Cold dogs: Sperm freezing, artificial insemination & non-invasive monitoring tools to facilitate a hybrid conservation management approach for endangered African wild dogs

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ARTICLE INFO

ABSTRACT

Given the ongoing fragmentation and decline of suitable habitat coupled with recurring disease outbreaks and pack eradications, a major rethink is required to manage the long-term genetic diversity of the endangered African wild dog. Competing financial demands in developing countries mean that conservation strategies need to yield the biggest genetic gains for the smallest economic cost. As such, we propose the development of a hybrid conservation approach to the metapopulation management of African wild dogs, which augments assisted dispersal and natural breeding initiatives with genetic gains afforded by sperm banking and artificial insemination. We discuss how such an approach would address current challenges faced by the species in the context of its unique social biology and how this could integrate with existing conservation management interventions. Moreover, during a period spanning nearly 20 years, we have developed many of the supporting reproductive technologies required to maximise genetic diversity of African wild dogs, in addition to a suite of non-invasive management tools to quantify, monitor and manipulate behaviour, vocalisations, hormones for reproductive function and responses to stressors, as well as immune function to improve the social cohesion, health and reproduction of packs.

1. The African wild dog

The African wild dog (AWD; *Lycaon pictus*) is a wolf-like canid native to sub-Saharan Africa and the sole species within the *Lycaon* genus [1]. They are a relatively large canid (75–110 cm tall and weighing 18–36 kg) with distinctive large rounded ears, long legs, and an individually identifiable mottled coat of yellow, black, brown and white. AWDs live in packs of typically 5 to 15 individuals and can survive to around 11 years of age in the wild [2,3]. Packs consist of an alpha pair, siblings and their offspring, with separate male and female dominance hierarchies conferring almost exclusive reproductive privileges to the alpha pair [4,

5]. Despite this, up to 10% of pups are sired by subordinate males and around 8% of pups are born to subordinate females, with infanticide or possession of the litter by the alpha female common in these instances [6]. Stable packs produce litters annually with pups raised in dens over a three-month period [7]. Pack members work cooperatively to raise young, protect territories, care for sick and injured individuals and hunt [4]. Young dogs disperse from their natal pack as a single-sex group at different ages (females: 21.8 and males: 28.1 months) and form a new pack with an unrelated opposite-sex group, facilitating genetic mixing within the species [8]. Formation of the new pack is often associated with dominance and aggression both between and within groups, and its

success depends on factors such as group size, individual compatibility and the establishment of the hierarchy [4,9]. In free-living populations, success of dispersal and new pack formation remains low due to restricted or highly fragmented habitats (often within fenced reserves) and high resource competition [10,11].

1.1. Conservation status, distribution and habitat loss

The AWD was once abundant throughout sub-Saharan Africa but is now the second most endangered canid on the continent, with fewer than 6600 individuals left, of which only 1400 are adults [6,12]. Today, AWD packs are limited to just 7% of their former range and exist in low population densities across large home ranges [13,14]. Free-living populations are now largely confined to protected areas such as game reserves and conservation parks, scattered across ever-decreasing fragmented habitat within Eastern and Southern Africa and a last stronghold in Botswana [6,12,13,15–17]. In South Africa, fewer than 8% (n ≈ 550) of animals persist in 77 packs across 14 highly fragmented populations [18,19]. With the possible exception of Botswana, none of these can be regarded viable self-sustaining populations because each have fewer than 500 individuals [13,20]. Thus, the reality of ongoing human expansion coupled with predicted effects of climate change on habitat suitability, as modelled recently in the Ethiopian wolf [21], means that AWD habitat will inevitably fragment and decline further. As a consequence, opportunities for natural dispersal will cease, thereby accelerating genetic loss [11,15,22,23].

2. Threats

Historically, human persecution was the biggest cause of population decline up until the 1990 s, when legislation was enacted in many African countries to try to save the species from extinction [24]. However, human-wildlife conflict remains a significant threat to AWDs, with targeted persecution still unacceptably high, especially in farming communities in which pre-emptive killing, more negative attitudes and greater perception of risk occur [24–26]. More recently, a combination of natural and human-induced factors appear to co-contribute to the species' continued decline and loss of genetic diversity. These include habitat loss and fragmentation, anthropogenic factors (snares, vehicle strike), increased exposure to infectious diseases and intra- and interspecies competition [6].

2.1. Loss of genetic diversity

Considerable loss of genetic diversity has occurred over the last 100 years in this species, resulting in a divergence of alleles across different locations in Africa [23]. This genetic decline is contemporaneous with extensive anthropogenic threats such as habitat loss and deliberate hunting throughout the 20th century [13]. Such human-related factors have limited the range of AWDs and as such, restricted the effective population size which can be maintained within remaining safe habitats. When populations become small and isolated, they face the prospect of genetic drift in which genetic divergence occurs between populations due to heightened levels of inbreeding [23]. This can result in reduced species fitness, as has been demonstrated in several other species of wild canid in which fertility and pup survival to adulthood is low when inbreeding is high [10]. The reduction in AWD habitat has already led to declines in both neutral and adaptive gene alleles due to population bottlenecks, with genetic variation below that of other free-living canids [15,22,23]. As such, without active management of AWD populations that face a lack of natural dispersal corridors or where genetic declines are already evident, inbreeding and reduced population fitness are expected to occur.

2.2. Disease

Increased exposure of wild dogs to infectious diseases and pathogens occurs as a result of proximity to human-used lands, domestic dogs and other wildlife host species [27–32]. Both historical and recent African wild dog extinction events and population crashes have resulted from known pathogens such as canine distemper virus (CDV), canine parvovirus (CPV) and rabies virus [33]. Both CDV and rabies outbreaks have caused entire pack and local population eradication events in reserves and wildlife parks throughout Africa [30,34–38]. Despite evidence suggesting some adult individuals can survive outbreaks [36], these pathogens pose a significant threat to the persistence of packs in regions in which the pathogen is endemic, threatening the important pool of local pack genetics. CPV appears to have low rates of mortality in adults but is associated with high pup mortality in dens [13,36], although direct evidence is lacking thus far [39]. However, high CPV titres in adults appear to coincide with greater than 60% juvenile mortality and a 38% decrease in normal litter size [36,39,40].

3. Current conservation strategies, realities and problems

The International Union for the Conservation of Nature Species Survival Commission (IUCN-SSC), with the cooperation of representatives from range countries, has established regional conservation strategies to aid the recovery of AWD populations [41,42]. Their core objectives include improving co-existence with humans, developing conservation capacity, and improving our understanding of the biology of AWDs. Community education programs have resulted in a decline in targeted persecution of wild dogs by humans [24], with people having formal education expressing more positive views towards large carnivores than poorly educated people [26]. However, despite data showing that livestock losses to wild dogs is low, farmers' attitudes towards them remain negative [26,43].

3.1. Captive breeding, conservation and captive stress

Captive populations of AWDs exist in zoos around the world and serve a number of purposes, including raising public awareness and funding for the species, safeguarding the species through breeding programs, providing animals to help supplement and reinstate populations in the wild, as well as playing a key role in research to understand basic biology, develop effective vaccines and improve welfare [13,44,45]. Captive institutions have been instrumental in advancing our understanding of AWD physiology and social behaviour, providing access for research that would otherwise be difficult to conduct in wild populations. This includes investigating stress management techniques [46–48], understanding important factors effecting reproductive physiology [49–51], and determining the intricacies of social behaviour and hierarchy [52–54]. Moreover, zoo education programmes appear to effectively increase conservation understanding and pro-conservation attitudes [55,56].

Unfortunately, both permanent and temporary captivity are known to cause higher rates of stress in AWDs [57], which may impact their ability to reproduce and maintain social stability [48,54,57,58]. Even with improved knowledge of husbandry and veterinary care, captive wild dogs have relatively high rates of pup mortality (up to 53% and 63% in the USA and South Africa respectively) largely attributed to infanticide by the dam [44,59,60]. The age at breeding, experience of the bitch and the degree of genetic relatedness between parents are reported as the main contributing factors to litter success in captivity [60]. Zoo populations are an important supplement to free-living populations. However, high rates of infanticide and inbreeding highlight the difficulties faced by captive management, including the need to translocate animals between institutions and form artificial packs to maintain adequate genetic flow. Such management interventions are costly and may not result in bonding between unfamiliar individuals to create

reproductive units.

3.2. Metapopulation management, genetic diversity and intervention stress

Metapopulation management involves the translocation and artificial formation of packs across multiple fragmented habitats as though they are a single large viable population [61]. It typically involves a range of conservation interventions that include capture and sedation, handling, movement of animals, temporary captivity, and the artificial bonding of opposite-sex groups of unfamiliar wild-caught animals to form a reproductive pack prior to release [61,62]. This human-assisted conservation management approach is thought to most closely mimic the natural dispersal behaviour of young African wild dog adults described in Section 1.0 [8], and can supplement declining populations in order to avoid genetic drift or can re-establish populations in specific habitats [10,22,63]. Caution is required given the current enthusiasm for 'rewilding' captive animals by the zoo community [64], because previous attempts to translocate captive-breed AWDs into the South African wilderness were largely unsuccessful because captive animals lacked the necessary hunting and survival skills [65]. Translocation of captive-bred and wild-caught artificially bonded packs however, are often as successful as between wild-caught packs [66].

In social carnivores, bonding of animals typically occurs in temporary holding enclosures to ensure not only that packs remain at release sites more often, but also to facilitate the social cohesion of packs and the establishment of hierarchies [10,17,67–70]. Larger groups require more time to form stable social bonds within the boma than smaller groups [54,71], and the occurrence of breeding in the boma can improve survival post release [72]. Formation of a stable unit may be indicated by: (i) spatial arrangement, in which animals of opposite sexes choose to regularly rest near each other [52,71]; and (ii) decreasing frequency of hoo-calling (long distance call) between animals in proximity [71]. That said, translocation, artificial pack formation and temporary captivity are highly stressful to social carnivores, with fighting and physical injury due to intra-pack aggression within pre-release enclosures being a major cause for mortality in some reserves [63,73–75]. AWDs also often experience chronic stress and heightened aggression during translocation, which can lead to significant morbidity and even mortalities. This is often caused by disruption of hierarchical bonds and temporary pack separation, resulting in pack instability [46,76,77]. While efforts have been made to both understand and mitigate stress and aggression in captive wild dogs [48,57], there is limited monitoring of stress parameters during the capture, handling and transport of African wild dogs undergoing metapopulation management - warranting further research to standardise management procedures in terms of care and welfare.

While success of natural dispersal outside fenced reserves is low due to human-wildlife conflict, the success of translocation and artificial pack formation is around 86%; although these figures are derived from preliminary measures of pack establishment within holding enclosures [72]. The metapopulation management approach to date, has been highly successful with over 250 dogs (38 packs) translocated across 19 fragmented reserves in South Africa and recently Mozambique, Zambia and Malawi [78]. This has enabled rapid growth of small, isolated populations of AWDs [79]. Sadly, few protected areas are large enough to maintain a viable self-sustaining wild dog population [13], meaning artificially translocated packs are necessary to maintain genetic diversity among metapopulations in which no natural dispersal corridors exist [61,75]. As it currently stands, to effectively maintain 95% genetic heterozygosity within South African AWD populations, at least two individuals need to be moved between each managed pack within fenced reserves every two years [80]. This would require the regular formation of artificial packs between individuals across all reserves – an expensive exercise that would require careful management to avoid inadvertent costs to the welfare of packs.

While the metapopulation management approach can help limit this loss of genetic diversity [22,61], it too is constrained by current conservation realities. The current practice of translocating young artificial packs into peripheral habitats does not directly infuse 'fresh blood' into existing established packs that dominate the core AWD range. This leads to the slow flow of valuable genes into a population, which may have detrimental implications for rapid dispersal of disease resistance genes typically needed to overcome epidemics. To add to this dilemma, translocation efforts are further constrained because many reserves are rapidly reaching carrying capacity for AWDs [78]; and whose prey stocks often struggle to accommodate the rapid expansion of new packs that are highly efficient and effective hunters [19,79]. In summary, given that both the financial and welfare cost of such conservation interventions are substantial [57], careful consideration is required to ensure the minimum number of animals that infuse the greatest genetic gains are relocated. In addition, complementary strategies (see Section 5.0 below) should be adopted that accelerate gene flow by introducing valuable traits (e.g. disease resistance or climate resilience genes) into established packs without disrupting existing hierarchies.

4. Tackling the problem

Unfortunately, the realities of AWD conservation mean that on their own, current conservation strategies struggle to overcome the ever-increasing rates of habitat loss, persecution and disease outbreaks that erode natural dispersal and genetic exchange. With reserves approaching carrying capacity, and the translocation itself being expensive, labour intensive, haphazard and stress-inducing for AWD packs, there needs to be a rethink about how to most effectively manage the metapopulation. Moreover, increasing competition for funding means cost-efficient conservation strategies need to be considered. Perhaps now more than ever is there a role for captive institutions, reproductive specialists, geneticists and reserve/metapopulation managers to work together to create a welfare-conscious strategy to link the existing captive and wild pool of genetic diversity within the species. In this vein, our African Wild Dog Program is helping to tackle these problems on multiple levels:

- maximising genetic diversity (through sperm collection and banking, non-invasive monitoring of female fertile period, and artificial insemination via a hybrid approach)
- improving social cohesion, pack health and reproduction (through application of appeasing pheromones, as well as non-invasive monitoring of behaviour, vocalizations, hormones and immune function)

We will discuss each of these in more detail in the coming sections.

5. Maximising genetic diversity

5.1. Benefits of artificial insemination

Given natural dispersal is extremely limited or no longer possible for many fragmented populations of AWDs, basic assisted breeding tools like artificial insemination (AI) could help overcome the problem of genetic loss and help maximise genetic diversity [81]. AI involves the introduction of sperm into the female reproductive tract without sexual intercourse [81] and has been successful in a wide variety of wildlife species to date [82,83]. When coupled with sperm banks, AI can benefit the metapopulation management of African wild dogs on multiple levels. Firstly, banks of frozen sperm could be used as a genetic insurance policy (a frozen repository of valuable genetics and genetic diversity) against disease outbreaks and local population eradications like that which occurred in Laikipia, Kenya in 2017 [20,84,85]. Sperm banking also permits gene rescue from valuable dead, injured animals that cannot exert dominance or breed, or highly rare dogs, and should be considered a priority for the 15 remaining adult animals that constitute
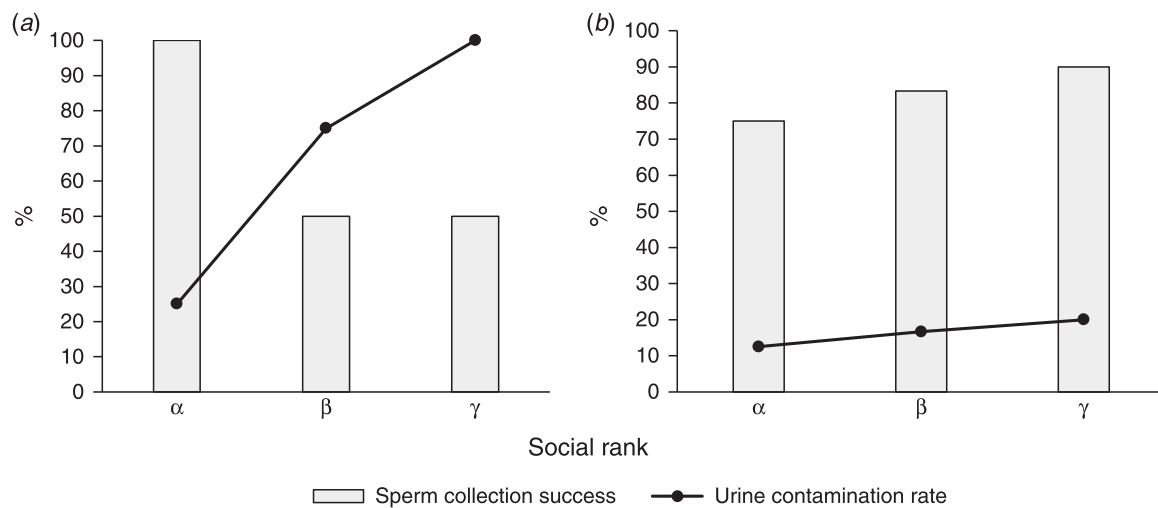
Fig. 1. : Collection success of electroejaculation and rate of urine contamination in sperm samples from African wild dogs (*Lycaon pictus*) grouped by social rank during the (a) prebreeding and (b) breeding seasons. α, alpha male; β, beta male; γ, gamma male ([51] with permission).

the last known West African population [86]. AI can accelerate gene flow by facilitating the rapid introduction of important genes (e.g. disease resistance) into established packs without moving animals or disrupting established hierarchies [81]. Moreover, moving semen rather than whole animals lowers the risk of introducing diseases into naïve populations, or moving naïve animals into a new area in which disease is endemic. Lastly, modelling has shown that genetic heterozygosity can be sustained with fewer animals and reduced cost (see Section 7.0 below; [87–89]).

However, in order for AI to yield maximum benefits, the development of several species-specific tools are needed for AWDs. These include the ability to (i) reliably collect and freeze spermatozoa; (ii) noninvasively monitor reproductive hormones and behaviour to predict the fertile period in the female; (iii) optimise the AI technique; and (iv) develop strategies to minimise pack instability during conservation interventions. Admittedly, progress in this space is slow going largely inhibited by limited funding to support such initiatives (that compete with traditional habitat protection/ecological approaches), but further impeded by rare access to captive and wild packs to develop and apply the tools, as well as a traditional resistance by reserve managers and field ecologists to adopt assisted breeding technologies that are regarded as 'unnatural' [90,91]. This is despite other 'unnatural' interventions like vaccination, translocation and artificial pack formation being widely embraced as part of metapopulation management strategies [54, 92–94]. Below, we will endeavour to provide an update on progress made to develop these tools (i) to (iv) in order to maximise genetic diversity for the species.

5.2. Sperm collection and banking

It is important to ensure semen for downstream use in sperm banking and AI can be collected at the right time from the right animals. While evidence suggests that males may be fertile throughout the year [81], previous studies in AWDs have shown a seasonal pattern in androgen levels, accessory sex glands and some sperm quality parameters [4,49, 77,95]. Spermatozoa had the highest progressive motility, viability and normal morphology in the pre-breeding season (January) as well as highest number in the breeding season (April) among n = 14 captive AWDs in South Africa [49]. Similarly, spermatozoa of good quality could only be collected in the breeding season among n = 7 captive AWDs in Australia [77]. Our own studies in n = 28 males across 8 different captive US and Namibian packs revealed a significant increase in androgens, prostate and testis size, as well as progressive sperm motility and total sperm number ejaculated during the breeding season [51].

Moreover, given that subordinate AWDs usually do not breed but help rear the pups [5], it is not clear whether they are under some form of physiological reproductive suppression, as seen in other species [81, 96–98]. Alpha male AWDs are known to have higher androgen concentrations compared to subordinate males in the breeding season [49, 77,95], which could enhance both spermatogenesis and accessory sex gland secretion [99,100]. When we compared semen collected by electroejaculation from n = 10 alpha vs. n = 18 subordinate male AWDs from 5 US and 3 Namibian packs during the breeding season, we found that although alpha males had larger prostates, there was no effect of social rank on sperm quality [51].

Interestingly, we also found the success of semen collection by electroejaculation declined from 83% in the breeding season to 67% in the prebreeding season, with collection failure exclusive to subordinate males (Fig. 1; [51]). At 67%, urine contamination also tended to be higher in subordinate males in the prebreeding season and significantly affected several sperm quality parameters (Fig. 1). Johnston et al. [77] also observed a decline in semen collection success from 71% in the breeding season to 0% in the non-breeding season. Collectively, these studies indicate that AWD spermatozoa may be of higher quality in the breeding season when its collection is less likely to be prone to electroejaculation failure and urine contamination. Pharmacological ejaculation has been shown to yield improved sperm quality (typically smaller volume but more highly concentrated motile spermatozoa with minimal urine contamination) in domestic dogs, crab-eating foxes and preliminary trials in African wild dogs and maned wolves [101–103]. Further research is necessary to determine whether pharmacological ejaculation via urethral catheterisation could improve the reliability and quality of semen collection outside the breeding season in AWDs. Moreover, we have shown that males of all social rank produce spermatozoa in the breeding season of suitable quality for downstream use in sperm banking and artificial insemination.

In order to effectively act as a genetic insurance policy for the species that can be re-infused into populations as needed, banks of AWD spermatozoa need to be frozen using a robust protocol that yields post-thaw sperm quality suitable for downstream use in AI of females. In domestic dogs, the chance of pregnancy and larger litter sizes increases when inseminated spermatozoa can maintain motility for extended periods of time [104]. This is because completion of oocyte maturation that facilitates fertilization in the dog is highly variable (60–108 h after ovulation; [105]). This requires selection of highly motile (70–75%) fresh spermatozoa for freezing that yield a post-thaw motility ≥ 40% to achieve whelping rates of ≥ 70% [106,107]. Freezing of AWD spermatozoa has only been reported in three previous studies [77,104,108].

Table 1

Mean ($\pm$ SEM) percentage of motile spermatozoa before and after cryopreservation using different freezing protocols in captive African wild dogs (*Lycaon pictus*) [77, 104,108].

Study	No. males	% Motile spermatozoa						
		Fresh	Post-thaw 0-10 min	Post-thaw 30 min	Post- thaw 2 h	Post- thaw 4 h	Post- thaw 6 h	Post-thaw 8 h
Hermes et al. 2001	n = 1							
	Fraction 1	50	0	0	-	-	-	-
	Fraction 2	90	30	5	-	-	-	-
	Fraction 3	95	40	10	-	-	-	-
Johnston et al. 2007	n = 7	72.6 $\pm$ 3.1	31.8 $\pm$ 5.8	-	0.2 $\pm$ 0.1	-	-	-
Van den Berghe et al. 2018	n = 4							
	Protocol 1	75.0 $\pm$ 4.4	18.3 $\pm$ 7.1	-	0.8 $\pm$ 0.8	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Protocol 2		33.3 $\pm$ 4.5[a]	-	30.2 $\pm$ 1.8[a*]	18.5 $\pm$ 3.2[b*]	9.6 $\pm$ 4.5[c*]	1.8 $\pm$ 1.2[c]

Values with different letters for a particular freezing protocol indicate a significant difference between times post-thaw. *significant difference between protocol 1 and 2 at a given time post-thaw.

Hermes et al. [108] froze 3 separate fractions of electroejaculated spermatozoa from n = 1 captive AWD diluted 1:3 in TEST buffer with 7.5% glycerol and 15% egg yolk using a programmable freezer. Unfortunately, sperm motility declined rapidly from 50%, 90% and 95% in fresh fractions to 0%, 5% and 10% 30 min after thawing and was associated with a marked increase in tail morphological defects (Table 1; [108]). Johnston et al. [77] froze electroejaculated spermatozoa collected from n = 7 captive AWDs diluted 1:1 in Tris-citrate buffer with 20% egg yolk that was cooled over 2 – 3 h to 5 °C, before a further 1:1 dilution in Tris-citrate buffer with 20% egg yolk and 8% glycerol (4% final concentration) and freezing over liquid nitrogen vapour. Unfortunately, compared to fresh samples, sperm motility (72.6 vs. 0.2%), motile rating (3.4 vs. 0.1), viability (81.4 vs. 8.1%) and intact acrosomes (98.5 vs. 92.1%) declined rapidly 2 h after thawing (Table 1; [77]).

There was clearly a need to improve sperm freezing protocols for AWDs given that spermatozoa were almost completely immotile 2 h after thawing in previous studies. Thus, we attempted to improve the post-thaw survival of AWD spermatozoa by testing two freezing protocols that routinely yield high quality frozen-thawed spermatozoa in domestic dogs [107,109]. In contrast to previous studies, one of these protocols also incorporated the two-step addition of an anionic detergent and other factors present in Equex STM, thought to stabilise the sperm plasma membrane and increase its permeability [109]. Our biggest challenge was that of the n = 24 AWD males in which spermatozoa was collected by electroejaculation, only ejaculates of n = 8 were of sufficient quality ($\geq$60% initial motility) for freezing (n = 4 good quality with 75.0 $\pm$ 4.4% motility and n = 4 poor quality with motility that rapidly declined to 3.3 $\pm$ 3.3% prior to freezing; [104]). Freezing Protocol 1 consisted of a one-step 1:1 dilution in TRIS extender with 20% egg yolk and 8% glycerol, cooled over 2.5 h to 4 °C, suspended in straws for 10 min in liquid nitrogen vapour then plunged into liquid nitrogen. Protocol 2 consisted of an initial 2:1 dilution in TRIS extender with 20% egg yolk and 3% glycerol, cooled over 2.5 h to 4 °C, then a second 3:1 dilution in TRIS extender with 20% egg yolk, 7% glycerol (5% final concentration) and 1% Equex STM (0.5% final concentration), suspended in straws for 10 min in liquid nitrogen vapour then plunged into liquid nitrogen. Amongst the n = 4 good quality samples, motility declined significantly from 75% in fresh samples to 18% and 33% after freezing in Protocol 1 and 2 respectively (Table 1). Post-thaw sperm viability was significantly higher in Protocol 2 than 1 but still significantly lower than fresh samples (58%, 28% and 91% respectively). Post-thaw integrity of the sperm acrosome did not differ between fresh samples and those of Protocol 2, but declined significantly in Protocol 1 (94%, 60% and 21% respectively). Interestingly, rates of normal morphology and DNA integrity did not differ before or after freezing [104]. When the longevity of frozen-thawed spermatozoa were evaluated over a period of up to 8 h, sperm motility and acrosome integrity declined rapidly to nearly zero within 2 h in Protocol 1. By contrast, the motility of sperm frozen in Protocol 2 only declined after 4 h but persisted for up to 8 h (Table 1). Post-thaw viability and acrosome integrity

were consistently 2- to 3.5-fold higher for Protocol 2 than 1 throughout the evaluation, with viability only declining significantly after 6 h (Fig. 2; [104]). Our study demonstrates that a sperm freezing protocol consisting of a two-step dilution in Tris extender with 20% egg yolk, 5% glycerol and 0.5% Equex STM provides post-thaw spermatozoa of sufficient quality and longevity for downstream use in sperm banking and AI for the first time in AWDs. Further work (modifying glycerol concentration, cooling rates and or using a defined substitute for egg yolk) may help to refine this freezing protocol. However, the current success is strongly dependant on consistently obtaining good quality spermatozoa for freezing – a challenge across all three studies to date [77,104,108]. In this regard, pharmacological ejaculation may improve the quality of AWD semen collected [101–103].

5.3. Predicting the fertile period for AI in females

Most free-roaming canids cycle once per year, with the breeding season controlled by environmental factors such as day-length and food availability. Domestication and captivity appear to decrease seasonality, with most breeds of dogs and some captive wild canids [110], including the AWD [81], cycling more than once per year. Seasonal reproduction is evident in most populations of female AWDs except those within 2° of the equator [111], with breeding typically occurring during summer (January to March) in the Southern Hemisphere or (May to July) in the Northern Hemisphere [81]. If unsuccessful in summer, occasionally packs will subsequently mate in spring.

The female reproductive cycle itself is similar in most canids so far studied, characterised by a mono-oestrous cycle with long pro-oestrus and oestrus (early and late) periods, a pregnant or pseudopregnant period of dioestrus, and seasonal anoestrus [81,112,113]. Being able to accurately predict the fertile period in canids is critical when performing AI, especially when using frozen semen, due to its reduced quality and longevity [104,109]. The fertile period occurs during late oestrus and corresponds with completion of oocyte maturation in the oviduct some 48 – 60 h after ovulation, and is the best time for AI in canids [105, 114–116]. As such, a detailed understanding of events and methods to monitor them around the periovulatory period in canids is essential for successful AI.

In the domestic bitch, pro-oestrus is characterised by vulval swelling, a serosanguinous vulval discharge and the attraction of male dogs. During this phase, lasting anywhere from 3 to 21 days, circulating concentrations of oestrogen rise and reach a peak. Standing oestrus, the period when the bitch allows mating by the male, lasts on average 9 days but may extend as long as 21 days [117]. Around the start of standing oestrus, declining oestrogen levels coincide with rising progesterone levels derived from luteinising hormone (LH)-induced luteinisation of preovulatory ovarian follicles [118]. Ovulation is considered spontaneous in the domestic bitch and most wild canids studied to date including AWDs [119]. In domestic dogs, ovulation occurs around 2 days after the LH peak, with ovulated oocytes requiring a further 48 –
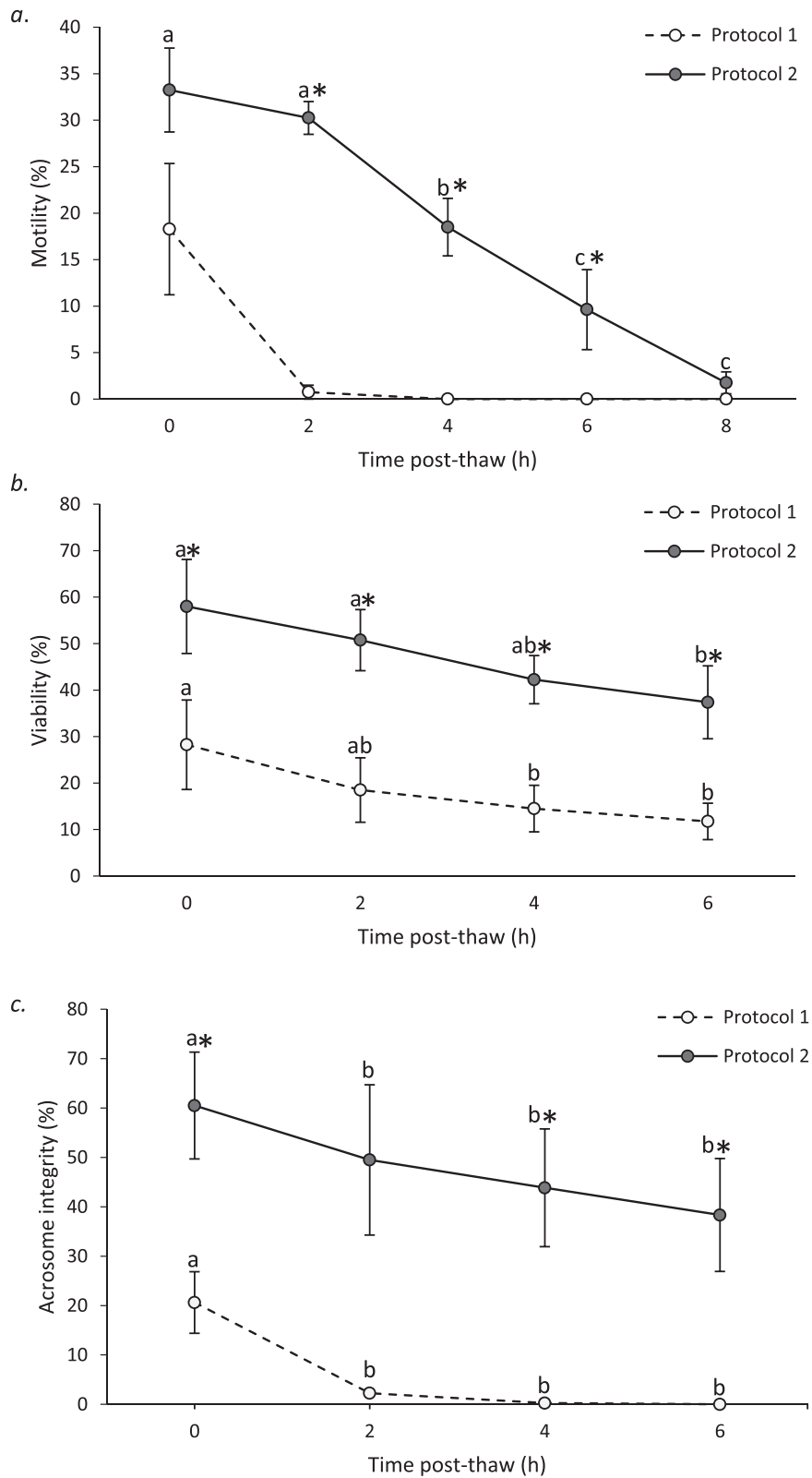
Fig. 2. : Post-thaw (a) motility, (b) viability and (c) acrosome integrity of African wild dog (*Lycaon pictus*) spermatozoa over a period of 6–8 h after cryopreservation using Protocols 1 and 2. * indicates a significant difference between both protocols at a specific time point. Different letters within each protocol indicate a significant difference between time points
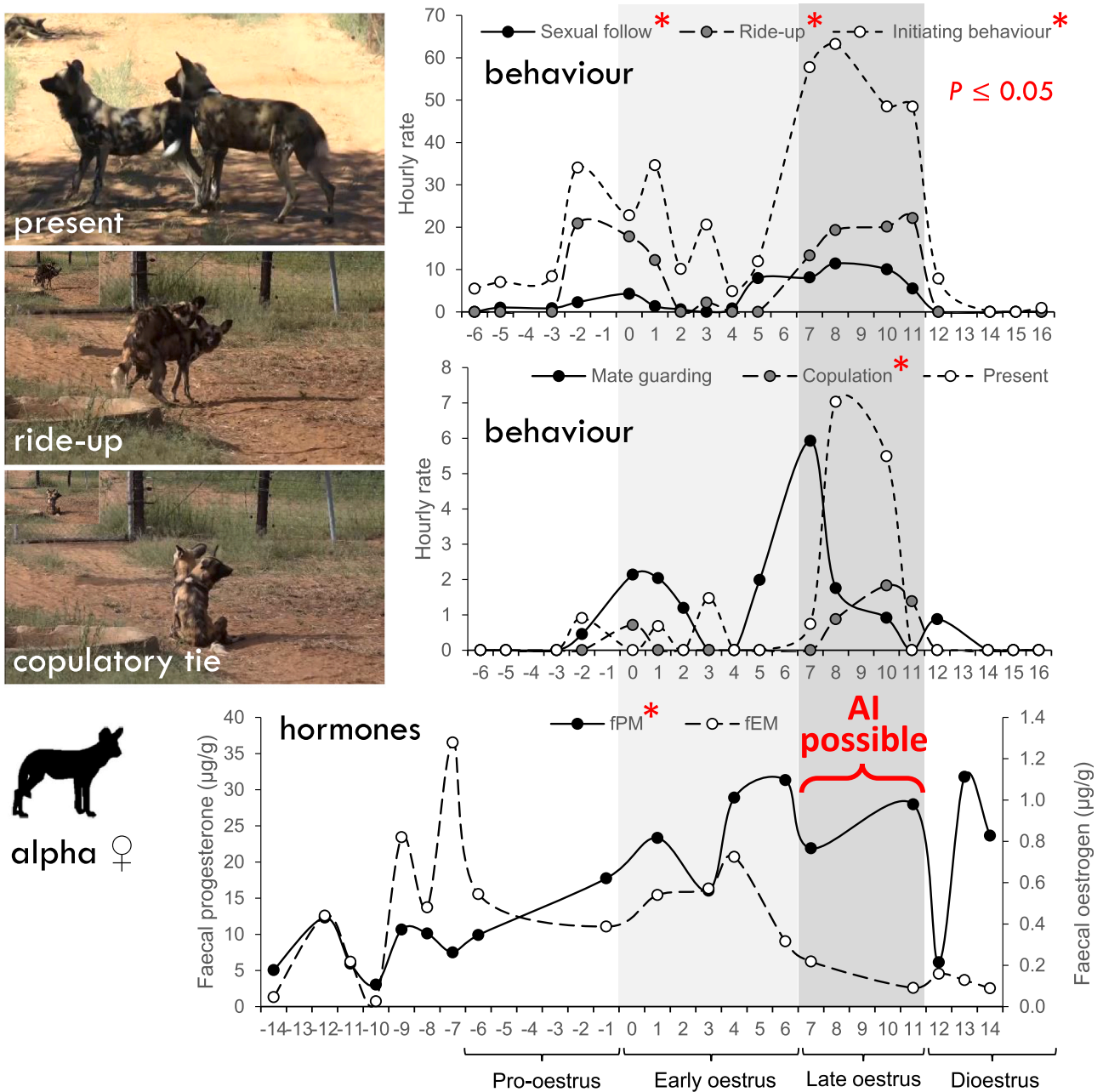(modified from [104] with permission).

Fig. 3. : A biphasic pattern with more than 2-fold increase in hourly rates of certain sexual behaviours (sexual follow, ride-up, initiation, copulation) coupled with a 2-fold increase in faecal progestogen metabolites can predict the fertile period for artificial insemination during late oestrus in female African wild dogs (*Lycaon pictus*). * indicates a significant increase; fPM, faecal progestogen metabolites; fEM, faecal oestrogen metabolites; AI, artificial insemination (modified from [116] with permission).

60 h to complete maturation before fertilisation can occur [120]. While fresh spermatozoa may remain fertile within the female reproductive tract for several days, studies using short-lived (12 – 24 h) frozen semen suggest that fertilization in the domestic bitch occurs 3.5 – 7.5 days after the LH surge [121], with the highest number of conceptuses conceived 6 days after plasma progesterone concentrations first reached a value of 6 nmol/L [122]. Moreover, conception rates decrease markedly when AI is performed one day prior to the onset of dioestrus, or later [121,123].

Techniques to monitor oestrus in canids are important to accurately pinpoint the fertile period. In the domestic bitch, oestrous monitoring encompasses some or all of the following: physical changes and behavioural cues, vaginoscopy, vaginal cytology and blood sampling to measure progesterone concentrations. Some practitioners also monitor LH concentrations once or twice daily in blood samples to detect the LH

surge [117]. While vaginoscopy and vaginal cytology are useful to confirm oestrus, they are of limited value in determining the optimal time for insemination, particularly when insemination opportunities or semen doses are limited, or when short-lived frozen semen will be used.

In clinical practice, prospective timing of an insemination of frozen semen in the domestic bitch is achieved primarily through the monitoring of circulating progesterone concentrations. Steckler et al. [122] showed that optimal fertility is achieved by the insemination of frozen semen on Day 6 following the LH surge, with the latter correlated to the first day that circulating progesterone concentration exceeded 6 nmol/L. However, traditional invasive methods to time AI in female domestic dogs by monitoring serum progesterone as well as cytological and endoscopic changes in the vagina, are not feasible without immobilization in wild canids. While there may be limited scope to train AWDs

for repeated blood sampling in a crush cage, behavioural observations coupled with non-invasive quantification of reproductive hormones or their faecal metabolites may be a more suitable alternative to determine the fertile period in order to time AI in this species [116]. Synchronisation techniques and induced ovulation could also help with accurate timing of AI.

We monitored sexual and non-sexual behaviours coupled with faecal oestrogen (fEM) and progestogen (fPM) metabolites before, during and after the mating period of n = 3 AWD alpha females across 3 captive Namibian packs [116]. We also tried to benchmark these parameters against observed mating, clinical signs of oestrus, serum oestrogen and progesterone concentrations, vaginoscopy and vaginal cytology. A distinct biphasic increase in hourly rates of most sexual behaviours was observed corresponding with late pro-oestrus/early oestrus phase and 2nd much higher late oestrus phase (Fig. 3). When grouped by reproductive phase, initiating behaviour, sexual follow (both particularly high), ride-up and copulation increased over three-fold, and alpha male-female affiliative behaviour and nonsexual follow increased over two-fold from early to late oestrus, and were higher than any other reproductive phase ($P \leq 0.05$). Moreover, all ceased abruptly on the day after last copulation — clearly indicating the first day of dioestrus. Similarly, there was also a monophasic increase in alpha female-male resting behaviour with a peak during late oestrus that declined significantly in dioestrus [116]. Faecal hormone concentrations were more variable between individual females. The alpha female of the Platform pack showed fPM concentrations similar to anoestrus females that did not change across phases, indicating anovulation (confirmed by failed conception). By contrast, the alpha female of the Brutus pack showed a more than 2-fold significant increase in fPM from pro-oestrus to both early, late and dioestrus (Fig. 3). Similarly, the alpha female of the San pack showed a trend for a 2-fold increase in fPM from early pro-oestrus to late oestrus and further 2-fold increase in dioestrus, but limited samples made statistical comparison impossible. Despite a distinct trend for declining fEM from pro-oestrus through to late oestrus in n = 2 alpha females, no significant difference between phases could be detected (Fig. 3; [116]). This is consistent with oestrogen concentrations in other canids, which are known to be less reliable indicators of ovulation and the fertile period [124]. In summary, we have demonstrated that a 2 to 3-fold increase in the rate of several core behaviours (male-female affiliative behaviour, male initiating behaviour, sexual and non-sexual follow, ride-up and copulation) can discriminate late oestrus (the fertile period) from other phases of the female AWD reproductive cycle for timed AI. However, some quantification of rates of these behaviours is necessary to detect the up to 4.8-fold increase at this time. Moreover, quantification of a 2-fold increase in fPM may provide a useful non-invasive indicator of the onset of the broader oestrus phase but without behaviour, should not be used alone to distinguish the fertile period in late oestrus. Moreover, while vaginoscopy and vaginal cytology have shown limited application in the AWD in our study, further research is warranted.

5.4. Artificial insemination technique

In the domestic dog, intrauterine insemination is widely considered superior to vaginal inseminations with frozen semen [106]. Due to the anatomical orientation and limited accessibility of the canid cervix, intrauterine insemination is performed either surgically via midline or flank laparotomy or laparoscopy, or with a specially designed catheter inserted under endoscopic guidance (transcervical insemination; TCI; [125]), or blindly, using a Norwegian catheter [126]. Use of the Norwegian catheter in an African wild dog would require specially trained personnel and the appropriate catheter, potentially adjusted in size or shape to suit the anatomy of this species. Similarly, TCI requires expensive, specialised equipment and an operator highly trained in this technique. In contrast, surgical insemination via laparotomy is within the scope of routine veterinary practice but carries risks associated with

abdominal surgery [127]. Surgical artificial insemination in domestic bitches has been banned in several countries, including the United Kingdom [128].

To date, there have been no reports of successful AI in the AWD [116]. In other wild canids, reports of successful AI using fresh or frozen semen are scant but include the use of laparoscopic intrauterine insemination and the Norwegian catheter [129]. Field laparoscopy could be utilised when frozen-thawed spermatozoa is highly valuable but of poor quality. However, given the endangered status of the African wild dog, any attempts at AI in this species should try to avoid surgical approaches where possible, with the aim to achieve intrauterine insemination using either TCI or the Norwegian catheter under field-friendly conditions. In situations where this is impossible, vaginal insemination with a simple canine insemination pipette may represent a more practical alternative in the field if sperm quality is high. Interestingly, excellent pregnancy rates were achieved with frozen-thawed semen inseminated vaginally in domestic bitches, following the addition of prostatic fluid to the inseminate [130,131]. The role of seminal plasma (primarily consisting of prostatic fluid in the canine) in aiding sperm survival and enhancing fertility is an area of growing research interest, in humans and mammals [132]. This suggest that the seminal plasma of AWDs should be preserved at the same time as spermatozoa is frozen, and reconstituted at the time of AI to enhance the longevity and fertilization success of frozen-thawed spermatozoa in the female tract. Moreover, efforts are needed to design a species-specific anatomically accurate Norwegian-style TCI catheter, suitable for non-surgical IUAI in the field.

6. Improving social cohesion pack health & reproduction

During our efforts to develop tools to maximise genetic diversity in AWDs, we came to realise that management of stress, aggression and the complex social hierarchy within the pack was critical to the success of any captive or wild conservation intervention. As such, with our collaborators we started to develop a suite of non-invasive tools to monitor and manage social cohesion, pack health and reproduction.

Stress causes changes to both physiology and behaviour of animals, the magnitude of which is regulated by the release of glucocorticoids including cortisol and corticosterone from the hypothalamic-pituitary-adrenal (HPA) axis [133,134]. Upregulation of glucocorticoids increases energy mobilisation and down regulates energy storage, growth-related hormones, reproduction and reproductive hormones, immunity, and the inflammatory response [134]. Additionally, in many animals stress occurs in combination with increased aggressive behaviours [135,136]. Aggression is often attributed to upregulation of testosterone and other androgens from the hypothalamic-pituitary-gonadal (HPG) axis [10,51,137,138]. Prolonged activation of the HPA and HPG axes are correlated with increased aggression and reduced levels of parental care, which negatively impact reproduction, juvenile survival and immunity [139–142].

6.1. Faecal hormone metabolite assays

As mentioned above, frequent invasive blood sampling to look at changes in reproductive and stress hormones is not feasible without sedation in wild canids like the AWD, with sedation and sampling being acute stressors in this species. Thus, monitoring of hormones and their metabolites in non-invasive faecal samples has become a viable alternative in AWDs. However, faecal hormone metabolite assays need to be optimised and reliably validated in a species-specific manner. Our African Wild Dog Program began nearly 20 years ago at a time when reproductive knowledge about this species was limited. Thus, our initial focus was to expand the limited existing knowledge about female reproduction. To achieve this, we first needed to develop a simple method to identify and collect faecal samples defecated by specific individuals in a socially complex group-housed species. Ultimately, using
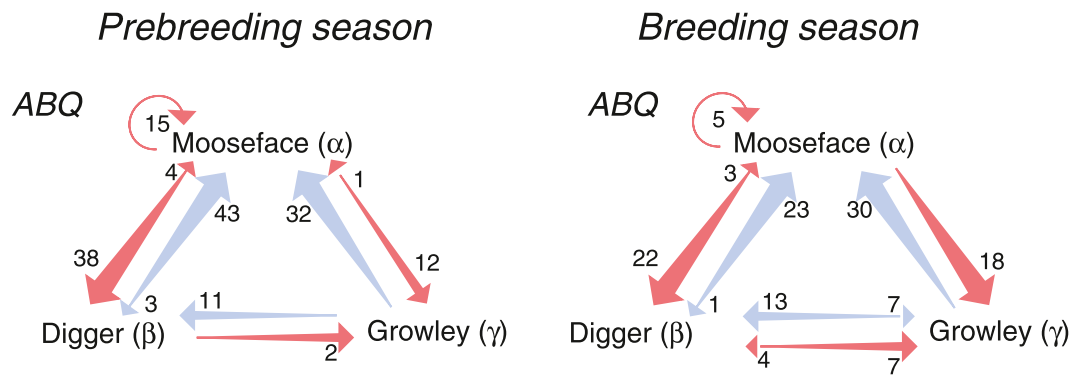
Prebreeding season

Breeding season

Fig. 4. : Classification of male African wild dog (*Lycaon pictus*) social hierarchy based on the frequency of dominant (red arrows), submissive (blue arrows) and scent marking (round red arrows) behaviour in the ABQ (Albuquerque BioPark, NM, USA) pack during the prebreeding and breeding seasons. Arrowheads indicate the direction of behaviour from actor to recipient. Numbers next to arrows indicate the frequency of a behaviour within the total observation time. α, alpha male; β, beta male; γ, gamma male
(modified from [51] with permission).

meat mixed with different coloured non-toxic plastic beads and targeted feeding, we demonstrated that: (i) female AWDs are not strictly mono-oestrus, and (ii) that oestrus occurs in the absence of males [119].

Initially radio-immunoassays were established for AWD faecal steroid quantification of oestrogens, progestogens, androgens and glucocorticoids [50,143]. Analysis of faecal samples regularly collected from captive females in European Zoos and wild females from Hluhluwe-iMfolozi Park (South Africa), showed that female AWDs, like other canids, exhibit spontaneous ovulation as well as obligate pseudopregnancy. Pseudopregnancy (as determined by a sustained rise in progestogens) was observed in almost all captive females, with most housed in single-sex groups – indicating spontaneous ovulation. Moreover, progestogen concentrations in both captive and wild individuals indicate that most adult females ovulate and thus, are fertile but their reproduction is most likely suppressed by behavioural rather than physiological mechanisms [50,81]. This study was the first to analyse detailed endocrine profiles of both captive and wild AWD females across different reproductive states of pregnancy, pseudopregnancy and acyclicity [50,143].

We were also able to investigate adrenocortical activity, in the form of glucocorticoid output, for the first time in both captive and free-ranging populations of AWDs using our validated faecal glucocorticoid metabolite (fGCM) radio-immunoassay [144]. Interestingly, we found that captive-housed females had higher and more individually variable fGCM levels compared to free-ranging females. This pattern could be confirmed with similar findings in captive male and female AWDs using an established fGCM enzyme-linked immunosorbent assay (ELISA; [51, 57]). Moreover, fGCM levels were highest in both female and male free-ranging individuals during pregnancy and denning, with the highest fGCM levels found in male yearlings [144].

Since one of the goals of faecal hormone metabolite analysis is to monitor reproductive function and responses to stressors in free-ranging AWDs, it is important to determine the stability of these steroid metabolites post-defaecation in samples subjected to elevated ambient temperatures in the field. In an initial study, fresh faeces from n = 7 captive AWDs in South Africa were subjected to ambient room temperatures (12 – 20°C) for 16 days, and showed no significant change in immunoreactive fGCM concentrations over a 48 h period [145]. Similarly, we found no significant effect of time on fGCM or faecal androgen metabolite concentrations over a 72 h period, when fresh faeces collected from n = 8 AWDs in Namibia were subjected to external ambient summer temperatures (11°C – 50°C; [48]). Collectively, these results suggest that reproductive and stress-related hormone metabolite concentrations remain relatively stable in ambient temperatures for at least 48 h, given respective steroid extracts are quantified with the appropriate assays, which is twice as long as required during typical daily collection regimes.

6.2. Faecal immunoassays

As previously stated, pathogens such as CDV and rabies are a major contributor to AWD mortality throughout Africa, and prolonged stress experienced by AWDs during periods of captivity may lead to decreased immune health [10]. Immunisation against these pathogens may form part of the metapopulation management of AWDs [72]. New-born pups rely heavily on maternal antibodies provided in the colostrum of lactating bitches, which gradually decline over time as the pup's own immune system begins to develop [146]. However, there is a gap in which the immune system is still under-developed, making pups highly susceptible to pathogens [147]. As such, immunisation can be highly beneficial to adults and their offspring, with vaccination during pregnancy conferring immunity against pathogens from the mother to new-born pups [147]. Furthermore immunisation of young immunologically naïve animals can increase their chance of survival if an outbreak occurs after maternally transferred immunity wanes [147].

Reduced efficacy and inconsistent antibody responses have been noted to both CDV and rabies vaccines in AWDs compared to domestic dogs [67,79,148,149]. In some cases, despite vaccine administration individuals have contracted rabies shortly after release into novel environments [149,150]. Currently, immune monitoring in response to stress and/or vaccination requires the repeated collection of blood or saliva, necessitating repeated sedation events that could further exacerbate stress in this species. Similar to the measurement of steroid hormones in faeces discussed above, antibodies such as immunoglobulins contained in faeces present a non-invasive alternative to measure immune responses to vaccination, with assays already developed in the domestic dog [151,152].

Immunoglobulins are normally upregulated in response to pathogen invasion. Immunoglobulin M (IgM) and G (IgG) upregulation are important indicators of the primary and secondary immune response respectively post-vaccination [153]. Recently, we have shown that the IgM, IgG and CDV-specific IgG immune responses to vaccination can be measured in the faeces of domestic dogs and captive Australian dingoes [154]. Furthermore, we are now able to detect IgM and IgG in the faeces of AWDs using the same technique [154], providing us with a non-invasive tool with which to measure immune responses to vaccination and detect any potential immune system suppression induced by stressful conservation management interventions.

6.3. Behaviour & vocalization monitoring

Communication between individuals within AWD packs is mediated

through a wide range of physical behaviours and vocalisations. Changes in behaviour or vocalisations can be used to monitor reproductive events like oestrus, pregnancy or presence of pups in the den, but also stress, aggression, hierarchy and social cohesion.

We have already shown how behaviour can be used as a highly precise non-invasive method to determine the fertile period for timed AI in AWD females (Section 5.3; [116]). However, it is also a very valuable tool for determining social hierarchy and dominance within packs [51]. When we scored the frequency of dominant, submissive and scent marking behaviours in 5 captive US packs, a clear dominance hierarchy was discernible, with (i) the alpha male exhibiting the greatest frequency of dominant behaviours directed toward subordinates, (ii) beta males directing frequent submissive behaviour toward the alpha while directing lower frequency dominant behaviour toward the gamma male, and (iii) gamma males directing the most frequent submissive behaviour toward alpha, then beta males while receiving dominant behaviour at different frequencies from both. Interestingly, while the dominance hierarchy remained unchanged there were differences in the frequency of these behaviours between the pre-breeding and breeding season (Fig. 4; [51]). This indicates that the frequency of certain behaviours could be a sensitive indicator of social cohesion during stressful conservation interventions such as separation/reintroduction, translocation, artificial pack formation or assisted reproductive procedures.

When we applied the behavioural ethogram of Van den Berghe et al. [48], to monitor social cohesion in AWD packs undergoing a separation, immobilisation then reintroduction (SIR) intervention for health checks, we saw a strong positive association between certain dominant and submissive behaviours and changes in physiological markers of aggression and stress in the form of faecal androgens and glucocorticoids respectively. Contact and non-contact dominance as well as passive and active submission behaviours increased significantly above baseline on the day of reintroduction in AWDs, indicating social disunity. This was concurrent with a rise in stress and aggression hormones. Rates of behaviour and hormone concentrations returned to baseline 24 h later as the pack hierarchy was re-established [48].

In addition to behaviour, changes in the rate and characteristics of certain AWD vocalisations may be sensitive indicators of social cohesion or even help pinpoint the fertile period in oestrus females. Changes in the harshness, amplitude and pitch of domestic dog barks differ between play and disturbance [155]. Furthermore, canid vocalisations are thought to mediate social behaviours including hierarchy maintenance [155–164]. AWDs are one of the most vocally complex species of canid, with 27 identified vocalisations in adults and 12 vocalisations in pups [158,165]. To date however, the use of vocalisations to monitor social cohesion or reproduction is limited in AWDs. The reduction of long distance contact calls (hoo-calls) over time during captivity in parallel pre-release enclosures, has been used to indicate growing familiarity between two unrelated dispersal groups before their introduction to form an artificial pack [54].

Additionally, vocalisations of different canids appear to change in the breeding season, with increased howls [157,166,167]. As such, changes in vocalisation in AWDs could be used to indicate onset of the breeding season as well as changes in emotional and/or physiological state. Recently, we have found that mob twitters occur at a higher rate during the breeding season. Moreover, a decline in the frequency range of twitters, begging cries, yelps/squeals and barks occurs during the breeding season (Riddell et al. in prep). Interestingly, the rate of vocalisation does not differ during stressful events, but audio characteristics of certain vocalisations do. Changes in frequency range, syllable length and intersyllable gap of spar twitters, social twitters, moans, begging cries, barks, growls, hoo-calls, whimpers and yelps/squeals occur in response to SIR. The type of change varies between call type but includes either a higher or reduced frequency range, increased syllable length or increased intersyllable gap, and appears to occur at the same time as increased dominant behaviours – indicative of social disunity (Riddell et al. in prep).

6.4. Appeasing pheromone mitigation

Unfortunately, most well-intentioned conservation interventions can induce unforeseen stress and aggression in AWDs, associated with increased faecal glucocorticoid and androgen concentrations and altered behaviours [48,57,144,168]. Even the application of assisted reproductive technologies (semen collection and artificial insemination) requires pack members to be separated, immobilised and reintroduced, which we know can causes acute stress and aggression in AWDs [48]. Since chronic stress and aggression can have serious consequences for social cohesion, immune function and reproduction, reliable strategies to minimise or mitigate these effects during captive and metapopulation management procedures is as important as the conservation action itself. Appeasing pheromones (naturally occurring olfactory signals produced by animals that modify behaviour or physiology of conspecifics) have been suggested to be a potentially powerful tool in canid conservation, providing a natural method of stress and aggression modification [10].

ADAPTIL is a commercially available synthetic form of the dog appeasing pheromone available in a number of application methods including imbibed collars, diffusers and aerolite sprays. ADAPTIL has been reported to reduce stress and aggression in domestic dogs in a range of settings, including during transport, veterinary visits, thunderstorms and in rescue shelters [169–173]. As such, we hypothesised that ADAPTIL could also minimise stress and aggression during conservation interventions (including assisted reproduction) in wild canids like the AWD. However, because of the species-specific nature of pheromones, we first needed to test whether AWDs could perceive domestic dog appeasing pheromone. When n = 10 captive AWDs across 4 South African packs were exposed to a 1 m^2 area of the enclosure treated with ADAPTIL, animals in 3 out of 4 packs showed increased sniffing, urinating, panting and licking behaviours, as well as increased time resting in ADAPTIL-treated areas [46]. Similar results were observed using a recently modified version of the pheromone – indicating that AWDs can detect and direct positive behaviours towards this domestic dog-derived pheromone (Riddell et al. in prep). In a follow-up experiment to test the effect of ADAPTIL on stress hormones, n = 3 individually housed females were fitted with 2.5% ADAPTIL collars and faecal samples collected from 3 days before to 5 days after treatment. Encouragingly, faecal glucocorticoid metabolites decreased by 32% and 18% respectively compared to baseline in 2 out of 3 females after ADAPTIL treatment – suggesting ADAPTIL may directly act on the HPA axis to reduce stress hormones in AWDs [46].

In a follow-up placebo-controlled double-blinded study, we sought to determine whether ADAPTIL could reduce stress and aggression during semen collection and sperm banking initiatives [48]. In total, n = 12 AWDs across 4 captive packs where alternatively treated with ADAPTIL or placebo spot-on solution during a separation, immobilization, reintroduction (SIR) procedure at 2 different times of the year. Behaviour (grouped as dominant, submissive, affiliative or aggressive) was observed and faeces collected 3 days before to 5 days after treatment and reintroduction. Interestingly, faecal androgens but not faecal glucocorticoids were suppressed at the time of reintroduction when pack members were treated with ADAPTIL, while faecal androgens and faecal glucocorticoids both increased significantly in placebo-treated animals. This was associated with a significant increase in contact dominance behaviour in placebo- but not ADAPTIL-treated packs, and a trend toward non-contact dominance and reduced active submission behaviours in ADAPTIL-treated packs [48]. Thus, we demonstrated that ADAPTIL may be a useful tool for the conservation management of AWDs because it appears to reduce hormones and behaviours associated with aggression. While the exact mechanism is unclear, ADAPTIL's role in limiting prolactin decline [174], suggests it may act by prolactin negative feedback on the HPG axis to limit androgen production.

Collectively, these pheromones as well as the non-invasive monitoring tools we have developed allow us to monitor and manage the health and welfare of packs undergoing both traditional but also hybrid

Table 2

Minimum population size and program cost to retain 90% genetic heterozygosity over 100 years under a natural breeding vs. hybrid (natural + assisted) breeding scenario in captive wildlife [87–89].

Species	Breeding scenario	Population size	Cost (AU$ million)				Study
			1st Year	100-Year captive colony	100-Year ART back-cross	100-Year total program	
Koala (*Phascolarctos cinereus*)	Closed captive breeding	223	5.3	73.1	-	73.1	Howell et al. 2022
	Captive breeding with ART back-cross (every 7 years)	17	0.9	5.4	5.2	10.6	
Orange-bellied frog (*Geocrinia vitellina*)	Closed captive breeding	400	1.2	466.1	-	466.1	Howell et al. 2021
	Captive breeding with ART back-cross (every 4 years)	17	0.068	20.1	0.95	21.1	
White-bellied frog (*Geocrinia alba*)	Closed captive breeding	400	0.72	284.5	-	284.5	
	Captive breeding with ART back-cross (every 4 years)	17	0.049	12.4	0.95	13.4	
Dibbler (*Parantechinus apicalis*)	Closed captive breeding	1397	3.3	70.1	-	70.1	Howell et al. 2023
	Captive breeding with ART back-cross (every 1 year)	14	0.062	0.729	0.282	1.0	
Numbat (*Myrmecobius fasciatus*)	Closed captive breeding	1583	8.4	185.4	-	185.4	
	Captive breeding with ART back-cross (every 1 year)	16	0.114	1.9	0.312	2.2	

conservation interventions. This should increase pack cohesion, disease resistance and litter success, and ultimately improve survival of packs released into the wild.

7. Hybrid conservation management approach

Hybrid conservation management is a strategy that incorporates both natural and assisted breeding to manage the long-term genetic diversity of a species [88]. Several important criteria need to be met for successful application of such a strategy to wild populations, including (i) a good understanding of the reproductive biology and life history of the target species, (ii) significant progress in establishing relevant assisted breeding technologies, (iii) a good network of captive facilities and potential for sperm banking infrastructure, and (iv) a good network of field populations with regular access to and movement of whole animals. In the case of the African wild dog, such a hybrid approach would most realistically rely on a combination of natural breeding and artificial insemination using frozen sperm banks. This practicality reflects current progress in assisted reproductive technology in the species coupled with the realities for potential field application into existing metapopulation and range expansion management regimes (see below; [54,61,81,104, 116]).

While the idea of a hybrid conservation management approach for African wild dogs might sound like science fiction to traditional wildlife managers, it has precedence in the success of the black-footed ferret (*Mustella nigripes*) recovery program in North America; in which the species was saved from extinction by a combination of captive breeding, AI, habitat restoration and pest control [175,176]. In 1981 the black footed ferret population was near extinct, with all n = 18 remaining individuals captured to facilitate intensive captive breeding. Approximately US$4.2 million was allocated in applied research funding to develop a protocol to augment lost genetic heterozygosity back into the captive population using frozen founder sperm and artificial insemination [88,177]. So far more than 6100 offspring have been produced by captive breeding and 139 kits conceived by AI from underrepresented, dead or sexually incompatible founders; whose genetics would have been lost to the population if efforts relied on natural breeding alone [175]. Currently, 4300 reintroduced black-footed ferrets survive across 29 sites in the wild [178].

Similarly, the iconic Australian koala (*Phascolarctos cinereus*) is currently listed as vulnerable nationally, and endangered across much of

its range in the eastern states due to habitat loss (urbanisation and bush fires), disease and vehicle strike [179]. The reproductive biology of the species is reasonably well studied with a long history of use as a research model for the development of assisted reproductive technologies in marsupials [180]. With an oestrous cycle of 33 days, evidence of induced ovulation, and a capacity for chilled spermatozoa to survive for up to 40 days, the koala makes an ideal candidate for the use of AI with chilled semen from captive or wild-caught individuals [181]. To date, n = 34 offspring have been conceived by AI using fresh and chilled spermatozoa [182,183]. Moreover, other reproductive life history traits are known (optimal breeding age, 7-year generation length in females), an extensive network of zoos/wildlife hospitals exists, and detailed costs for biobanking, captive and assisted breeding are available – making it a suitable candidate for hybrid conservation management [88].

The goal of current conservation strategies should be to maintain 90% of the genetic heterozygosity of a population for 100 years [184, 185]. Howell et al. [88] compared population size and cost needed to sustain 90% heterozygosity for 100 years in captive koalas across 16 Australian sites under two different modelling scenarios:
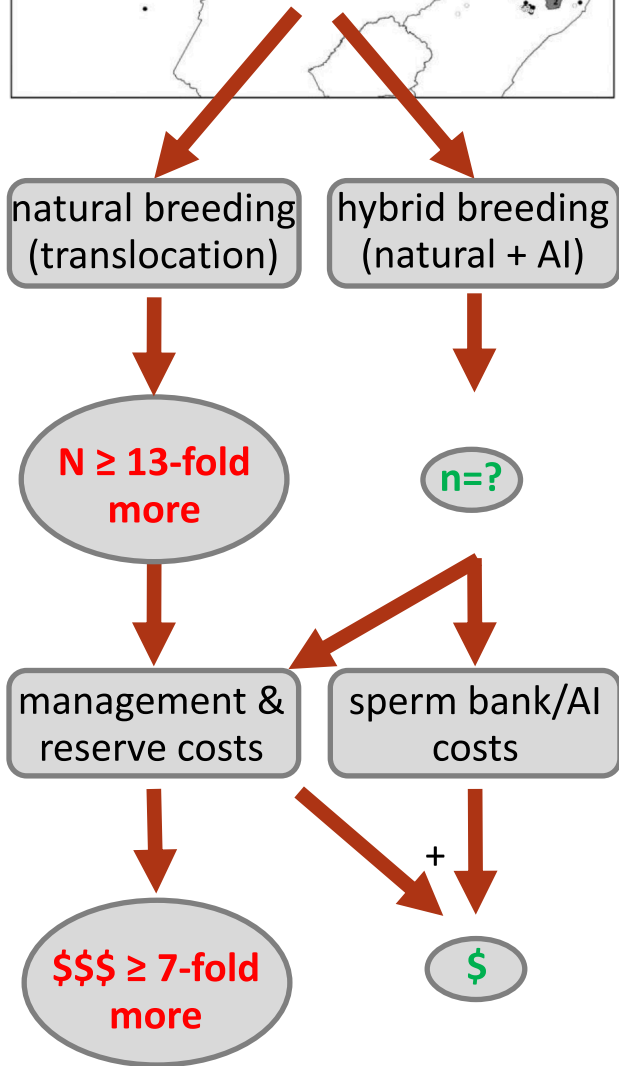
(i) closed captive breeding conditions (i.e. natural breeding)
(ii) captive colonies where artificial insemination is used to backcross the colony to founder males using frozen spermatozoa every generation (7-year intervals; i.e. natural breeding + sperm banking/AI)

The economic holding cost per animal (including husbandry, veterinary, food) plus on-site infrastructure and facilities costs were calculated and these were augmented with costs associated with sperm banking/AI (equipment, labour) for scenario (ii) above. Surprisingly, 13-fold fewer animals (n = 223 down to n = 17) and 7-fold lower costs (AU$73.1 down to AU$10.6 million) are needed to maintain 90% heterozygosity targets for 100 years when natural breeding is augmented with sperm banking/AI (Table 2; [88]). The cost savings in the hybrid management model were primarily attributed to substantial reductions in captive colony size, while still maintaining 90% heterozygosity. Moreover, while n = 17 animals may be suitable to retain genetic diversity, they may pose the risk of being too small to act as an insurance population in the event of a sudden catastrophic decline in wild populations – and so insurance populations closer to the average proposed by multiple Species Survival Plans of n = 137 distributed across multiple
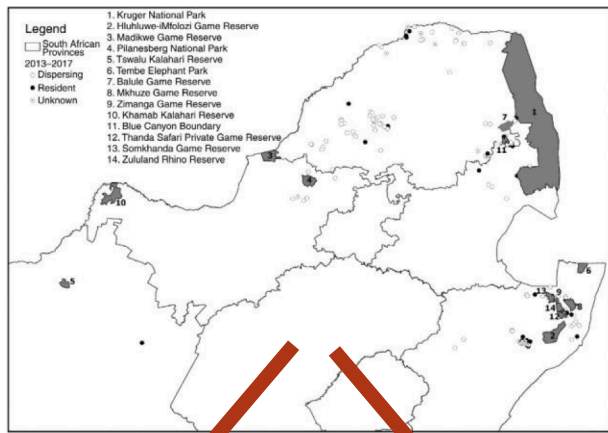
Fig. 5. : Flow diagram to model minimum population size and total project cost to retain 90% genetic heterozygosity over 100 years using a natural breeding vs. hybrid (natural + artificial insemination) breeding approach for the metapopulation management of wild fragmented populations of African wild dogs (*Lycaon pictus*) in South Africa. South African metapopulation map
modified from [18] with permission.

locations might be prudent [88].

Similar reductions in population size and program cost were observed when hybrid management approaches were modelled in other Australian marsupials and amphibians including the endangered dibbler (*Parantechinus apicalis*) and numbat (*Myrmecobius fasciatus*) as well as orange-bellied frog (*Geocrinia vitellina*) and white-bellied frog (*G. alba*; Table 2; [87,89]). Among the marsupials, on average 100-fold fewer animals and 70- to 84-fold lower costs are needed to maintain 90% heterozygosity targets for 100 years when natural breeding is augmented with sperm banking and assisted breeding (Table 2; [89]).

While among the amphibians, on average 24-fold fewer animals and at least 21-fold lower costs are needed (Table 2; [87]). In summary, the above studies demonstrate how the integration of assisted reproductive technologies (like sperm banking and AI) into natural breeding programs could dramatically reduce inbreeding and the number of breeding animals needed; which in turn significantly reduces program costs [88].

Table 3
Integration of a hybrid conservation approach to the metapopulation management of African wild dogs (*Lycaon pictus*).

Action	Captive development & application	Wild application
1	Partner with local network of sperm banks (zoos, government, NGOs, universities)	Partner with local network for AWD translocations (NGOs, government & private reserves)
2	Bank sperm from all adult males during breeding season when sedated for other reasons	
3	Research to perfect AI timing & technique (zoos, rescue/ conservation centres):	Apply AI during wild translocation & artificial pack formation (government & private reserves):
	a) appeasing pheromones to manage intervention (stress & aggression)	
	b) non-invasive tools to monitor social bonding/reproduction (faecal hormones, behaviour, vocalisations) & immune status post-vaccination (faecal Ab)	
	c) hormone & behaviour monitoring to predict fertile period in female (captive enclosure or temporary boma)	
	d) AI at same time as natural mating (mixed paternity litter okay)	
4	Transfer genetic diversity between captive institutions as proof-of-principle (fresh then frozen-thawed sperm)	Transfer genetic diversity between wild populations as proof-of-principle (fresh then frozen-thawed sperm)
5	Improve genetics of captive population (AI with wild-sourced sperm)	Improve genetics of wild population (AI with captive or wild-sourced sperm from genetically valuable individual)
6		Release of genetically heterogeneous, socially cohesive, immunologically robust pack into wild

AWD, African wild dog; NGOs, non-government organisations; Ab; antibodies; AI, artificial insemination

7.1. Integrating sperm freezing/AI into a hybrid conservation management strategy for AWDs

Given the clear benefits demonstrated in the above models, how do we integrate a hybrid conservation approach to the metapopulation management of wild fragmented populations of African wild dogs in South Africa? To assess the costs/benefits for this species, we first need to undertake genetic modelling to determine the minimum effective population needed to retain 90% heterozygosity over 100 years across the 14 in situ fragmented populations, under two different modelling scenarios (Fig. 5):

(i) the current natural breeding (translocation & artificial pack formation) strategy
(ii) the proposed hybrid breeding (natural + sperm banking/AI) strategy

We then need to determine the management costs (capture, sedation, translocation, veterinary, equipment, and temporary captivity/food/ husbandry/facilities) as well as reserve costs (prey consumption, disease management, fence and habitat maintenance, pack monitoring and escape) per animal. These need to be augmented with costs associated with sperm banking and AI (equipment, facilities and labour) for each animal involved in the hybrid scenario (ii) above. The total project cost for each scenario can then be calculated by scaling up these costs for each of the two different sized populations (Fig. 5). Based on the limited number of previous studies [87–89], we predict that, given the generation interval and time to sexual maturity of AWDs is more similar to the koala, the minimum population size will be at least 13-fold fewer and total project cost at least 7-fold lower if the hybrid conservation management approach is adopted. This could alleviate pressure on the carrying capacity of reserves and provide economic savings without reducing genetic diversity of the species – freeing-up funds for other important conservation priorities. However, the strength of any model depends on the quality of the data used to generate it. This requires

collaboration with game reserve and/or metapopulation managers with extensive wild dog management experience to help develop accurate models.

Table 3 illustrates the steps needed to implement a hybrid conservation approach to the metapopulation management of AWDs. Of crucial importance is the establishment of partnerships with zoos, governments, NGOs, universities and state/private reserves to facilitate further refinement of the techniques and their application to in situ conservation. The table also shows how the different elements we have developed and discussed in this paper can be applied to advance conservation of the species; be it the banking of sperm from sedated male AWDs, the use of pheromones and non-invasive tools to manage and monitor the AI intervention, or the transfer of genetic diversity by AI between captive – captive, wild – wild, wild – captive or captive – wild individuals to increase survival of the next generation of pups. It should be stressed that the goal of AI in conservation of captive and wild AWDs is to supplement rather than replace the genetics of the alpha male – negating the requirement to vasectomise this male (which is undesirable for any endangered species). Thus, the alpha female is naturally mated by the alpha male in parallel with AI, theoretically resulting in litters of mixed paternity. AI serves to introduce valuable foreign genes (e.g. disease resistance) into some pups, while locally-derived survival genes are naturally introduced into other pups by the alpha male – thus increasing the chance that some offspring will survive if conditions change adversely one way or another. Moreover, mixed paternity co-sired by beta and lower ranking males occurs naturally in 10% of African wild dog litters [186–188]; increasing our confidence that such a hybrid AI approach will support successful rearing of litters by the whole pack [116].

8. Conclusion

The complex social structure of AWDs presents significant challenges to conventional conservation management of this species. AI coupled with sperm banking offers the potential to maximise genetic gains with fewer animals at lower cost when applied to augment traditional metapopulation management practices. To this end, we have (i) identified that males of all social rank yield good quality semen during the breeding season, (ii) developed a robust sperm freezing protocol that enables the indefinite preservation of this genetic diversity, (iii) determined the optimal time for its downstream insemination during the female fertile period using behavioural and hormonal cues, (iv) developed a suite of non-invasive tools to monitor stress, aggression, social cohesion, immune health and reproductive status of packs, and (v) validated the use of appeasing pheromones to help mitigate these deleterious effects during conservation interventions. This paves the way for the use of AI to manage the valuable genetics of both captive and wild packs of African wild dogs as one contiguous metapopulation. This requires the trust, good-will and collaborative effort of captive institutions, reproductive biologists, geneticists, ecologists as well as reserve and metapopulation managers if it is to emulate the success of previous hybrid programs like that for the black-footed ferret. Moreover, such techniques may be directly applicable to other socially complex species, like the Ethiopian wolf – one of the world's most endangered canids.

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.

Acknowledgements

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