been studied in relation to *ex situ* black rhino cycle irregularity including adrenal activity [17,25,32] body condition [24], temperament [24] and microbiome [28], similar irregularity has been documented in wild black rhinos [31,106,107] and does not appear to reduce reproductive success [31,107]. Furthermore, serial ultrasound monitoring has revealed persistent anovulatory follicles in fertile female black rhinos that produced calves before and/or after these observations (M. Schook, personal communication). The variable duration of such persistent or luteinized follicles is likely to disrupt cycle length if rhinos are not treated with prostaglandin that effectively induces luteolysis [59]. Together, these data may indicate that anovulation and cycle irregularity is somewhat "normal" for this species and not a side effect of captive management or associated physiological adaptation. However, it could be exacerbated under *ex situ* conditions, especially if females are not introduced to males regularly, and further research is certainly warranted to better understand the physiological or social mechanisms driving it. Given its prevalence, one might even suggest there is an element of induced ovulation to this species, though it could not be classified as an obligate induced ovulator like the Sumatran rhino since spontaneous ovulation has been well documented via serial ultrasound monitoring [42].

ART

Although the first IVF embryos produced following OPU [50] and oocyte rescue/IVM [96] in any rhino species were from black rhinos, those embryos cleaved only once or twice before development ceased, and there have been no additional reports of black rhino IVF suggesting the research has not progressed significantly. Because animal managers believe black rhinos are reproducing naturally at a rate that will sustain the *ex situ* population, it is not surprising that ART is not a high priority for black rhinos, but it is desired to reduce rhino transfers among facilities (L. Smith, personal communication). Furthermore, for long-term management of a population facing potential genetic skew [105], future infusion of cryopreserved sperm may be beneficial. Therefore, black rhino AI efforts have continued over the years and now have been attempted by multiple groups at numerous zoos in several countries, and in females known to be fertile, but success has eluded all efforts to-date. Discovery of the high incidence of anovulation in this species is one by-product of these efforts and could have been a factor hindering AI success in some cases [59].

GOH rhino reproductive discoveries and scientific achievements of the last quarter century

In the 1980 s, urinary hormone metabolite monitoring data in the GOH rhino indicated the species experienced highly variable reproductive cycle lengths ranging from 39 to 64 days [21], with estrogen metabolite data uniquely informative in this species compared to that of the black rhino [12]. By the end of the 1990 s, the unusually large pre-ovulatory follicles (>10 cm) produced by this rhino species had just been discovered during a case study that employed year-long serial rectal ultrasound monitoring in conjunction with urinary estrogen assessments in a single, young, female rhino [41], but the data were questioned by some who suspected the female had cystic ovaries. Although AI was the targeted goal of the project, it had not yet been attempted.

Reproductive cycle irregularities

Over the past two decades, our understanding of the complex GOH rhino reproductive physiology has increased substantially from those early beginnings. Due to multiple studies involving the powerful combination of hormone monitoring and serial ultrasonography, it is now well established that GOH rhinos produce the largest pre-ovulatory follicle of any mammalian species studied to-date, with it commonly reaching 10–12 cm in diameter [44] and sometimes up to 20 cm [59]. In contrast to other rhino species, the follicular phase is long, averaging > 13 days, and the dominant follicle reaches maximum size a week or more prior to ovulation [44]. GOH rhinos also often develop anovulatory luteinized follicles [44,59]. All of the above contribute to the high variability in inter-ovulatory interval which can be as short as 36 days or as long as 61 days [44] and explains previous reports based only on urinary estrogen metabolites in the 1980 s [21].

In addition to excessive follicle size and follicular phase, GOH rhino estrous behavior can be peculiar. This rhino species is known for its typical flamboyant display of pacing, vocalizing, urine squirting, and vulva winking when in estrus, all of which typically last one day [44]. However, with some cycles, females exhibit behavioral estrus on two days separated by a few days of normal behavior [44], and in other cases, the females exhibit no estrual behaviors at all despite hormonal patterns indicating regular cyclicity [56] and ovulatory responses to hormone injections [108]. These periods of silent estrus can be misinterpreted by animal staff who often believe the female is pregnant or acyclic. Thankfully, this species' high hormone concentrations make it possible to monitor progesterone, estrogen, androgens, and LH non-invasively in urine, feces and/or saliva, which allows informative characterization of the reproductive status when behavior is unreliable [44,109].

ART

There are no reports of IVF or OPU in GOH rhinos, but there has been substantial progress with AI in this species. GOH rhino AI techniques were developed in parallel with those for white rhinos at the start of the 21st century with both achieving term pregnancies in close succession (white rhino with fresh sperm in 2005 [48] and GOH rhino with frozen sperm in 2008 [80]). Unfortunately, these first term pregnancies in each species ended with stillbirths but did demonstrate the feasibility of AI in rhinos, and subsequent efforts resulted in live calves. Stillbirths are not uncommon in rhinos [110–112] so there is no reason to believe the AI procedures were responsible. As with white rhino AI, a GnRH ovulation-inducing reagent was used to ensure timely ovulation relative to insemination in GOH rhinos since anovulation had been documented repeatedly during natural cycles, and a minimum dose of 500 x 10^6 motile sperm was targeted for the procedures [51]. In some cases, additional therapies were employed to support conception and pregnancy maintenance including oxytocin for treating intraluminal uterine fluid accumulation and altrenogest supplementation, respectively [51, 52]. Although GOH AI calves have been produced with fresh and chilled sperm, the majority have resulted from frozen-thawed semen, thereby proving the value of sperm banking for the genetic management of rhinos [51,52]. Furthermore, sex-sorted samples have been available for AI trials since 2017 [83], offering another potentially useful tool for population management. Of the seven GOH AI calves, four are still alive, and one just produced her own calf after breeding naturally (Fig. 2). She is the first AI calf of any rhino species to successfully reproduce and represents an important milestone as it is imperative ART offspring become productive additions to *ex situ* populations and not just scientific novelties.

Sumatran rhino reproductive discoveries and scientific achievements of the last quarter century

Early reproductive studies in Sumatran rhinos were not encouraging with reports of serious reproductive tract pathologies [39] and dangerous, aggressive interactions resulting from randomly timed introductions between male and female pairs [113]. However, by the late 1990 s, intensive serial ultrasound and serum hormone monitoring of one reproductively active female led to the discovery that the species is an induced ovulator with a 21-day cycle and 20 mm preovulatory follicle [22]. This information was immediately incorporated into a science-driven breeding strategy that relied on serial ultrasound exams to time male-female introductions, confirm ovulation, and detect early embryos. The strategy resulted in five conceptions over three years, but the embryos were all lost within the first 3 months of gestation [57].

Science-based breeding and pregnancy maintenance

Twenty-three years later, Sumatran rhino breeding has succeeded in both the U.S. and Indonesia. Altrenogest supplementation proved instrumental in overcoming EED and supporting the first term pregnancy in the U.S. in 2001 [57], and has appeared similarly beneficial when administered to Sumatran rhinos in Indonesia that also seem prone to EED [61]. However, serum progesterone data fail to support luteal insufficiency associated with EED in this species, so altrenogest's beneficial mechanism of action remains a mystery [60]. Regardless, eight critically endangered Sumatran rhino calves have been produced via the science-based breeding strategy, four of which already are of proven fertility themselves. Given that the wild population is down to

Fig. 2. The first rhino conceived by artificial insemination to successfully reproduce is a female GOH rhino that bred naturally at the Tanganyika Wildlife Park, Kansas, USA, delivering a healthy male calf in February 2023 (photo courtesy of Hoofstock Keeper, Sierra Smith).

just ~50 individuals [2], and wild females are often already infertile when captured [67], every new calf is a lifeline for the species. It is not known if the prevalence of EED in this species is associated with early uterine pathology that evades diagnosis via ultrasonography, but a recent successful term pregnancy in a female with a 3 cm leiomyoma and multiple small cysts throughout her uterus is encouraging evidence that some pathology can be tolerated [114].

ART

Despite natural breeding achievements of the past quarter century, many conservationists do not believe the approach will be enough to save the species, and ART is being heralded as essential for saving Sumatran rhinos [115]. Unfortunately, Sumatran rhino ART is still in its infancy, though some work has been performed. AI in this species is not difficult to perform manually with little or no sedation, and an ovulation induction protocol, essential for induced ovulators, is established [53,59]. However, pregnancies have not yet been achieved despite numerous attempts in both the U.S. and Indonesia. In one case, a young female rhino was inseminated 14 times over 18 months. GnRH was administered im or sq [53,59] 24–37.5 hr prior to AI, and ovulation was confirmed within 24 hr of the insemination in 12 of those cases, but conception was never confirmed by ultrasound (T. Roth, unpublished data). One likely culprit in these failed AI attempts was sperm quality/quantity. The median number of sperm inseminated was just 31.7 x 10^6 motile sperm, and in only one case did the dose exceed 100 x 10^6 motile sperm, far less than the 500 X 10^6 motile sperm used in successful GOH or white rhino AI [47,48,51,55].

Sumatran rhinos are notorious for poor sperm/semen quality. Samples collected post-coitum following natural ejaculation average 60% motility and 40% structurally normal sperm [70], and samples collected by electroejaculation or urethral catheterization generally are worse. Twelve semen collection attempts on four adult male rhinos (most proven sires) yielded only four samples with > 100 x 10^6 sperm, but two of them contained ~1.5 billion sperm demonstrating that recovery of higher sperm numbers is possible. Regardless, ≥ 50% of the sperm cells collected were immotile with a similar percentage morphologically abnormal. Furthermore, sperm banking efforts are plagued by high sample viscosity and/or urine contamination that results in low post-thaw motility (Z. Arsan, B. Bryant, D. Candra, S. Citino, M. Ferawati, J. O'Brien, and T. Roth, unpublished data). However, efforts to improve Sumatran rhino sperm collection/quality continue with new rectal probe designs, modifications to urethral catheterization protocols, and media components/supplements that may neutralize damaging effects of urine exposure on sperm cells or facilitate sperm recovery from viscous samples [78].

IVF has been attempted in Sumatran rhinos following both oocyte rescue/IVM post-mortem [116] and OPU [93] on several occasions, but no viable embryos have resulted, even though ICSI was employed to assist fertilization with the latter. Meanwhile, induced pluripotent stem cells (iPSCs) have been developed as a potential future source of sperm and oocytes [117]. Desperation is driving the Sumatran rhino ART effort given the dire situation of the species, and there is likely to be more progress in the near future. However, with so few fertile individuals remaining and no abundant, closely related species in existence to provide appropriate surrogates, it is unlikely that ART will save the day for Sumatran rhinos in the event the breeding population disappears.

Table 2
Notable scientific advancements and achievements in both rhino reproductive science and ART over the past quarter century and potential future directions or questions to address.

Species	Subject	2000	2024	Potential next steps/questions
White rhino	Acyclicity	Rhinos considered non-reproductive, infertile, asymmetric aging[65,66]	Many rhinos are producing follicles/oocytes; strategies available that lead to reproductive success[54,59,65,98,101]	Why does this happen and how can we stop it from happening?
	Long vs. short cycles	Only short cycles proven to be fertile[18,20]	Both cycles are fertile[55,98]	Why is the luteal phase sometimes extended?
	AI	Attempted but not successful[43]	Successful numerous times in several countries[47,48,55]	Female calves born from sex-sorted sperm
	IVF	No success[92]	Repeated blastocyst production by one lab[49,95]	Healthy calves from IVF and ET
	Stem cells	Not yet attempted	Stem cell and primordial germ cell lines established[49,104]	Functional gamete generation *in vitro*
Black rhino	Irregular cycles	Documented via fecal hormone metabolite monitoring[17]	Prevalence of HAFs following anovulation may be related[42,59]; also occurs in wild rhinos w/o impacting reproduction[107]	Is induced ovulation involved to some degree?
	AI	Attempted once[43]	Attempted many times in fertile females without success [M. Schook, unpublished]	Develop successful AI protocol for species
	IVF	Not yet attempted	Two (2 and 4-cell) embryos reported[50,96]	Production of blastocysts
GOH rhino	Reproductive cycles	Significant cycle length variation revealed by urinary hormone metabolite monitoring [21]	Lingering pre-ovulatory follicles associated with variable cycle length[44]	Develop methods of controlling cycle for fixed-time AI
	Follicle size	First report of very large pre-ovulatory follicles in one rhino[41]	Species declared to have the largest pre-ovulatory follicles of any mammal[44,51]	
	AI	Need identified but not yet attempted[8]	Successful numerous times and used to infuse lost genes into population[51]	Produce female calves with sex-sorted sperm
Sumatran rhino	Induced ovulation	Strong evidence in one rhino[22]	Well-proven across numerous rhinos in several locations [53,58,61]	Is induced ovulation unique to the Sumatran species?
	EED	Documented repeatedly in one zoo rhino [22]	Documented repeatedly in all three proven females, two in an Indonesian forest sanctuary[67]	Why is EED so prevalent in this species even when gross pathology is absent?
	Pregnancy maintenance	One pregnancy progressing with altrenogest supplement	Six, term altrenogest-supplemented pregnancies[57,59]	Can more females carry pregnancies naturally?
	Breeding	No success[113]	Eight calves born[57,59,61]	Shorten calving intervals; increase genetic diversity
	ART	Post-coital sperm collection/cryopreservation in one rhino[70]	Sperm collection/cryopreservation, AI, OPU, and IVF attempted numerous times with minimal/no success [117]	Improvement in any/all ARTs

Acronyms: ART = assisted reproduction technology; AI = artificial insemination; ET = embryo transfer; HAF = hemorrhagic anovulatory follicle; EED = early embryo death; OPU = ovum pick-up

The science-conservation connection

Key advances in reproductive science and technology for each rhino species are summarized in Table 2 for quick reference, but in closure, highlighting how all of this progress directly impacts rhino conservation efforts is warranted. We now have a variety of husbandry/management tools for overcoming reproductive failure in white rhinos. Acyclic animals are not necessarily infertile, nor are individuals with long reproductive cycles. Several strategies have proven effective in overriding these challenges to some extent, including hormonal stimulation followed by natural breeding or AI, reducing dietary phytoestrogens, and changing the social environment by moving bulls or cows among facilities. Additionally, advanced white rhino ART technologies now offer a strategy within reach for ensuring genes from the Northern white rhino contribute to future calves. Although black rhinos may exhibit irregular reproductive activity, it is not necessarily cause for alarm, and may simply be resulting from frequent anovulatory cycles. Introducing females to bulls daily for breeding is likely the best remedy if the pair is behaviorally tolerant. For GOH rhinos that are overly aggressive or for infusing new genes from cryopreserved white or GOH rhino sperm banked years ago, AI now is available as an alternative to natural breeding and/or loss of genetic diversity and can be conducted manually using simple equipment with females in standing sedation. For both GOH and white rhinos, it may soon be possible to skew the offspring sex ratio towards females which are generally more desirable for ease of management by selecting for x-bearing sperm when performing these AI procedures. The Sumatran rhino that faces imminent extinction in the wild now has a chance of surviving because a science-based approach to breeding them *ex situ* has been developed and is employed diligently with proven, repeatable success at the only remaining breeding center for this species. Each of these examples provides tangible evidence of the reproductive science-rhino conservation connection. Admittedly, reproductive science alone is not enough to save rhinos from extinction, but progress made in this field over the past quarter century offers a plethora of approaches for bolstering *ex situ* populations, thereby providing a sound counter-offense to species' declines and loss of genetic diversity in wild populations.

CRediT authorship contribution statement

Roth Terri L.: Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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