# Improving the Performance of Mpwapwa breed cows in Tanzania: A Fertility Management Approach

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**KABUNI THOMAS KABUNI**

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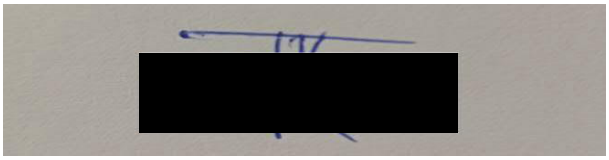
**Declaration**

I Kabuni Thomas Kabuni, hereby declare that I am the sole author of this thesis, entitled: "Improving the Performance of Mpwapwa breed cows in Tanzania: A Fertility Management Approach", submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Science.

This work is the result of my own research, except where otherwise acknowledged, correctly and completely.

Tanzania Livestock Research Institute Livestock Research (TALIRI) Ethical Clearance Committee approved all the studies that required the handling and manipulation of animals on 12th Feb 2021.

KABUNI THOMAS KABUNI



## Abstract

Beef cattle farming is an important part of Tanzania's economy being widespread throughout Tanzania. It is commonly practiced by small-scale farmers, often in conjunction with other agricultural and commercial activities. As part of the colonial government's efforts to develop agriculture in Tanzania in the 1940's, the Mpwapwa breed was developed, largely from *Bos indicus* stock, as a dual-purpose breed to provide better beef and milk production. The Mpwapwa TALIRI research centre, in the Dodoma region of Tanzania, maintains a nucleus herd of Mpwapwa cattle from which it has provided breeding stock and, prior to 1973, an artificial insemination (AI) program to farmers. These programs fell into abeyance during the post-colonial era. Critically, the AI service was discontinued until TALIRI Mpwapwa started to re-equip its facilities in 2018. The major limiter to re-establishing the AI service has been the high cost and poor availability of liquid nitrogen, which means that it is not feasible to develop a service that is dependent on cryopreserved semen. In the past globally, and in New Zealand currently, bovine AI services have been based upon chilled or ambient temperature (AT) diluents, so the feasibility of developing a new AI service for Mpwapwa cattle using ambient-temperature semen was investigated. Most of the use of AT diluents has been in temperate climates, so an important component of re-establishing the AI service was to determine whether AT diluents could sustain sperm viability at the high (>30ºC) temperatures that pertain in Tanzania.

The first step was to survey farmers who kept Mpwapwa cattle about their management and breeding practices using a cross-sectional survey. This was undertaken to determine whether they were interested in an AI program for their cattle and whether they thought it would be a useful method of breeding for their cattle. A survey was administered across 100 farmers in the Mpwapwa region. Median herd size was 7 (range 1-150) and median farm size was 12 acres (0.4-500), milk yields were generally under 5 L/cow/day and carcass weights were typically 112-142 kg. The Mpwapwa breed was regarded as better than other local breeds. Only 17 farmers had used AI, almost always after single-$PGF_{2\alpha}$ synchronisation. However, 69 farmers who had not used AI were interested in doing so and 61 considered that AI was better than natural bull mating being aware of the significant of AI on livestock breeding. The conclusions from the survey were that an AI service would have to be tailored around small herds, probably with fixed-time AI (FTAI) after single-$PGF_{2\alpha}$ synchronisation, and would have to offer farmers significant improvements in animal genetics and fertility and/or easier management of breeding than at the present.

As the Mpwapwa bulls had never been evaluated to determine whether their semen was of adequate quality to use in an AI service, the next step was to undertake breeding soundness examination (BSE) of the bulls in the TALIRI Mpwapwa stud. The 53 heaviest bulls out of the total stud of 120 were subjected to a full BSE including semen examination (collection by electroejaculation). Scrotal circumference was similar in bulls that were 24-36 months old (mean: 27.1 cm, SD: 1.6 cm) and those that were >36 months old (mean: 27.8 cm, SD: 2.0 cm). Semen was successfully collected from 44 bulls. Mean ejaculate volume was 5.5 mL (SD: 2.7 mL). Only 4 bulls did not have ≥75% morphologically normal sperm. Mean ejaculate density was relatively low at 303 $x10^6$ sperm/mL (range: 57-966, SD: 258 $x10^6$ sperm/mL). Density was >400 $x10^6$/mL in 31 bulls, >700 $x10^6$/mL in 2 bulls and >800 $x10^6$/mL in 6 bulls. These 8 bulls were considered suitable for use in an AI program. The variation of semen quality was largely as expected for a breed that had not been subjected to any form of selection for breeding ability. Findings for these Mpwapwa breed bulls largely align with those of similar low body-weight breeds of *B. indicus* that are found in East Africa and South-East Asia, but are significantly less than would be expected from the improved *indicus* breeds of South America, Australia and southern Africa. DNA fragmentation was examined in the same 53 bulls, again, as the status of these hitherto unselected bulls was unknown. Most bulls had unfragmented sperm (mean: 94.7%, SD: 6.8, Mode: 100%), with only 5 bulls having <90% normal sperm. Fragmentation was therefore not sufficiently widespread in the bull stud to cause concern about their use in AI.

The next step was to assess the survival of sperm at ambient temperatures. Ejaculates from 35 bulls were diluted 1:1 in Tris-egg yolk (TE), Optixcell and coconut water, loaded into 0.25 mL mini-straws and incubated in water baths at 20°C, 27°C and 33°C (Year 1) or 8°C, 17°C and 33°C (Year-2). Motility was evaluated using computer-assisted analysis after 6, 24, 48, 72 and 120 h. Sperm survival was related to temperature, such that survival at 8°C was better than at other temperatures. Survival in coconut water was poor, with limited survival even at 24 h. Mean survival at 72 h was <40% in both TE and Optixcell, but was >40% at 48 h except at 32ºC. These results suggested that storage at <20ºC for up to 48 h would give acceptable motility (i.e. ≥50%) for use in AI. Sperm from some (n=4) individual bulls survived, however, for up to 120 h at 8ºC and from 2 bulls at 17ºC. Semen from 3 bulls survived for 120 h at 32ºC. These results showed that storage for 48 h at 17°C in TE or Optixcell was feasible for most bulls, and individuals could be identified whose semen survived longer and/or at higher temperatures for at least 72 h. Thus,

maintaining semen at ambient temperatures of 32°C was difficult for >24 h, but was readily achievable in an AT diluent with a modest amount of cooling for 48 h. This survival would permit an AI service based on AT semen, provided inseminations could be performed soon after collection, or after a period of modest refrigeration.

Finally, a proof-of-concept AI trial was undertaken, in which 303 cows were inseminated with cryopreserved or AT semen after a double-$PGF_{2\alpha}$ synchronisation (to detected oestrus after $PGF_{2\alpha}$-1 and by FTAI after $PGF_{2\alpha}$-2). Conception rates to AI were 62% to AT and 38% to cryopreserved semen (final pregnancy rates were 99% and 97% respectively after 12 weeks of bull mating). Whilst the trial was not undertaken to demonstrate that one method was superior to the other, it did demonstrate that FTAI with AT semen was at least as good as with cryopreserved semen.

In terms of developing an AT AI service, this thesis has shown that it would likely be well-received by smallholder farmers, that there is a cohort of bulls which are of satisfactory breeding quality for use in AI, that their semen survives for long enough for AT semen to be the basis of an AI service, and that results to FTAI are at least as good as with cryopreserved semen. In summary, the protocol itself is cost-effective and, as such, it could be used within the Tanzanian beef cattle breeding programmes. Being simple to schedule should make it easily accessible and implementable by poor smallholder farmers in the Mpwapwa region and, hence more accessible than other expensive protocols with more hormones. Even compared to natural mating, the costs of AT AI are still cheaper, making it more affordable than the costs of buying and managing a high genetic merit Mpwapwa bull. Wider screening of bulls for those whose semen survives longer and/or at higher ambient temperatures would allow for the selection of bulls with the highest quality semen. Addition of a limited degree of refrigeration (maintaining semen at 15-20$^{o}$C) would allow more flexibility to the AI collection/processing centre.

**Keywords:** ambient temperature artificial insemination, oestrus synchronisation programme, bull fertility evaluation, smallholder farmers.

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# Chapter One: General Introduction

The Mpwapwa breed of *Bos indicus* cattle is a synthetic breed which was developed from improved Indian dairy breeds (about 60%), African zebu breeds (30%) and European dairy breeds (10%), to create a dual-purpose breed which was suitable for low to medium production Tanzanian farms. Compared to local Tanzanian zebu cattle, Mpwapwa cattle have higher growth rates and produce four times as much milk, while still having the same level of disease resistance and producing bulls that can be used for draught power. However, the potential of Mpwapwa cattle has never been properly realised. For example, the average milk yield of Mpwapwa cattle is currently no better than it was in the late 1970s. The principal driver of this lack of change is that in the 1980s and 1990s, structural adjustments to International Monetary Fund (IMF) and World Bank (WB) policies led to significant reductions in spending in Tanzania on agricultural research, and the privatisation or defunding of the parastatal farms, which were crucial to maintaining the development of the Mpwapwa breed. This meant that rather than artificial breeding on research farms, genetic development of the Mpwapwa breed was primarily left to semi-commercial smallholder Tanzanian farmers who used natural mating. Reduced selection pressure thus led to reduced performance, which was accompanied by reduced numbers of Mpwapwa cattle. Although in recent years, this policy has changed and there is now increased government support for research institutions, parastatal farms and extension services, particularly related to their role in development of cattle breeds designed for Tanzanian conditions, the lack of support in the 80s has meant that the current performance of Mpwapwa cattle is no better than it was in the 1970s.

This means that, despite changes in policy and government support, significant issues still remain in getting Mpwapwa cattle to achieve their production potential. For example, a recent project which evaluated the potential of the Mpwapwa breed for genetic improvement (Chawala *et al.,* 2017) concluded that selection to improve both production and reproduction was feasible and that such improvements could be transferred to smallholder production systems. The project also concluded that, achieving these goals would require a systematic programme of genetic improvement, with cows being mated to the best bulls on both research stations and on smallholder farms. To achieve the latter would require artificial mating and fertility management programmes, both of which are rare on Tanzanian farms. Artificial insemination (AI) was commonly used in the 1970s, but budgetary cuts (especially the reduction in funding of AI services) have significantly reduced its use. Recent funding increases (both government and aid organisations) have enhanced

the feasibility of AI (through funding for laboratory resources, especially at TALIRI Mpwapwa, and through the training of technicians) but, if it is to be used on wide-scale, AI will need to be used alongside fertility management schemes that have been specifically designed and tested in Tanzanian systems.

Liquid nitrogen availability is one of the key constraints to the use of AI in Tanzania (MLFD, 2010, 2011; Msalya *et al.,* 2017). It is both expensive and at times difficult to source and handle by most AI service providers. Preservatives that allow the survival of sperm at ambient temperatures are available. Their use could significantly reduce the costs of AI, making it much more affordable for smallholders and thus increasing uptake. However, ambient AI has not been used widely in Tanzanian cattle, so the technology needs testing before it can be recommended.

Additionally, in contrast to AI using cryopreserved sperm, where a relatively small number of bulls distant from the farm using AI can be used to provide semen, using semen preserved at ambient temperatures requires bulls to be more locally sourced. Most farms in an area currently have communal bulls on site (Kabuni & Laven, 2021). So this would not require major on-farm changes (using ambient AI will reduce the number of bulls needed as fewer better bulls could be selected), but all selected bulls would need to be tested for fertility to ensure that they are suitable for use. Thus, before AI based on semen preserved in ambient-temperature diluents is widely used it is important to assess the general fertility status of Mpwapwa bulls. This is in contrast to AI with cryopreserved semen, where a small number of bulls can be used (and thus be selected based on having good fertility), while for ambient AI the selection pool has to be greater to enhance the ability to choose bulls based on having good fertility.

Oestrus observation is a limiting factor in the use of AI in Mpwapwa cattle (Kabuni *et al.,* 2022). Low body condition and low quality feed reduce the chance of cows showing oestrus. In addition, herd sizes are small, so the sexually active group will be small – further limiting the chance of observation. Furthermore, inseminating small numbers of cows on a daily basis is much less cost-effective than the planned AI of multiple cows on a single day. The development of a cheap and effective synchronisation programme, preferably using a prostaglandin-based protocol as it was used on Mpwapwa cows during my MSc. research project (Kabuni, 2017), could thus increase the use of AI in these cattle.

This PhD thesis sets out to address the following hypotheses;

i. Smallholder farmers' knowledge, attitudes and practices are presently limiting the uptake of AI technology.
ii. Mpwapwa breed bulls are suitable for use in local AI schemes.
iii. Prostaglandin-based synchronisation protocols could be used in the implementation of an AI programme in Mpwapwa cattle.

## Objectives and thesis outline

### Objectives

To address these hypotheses, the following objectives were set;

i. To understand smallholder farmers' knowledge, attitudes and practices regarding fertility and AI programmes.
ii. To understand the fertility of Mpwapwa breed bulls and the potential for their use in a local AI scheme.
iii. To evaluate the survival of sperm from Mpwapwa bulls in simple ambient-temperature diluents, as the basis for a low-cost, ambient-temperature AI service.
iv. To evaluate the fertility outcomes of ambient-temperature AI after a prostaglandin-based oestrus synchronisation protocol in Mpwapwa cows on smallholders' farms.

### Thesis Outline

The aim of this thesis was to develop and test a scheme designed to optimise the use of semen from quality Mpwapwa bulls on Mpwapwa cows. It is structured into seven chapters: chapter one (general introduction), chapter two (review of the literature on cattle breeding and research in Tanzania and global advancement in breeding technologies in the cattle industry), chapter three (evaluation of smallholder farmers' knowledge, attitudes and practices in regard to fertility and AI programmes at farm level in Mpwapwa district of the Dodoma region in Tanzania), chapter four (presentation of the results of fertility testing of Mpwapwa breed – comprehensive bull soundness examination with semen testing and DNA fragmentation assessment - and the impact of those findings on the likely value of Mpwapwa bulls involvement in local AI schemes), chapter five (presentation of the impact of different diluents/extenders and storage temperatures on sperm

survival to determine suitable diluent and ambient temperature for storage of Mpwapwa bulls' semen), chapter six (pregnancy rates after fixed time insemination using ambient and frozen se- men in synchronised Mpwapwa cows), and chapter seven (general discussion and suggestions for future studies and programs).

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# Chapter Two - Literature Review

## Cattle Breeding and Research in Tanzania: A History

### *Pre-Independence Era*

Modern cattle breeding started in Tanzania in 1905 whilst Tanganyika was a German colony (LPRI, 1983; Rushalaza & Kasonta, 1992; Kyomo & Kifaro, 2005). At that time, the Mpwapwa district of the Dodoma region (6°20′54″S 36°29′12″E) was selected to be the centre for veterinary activities in Tanganyika and to undertake research on animal husbandry and diseases (LPRI, 1983; Kyomo & Kifaro, 2005). The presence of mixed tropical (i.e. arid and semi-arid areas) and temperate climatic conditions within the district was one of the key drivers for the establishment of the Livestock Veterinary Centre in the town of Mpwapwa, with farms established at Ilolo (pasture research farm), Vianze, Mjitu, Chibwe-Changula (breeding tropical animals), Kiboriani (breeding temperate animals) and a main office at Kikombo (Pratt & Gwynne, 1977).

Another driver was that both Koch and Theiler had undertaken research on East Coast Fever (ECF) in the area (Lawrence, 1992). This ECF research stimulated animal disease research, particularly on tick-borne diseases within Tanganyika, and led to the construction of the first cattle dip in the entire East African region within the Kikombo main office in 1907 (LPRI, 1983; Kyomo & Kifaro, 2005).

Research at the Livestock Veterinary Centre ceased during the First World War (1914 – 1918) but, then, after the British took over Tanganyika, research was restarted and revitalised. Except for another break from the Second World War, the Livestock Veterinary Centre was an active research centre for Tanganyika until independence in 1961 (LPRI, 1983; Kyomo & Kifaro, 2005); with British and European scientists undertaking a range of research there. These included Dr. H.M French, who was actively involved in animal breeding and husbandry research and Drs. CJ Buckley and HG Hickson, who researched animal breeding (French, 1940; Katyega, 1987). During this period, there was a considerable focus on animal breeding, particularly of cattle. In 1951, the Central Livestock Registry was established to store records for all the livestock on all farms owned by the British colonial government. This registry, alongside the distribution of improved animals to government livestock farms and individual livestock farmers, gave an impetus to co-ordinated breeding programmes for cattle and other livestock species across Tanganyika.

### *Post-Independence Era*

Tanganyika became independent in 1961. That was the same year that liquid nitrogen-based Artificial Insemination (AI) services were introduced, for the first time, into the Northern Zone of Tanganyika (primarily in Kilimanjaro and Arusha regions) (LPRI, 1983; Kyomo & Kifaro, 2005). The intention for the introduction of AI services was to improve reproductive performance and productivity of dairy cattle. The AI service centre was based at the Tengeru Livestock Training Institute in Arusha, with Tengeru acting as both an AI service provider and a training centre for AI technicians. However, in the middle of the 1960s, the AI programme failed, due to insufficient funds and the lack of support from the government (LPRI, 1983; Kyomo & Kifaro, 2005).

In 1967, AI services were re-introduced at Mpwapwa Research Centre, but this time using ambient temperature, rather than cryopreserved, semen. In 1968, AI services using frozen semen re-started, following the installation of a liquid nitrogen plant in its Animal Biotechnology Laboratory. Bulls of different breeds (dairy, beef and dual purpose) were kept at the centre for semen production for the AI service. However, in 1973, semen production activities at Mpwapwa were shifted to the newly developed National Artificial Insemination Centre (NAIC) at Usa River in Arusha (Mejool, 1977). This remained the sole site of frozen semen for AI in Tanzania until 2018, when the Tanzania Livestock Research Institute, with government support, refurbished its Animal Biotechnology Laboratory at Mpwapwa, by re-equipping it with equipment necessary for semen and embryo collection, processing, packaging, storage and utilisation.

## Current Use of Assisted Reproductive Technologies in the Tanzanian Cattle Breeding System

Assisted Reproductive Technologies (ARTs) are modern reproductive technologies developed with a focus of enhancing animal productivity (Mapletoft & Hasler, 2005; Velazquez, 2008; Rodriguez-Martinez, 2012) through improving reproductive performance and enhancing genetic improvement (Faber *et al.*, 2003; Thibier, 2004; Sartori *et al.*, 2016; Mwanga *et al.*, 2019). The use of these technologies, especially in high-income countries, has positively contributed towards the transformation of the livestock sector and to food security. Examples of the most common ARTs are shown in Table 1. The most commonly used ART in Tanzania is AI; which may be accompanied by oestrus synchronisation, especially on large-scale public and private farms (MLFD, 2011a, b; Ojango *et al.*, 2016). The use of MOET in Tanzanian cattle, sexed semen, *in vitro* embryo production (*in vitro* maturation (IVM)), *in vitro* fertilisation (IVF), and *in vitro*

culture (IVC)), trans-vagina ovum pick-up (OPU) and cloning are all exceedingly rare, to the extent that, other than AI, these procedures can best be described as being 'in their infancy' in Tanzania, with only a few trials of MOET and sexed semen on public and large private farms. Natural mating is still, by far, the most common breeding method used by smallholder farmers. This significantly limits genetic improvement and, because the vast majority of breeding bulls are neither proven nor tested, results in an increased risk of venereal disease and high rates of inbreeding within a herd. These issues then lead to an increased incidence of infertility, poorer reproductive performance and reduced productivity.

The use of AI as a method of breeding cattle in Tanzania has always been limited (Ogutu *et al.*, 2014). The reasons for this are: centralisation of AI services at the NAIC in Arusha and NAIC's stations in different zones meant for local distribution only (MLFD, 2011b; Katjiuongua & Nelgen, 2014). This centralisation also resulted in a reduction in the number of trained AI technicians in the country, which together with the number of non-practising AI technicians further reduced the utilisation of AI services (Figure 1), especially outside of the northern regions of Arusha, Kilimanjaro and Tanga (Chawala, 2020).

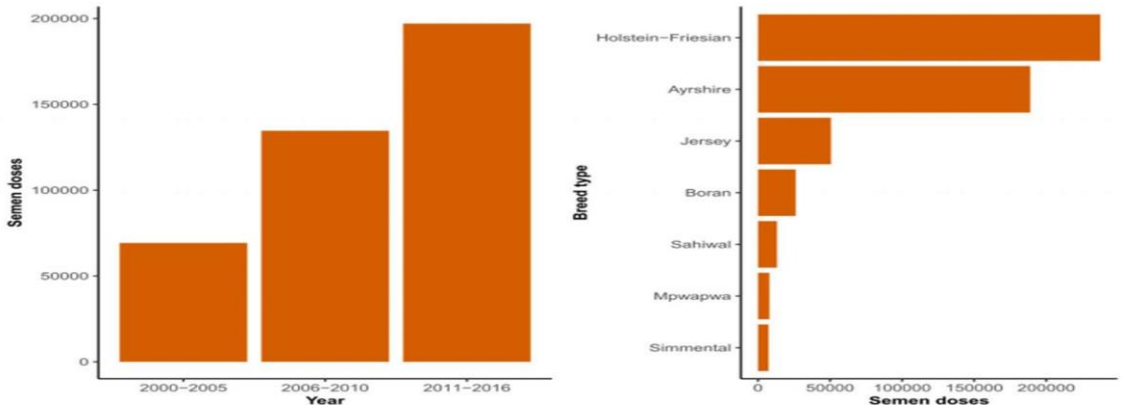


**Figure 1.** National Artificial Insemination Centre in Tanzania (NAIC) production trends and distribution of semen doses by breed from the year 2000 - 2016 (adapted from Chawala, 2020).

**Table 1.** The most common Assisted Reproductive Technologies (ARTs) used in the cattle breeding industry

| ART | Description | Reference |
|---|---|---|
| **Artificial Insemination (AI)** | The process of collection of sperm from a donor bull, separation of the ejaculate into multiple doses, preservation of the sperm (ambient or cryopreservation) and insertion into a cow at the correct stage of the oestrous cycle | Thibier & Wagner, 2000; Vishwanath, 2003. |
| **Sexed semen** | Semen of either X or Y chromosome sperm separated by the flow cytometry machine via sorting and selection procedure. | Weigel, 2004; Seidel, 2007 |
| **Embryo Transfer (± multiple ovulation (ET/MOET)** | The process of collection of embryos from either super ovulated or non-super ovulated donor cows, evaluation of the embryos into different grades, preservation of the embryos (ambient or cryopreservation) and transferring into a recipient cow at the exact stage of the oestrous cycle | Mapletoft *et al.*, 2002; Mapletoft & Hasler, 2005. |
| ***In vitro* Maturation (IVM)** | The first step of *in vitro* embryo production involving *in vitro* maturation of the collected oocytes | Galli *et al.*, 2003; Velazquez, 2008. |
| ***In vitro* Fertilisation (IVF)** | The second step of *in vitro* embryo production involving incubation of oocytes and sperm under fertilisation medium | Galli *et al.*, 2003; Blonding *et al.*, 2009. |
| ***In vitro* Culture (IVC)** | The third step of *in vitro* embryo production involving embryo culture prior to transfer into recipient cows | Galli *et al.*, 2003; Velazquez, 2008. |
| **Ovum Pick Up (OPU)** | The method of collection of oocytes from cows' ovaries prior to *in vitro* maturation, fertilisation and culture during invitro embryo production | Merton *et al.*, 2003; Velazquez, 2008. |
| **Cloning** | The process of producing genetically alike individuals by either splitting of embryos or transferring of nucleus | Wheeler, 2003, 2007; Velazquez, 2008. |

The majority of AI uses semen from imported dairy cattle breeds (Holstein-Friesian, Ayrshire and Jersey; Figure 1) with much lower usage in beef/dual purpose breeds (Boran, Sahiwal, Mpwapwa and Simmental). The pattern of use of AI, and the regions in which it is used are reflected by the location of the dairy industry in the northern part of the country (i.e. where the climate is temperate: Kurwijila & Boki 2003; Michael *et al.*, 2018: Figure 2) and in the highland zones in the southern part of the country (again, where a temperate climate permits dairy farming). The pattern of breeds used in AI similarly reflects that the majority of inseminations are in dairy cattle.

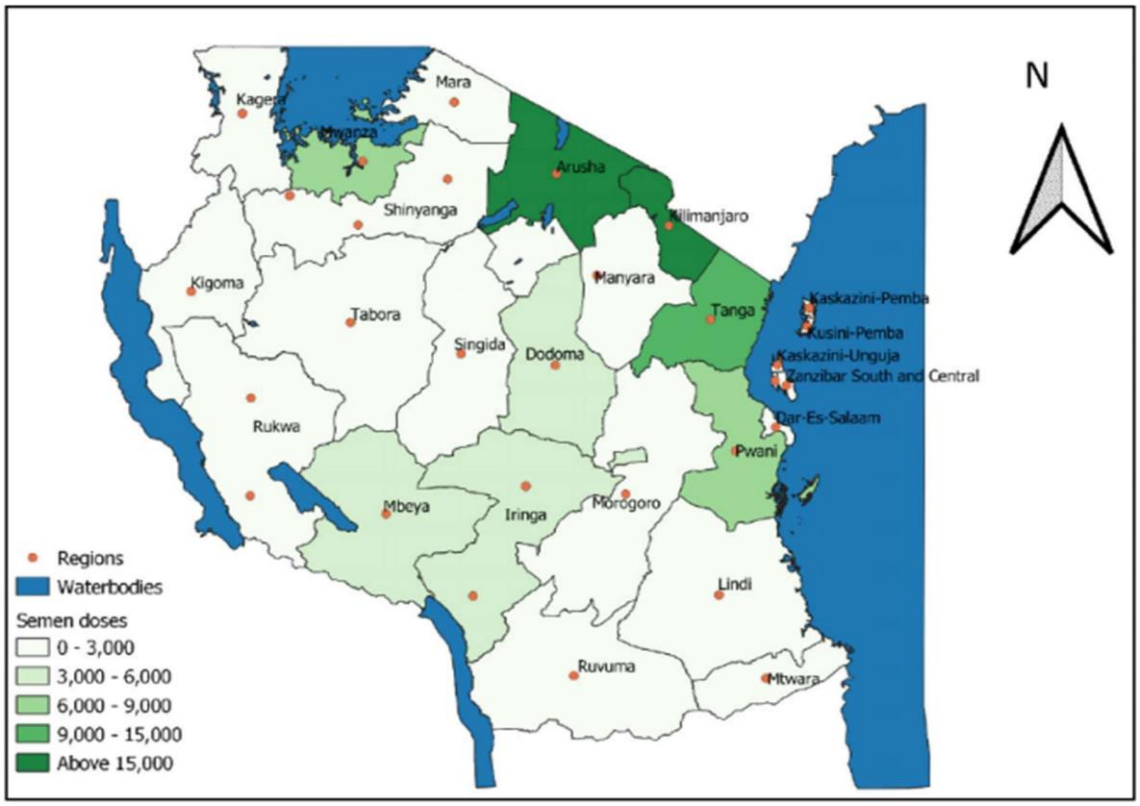


**Figure 2.** Adoption of Artificial Insemination (AI) technology in Tanzania based on distribution of semen doses from 2012 – 2016 (adapted from Chawala, 2020)

Alongside the centralisation of AI services and the lack of skilled AI technicians, the high cost of AI services (compounded by very low conception rates and the need for subsequent natural service or repeat insemination) have also been major barriers to the uptake of AI technology. The absence of sound herd fertility and health programmes (MLFD, 2011a, b; Ogutu *et al.*, 2014) has also impeded the uptake of AI.

The AI delivery system in Tanzania is not currently meeting the production needs of the Tanzanian cattle industry. Although there are ongoing efforts to improve this situation through the efforts of non- governmental organisations (e.g. Land O'-Lakes Public Private Partnership for Artificial Insemination Delivery (PAID) programme, which has a focus on increasing the number of AI technicians), trans-national organisations (e.g. International Livestock Research Institute (ILRI) African Dairy Genetic Gains programme) and the Tanzanian government (e.g. upgrade of Mpwapwa Animal Biotechnology Laboratory), further investment and development is required to enable effective and efficient utilisation of AI within the Tanzanian cattle breeding system. There is little impetus for the use of other ARTs.

## Challenges Affecting Utilisation of Assisted Reproductive Technologies (ARTs) in the Tanzanian Cattle Breeding System

### *The use of indigenous cattle breeds*

In Tanzania, as in most other East African countries (Mwai *et al.*, 2015), indigenous cattle breeds are principally derived from Zebu cattle *(Bos taurus indicus)* (*Bos indicus*), rather than Eurasian or African (*Bos taurus taurus*) (*Bos taurus*) cattle. These indigenous breeds predominate in the traditional pastoral and agro-pastoral systems of the mixed tropical (i.e. arid and semi-arid areas) and temperate areas of the country (Kanuya *et al.*, 2006a, b; Matiko *et al.*, 2008; MLFD, 2011a). The most important indigenous breed in Tanzania is the Tanzania Shorthorn Zebu (TSZ), which accounts for 95% of the cattle in the country (Msalya *et al.*, 2017a). This breed has multiple strains, such as Singida White, Tarime, Sukuma, Chagga and Iringa Red, many of which are rare and geographically confined (Msalya *et al.*, 2017a). Several other Tanzanian breeds, although principally derived from *Bos indicus* also have some *Bos taurus* genetics (e.g. Mpwapwa, Ankole and Boran breeds: Msanga *et al.*, 2001; MLFD, 2011a; Msalya *et al.*, 2017a).

Tanzania Shorthorn Zebu and strains/crossbreeds derived from them generally have slow growth rates, low mature weights, low milk yields and generally low productivity (MLFD, 2011a, b; Msalya *et al.*, 2017a). Nevertheless, TSZ and related cattle supply 95% of the beef and 70% of the milk consumed in Tanzania (Msalya *et al.*, 2017a).

The low genetic merit of the TSZ and related breeds is compounded by variable, and often-low, planes of nutrition and shortages of water, all of which further depress cattle productivity (LPRI, 1986, 1991; Msalya *et al.*, 2017a). All of these factors also affect fertility by reducing the

functionality of the reproductive system (Mukasa-Mugerwa, 1989). Productivity is dependent on fertility (Kanuya *et al.,* 2006a, b), so the impact of these factors on fertility exacerbates and accentuates their direct effects on productivity. Addressing the challenges that affect the fertility of cattle breeds in Tanzania is necessary for improving reproductive performance and for improving productivity, sustainability and owner incomes.

In order to improve the reproductive performance in a system, baseline data on current performance are needed (Brownlie *et al.,* 2015). However, whilst it is generally accepted that *Bos indicus* cattle characteristically show poor reproductive performance (Kanuya *et al.,* 2006a, b); there have been few published reports on the reproductive performance and fertility of Tanzanian cattle breeds. The only published large-scale study of fertility of TSZ that was found in the literature is that conducted by Kanuya *et al.* (2006a, b). Those authors conducted a study at Gairo (36°45′E 6°30′S) from 2001 to 2004 in three villages where cattle were usually grazed on communal land during the day and kept in kraals during the night. The study area normally gets rainfall of about 600 mm/year with ~90% falling between December and April. Over the study period, data were collected from 275 lactations from 177 cows. Pregnancy or cyclic status was assessed every two weeks via transrectal palpation. They reported calving frequencies and the percentage of cows that were pregnant on a month-by-month basis. Using the later data in comparison to an expected 90-day calving to conception interval, they created an 'expected percent pregnant' model. Comparison of the expected and actual data (Figure 3) highlights the underperformance of TSZ cattle in that study.

One of the aims of developing the Mpwapwa breed cattle was to develop a Tanzanian-adapted breed that had better productivity and fertility than the TSZ. At first at least, this was successful (Rushalaza & Kasonta, 1992), but the performance of Mpwapwa cattle has subsequently declined in terms of fertility as well as productivity. For example, Kabuni (2017) in a study of prostaglandin use in Mpwapwa cattle, reported that of 100 untreated cattle only 49 became pregnant after natural mating during a 12 week breeding season.

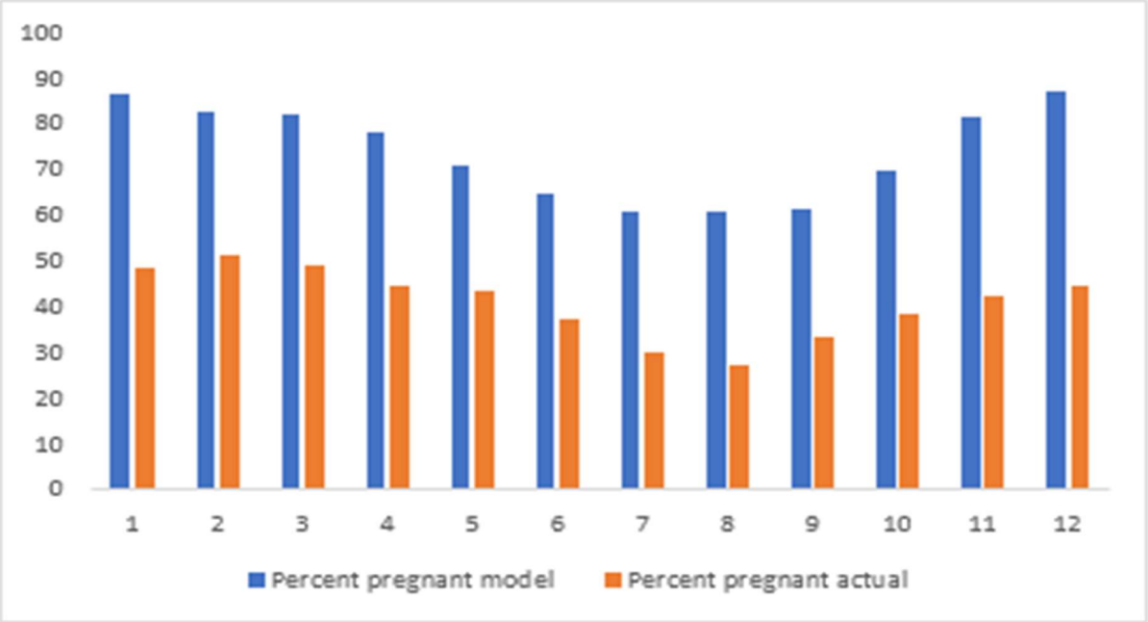


**Figure 3.** Expected proportion pregnant (based on monthly calving proportion and ~365-day calving interval) compared to actual proportion pregnant (data from Kanuya *et al.*, 2006a)

### *Dominant use of natural mating*

Natural mating is thus by far the most common method of breeding, especially outside the main dairying areas, on farms where the main breeds are *B. indicus* based, and on smallholdings. These bulls are very rarely tested either for disease or breeding soundness, which almost certainly contributes to the generally poor fertility of Tanzanian cattle (Parkinson, 2004). Nonetheless, their use requires little input from the farmer, whereas, especially for smaller farms, using any ARTs (even AI) would incur significant costs and would require significant changes in routine management.

### *Infrastructure, expertise, breeding policy and breed associations*

Because of the lack of support for their development, current ART-related infrastructure and expertise is not sufficient to effectively and efficiently accommodate even the current demand for ART in Tanzania - let alone its potential use (Msalya *et al.*, 2017b). Even for medium to large- scale dairy farms, which are the main users of AI, its use is strongly associated with proximity to NAIC (Chawala, 2020: Figure 2). To get ART to the majority of Tanzanian cattle farmers (who are small-scale pastoralists) would require significantly better infrastructure and a substantial increase in the number of people with relevant expertise. The government has developed a strategy

towards doing so (MLFD, 2010) but so far with little obvious effect beyond the refurbishment of NAIC and re-equipping of the Animal Biotechnology Laboratory at TALIRI Mpwapwa.

Further, having a nationally coordinated livestock breeding policy, as well as active breed associations/societies, would provide valuable support for increasing the use of ART. There has been government support for such developments (MLD, 2006; MLFD, 2010), but implementation has been lacking.

### The Advantages of Integrating ARTs into the Tanzanian Cattle Breeding System

Using ARTs in cattle has been shown to increase the rate of genetic gain, reduce the transfer of venereal diseases, improve fertility and increase progeny value (Baruselli *et al.*, 2017; Moore & Hasler, 2017). All of these advantages would be of significant benefit if realised in the Tanzanian cattle breeding system and would increase farmer incomes and contribute to the country's GDP as well supporting the sustainability of Tanzanian cattle farming and reducing reliance on imports (MLFD, 2010; 2011a, b).

### Future Investments in the Tanzanian Cattle Breeding System

The Tanzanian cattle breeding system has plenty of investment opportunities, as the government has created an enabling environment for both domestic and foreign investors in a so-called 'win – win' situation (MLFD, 2010, 2011a, b). Currently, the demand for ART services, especially AI, is high, but the available service providers are few and cannot satisfy the market. The key investment areas that are necessary for improving the performance and efficiency of the Tanzanian cattle breeding system are: 1) provision of sufficient and reliable AI delivery systems; 2) production and distribution of quality fresh and frozen bovine semen; 3) if cryopreserved semen is to be used, the production and distribution of liquid nitrogen (LN) gas to stakeholders; and, 4) investing in capacity building to the AI service providers and consider only those with at least a certificate in animal health and production or above those qualification.

## Artificial Insemination

### Measures of semen quality

Generally, assessment of semen quality is conducted inside the laboratory after collection of the semen samples. Semen samples are assessed for motility, morphology and concentration (Tanga *et al.*, 2021). The minimum value for sperm concentration for use in AI is typically $\geq 800 \times 10^6$/mL per ejaculate and $25 \times 10^6$ per cryopreserved AI dose (Indriastuti *et al.*, 2020). The classification of semen motility and morphology measures are as shown in Tables 2 and 3.

**Table 2.** Microscopic examination of semen mass motility

| Gross motility | Individual motility | Classification |
|---|---|---|
| Rapid/vigorous dark swirls and eddies | Rapid straight | Very good |
| Slow swirls and eddies | Moderate straight | Good |
| No swirls, some oscillation | Slow, straight | Fair |
| Little movement, sporadic oscillation | Very slow, erratic | Poor |

**Table 3.** Microscopic examination of semen morphology

| Primary abnormalities (%) | Secondary abnormalities (%) | Classification |
|---|---|---|
| <10 | <25 | Very good |
| 10 - 19 | 26 - 39 | Good |
| 20 - 29 | 40 - 59 | Fair |
| >29 | >59 | Poor |

Assessment of semen samples against these criteria helps to ensure that ejaculates are screened for those that are unlikely to result in acceptable conception rates. Similarly, semen assessment is also an essential tool in evaluating the fertility status of natural service bulls (Vincent *et al.*, 2012).

The motility, morphology and viability of sperm is affected by storage time, temperature and the use of extenders. For example, raw semen is only viable for a short period of time at ambient temperatures, whereas, extenders can be used to maintain semen viability for 48 hours or more. Cryopreservation preserves the lifespan of sperm indefinitely. However, a significant proportion of sperm do not survive the cryopreservation process, whilst those that survive may have altered motility and capacity to undergo the acrosome reaction (Shannon & Curson, 1984; Vishwanath & Shannon, 2000; Raseona *et al.*, 2017; Anzar *et al.*, 2019).

### Factors influencing success of artificial insemination

Several factors influence the success of AI: fertility of the cow, the level of feeding, age of animals, accuracy of oestrus detection, use of properly processed and handled semen, correctly timed insemination and appropriate insemination technique. When these factors are correctly applied the outcomes are better conception and pregnancy rates, with a decrease in the rate of returns to oestrus and animals that fail to conceive (Jemal & Lemma, 2015).

### Development of room temperature diluents for storage of liquid/fresh semen

The development of egg yolk-phosphate semen extender in the United States was the discovery which marked the first great achievement in the history of semen storage (Phillips & Lardy, 1940). The extender was then improved when sodium citrate replaced phosphate as a buffering agent (Salisbury *et al.*, 1978). Semen storage methodology was one of the challenges which affected the time required for semen to be transported and used under field conditions (Salisbury *et al.*, 1978; Vishwanath & Shannon, 2000). Thus, the first guiding principle for the storage of liquid semen was cooling it to below body temperature (5°C in the first instance) which helped to control sperm metabolic rate in order to extend their survival under ambient/cooled temperatures (Salisbury *et al.*, 1978; Vishwanath & Shannon, 2000). The survival of sperm at 5°C enabled liquid semen to be utilised for up to four days. Concurrently, microscopic examination of semen was made much easier when sodium citrate was found to disperse the lipids/fats globules in the egg-yolk extender. Later, it was discovered that glycerol could be added to semen as a cryoprotectant, allowing for successful cryopreservation (Salisbury *et al.*, 1978; Vishwanath & Shannon, 2000).

#### *Development of Illinois variable temperature (IVT) diluents*

*In vitro* preservation of bovine semen as applied to AI practices involves the technique of dilution and/or the use of temperatures below normal body temperature (Salisbury, 1957). The widespread use of refrigerated temperatures to control the metabolic rate of sperm played a significant role in extending the lifespan of semen in early AI practice. Early simple diluents allowed for the survival of sperm for up to four days (Salisbury, 1957). Two schools of thought emerged: the use of chilled (4$^{o}$C) semen *versus* the use of ambient temperature diluents. Pivotal to the development of ambient temperature diluents was the Illinois Variable Temperature diluent (IVT), which used the bicarbonate/$CO_2$ system to slow sperm metabolism. Other components of IVT diluent were a mixture of antimicrobial agents and salts (VanDemark, 1957). Examples of IVT diluents and its modifications are shown in Table 4: Original IVT (1G-1B); IVT diluent clones: IVT(4G-1B); IVT(4G-2B); IVT(4G-4B); Arkansas no.2; Tenn. YCCG; Germany EIBL; and Cornell University Extender (CUE). Much of the impetus for development of ambient temperature diluents came from work with pig AI, since boar semen is very intolerant of cryopreservation. The onset of successful cryopreservation for bovine semen somewhat arrested the development of ambient temperature

diluents for cattle AI, until their later revival in diluents such as the Caprogen diluent (Shannon, 1964, 1965, 1968).

### *Development of Caprogen diluent*

Originally, the development of Caprogen diluent was focused on preserving liquid bull semen chilled at 5°C. However, when it was stored at 15-27°C, the semen yielded better fertility performances, and thus, its utilisation was then widely applied at ambient temperature (Shannon & Curson, 1984). Caprogen diluent is a variant of IVT, which also includes catalase, which is responsible for removal of peroxide produced in the media by sperm metabolism and/or sperm death. Additionally, oxygen is removed from the Caprogen diluent by saturating it with nitrogen gas, which is further responsible for the reduction of sperm metabolic rate (Shannon, 1964, 1965, 1968).

In New Zealand, a very substantial proportion of dairy cattle inseminations uses liquid/fresh semen preserved in Caprogen diluent. Caprogen has been extensively utilised in the New Zealand AI industry over decades and has produced good fertility outcomes. Specifically, it allows for a smaller number of sperm in an insemination dose (as low as $2 \times 10^6$ total sperm/insemination: Shannon *et al.*, 1984), which allows each ejaculate to be divided into more insemination doses. Secondly, at least in the short term, sperm do not undergo the acrosome damage associated with cryopreservation, which may enable higher conception rates and/or greater leeway in the timing of insemination to ovulation. It is also less expensive than cryopreservation, as it does not require the industrial infrastructure required for producing liquid nitrogen; and hence there is potential for simplification and cost reduction in the supply chain between production and insemination. These benefits could improve the uptake of cattle AI in situations where cost and supply chain difficulties are presently prohibitive.

**Table 4.** Composition of the original IVT diluent and its later modifications (adapted from Bartlett & VanDemark, 1962)

| Ingredients | Original IVT (1G-1B) (Ref. 19) | IVT 4G-1B ….. | IVT 4G-2B ….. | IVT 4G-4B ….. | Arkansas No.2 (Ref. 6) | Tenn. YCCG (Ref. 15) | Germany EIBL (Ref. 2) | Cornell CUE (Ref. 4, 5) |
|---|---|---|---|---|---|---|---|---|
| Buffer (g/100ml unless otherwise noted)[a] | | | | | | | | |
| Sodium bicarbonate | 0.21 | 0.21 | 0.42 | 0.83 | 0.21 | 0.21 | 0.18 | 0.21 |
| Sodium citrate | 2.00 | 1.48 | 1.00 | 0.09 | 1.00 | 1.60 | 1.67 | 1.45 |
| Potassium chloride | 0.04 | 0.04 | 0.04 | 0.04 | - | 0.04 | 0.03 | 0.04 |
| Glucose | 0.30 | 1.20 | 1.20 | 1.20 | 0.30 | 1.00 | 0.25 | 0.30 |
| Sulphanilamide | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.25 | 0.30 |
| Penicillin (IU/ml) | 1,000 | 1,000 | 1,000 | 1,000 | 1,000 | 500 | 500 | 1,000 |
| Streptomycin (r/ml) | 1,000 | 1,000 | 1,000 | 1,000 | 1,000 | 500 | 500 | 1,000 |
| Catalase | - | 0.01 | 0.01 | 0.01 | - | - | - | - |
| Glycine | - | - | - | - | 1.00 | - | - | 0.937 |
| Glutathione (reduced) | - | - | - | - | 0.154 | - | - | - |
| Gassed with | $CO_2$ | $CO_2$ | $CO_2$ | $CO_2$ | $CO_2$ | $CO_2$ | $CO_2$ | [b] |
| Egg yolk (% of final mix.) | 10 | 15 | 15 | 15 | 10 | 20 | 30 | 20 |
| Buffer portion (% of final mix.) | 90 | 85 | 85 | 85 | 90 | 80 | 70 | 80 |

[a] Proportion of buffer reduced in final diluent according to percentage of egg yolk added

[b] 0.087g citric acid was added to correct pH, thus resulting in release of $CO_2$ from the bicarbonate

### *Preservatives for semen: ambient vs frozen*

Semen extenders or diluents are media that are used to preserve, extend and protect sperm against several shocks during processing and storage (Raheja *et al.*, 2018). Preservation of semen is generally performed at either ambient (room) temperature, chilled to ~4°C, or frozen to -196°C using appropriate extenders for each category (Vishwanath & Shannon, 2000; Raseona *et al.*, 2017). Specific extenders are required for each technique, although some common formulae (such as egg yolk-citrate), can form the basis of extenders for different methods of preservation (Vishwanath & Shannon, 2000). Examples of extenders for room temperature are shown in Table
5. For cryopreservation, a cryoprotectant is needed to protect the sperm from the effects of intracellular ice and/or osmotic damage. Cryoprotectants can be penetrating (i.e. having their effect by penetrating the cell and causing changes to the properties of the intracellular environment: glycerol is by far the most common of these), or non-penetrating (i.e. having their effect upon the extracellular matrix). Examples of non-penetrating cryoprotectants include disaccharides and proteins: egg yolk-citrate-glycerol, egg-yolk lactose, skimmed milk-egg yolk, and tris-buffer based (Vishwanath & Shannon, 2000).

### *Advantages and disadvantages of ambient vs frozen techniques for semen preservation*

Preserving semen at ambient temperature or chilled to ~4°C has some advantages over cryopreservation (-196°C), as it is a much cheaper technique of preserving semen and requires much less expensive equipment and, hence, is much easier to use in the field (Raseona *et al.*, 2017). However, semen preserved at ambient temperatures cannot be stored much beyond 3-4 days, which is a much shorter period than cryopreservation (Öztürk *et al.*, 2019), in which life span appears to be prolonged indefinitely. The length of time over which cryopreserved semen can be stored and used is thus its main advantage, which also helps in disease control as the semen can be quarantined until the donor bulls are proven to be free of diseases transmissible via semen. However, in addition to being expensive, time consuming and requiring significant infrastructure, cryopreservation can adversely affect the function of sperm, particularly in terms of swimming characteristics and the time-course of sperm capacitation. Further, the semen of some bulls does not survive cryopreservation very well (Mostek *et al.*, 2017). In consequence, the dilution rates of semen for cryopreservation is much less than for ambient/chilled diluents. For cryopreservation, semen is usually diluted to 80-100 x $10^6$ sperm/mL (20-25 x $10^6$ sperm/insemination dose), whereas

**Table 5.** Composition of diluents for storage of bovine semen at low (5°C) and ambient temperatures. Ingredients are in g/100ml of medium (adapted from Vishwanath & Shannon, 2000)

| Ingredients | Egg yolk - phosphate (Phillips, 1939) | Egg yolk - citrate (Salisbury *et al.*, 1941) | Original IVT diluent (Van Demark *et al.*, 1957) | CUE (Foote *et al.*, 1960) | Tris medium for ambient storage (Foote, 1970) | CAPROGEN (Shannon, 1965) |
|---|---|---|---|---|---|---|
| Temperature of storage | 5°C | 5°C | 5°C | 5°C and ambient | 5°C and ambient | ambient |
| Tris | - | - | - | - | 3.028 | - |
| Potassium hydrogen phosphate | 0.2 | - | - | - | - | - |
| Sodium hydrogen phosphate | 2.0 | - | - | - | - | - |
| Sodium citrate | - | 3.6 | 2.0 | 1.45 | - | 2.0 |
| Sodium bicarbonate | - | - | 0.21 | 0.21 | - | - |
| Potassium chloride | - | - | 0.04 | 0.04 | - | - |
| Glucose | - | - | 0.3 | 0.3 | 1.25 | 0.3 |
| Citric acid | - | - | - | 0.087 | 1.675 | 1.0 |
| Glycine | - | - | - | 20.0 | - | 0.014 |
| Glycerol | - | - | - | - | 8.0 | 1.25 |
| Catalase | - | - | - | - | - | 0.003 |
| Caproic acid | - | - | - | - | - | 0.025 |
| Egg yolk (%) | 50.0 | 50.0 | 10.0 | 20.0 | 25.0 | 5.0 |
| Antibiotics | - | - | Yes | Yes | Yes | Yes |
| Gas phase | - | - | $CO_2$ | self carbonating | - | $N_2$ |

insemination doses for semen extended in Caprogen diluent can be as low as 5 x $10^6$ sperm/dose (Shannon *et al.*, 1984).

### Preparation of semen extenders

Generally, semen extenders, including those for chilled, ambient and frozen semen, are prepared using similar components with the exception of cryoprotectants (such as glycerol) which are only used with frozen semen. Semen extender components can be categorised as follows:

#### *Energy substrate*

These are primarily monosaccharides that are needed for sperm to undertake metabolic activities. Examples of energy substrate used in semen extenders are arabinose, glucose and fructose. A more complex energy substrate that is commonly used is egg yolk. For frozen semen, energy provision is important only for the short period between collection and the sperm becoming frozen. Once sperm are frozen, metabolic activity ceases. In contrast, energy provision is more important in chilled or ambient temperature diluents as sperm metabolic activity continues, so provision of sufficient energy substrates is required to ensure sperm survival for several days (Salisbury *et al.*, 1978).

#### *Buffering agent*

Sperm can tolerate only a narrow range of pH, so provision of buffering capacity is an important role of extenders. Examples of buffering agents used in semen extenders are TES, Tris, Hepes and MOPS. Bicarbonates and sodium citrate are simple buffers which are used as buffering agents in some semen extenders. Phosphate buffers have also been used in semen extenders, but their use is currently limited because they risk causing head-to-head agglutination (Rehman *et al.*, 2013).

#### *Maintenance of osmotic pressure*

Sperm can swell or shrink as a result of the water movement across the plasma membrane when extended in either hypotonic or hypertonic solutions (Guthrie *et al.*, 2002; Mughal *et al.*, 2013). Again, sperm are exposed to a series of changes of osmotic pressure during freezing and thawing which can affect their survival. As reviewed by Guthrie *et al.* (2002) and Mughal *et al.* (2013), maintenance of the osmotic pressure becomes critical for the sustenance of sperm motility. In order to achieve high sperm motility rate and acrosomal integrity, the osmotic pressure of the extender needs to be towards 280 mosm/kg (Guthrie *et al.*, 2002; Mughal *et al.*, 2013). The motility of

sperm is adversely affected by the osmolality changes in the extracellular environment caused by either hypotonic or hypertonic solutions. As such, once exposed to hypotonic or hypertonic solutions, the loss in motility becomes irreversible even if the sperm are reverted to an isotonic environment (Guthrie *et al.,* 2002; Mughal *et al.,* 2013).

### *Antimicrobial agents*

Antibiotics are usually added to semen extenders for two purposes: 1) to reduce the risk of transmission of pathogenic bacteria via semen and 2) to reduce the load of non-pathogenic bacteria, which can potentially contaminate the semen throughout the process from collection, through to processing and storage (Santos & Silva, 2020). A wide range of antimicrobial agents have been used in extenders for bovine semen, with the most common being β-lactams (penicillins and cephalosporins) and aminoglycosides (e.g. streptomycin and gentamycin), especially in combination (Santos & Silva, 2020). More recently, spectinomycin has also been used. Streptomycin is used primarily for anti-*Campylobacter* activity, penicillin for non-specific contamination, whilst spectinomycin is used for its anti-mycoplasma activity. More importantly, the need for better antimicrobial stewardship is an important consideration for setting up a new AI industry.

### *Cryoprotectants*

Cryopreservation of sperm requires a careful process of reduction in temperature, dehydration of the cell, freezing and storage, with equivalent care applied to the thawing process (Ugur *et al.,* 2019). Nevertheless, even if best practice is followed, the freeze-thaw process will result in sperm damage, with cryopreservation of semen being associated with sperm membrane damage, motility impairment, oxidative damage, decapacitation and the inability of sperm to sustain embryonic development (Ugur *et al.,* 2019).

Cryoprotectants reduce this damage, thus maintaining the viability of sperm after insemination, and thus the success rate of that insemination. Some products used in extenders for other reasons (primarily protection against cold shock) have some cryoprotectant properties (e.g. egg yolk and milk; Moor *et al.*, 1961; Ugur *et al.*, 2019), but other cryoprotectants are solely for that purpose (e.g. dimethyl sulfoxide (DMSO), butaine, glutamine and pyrollidone; Parkinson & Whitfield, 1987; Watson, 1990). Cryoprotectants can be penetrating or non-penetrating. The former are generally small molecules and can exert an impact on both the external and the internal environment of the sperm. For example, glycerol, which is the most commonly used cryoprotectant, acts by preventing the formation of large ice crystals during the freezing process,

by stabilising solutes and increasing the glass-forming tendency of the medium (Öztürk *et al.,* 2019). Glycerol also acts to reduce the membrane fluidity by binding to plasma membrane phospholipid groups (Anchordoguy *et al.,* 1987). The choice of cryoprotectant in early studies was largely a matter of trial and error because a complete and satisfactory explanation for the action of cryoprotectants did not exist (Ugur *et al.,* 2019). But cryoprotectants are intrinsically toxic, so the concentration at which they are used is a compromise between toxicity and protection. Their toxicity can be observed during equilibration and cooling/freezing stages causing osmotically induced volume changes which consequently lead to destabilisation of the plasma membrane structure and loss of its functionality. Temperature of addition also affects toxicity, such that many diluents are either added in 2-stages or add the glycerol at 4°C. Non-penetrating cryoprotectants are higher molecular-weight molecules which exert an impact on the internal environment of the sperm or on the external medium without penetrating the sperm cell. Examples of non-penetrating cryoprotectants include disaccharides such as sucrose and lactose (Öztürk *et al.,* 2019).

### *Protection against cold shock*

The cryotolerance of the sperm during cryopreservation is enhanced by the presence of cholesterol within the sperm plasma membrane. Cholesterol helps to support the sperm plasma membrane, defending the membrane structure during cold shock by preventing changes in the structural composition of the chains of the phospholipid. Thus, cholesterol has been used in extenders for sperm dilution as a protection against cold shock during cryopreservation and post-thawing (Anzar *et al.,* 2019).

The use of products of animal origin for cryopreservation is an increasing concern (Anzar *et al.,* 2019; Santos & Silva, 2020). The principal reason for this concern is that products of animal origin, such as egg yolk and milk, may pose a biosecurity risk, particularly as a source of microbial contamination (Anzar *et al.,* 2019). The World Organisation for Animal Health (WOAH, formerly OIE) thus recommends that these products should be confirmed to be microbe-free before they are used as extenders (Anzar *et al.,* 2019; Santos & Silva, 2020). To support this, there has been extensive research conducted to replace egg yolk and milk in semen extenders with products of plant origin, such as soy lecithin and liposomes (Anzar *et al.,* 2019; Santos & Silva, 2020). However, these products have limitations compared to animal-based products. For example, soybean-based diluents give inconsistent fertility results, probably because of inconsistencies in

production, while liposomes, although effective, are very expensive. Commercial chemically-defined semen extenders are playing an increasing role in standardizing the composition of semen extenders. Examples include skim milk based laciphos, egg yolk based media-Botu-Bor, BullXcell, Bovidyl, Triladyl and powdered coconut water based-ACP-111, Tris based-Tris concentrate-Gibco BRL, lecithin-phospholipids and phosphatidylcholine.

### Storage of bovine semen

#### *Chilled and ambient temperature*

Stored semen chilled at 4°C or at ambient temperature has a short storage lifespan of about 1- 4 days, which poses some limitations to its use. Further limitations are observed when semen is stored at ambient temperature (15°C – 27°C) giving a shorter lifespan of about 1-2 days as compared to storage at chilled temperatures (Shannon & Curson, 1984; Vishwanath & Shannon, 2000). The fertility of sperm preserved at ambient temperatures declines with time, mainly due to physiological changes such as extracellular oxidation, energy depletion and pH/osmotic damage. The limited lifespan of semen preserved at ambient temperatures or at 4°C means it is best used alongside accurately detected oestrus or fixed-time AI using a relatively low sperm dose/insemination in a short period of time, as it is conducted in New Zealand (Raseona *et al.*, 2017).

#### *At freezing temperature (cryopreservation of semen)*

In comparison to fresh semen, frozen semen can be stored for several years. The main issue is that the resources to store semen at this temperature of -196°C of liquid nitrogen can be difficult and expensive to maintain. This technique is manly used in cryopreservation of livestock germplasm and application of ARTs (Anzar *et al.*, 2019).

#### *Disease control in AI centres*

Last but not least, disease control in ambient temperature semen requires that AI centres comply with adequate biosecurity conditions as laid down by the WOAH: they must apply biosecurity measures to the semen collection facilities, semen laboratory, management of bulls, and disease control. Examples of the most common diseases screened in bulls in AI centres include bovine tuberculosis, brucellosis, leptospirosis, bovine viral diarrhoea virus, campylobacteriosis and venereal trichomonosis. Physical examination of bulls is also part of the biosecurity measures

required by the WOAH of AI centres. Nonetheless, as for the TALIRI Mpwapwa research centre's AI and fertility facility, a proposed AI and fertility programme would normally start based on the currently available facilities, thereafter, biosecurity measures would be regulated to fit the standards of the IAO. The most common disease that would be screened at the start of the programme are foot-and-mouth disease (FMD), brucellosis, and tuberculosis (TB). The AI facility and operating personnel would comply to the IAO standards at the start of the programme.

## Summary and conclusion

The presence of weak AI programmes accompanied by the high costs of the liquid nitrogen and AI delivery services, have created the need for the re-introduction of an ambient temperature AI programme into the Tanzanian cattle breeding system. In fact, liquid nitrogen availability is one of the key constraints to the use of AI in Tanzania; given that it is both expensive and, at times, difficult to source. Preservatives that allow survival of sperm at ambient temperatures are available, and their use could significantly reduce the costs of AI, making it much more affordable for smallholders and thus increasing uptake. The benefits of available ambient temperature diluents allow the potential for application of AI using fresh/liquid semen, which could help to improve and strengthen the AI programme in the Tanzanian cattle breeding system. However, ambient temperature AI has not been used widely in Tanzanian cattle, especially in recent years, so the technology needs testing before it can be recommended. Additionally, in contrast to AI using cryopreserved semen, where relatively small number of bulls distant from the farm can be used as semen donors, AI using ambient temperature semen requires bulls to be more locally sourced. Most farms in an area currently have communal bulls on site, so this would not require major on- farm changes (using ambient AI will reduce the number of bulls needed as fewer better bulls would be selected), but all selected bulls would need to be tested for fertility to ensure that they are suitable for use. Due caution will, of course, be needed to maintain adequate biosecurity of these bulls.

Oestrus observation is a further limiting factor in the use of AI in Mpwapwa cattle. Low body condition and low quality feed reduce the chance of cows displaying oestrus. In addition, herd sizes are small, so the sexually active group is small - further limiting the chance of observation. Furthermore, inseminating small numbers of cows on a daily basis is much less cost-effective than planned AI of multiple cows on a single day. The development of a cheap and effective

synchronisation programme, preferably using a prostaglandin-based protocol as it was used on Mpwapwa cows during my MSc. research project, could thus increase the use of AI in these cattle.

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# Chapter 3: Knowledge, Attitudes and Practices of Smallholder Farmers Regarding Fertility Programmes and AI in Mpwapwa District of the Dodoma Region in the Central Zone of Tanzania

## Introduction

Effective breeding practices can result in significant genetic gains and, thereby, improve livestock performance and productivity (Biscarini *et al.*, 2015). However, in Tanzania, the breeding practices of the smallholders, who raise beef and dual-purpose cattle in many parts of the country, are often ineffective and result in negligible genetic gain (Mwatawala & Kifaro, 2009; Mwaipopo & Mbaga, 2022). This can be observed in the central zone of Tanzania, a semi-arid agro-ecological zone, which has traditional pastoral and agro-pastoral communities (Kanuya *et al.*, 2006). In these communities, traditional breeding with communal home-bred bulls is by far the most common breeding method (Matiko *et al.*, 2008; Kabuni *et al.*, 2022). It is well-recognised that prolonged use of communal bulls increases the risk of spreading venereal diseases (Tekleye *et al.*, 1988), significantly increases inbreeding, and reduces genetic diversity thereby severely limiting genetic gain (Chawala *et al.*, 2017; Opoola, 2019).

The only cattle breeding technology that is currently available in Tanzania on a commercial basis is artificial insemination (AI). The use of AI, even when used alongside natural mating, should result in improvements in genetic gain (Chawala, 2020), so increased use of AI in smallholder communities could be significantly beneficial. However, the availability of AI services in Tanzania is limited by accessibility issues and the lack of trained AI technicians (Mwaipopo & Mbaga, 2022). It is increasingly being recognised by the government and other funders that these issues are limiting the performance of Tanzania beef and dual-purpose cattle (i.e. those which are principally owned by smallholders: Mwatawala & Kifaro, 2009) and that supporting the use of AI by increasing access and training additional technicians is necessary to meet country-level production goals (Mwatawala & Kifaro, 2009; Mwaipopo & Mbaga, 2022).

However, access to AI is not the only factor limiting its use by Tanzanian smallholders. Issues at farm level, including the generally poor fertility of smallholder cattle and, particularly, farmer awareness and understanding of the key issues around the use of AI (Kabuni, 2017; Chawala, 2020) also limit uptake, and would still be present if AI services became more available. Understanding farmers' knowledge, attitudes and practices in relation to fertility programmes is

thus imperative if programmes to increase AI uptake are to be successful in their goal of improving the productivity and performance of smallholder cattle.

The Tanzania Livestock Research Institute (TALIRI) central zonal office located in Mpwapwa district, has had a long-term programme of development of cattle that are suitable for smallholders and for the environmental conditions which predominate in Mpwapwa district. These projects have included an open nucleus breeding scheme for the development of the Mpwapwa breed of cattle (Chawala, 2020). TALIRI Mpwapwa has recently been funded by the Tanzanian government to develop AI services for cattle in that district. As part of this process, a study was undertaken to assess the knowledge, attitudes and practices of smallholder farmers within Mpwapwa district in relation to the application and uptake of fertility programmes and AI technology. The intended outcome of this survey is to understand smallholders' knowledge, attitudes and practices to underpin the development of fertility and AI programmes. Success in understanding how an AI programme could be implemented by smallholder farmers should lead to the overall goal of increased genetic gain and, hence, more efficient and effective beef cattle breeding.

## Methods

Research Ethical Clearance was obtained from the Tanzania Livestock Research Institute (TALIRI) (Feb 2022).

### *Selection of participants*

The selection process started at the village level, with 14 villages in the Mpwapwa district being selected for the survey (Figure 1). These villages were a purposive selection, inasmuch as, all of the selected villages had previous involvement with the TALIRI Mpwapwa open nucleus breeding programme. Participants were recruited from within those villages. The selection of villagers was again a purposive selection made with the assistance of the ward livestock extension officer. A total of 100 farmers were selected over the 14 villages: Chipogoro (11), Wiyenzele (17), Chinoje (3), Winza (25), Kisokwe (1), Iyoma (11), Lupeta (1), Ilolo (4), Igovu
(3), Ng'ambo (10), Mji Mpya (6), Muungano (4), Kileleni (3), Namba thelathini (1). Ten (10) more farms were recruited for the pilot survey, but these were not further used in the main study.

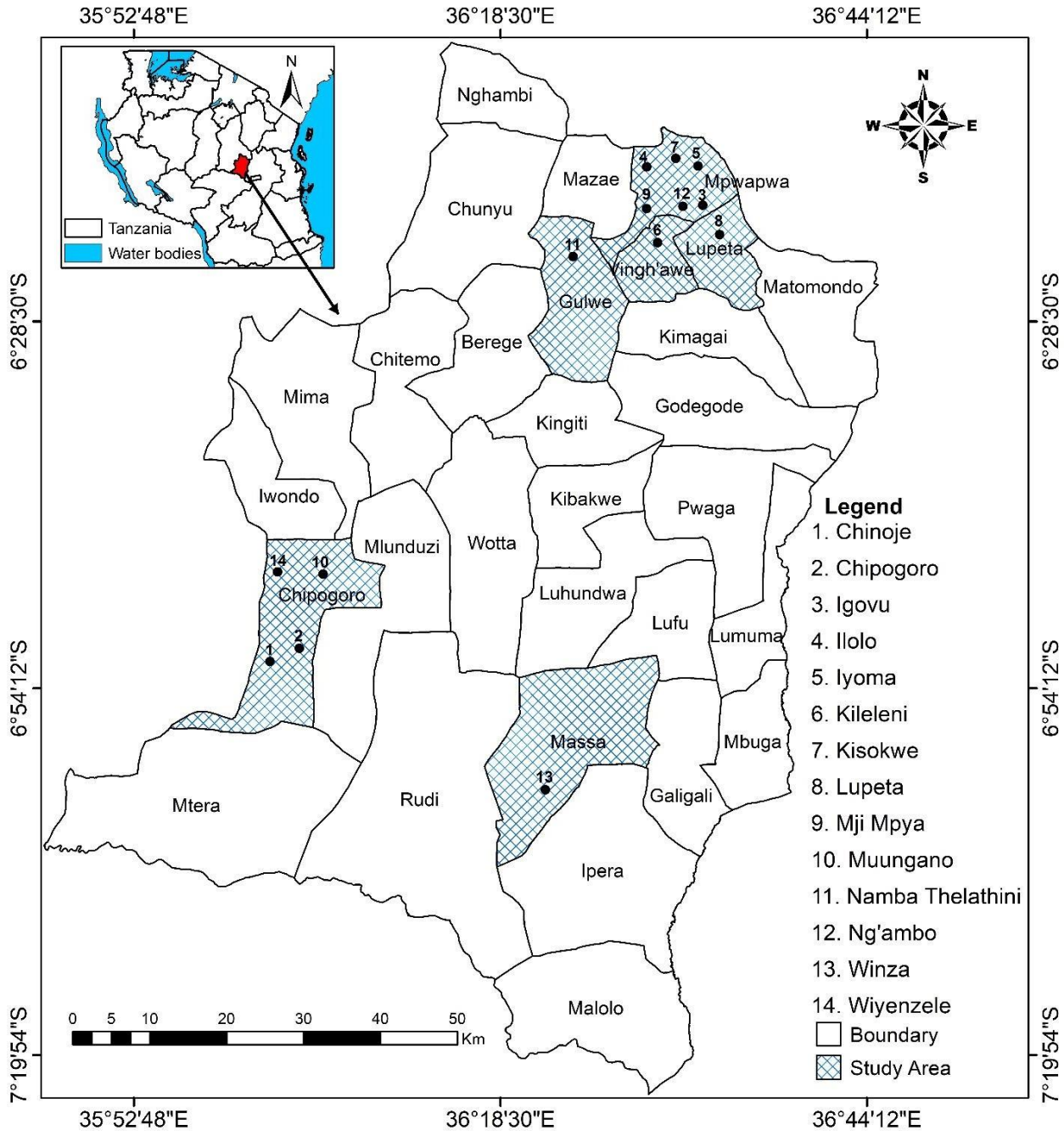


**Figure 1:** A map of Mpwapwa district showing the villages where the survey was conducted

### *Development of the questionnaire*

The questionnaire was designed to cover key factors such as farm details, breeding programme, management, nutrition, adaptation, production and growth performance (Solans-Domenech *et al.*, 2019; Aithal & Aithal, 2020) and contain both structured and unstructured questions.

### *Pre-testing and piloting of the survey questionnaire*

The survey questionnaire (see Appendix 1) was piloted with 10 farms that had been identified as being suitable for the main study. The data obtained in the pilot study were not included in the main study, nor were the farmers resurveyed. This pilot process was designed to train the enumerators in the process and to identify the questions in the questionnaire that needed modification to improve farmers' understanding of them.

### *Data collection*

Data collection followed after the completion of the pretesting and piloting of the survey questionnaire. It involved the author and four enumerators who had received training on how to administer the questionnaire during the pilot stage. The questionnaire was administered for two weeks starting on March 2022. Data were collected through individual face-to-face interviews with the farmers on each of the 100 farms.

**Figure 2:** Data collection in one of the selected farm at Winza village in Mpwapwa District. The enumerator (with a black hat) is interviewing two smallholder farmers, and recording responses on the questionnaire document. The interview is taking place at an area where cattle are kept so that responses can be verified with respect to the animals themselves

### *Statistical analysis*

Descriptive statistics for each survey question were collated and presented as proportions of respondents, or where suitable as medians (ranges). Most responses are presented at the univariate level, but where there was thought likely to be meaningful associations between responses they are presented at the bivariate level (relative risk [RR]), with logistic regression used for evaluating factors affecting intention to collaborate with TALIRI.

## Results

### *Respondents demography*

Of the 100 respondents, 92 were males, 8 were females, 95 were married/widowed and 5 were single. The median age was 46.5 years (range: 20-76) and the median number of people in each household was 8 (1-20). The highest education level of the respondents is summarized in Figure3. Of the 100 respondents, 88 had primary education, 13 had secondary education, 3 had went to primary school but didn't complete studies, 7 had never attended formal education, 2 had attended adult education (i.e. adults who attended formal education), 6 had attended college (i.e. certificate and diploma levels), and 1 had attended university.

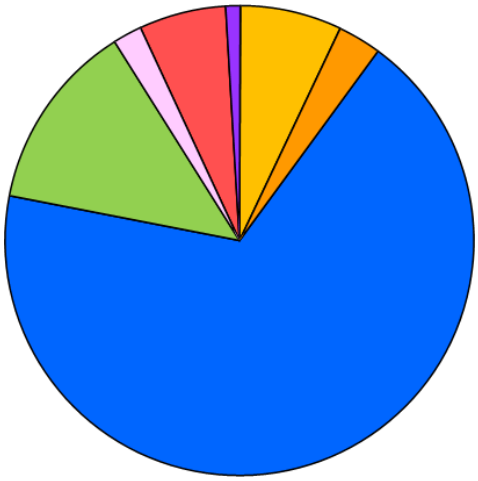

**Figure 3:** Maximum education level of farmers from the 14 selected villages of the Mpwapwa district. Never attended school, Never completed primary school, Completed primary school only, Completed secondary school, Adult education, College University.

### *Farm Characteristics*

a) Acreage

The median farm size of all the farms was 12 acres (range: 0.4-500).

b) Mpwapwa Breed Cattle

Thirty-two of the respondents kept Mpwapwa breed cattle In those herd, the median number of Mpwapwa cattle was 7 (range: 1–150). Cattle had been sourced through purchase from TALIRI

Mpwapwa farm, livestock markets, neighbours and through the on-farm open nucleus breeding scheme. All 32 respondents who kept Mpwapwa cattle reported that the reproduction and production performance was generally better (both cows and bulls) than for other local native breeds.

c) Other Breeds of Cattle

All 100 respondents also kept Tanzania Short-horned Zebu (TSZ) strains (i.e. Gogo, Hehe, or Sukuma) as well as other breeds including Ankole, Friesian (including Friesian crosses), and Jersey cross, with Gogo being the most common (54) (Figure 4). Apart from cattle farming, all respondents were also engaged in other agricultural and economic activities such as crop cultivation, entrepreneurship and other livestock business. Respondents had been keeping cattle for up to 40 years, with individuals starting to do so from 1982 to 2022. A range of reasons for starting keeping cattle were recorded (Figure 5) with family economic activity (70), family need (11), and inheritance (10) being the most common.

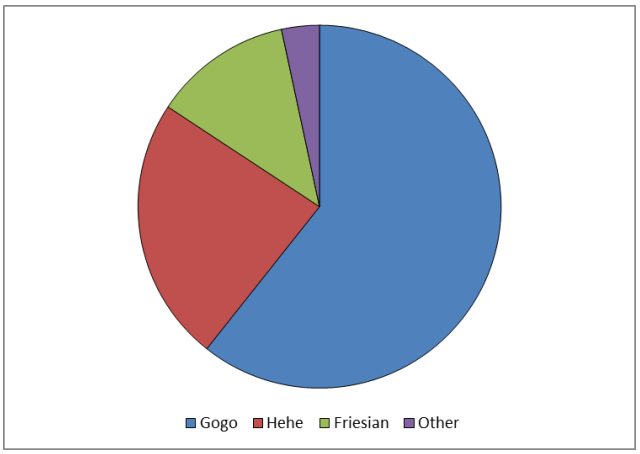


**Figure 4** Breeds of cattle kept by farmers from the 14 selected villages of Mpwapwa district other than Mpwapwa breed cattle. Other includes Sukuma, Ankole and Jersey (1 farm each).

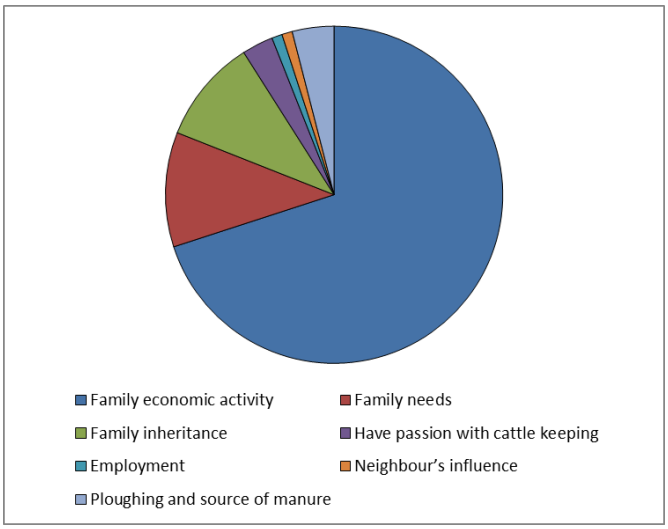


**Figure 5** Reasons for keeping cattle reported by farmers from the 14 selected villages of Mpwapwa district

d) Livestock Keeping Activities and Decision Making

Livestock keeping activities such as grazing, milking and dipping were carried out by the children, father, mother, younger brothers, members of the whole family, labourers and other relatives. The father was mostly responsible for making decisions about cattle farming (82 of respondents); more rarely the mother (1) or the whole family (17) were involved in decision making.

### *Livestock Performance*

a) Cows

Most respondents had data for milk yield (Figure 6a). Yields were generally under 5 L/cow/day, with most (61) respondents recording yields of 0.5-2.0 L. Yields of 2.0-4.0 litres/cow were reported from a further 26 farms, but only 4 farms recorded yields of >7 L/cow day. Reported body weight at first breeding (Figure 6b) was widely variable. Most respondents (63) reported that they did not know the weight of their heifers at first breeding; and, of those who reported that they did know, remarkably, 8 respondents estimated that they were ≤100 kg at first breeding. Most of the respondents (14) who reported knowing the animals' weights at first breeding estimated that they were >150 kg. Age at first oestrus (Figure 6c) was known by 78 respondents. Interestingly, there were two peak ages: either 2.6-3.0 years (43) or 3.6-4.0 years (24), corresponding to the first breeding season after they had reached adequate weight/sufficient age.

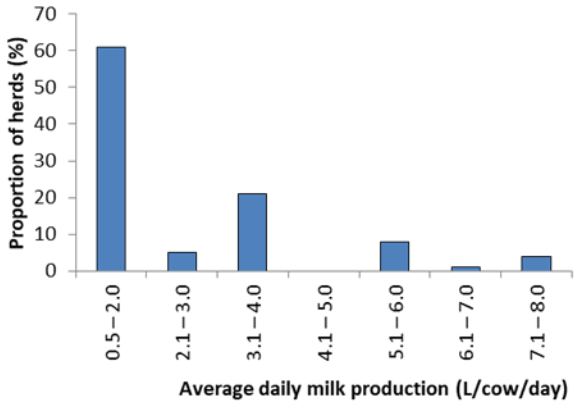


Figure 6 (a) Daily milk production

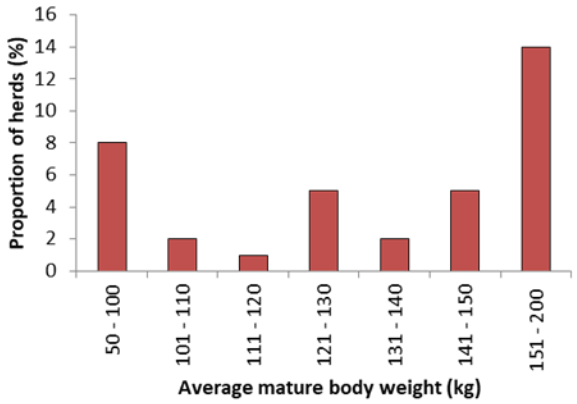


Figure 6 (b) Weight at first breeding


Figure 6 (c) Age at first oestrus

b) Bulls

Respondents were asked about the average carcass weight of bulls: 57 did not know, 6 estimated that weights were 59-80 kg, 22 estimated that weights were 112-142 kg and 5 >142 kg. Most respondents (85) knew the age at which bulls were first used for breeding: ≤2.5 years (9), 2.6-3.0 years (37), or 3.6-4.0 years (39). Most bulls were said to be mature enough when first used for breeding, with desirable body size, and expressing mounting and aggressiveness behaviours.

### *Farm management*

a) Animal health regimens.

Eighty-five respondents reported treating their cattle for intestinal worms. The most common regimen used was treating cattle once cases were observed (32 respondents), with 17 treating based on cattle performance and health condition. Treatment on a routine basis was reported by 36

respondents. Reported intervals between doses ranged from 1 to 6 months, with treatments every 3 months being the most commonly reported regimen (32 respondents).

All respondents reported treating their cattle for ectoparasites using dips. Again, the regimens used could be divided into reactive and routine with 42 respondents dipping once cases were observed and 58 dipping routinely. Intervals between treatments on farms that used routine dipping ranged from every 2 weeks to every 3 months, with the most popular being monthly (45).

A majority of respondents (74) vaccinated their cattle against foot and mouth disease (FMD), Rift valley fever (RVF), East coast fever (ECF), and Contagious bovine pleuropneumonia (CBPP). There were a range of vaccination regimens, with the two most common being: once cases were observed (41), and once per year (27). Other respondents vaccinated once every 3 months (1); or once every 3 years (5).

b) Grazing land, feeds and water conservation

Grazing was the principal feed source for all respondents with most (73) using grazing only. The remaining 27 fed concentrates in addition to grazing. The proportion of farmers grazing crop residues in addition to pasture, owning their own land and using conserved forage in the dry season (split by whether they used concentrates in addition to grazing) is shown in Table 1.

**Table 1**: Reported feed sources for 100 cattle farms in the Mpwapwa region of Tanzania

| *Principal feed source* | | *Feeds grazed* | | *Own grazing land* | | *Conserved forage in dry season* | |
|---|---|---|---|---|---|---|---|
| Grazing | 73 | Natural pastures | 38 | Yes | 40 | Yes | 44 |
| | | Natural pastures / crop residues | 35 | No | 33 | No | 29 |
| Grazing plus concentrate | 27 | Natural pastures | 3 | Yes | 16 | Yes | 17 |
| | | Natural pastures /crop residues | 24 | No | 11 | No | 10 |

Most respondents (59) grazed a combination of natural pastures and crop residues. Farmers who grazed crop residues were over 6 times more likely to feed concentrates (RR 5.6; 95%CI 1.8 -17.2) than farmers who only grazed natural pastures. A majority of farmers (56) reported owning grazing land; the proportion of grazing-only farmers who owned grazing land (40/73; 54%) was similar to the proportion of grazing landowners amongst farmers who used both grazing and concentrates

(16/27; 59%) (RR 0.93; 95%CI 0.63 - 1.3). A majority of farmers (61) reported feeding preserved forage over the dry period. Of the 61 farmers, 12 reported using hay alone, 21 fenced off pastures or crop residues for use during the dry period, and 26 used hay and fencing, while 2 reported purchasing hay and crop residues. The proportion of farmers who used both grazing and concentrates who reported using preserved forage (16/27; 63%) was similar to the proportion of grazing only farmers who reported feeding preserved forage (44/73; 59%) (RR 1.07; 95%CI 0.74 to 1.47). In contrast, there was an association between owning grazing land and feeding preserved forage. Farmers who owned their own grazing land were more likely to use preserved forage than farmers who did not (41/56 vs 20/44, respectively) (RR 1.61; 95%CI 1.12 to 2.31).

### *Livestock Breeding*

a) Breeding and Calving Seasons

Breeding data and outcomes are summarised in Table 2. Most respondents (82) reported that they bred their cows all year round, with only 14 and 4 reporting breeding exclusively in the rainy season or dry season, respectively. The average number of times that cows had calved over the three-year period was not known by 22 respondents. The most common estimate (51 respondents) was that cows had calved, on average, twice in three years (Table 2b). The number of calves born per farm over the last 12 months is summarised in Table 2c. The most frequent range of calves born per farm per year was 1-5 calves (67 respondents), with the highest reported number on a single farm being 80 calves. Of the 88 farms which reported having calves in the last year, 76 were able to state when the calves had been born. Of the 358 calves born on these farms in the previous year, 204 had been born in the rainy season (49/76 farms; 64%) and 154 in the dry season (42/76 farms; 55%). Twenty-four of the 76 farms (32%) reported calves being born in both seasons, much less than the 82% of all respondents who reported breeding cows all year round.

**Table 2.** Season of breeding and outcomes (calvings and calves) as reported by farms in 14 selected villages in Mpwapwa district (n = 100)

| a) *Season in which cows are bred* | |
|---|---|
| Season | Farms |
| All year round | 82 |
| Dry season (Jun – Dec) | 4 |
| Rainy Season (Jan - April) | 14 |
| Total | 100 |

| b) *Average calvings/cow in last 3 years* | |
|---|---|
| Range | Farms |
| 1 x | 17 |
| 2 x | 51 |
| 3 x | 10 |
| Don't know | 22 |
| Total | 100 |

| c) *Calves born/farm (last 12 months)* | |
|---|---|
| Range | Farms |
| None | 12 |
| 1 – 5 | 67 |
| 6 – 10 | 15 |
| 11 – 15 | 1 |
| 16 – 20 | 3 |
| ≥21 | 2 |
| Total | 100 |

b) Breeding cows and bulls

The mean reported lifespans of breeding cows and bulls were 8.2 and 7.8 years respectively, and the average age of the bulls currently servicing the herd was 5.7 years. Most respondents (76) used homebred bulls for breeding, with the remainder responding that they purchased bulls from neighbours (11) and livestock dealers (13).

### *Breeding Programme*

a) Breeding Goals, and BSE

Table 3 summarizes the respondents breeding goals and their practices in relation to bull soundness examinations (BSE). The vast majority of farmers (95) aimed for one calf per year from a cow. Of the 100 respondents, 55 stated that they used BSE, with 54/86 (63%) farmers who only used bulls reporting they used BSE compared to 1/14 (7%) of farmers who used AI in addition to bulls.

Across the 55 farmers who stated that they used BSE, the most common reasons for doing so were to check mating ability (33; 61%) and checking body and structural soundness (20; 37%).

b) Natural Mating (NM) and Artificial Insemination (AI)

Table 4 summarizes the knowledge, attitudes and practice of the respondents to AI. A majority of respondents had heard of AI (58 vs 42) but only 18 reported using used it (31% of those who had heard of AI). Of the 40 respondents who had heard of AI but hadn't used it, the most common reasons for not using AI were lack of access to AI services (including education about AI) (26 respondents; 65%) and cost (9 respondents; 23%). Of the 82 respondents who had not used AI, 52 (63%) would be interested in using it in the future. This includes 23 (54%) of the 42 respondents who had not previously heard of AI and 29 (73%) of the 40 who had heard of AI but had not used it previously on their farm. These data suggest that, in farmers who had not used AI but knew about it, knowledge of AI could increase their likelihood resulted in them being interested in using it in the future (RR 1.3; 95%CI 0.95 to 1.9). Of the 18 respondents who had used AI before, 17 (94%, stated that they would be interested in using AI again, resulting in a total of 69 respondents stated that they were interested in using AI in the future. Of these respondents, the main reason for using AI was to improve the genetics of their herd (55/69; 80%). For the 31 respondents who expressed no interest in using AI in the future, the three most important reasons were not believing it works (11; 35%), lack of availability of AI education (10; 32%), and cost (9; 28%).

**Table 3** Reported breeding goals, reliance on bulls and use of bull soundness examination (BSE) by Mpwapwa district farmers

| *Breeding goal for your cattle* | | | *Do you rely only on bulls for breeding?* | | | *Do you conduct BSE on your bulls?* | |
|---|---|---|---|---|---|---|---|
| Goal | Response frequency | | Response | Response frequency | | Response | Response frequency |
| 1 calf/cow/year | 95 | | Yes | 86 | | Yes | 55 |
| No goal | 5 | | No | 14 | | No | 45 |
| *Total* | 100 | | *Total* | 100 | | *Total* | 100 |

**Table 4** Knowledge, use, perception and interest in relation to using artificial insemination (AI) to bred cattle of Mpwapwa district farmers

| *Have you heard about AI?* | | | *Have you tried AI to breed your cattle?* | | | *If you have heard of AI, do you prefer natural mating over AI?* | | | *If you have not used AI, would you be interested in trying it?* | |
|---|---|---|---|---|---|---|---|---|---|---|
| Response | Response frequency | | Response | Response frequency | | Response | Response frequency | | Response | Response frequency |
| Yes | 58 | | Yes | 18 | | Yes | 32 | | Yes | 52 |
| No | 42 | | No | 82 | | No | 25 | | No | 30 |
| | | | | | | Both | 1 | | | |
| Total | 100 | | Total | 100 | | Total | 58 | | Total | 82 |

c) Oestrus Synchronization

Of the 18 respondents who had previously used AI, 13 (71%) had used oestrus synchronization before AI. All 13 had used synchronisation principally to facilitate mass breeding, and all reported that they had synchronised their cows using a single dose of prostaglandin F2α (PGF) protocol. Of the 13 respondents who reported having used synchronisation, 12 (92%) reported that the protocol had been effective.

d) Cooperation with TALIRI Mpwapwa

Of the respondents 58 stated that they had had a previous relationship with TALIRI Mpwapwa. Nevertheless 84 respondents were interested in collaborating with TALIRI Mpwapwa, with 79 of being interested in participating in a trial of AI methods run by TALIRI Mpwapwa, and 83 interested in being involved with an open nucleus breeding scheme undertaken by TALIRI Mpwapwa.

The effect of previous relationship with TALIRI Mpwapwa on interest in participating in reproduction-related research is summarised in Table 5. Irrespective of previous relationship the majority of respondents in both groups were interested in both research options, but the odds of a respondent with a previous relationship being interested were ~4 times higher than the odds of a respondent without a previous relationship (OR 3.67; 95%CI 1.1 to 12.2).

**Table 5:** Association between previous relationship with TALIRI Mpwapwa and interest in reproduction-related testing

| | | *Interested in a test of AI methods* | | | *Interested in an open nucleus breeding programme* | |
|---|---|---|---|---|---|---|
| | | No | Yes | | No | Yes |
| *Previous relationship with TALIRI* | No | 16 | 36 | | 13 | 39 |
| | Yes | 5 | 43 | | 4 | 44 |

When asked what topic (not specifically reproduction-related) they would most like to collaborate on with TALIRI Mpwapwa, the overwhelming favourite (89 respondents) was improving pasture productivity. The remaining 11 respondents did not state any topic.

## Discussion

The Mpwapwa breed was developed to fit the environmental conditions encountered by smallholder farmers across the different agro-ecological zones of Tanzania, particularly those areas not suitable for dairy farming (Chawala *et al.,* 2017; Chawala, 2020). The present survey targeted a group of farmers who were local to the TALIRI research centre and recommended by the local ward livestock extension officer. These farmers are thus the part of the population of farmers that will be targeted by TALIRI Mpwapwa, at least initially, for participation in an AI and fertility program.

No survey of this nature has ever been done within Mpwapwa district, nor indeed anywhere in the entire central zone, or even in the other zones found in Tanzania. The present survey, unlike other surveys undertaken elsewhere in Africa (Ingabire *et al.,* 2018; Nengovhela *et al.,* 2021; Gebre *et al.,* 2022; Nkadimeng *et al.,* 2022; Lemma *et al.,* 2023), addresses the current situation of fertility and AI programmes in beef cattle production, covering aspects which are generally under-reported in the African continent (Muller *et al.,* 2015; Mutenje *et al.,* 2020) as well as within the Mpwapwa region in the Tanzanian context. In this regard, limited studies exist on how institutions and social dynamics affect smallholder farmers breeding preferences. As such, there is much work to be done in order to understand the perceptions and preferences of smallholder farmers in breeding programmes with regard to the present institutions and social dynamics. If this is not properly addressed, the result could be continued low adoption and utilisation of AI technology by smallholder farmers (Muller *et al.,* 2015; Mutenje *et al.,* 2020). Thus, this survey was designed to identify potential issues which could affect the dissemination of TALIRI Mpwapwa AI and fertility program to these farmers, and thus the impact of that program.

Of the 100 respondents, 53 were aware of AI technology. Given that there has been no active AI programme in the region for 23 years apart from previous studies by the first author (Kabuni, 2017) and the TALIRI Mpwapwa herd breeding programme, this was considered to be a significant achievement. Previous use of AI was very strongly associated with willingness to use it again in the future (17/18 respondents), with the principal issue for these respondents being the prevailing challenges affecting the delivery of AI services. For the remaining 82 respondents it is clear that improving knowledge and understanding of AI in farmers is likely to be crucial for any successful program, alongside ensuring that the program is reliable and cost-effective. It is thus clear that

TALIRI Mpwapwa will need to provide AI education/training to farmers prior to them being involved in the AI and fertility program (or ensure that such education is provided). Nevertheless, it is clear that there is support among target farmers for such a program, and that the re-equipped animal biotechnology laboratory at TALIRI Mpwapwa research centre will be able to leverage smallholder farmers' willingness to adopt and utilise AI technology into their production systems. One important area of focus for future research is the use of a fresh/liquid semen AI programme as this will reduce costs (as has been for the case of New Zealand: Shannon and Curson, 1984; Raseona *et al.,* 2017) as well as the reliability of AI services (as it does not rely on the use of liquid nitrogen which has inconsistent availability).

Nevertheless, despite the interest of the farmers in this survey in adopting and utilising AI technology into their breeding programmes, it is important to recognise where AI services presently sit in Tanzania, particularly in non-dairying regions such as Mpwapwa. Currently, AI services are only partially accessible and mainly confined, as a result of high delivery costs and centralisation of AI services (Chawala, 2020) to the northern zone of the country. This means that adoption and utilisation of AI technology within Mpwapwa district and the entire central zone of Tanzania is still in its 'infancy', and progress is likely to be incremental (at least at first).

Management practices on-farm have a large impact on fertility outcomes, and it is important to acknowledge that, as identified by this survey, the level of management on many farms is likely to be a significant constraint to the success of an AI and fertility programme. As such, for effective adoption and efficient performance that program, we need to focus on improving is management practices on smallholder cattle farms. These improved management practices (e.g. nutrition, health and fertility) will not only enhance the performance of the AI technology, but also improve beef cattle productivity more generally (Msalya *et al.,* 2017a, 2017b). This is likely to require education and significant, either through TALIRI or from partner organisations.

It is clear that the use of AI in cattle can result in improved reproduction and productivity through increased genetic gain, reduced generation interval, and control of the spread of venereal diseases (Johan & van Arendonk, 2011; Baruselli *et al.,* 2018; Marrella *et al.,* 2021). The contribution of AI technology to cattle improvement is evident in countries within and outside the African continent that have similar climatic conditions to Tanzania. For example, countries in Africa (e.g. Kenya, Ethiopia and South Africa) and South America (e.g. Brazil, Argentina, Mexico and Peru)

have used AI technology widely and managed to utilise it within smallholder farmers' settings (Muller *et al.*, 2015; Lemma *et al.*, 2023). Many studies have evaluated the effect of adoption and utilisation of AI technology on smallholder farmers (e.g. Murage & Ilatsia, 2011; Dehinenet *et al.*, 2014; Yohannes, 2014; Gebre *et al.*, 2022; Lemma *et al.*, 2023). Generally, almost all studies of AI show the positive impact of its use on livestock genetic gain and productivity and, hence, increased income and improved livelihood of the smallholder farmers. However, getting smallholders to fully adopt the use of AI can be complex and difficult as research from India shows (Rathod *et al.*, 2017). The principal limitations identified by that study were, inadequate number and training of AI technicians, inefficient oestrus detection methods, low conception rates, high charges for AI services, undeveloped AI facilities/infrastructure, ineffective post-AI nutritional management plans, as well as the unreliability of AI services and AI service providers. The same limitations also apply within the Mpwapwa district (Kabuni *et al.* 2023), and although it is anticipated that the TALIRI Mpwapwa AI and fertility programme will solve most of the reliability and infrastructure issues, the adoption and progress of AI technology by farmers within Mpwapwa district will still be limited by the lack of appropriate herd health, fertility, and nutrition programs alongside the lack of proper record keeping.

One key area identified by the present survey is the variability in routine management procedures (such as drenching and dipping) across respondents, which is also matched by the variability in nutritional arrangements. Optimising herd health and nutrition are both critical for achieving the goals of the TALIRI Mpwapwa AI and fertility program.

At the time of the survey, the previous breeding programme for smallholder farmers led by TALIRI, which used an open-nucleus breeding scheme with Mpwapwa breed bulls, had fallen into disuse. Instead, farmers had reverted to using unproved and untested homebred bulls to breed their cattle with no specific focus on a breeding programme. This has led to significant inefficiencies in breeding. For example, although 95% of respondents had a target of having one calf per year per cow as their breeding goal, only 10% of respondents reported that they achieved this. Similarly, although 82% of respondents reported breeding cattle throughout the year, only 32% of farms who knew when calves were born reported that calves were born in both the rainy and dry seasons.

## Conclusion

The Mpwapwa breed is specifically designed to be suitable for smallholder farms in non-dairying areas of Tanzania. However, in recent years there has been negligible genetic progress of the breed, and even local farmers are no longer benefiting from a continually improving productive cow designed for their conditions. It is clear that a different strategy is needed if I have to improve productivity of beef and dual-purpose cattle in Mpwapwa and other similar districts. Recent developments at the TALIRI Mpwapwa laboratory mean that it can act as a focus and base for the development of a new AI and fertility program designed for smallholder cattle. However, for such a program to be successful, I need to understand the perceptions and preferences of smallholder farmers towards such a program. This survey has shown that there is clearly an appetite within the target population for such a program, but for it to be successful I need to focus on education and training around AI and breeding management, and that this needs to be combined with improving the standard of animal health and nutrition management on target farms.

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*Appendix 1*

## QUESTIONNAIRE ON SMALLHOLDER FARMERS' ATTITUDES ON DEVELOPED FERTILITY MANAGEMENT PROGRAMME AT TALIRI MPWAPWA FOR MPWAPWA BREED CATTLE

Questionnaire No _____

Date _____

Interviewer: name _____

Region _____

District _____

Division _____

Ward _____

Village _____

Interviewer: mobile _____

### Introduction

My name is Kabuni T. Kabuni. I am a Research Officer at TALIRI Mpwapwa and I am a PhD student at Massey University in New Zealand. For my PhD programme, I am studying the use and development of Mpwapwa cattle in Tanzanian farms, and the breeding services provided for Mpwapwa cattle by TALIRI Mpwapwa.

This questionnaire has been developed to help TALIRI understand your management and breeding practices of Mpwapwa cattle, whether the fertility and artificial insemination (AI) breeding programmes at TALIRI Mpwapwa are of benefit to your farming, and whether any changes (more/different) to those programmes would make them of greater benefit to you.

The findings from this study will help improve the fertility and AI programmes at TALIRI to enhance the performance of Mpwapwa breed cattle on-farm, and will facilitate their application and utilisation on-station at TALIRI Mpwapwa farm and in other agro-ecological zones in the country where Mpwapwa breed cattle and other beef cattle eco-types are farmed.

This questionnaire will last for about 20 minutes

To identify this interview, and in case I need to have further correspondence with you, your name and address will be recorded.

You may decline to answer any question; and you may decide to end the interview at any point.

Do you agree to be part of this questionnaire? YES/NO

If the answer is NO, please do not proceed further with this questionnaire.

## Section A: Personal Details

### *Personal*

1. Name _____
2. Sex _____
3. Age _____
4. Marital Status _____
5. Education Level _____
6. Number of household members _____
7. Phone number: _____

## Section B: Farm Details

### *Your farm*

1. Farm Size _____
2. Do you keep Mpwapwa breed cattle? YES/NO
3. If YES, how many Mpwapwa breed cattle do you have? _____
4. How did you acquire cattle of this breed and what was the source? _____

   _____
5. Do you keep other breeds or types of cattle as well? YES/NO
6. If YES mention them _____
7. Apart from livestock keeping, are you engaged with other agricultural and economic activities? YES/NO
8. If YES, what are they?_____

   _____
9. When did you start keeping cattle? _____
10. Why did you start keeping cattle? _____

### *Decision-making*

1. Which members of your household normally participate in the following livestock keeping activities?

   E.g. grazing _____

   E.g. milking _____

E.g. dipping _____

2. Does anyone else help look after your cattle? If yes, who?_____

_____

3. Who is responsible for making decisions about cattle farming activities (i.e. purchase of new animals, selling of animals and animal products) at your farm?_____

_____

*Your livestock*

1. How do Mpwapwa breed cattle generally perform at your farm? _____

_____

2. What is the average daily milk production per cow at your farm?

   (Taliri Mpwapwa farm average is 5 litres/cow/day) _____litres

3. What is the average carcass weight of a 4 years old bull?

   (Taliri Mpwapwa farm average is 150 kg) _____kg

4. What is the average mature breeding weight of your cows?

   (Taliri Mpwapwa farm average is 180 kg) _____kg

5. How old are your cows when you first see them in heat/being ridden by other cows?_

_____

6. What is the average mature breeding weight of your bulls?

   (Taliri Mpwapwa farm average is 300 kg) _____kg

7. How old are your bulls when you first use them for breeding?_____
8. Are they mature enough at that age? How do you know?_____
9. In which seasons do you breed your cows?_____
10. Which months? _____
11. How many times do your cows calve down within three years?_____

    E.g. cow 1: last time calved_____time before that_____time before that_____

    E.g. cow 2: last time calved_____time before that_____time before that_____

    E.g. cow 3: last time calved_____time before that_____time before that_____

12. How many calves were born over the last 12 months (since this time last year)?_____

_____

13. How many calves were born in each calving season? _____

14. How many years do you keep cows for breeding? Give examples of the last 3 cows you culled

    E.g. cow 1:_____
    E.g. cow 2:_____
    E.g. cow 3:_____
15. How many years do you keep bulls for breeding? Give example of the three last bulls you culled
    E.g. bull 1:_____
    E.g. bull 2:_____
    E.g. bull 3:_____
    How many years have you been using your present herd bulls?
    E.g. bull 1:_____
    E.g. bull 2:_____
16. Where do you source your breeding bulls?
17. Breed yourself_____Neighbour_____Dealer_____Other (what?)_____

## Section C: Breeding Programme

1. What are your goals for breeding your cattle (e.g. one calf per cow per year, one calf every other year etc.)? _____

    _____
2. Do you rely only on bulls for breeding? YES/NO

    Why/why not?_____
3. Do you normally conduct breeding soundness evaluation (BSE) for your bulls? YES/NO

    Why/why not?_____

    _____

    If YES, how do you do it/which assessment do you make?_____

    _____
4. If YES, have you heard about artificial insemination (AI) as an alternative breeding method against natural mating? YES/NO
5. If YES to question 4, have you ever tried using AI to breed your cattle at your farm? YES/NO

    Why/why not?_____
6. If YES, do you prefer natural mating over AI? _____

    Why?_____

7. If NO, would you be interested in trying AI? YES/NO

   Why/why not?_____

8. If YES, do you normally synchronize your cows before conducting artificial insemination? YES/NO

   Why/why not?_____

9. If YES, which oestrus synchronization protocol do you normally use? _____

   _____

10. Is your oestrus synchronization protocol effective? YES/NO_____

    Please explain_____

*Cooperation with TALIRI Mpwapwa*

- Have you previously had any relationship with TALIRI Mpwapwa? YES/NO
- TALIRI Mpwapwa would like to undertake a trial of different methods of AI (frozen semen versus non-frozen semen). Would you be interested in participating? (This is an expression of interest, not a binding commitment). YES/NO
- TALIRI Mpwapwa would like to establish an open nucleus-breeding scheme using Mpwapwa breed cattle. Would you be interested in your farming being part of that scheme? (This is an expression of interest, not a binding commitment) at your environment? YES/NO

Comments on either of the above_____

_____

_____

_____

## Section D: Management, nutrition, adaptation, production, and growth performances of cattle

1. What is the deworming regimen of your farm? _____

   _____

2. What is the dipping regimen of your farm? _____

   _____

3. What is the vaccination regimen of your farm? _____

   _____

4. What is the feeding regimen of your farm? _____

   _____

5. What are the sources of feed for your cattle? _____
6. Do you have land for grazing your cattle? YES/NO
7. Do you conserve feeds/pastures for use during the dry season? YES/NO
8. If YES, which feeds conservation strategies do you normally use? _____

9. Do you experience water shortage for your cattle? YES/NO

10. If YES, in which season do you normally face the challenge of water shortage? _____

11. Which strategies do you normally apply to fight against water shortage? _____

*Cooperation with TALIRI Mpwapwa*

12. If you have land for grazing your cattle are you interested in developing it with pastures? YES/NO

13. If you do not have land for grazing your cattle If no, are you interested in allocating some portion of your land to establish pastures? YES/NO

Comments on either of the above_____

# Chapter 4: Is the semen from Mpwapwa bulls of sufficient quality for use in local AI schemes?

## A. Breeding Soundness Examination (BSE)

### Study Introduction

The Mpwapwa breed of *Bos indicus* cattle was initially developed as a locally adapted strain during the 1940s to improve the quality of beef cattle raised in Tanzania. Mpwapwa cattle were initially disseminated around the region by direct sales of breeding animals (including bulls) and by the use of artificial insemination. The demise of Artificial Insemination (AI) services in the central zone of Tanzania has resulted in a marked attenuation of this program, such that most breeding of Mpwapwa cattle on smallholder farms is primarily through natural service by locally-bred bulls. There is very limited use of AI or genetically improved natural-service sires, even though bulls of the Mpwapwa breed have been continuously maintained at the TALIRI Mpwapwa research centre since 1973, and have been the subject of (limited) genetic selection for the productivity characteristics of conformation, growth rate and feed conversion efficiency.

The dearth of AI breeding in the Mpwapwa region reflects the fact that the delivery services of artificial insemination in Tanzania are inadequate to meet the requirements of the cattle industry. Despite recent efforts to improve the situation by both governmental and non-governmental institutions, further investment and development is still required to allow sustainable utilisation of AI services within the cattle breeding system in the country. The problems of providing AI services to Tanzanian farmers are illustrated by the history of AI at TALIRI Mpwapwa research centre. Initial efforts to provide an AI delivery service by the Mpwapwa research centre from Mpwapwa breed bulls, using ambient temperature semen, date back to 1967. A year later, in 1968, frozen semen was included in the AI delivery services of the centre. However, in 1973, semen production activities were shifted to Usa River in Arusha at the National Artificial Insemination Centre (NAIC) (Mejool, 1977) and services outside the Arusha region largely ceased. Thereafter, the NAIC remained the sole site of production of semen for AI delivery services in Tanzania until 2018, when TALIRI re-equipped its animal biotechnology laboratory at the Mpwapwa research centre with the equipment necessary for semen and embryo production.

The period of centralization of the AI services at the NAIC in Arusha resulted in a significant decline in the use of AI across most of the rest of Tanzania, and entrenched natural mating as the most common breeding method used by the majority of the smallholder farmers (MLFD, 2011; Katjiuongua & Nelgen, 2014; Ogutu *et al.,* 2014). It also limited the use of the Mpwapwa breed across the beef farms of the region

that are used by farmers as natural service breeding, the consequence has been that most bulls used for breeding are of unknown fertility status, and are not genetically selected for any production traits. Inevitably, this has significantly limited cattle genetic gain, whilst also resulting in an increased incidence of venereal disease, inbreeding, infertility, low reproductive performance and reduced productivity (Kanuya *et al.*, 2006a, b). The demise of AI services in the central zone of Tanzania has consequently limited the use of Mpwapwa bulls within the region. Nonetheless, the herd of bulls at TALIRI represent a potential nucleus herd from which an AI service could be developed, if it were possible to create the infrastructure needed for reliable on-farm delivery of an AI service to smallholder beef farmers. In other words, development of an AI programme, run by the TALIRI Mpwapwa research centre, could both increase the quality of livestock on the smallholder firms by the use of genetically improved bulls in the AI service itself, and by increasing the genetic quality of bulls that are used by farmers as natural service sires.

It is clearly imperative for bulls that are used in an AI service to have semen that is of adequate quality (i.e. in terms of potential fertilizing capacity) and quantity (i.e. in terms of their ability to produce quantities of semen that are compatible with the establishment of a reliable AI service). Fertility traits have not, however, been assessed in the animals in the TALIRI Mpwapwa bull stud since the demise of the former AI service, so such evaluations are a pre-requisite for the establishment of a new AI service. Bull fertility is a complex trait, but is one of the most critical factors to be considered in a breeding programme. Consequently, alongside performance traits, fertility needs to be one of the criteria in bull selection for use in an AI service (Parkinson, 2004; Barth, 2018).

### Breeding Soundness Examination

Assessing the potential fertility of the bulls at the TALIRI beef study is an essential prerequisite for the (re)establishment of an AI service. Generally, before entry to an AI stud, bulls are assessed for the structural soundness of their genital system and the quality of their semen. There are several, broadly similar, BSE protocols for assessing bull fertility: commonly used schemes include the Australian Bull Check and the American Society for Theriogenology (Chenoweth *et al.*, 1992; Fordyce *et al.*, 2006). These two BSE schemes provide simple, repeatable and unambiguous procedures for bull fertility evaluation. Under the Australian Bull Check scheme, a full bull check consists of five assessment components; general physical examination (i.e. structural and reproductive soundness), testes examination and scrotal size/circumference measurement, libido and mating ability assessment, semen collection and

quality assessment, and sperm morphology examination (Fordyce *et al.,* 2006). The American Society for Theriogenology published criteria for BSE of bulls in the 1990s (Hopkins & Spitzer, 1997), and has recently updated the minima expected for various classes of bulls (Koziol & Armstrong, 2018). These criteria particularly focus on scrotal circumference, percentage motile sperm and percentage of sperm with normal morphology. Depending on the score of the bull with respect to the minimum standards of bull fertility, are therefore considered to be classified as satisfactory, unsatisfactory or deferred (Chenoweth *et al.,* 1992, Kennedy *et al.,* 2002; Fordyce *et al.,* 2006; Koziol & Armstrong, 2018).

The evaluation of bovine semen during a breeding soundness examination is generally confined to motility, concentration and morphology (Rodriguez-Martinez, 2003; Chenoweth & McPherson, 2016; Tanga *et al.,* 2021), for which there are well-established acceptance criteria for different classes (e.g. *B. taurus* vs *B. indicus*; age, breed) of bulls. Such methods, regardless of whether they use subjective (visual) or objective (computer - assisted) evaluations, or whether the semen samples to be evaluated are fresh, cooled or frozen, the procedures for semen analysis were principally developed to try to predict the fertilizing ability of the ejaculate and/or bull (Graham, 2001; Moce & Graham, 2008). Thus, whilst meeting these acceptance criteria does not correlate highly with conception (or non- return to service) rates in an AI service, bulls that do not meet the minimum criteria are unlikely to perform adequately, so can be screened out of use in the programme.

## Materials and Methods

This study was undertaken at the TALIRI Mpwapwa research centre in the Dodoma region of Tanzania (Figure1). The climate of Mpwapwa is subtropical but is modified by altitude. Weather is warmer between July and November (i.e. five months on average) and cooler between June and August. Most rainfall occurs between December and April (i.e. five months on average), while there is a long dry season from May to October. There are two breeding seasons for cattle which are management driven: the first between March to May (at the end of the rainy season) and the second between September to November (at the end of the dry season). The breeding soundness examination of the Mpwapwa breed bulls at the TALIRI research centre was undertaken in March 2021.

All animal-related manipulations had received Livestock Research Ethical Clearance from the Tanzanian Livestock Research Institute (TALIRI) (12/02/2021) prior to the start of the study.

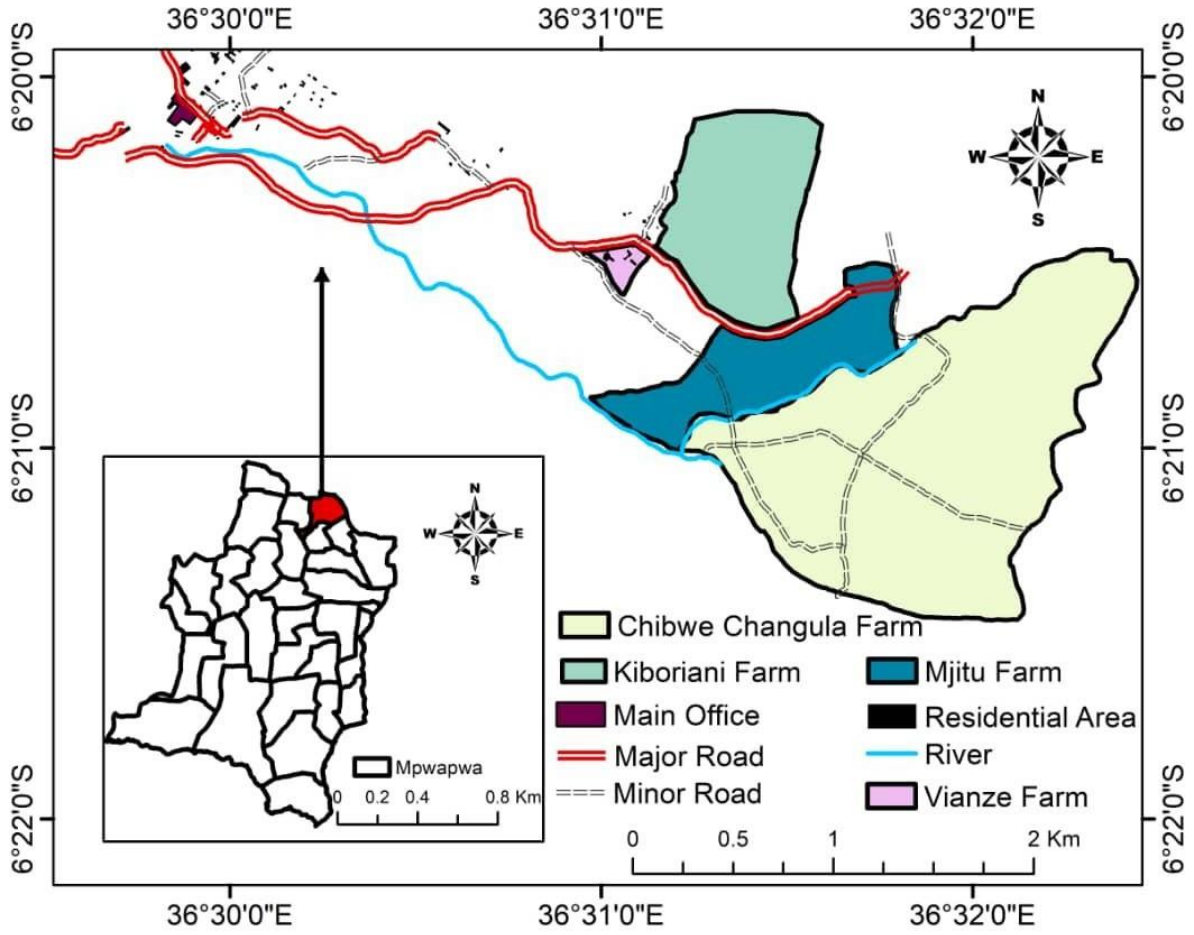


**Figure 1:** A map of TALIRI Mpwapwa research centre showing the farm where the BSE study was conducted

### *Selection of bulls*

All bulls came from the TALIRI Mpwapwa research herd (Figure 2). There were 120 mature (≥2 years old) Mpwapwa breed bulls available for selection. Bulls were weighed and body condition scored (1-5 scale; Nicholson & Sayers, 1987) and the heaviest 53 bulls were selected for the BSE study.

**Figure 2:** A group of Mpwapwa breed bulls at TALIRI Mpwapwa farm

### *Animal health management and nutrition prior to BSE*

Bulls were treated with 5 mg/kg levamisole (Levamizole, Eagle Vet. Tech Co. Kenya) for the control of endoparasites. For a period of four weeks before examination, all animals were given access to unrestricted grazing (stocking rate of 0.36 livestock units/ha) in 15 ha paddocks (Figure 3). The principal grass species in these paddocks were *Cenchrus ciliaris* (buffel grass), *Hyperrhenia rufa* (giant thatching grass), *Themeda spp* (kangaroo grass), *Cynodon dactylon* (Bermuda grass) and *Chloris gayana* (Rhodes grass). In addition, bulls were trough-fed 0.6 kg/bull/day of a compound ration made from 600 g/kg maize bran, 390 g/kg sunflower seed cake and 10 g/kg salt, and had access to mineral lick blocks (Farmers Centres Ltd, Tanzania) at an allocation rate of 400 g/bull/week. Details of the mineral content of the blocks are in Appendix 1. Bulls were dipped weekly in a bath containing 100 g/L of alphacypermethrin (Paranex, Farm Base Ltd, Tanzania) for the control of ectoparasites. In March 2021 and 2022, BCS was assessed and weight measured as each bull left the crush after the four-week management period. No mineral testing was undertaken at any stage of the study.

**Figure 3:** A group of selected Mpwapwa breed bulls grazing in one of the paddocks at TALIRI Mpwapwa farm

### *Allocation to treatment*

The 53 selected bulls were grouped based on number order and ear tag number. They were then divided into five BSE Groups (n=10/11) based on that order, with the first bull going to BSE Group 1, the second bull going to BSE Group 2 and so on.

Ten or eleven bulls were examined per day over a period of five days. The BSE component was based on the procedures outlined by Chenoweth *et al.* (1992), Kennedy *et al.* (2002), Fordyce *et al.* (2006), and Kastelic & Thundathil (2008):

a) Examination of structural and reproductive soundness (physical examination)

   Bulls were observed while walking on a hard surface. Legs, joints and muscles were checked for lameness and locomotion problems that could affect mating ability. Sight was then checked by walking towards their front view.

   For examination of reproductive soundness, bulls were confined in a crush. The scrotum and its contents were palpated to ensure that the testes were symmetrical, non-painful and smooth and that they moved freely within the scrotum. Similar checks were undertaken on the epididymides and spermatic cords. The prepuce and penis were then checked for thickening, adhesions and the presence of discharge.

b) Measurement of scrotal circumference (SC)

Scrotal circumference (SC) was measured by pushing the testes to the bottom of the scrotum (Figure 4). A standard cloth tape was placed around the scrotum at its widest point with moderate tension.

**Figure 4:** Measurement of scrotal circumference on Mpwapwa breed bulls after the testes had been pushed down

c) Collection and examination of semen

Semen was then collected by electro-ejaculation (Ejakulator, Minitube GmbH, Germany) (Figure 5)**.** One person was responsible for inserting the probe, one for regulating the voltage machine and one for collecting the semen. During the process, the bull was observed for erection and ejaculation, and electrical stimulation ceased immediately upon ejaculation. Semen samples were collected into vials using an artificial vagina (AV) cone. Samples were collected and maintained at ambient temperature and taken to the laboratory within 1 to 2 minutes of collection.

A one-step dilution protocol/method was applied. The working concentration of extenders/diluents was initially prepared by making 4:1 dilution in distilled water. Thereafter, semen samples were diluted in Optixcell extender at a fixed ratio of 1:1 (i.e. 2mL of collected semen against 2mL of the prepared semen extender). When required for microscopic examination, the semen was further extended, at a ratio of 5:1. Semen samples were diluted at an ambient room temperature of 32°C.

Semen samples were evaluated for:

i. Volume and colour

The volume of the ejaculate was recorded, and its colour assessed. The absence of blood and urine staining was confirmed and colour then recorded. Semen samples were then placed into a water bath set at 32°C.

ii. Motility

Mass activity/gross motility was assessed using undiluted semen placed on a warm slide without a coverslip (Figure 6). Aliquots of semen were also diluted (1:1) in Optixcell diluent (IMV, L'Aigle, France) and individual progressive motility assessed. Both motility measures were undertaken using a phase-contrast microscope (MBL2000 Kruss Optronic GmbH, Germany) at x10 (mass) and x100 – x400 (individual) magnifications. Motility was expressed as a percentage of progressively motile sperm.

**Figure 5:** Collection of semen from Mpwapwa breed bulls

iii. Morphology

Morphology was assessed using unstained diluted semen specimens using a phase-contrast microscope (MBL2000 Kruss Optronic GmbH, Germany) at (x1000 magnification). A drop of diluted semen was placed on a glass slide, gently covered with a cover slip, and placed on a microscope stage for examination. 100 sperm were counted in a random sampling pattern.

iv. Concentration

The concentration of sperm in the ejaculate was measured using spectrophotometer (Accuread Photometer, Biochrom Ltd, USA). Semen (10-μL) was mixed with 1 mL of sodium chloride solution (0.9% w/v) in a cuvette. The mixture was then thoroughly mixed and placed into spectrophotometer for concentration reading. The spectrophotometer had been previously calibrated by haemocytometer counts.

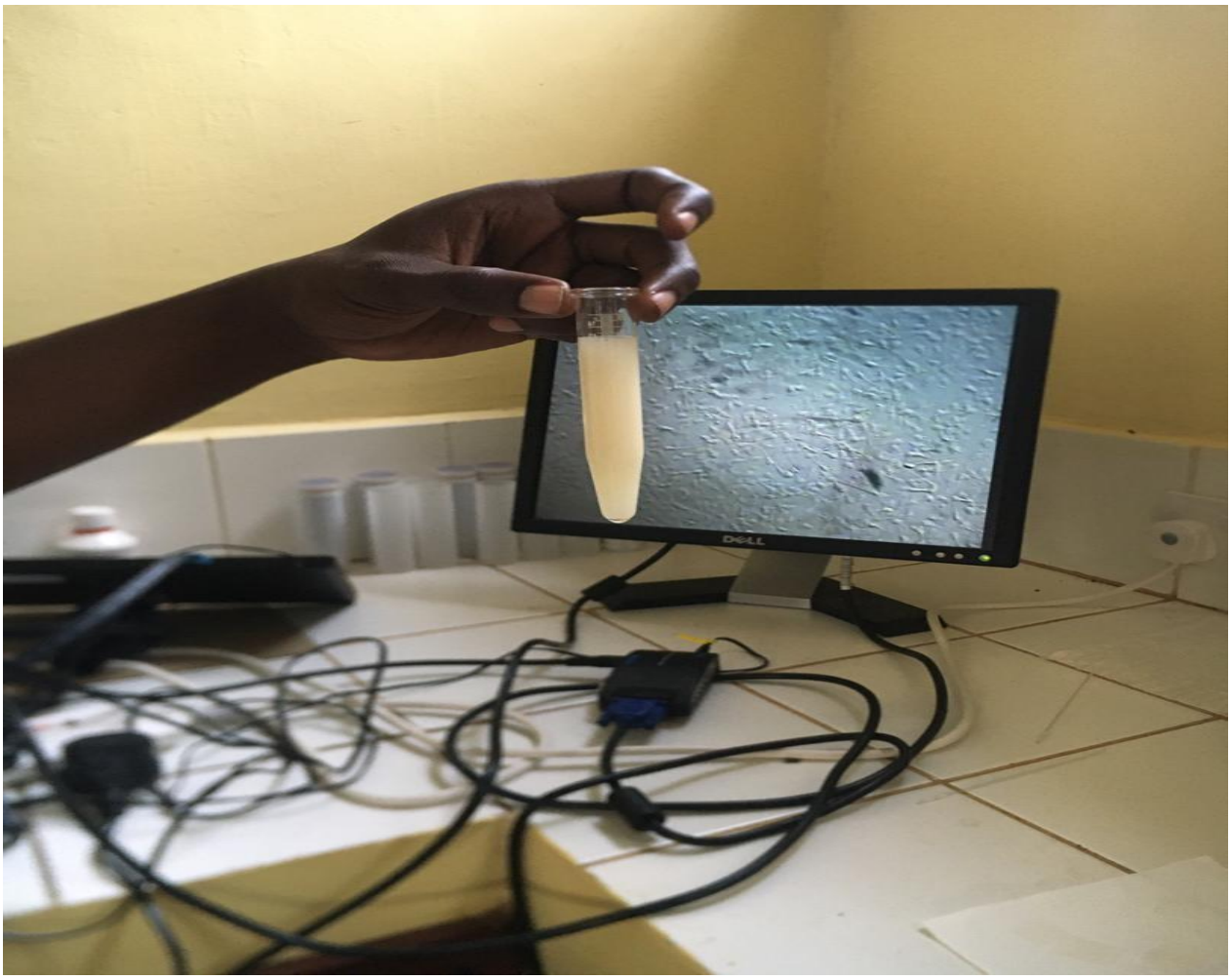


**Figure 6:** Assessment of mass motility of semen samples collected from Mpwapwa breed bulls

Semen was successfully collected from 44/50 bulls: only seminal plasma was collected from the remaining 6 bulls (see Appendix 2).

### *Statistical methods*

Descriptive analysis was used to assess semen quality data. Box plots were used to illustrate the effect of SC, concentration and age on bulls' semen quality and their relationships.

## Results

The age of bulls at the start of the study ranged from 28-49 months (except for one bull which was 122 months old) (Mean: 40.7, SD: 13.6 months). Mean body weight at the start of the study was 226.4 kg (range: 141-320 kg, SD: 35.6 kg) and, after the period of unrestricted grazing, was 266.6 kg (range: 185-365 kg, SD: 32.9 kg). The mean weight gain was 40.2 kg (median: 40 kg, range: 15-72 kg, SD: 6.8 kg). Body condition score at the start of the study was 3.0 across all bulls, and after the period of unrestricted feeding had increased to 4.0. The range of scrotal circumferences is shown in a box plot in Figure 7a. Scrotal circumference was unrelated to age (P>0.50) (Figure 7b). When separated into bulls that were under 36 months versus those over 36 months, there was no difference in mean scrotal circumference (27.1, SD: 1.6 cm *versus* 27.8, SD: 2.0 cm).

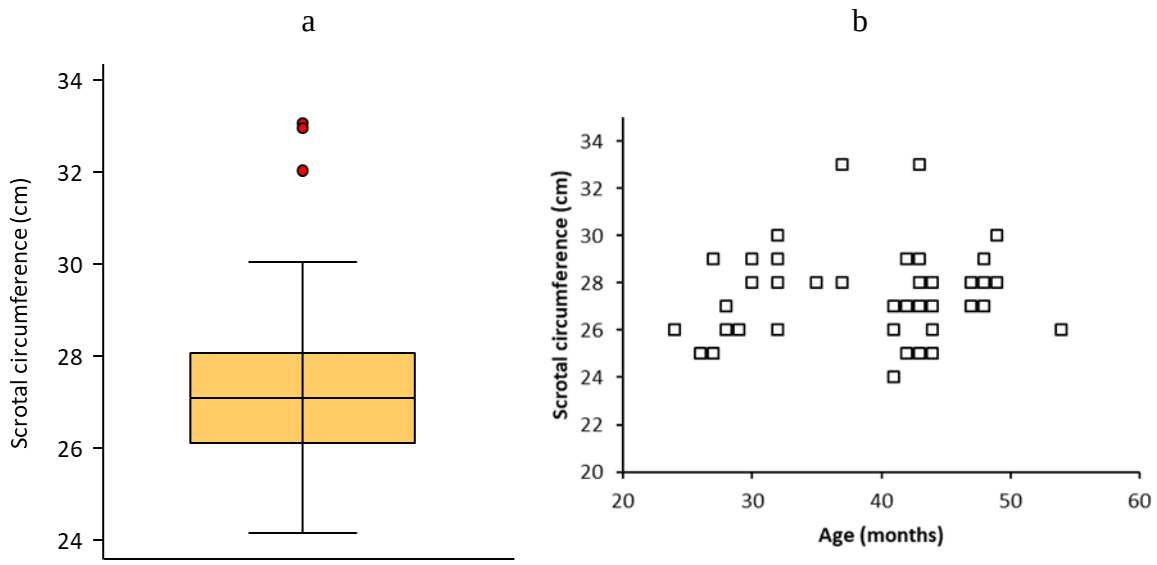


**Figure 7:** Scrotal circumference: (a) mean and range, (b) relationship with age

Semen was successfully collected from 44/50 bulls: only seminal plasma was collected from the remaining 6 bulls (see Appendix 2). Mean ejaculate volume over all bulls was 5.5 mL (range: 1.9-14.9 mL, SD: 1.9 mL). When bulls that produced seminal plasma were excluded, there was little change in the mean volume (mean: 5.5 mL, SDL 2.7 mL).

Mean and range of sperm concentrations are shown in Figure 8a. Mean ejaculate density was relatively low, at 303 $x10^6$ sperm/mL (range: 57-966, SD: 258 $x10^6$ sperm/mL). Figure 8b shows the numbers of bulls producing ejaculates of different densities. Overall, 31/47 (66%) bulls produced ejaculates with ≤400 $x10^6$ sperm/mL (excluding those from which only seminal plasma was collected: 37/53 (70%) if those bulls are included). Six bulls produced ejaculates that were of sperm density ≥800 $x10^6$ sperm/mL, and two others produced ejaculates ≥700 $x10^6$

sperm/mL. These 8 ejaculates would meet established criteria for use in an AI program. All other ejaculates were ≤600 x$10^6$ sperm/mL.

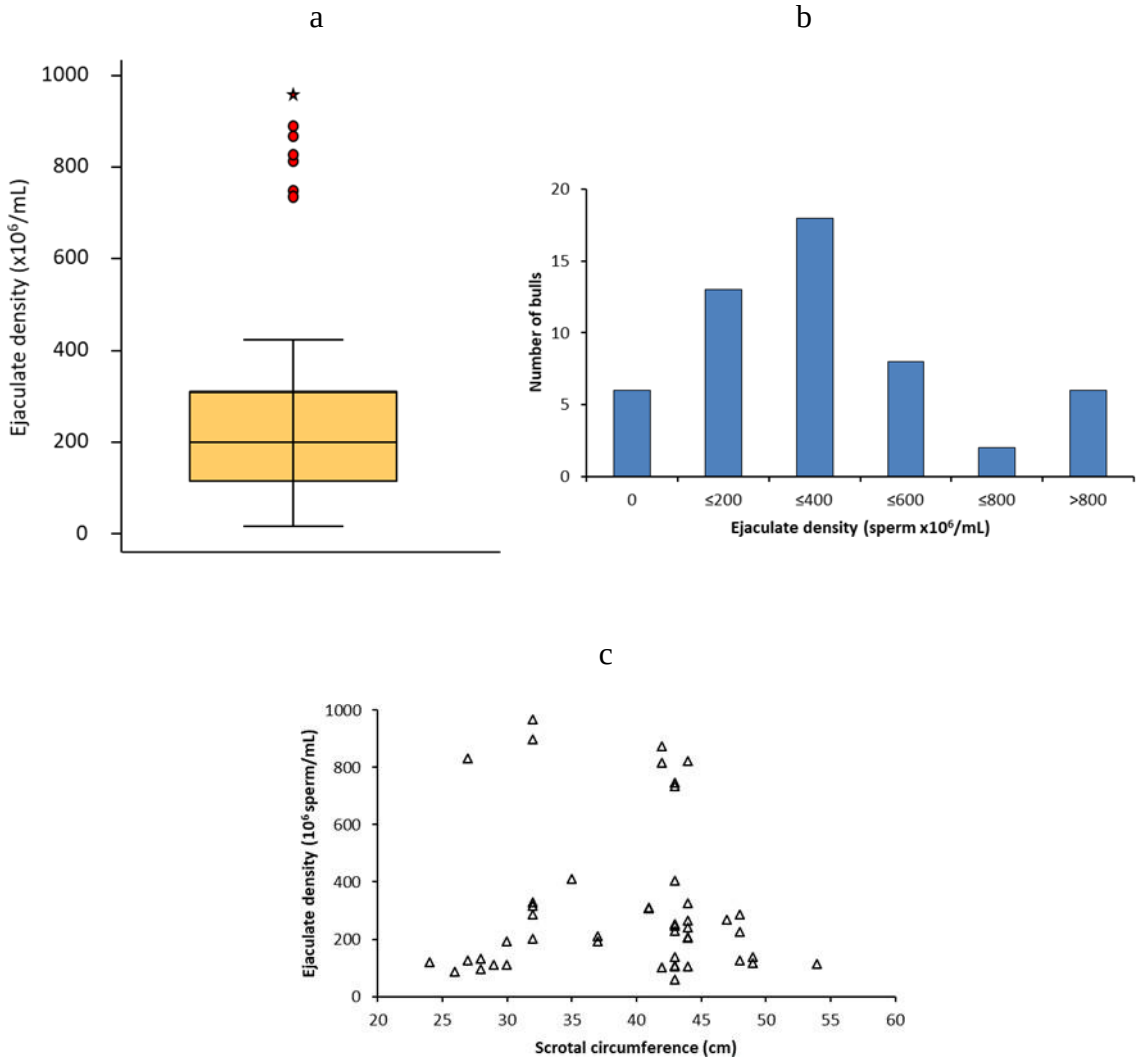


**Figure 8:** Ejaculate density (x106 sperm/mL) (a) box plot, (b) number of bulls producing ejaculates of different densities, (c) relationship between scrotal circumference and ejaculate density (R=0.33).

Mass motility scores are summarised in Table 1. Mean numbers of progressively motile sperm were not well correlated with mass motility scores, although +++ scores had slightly higher numbers of motile sperm than + or ++. Proportions of progressively motile sperm are illustrated in Figure 9. Most (41/47: 87.2%) bulls had ≥70% motile sperm, but only 19 bulls had ejaculates that contained ≥80% motile sperm. These 19 bulls would meet generally-accepted criteria for inclusion in an AI program. Proportions of morphologically normal sperm are shown in Figure

10. Only 4/47 (8.5%) of bulls had less than 70% morphologically normal sperm in their ejaculates.

**Table 1.** Proportions of bulls with different mass motility scores

| Motility score | Number of bulls | Proportions (%) | Mean individual progressive motility (%) |
|---|---|---|---|
| + | 12 | 26 | 71.3 ± 8.8 |
| ++ | 24 | 51 | 75.8 ± 6.7 |
| +++ | 11 | 23 | 83.4 ± 5.2 |

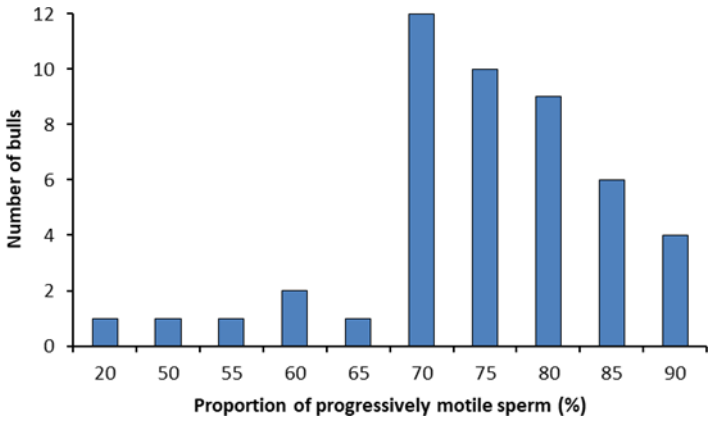


**Figure 9:** Proportions of progressively motile sperm in the ejaculates of individual bulls. Aspermic ejaculates are omitted.

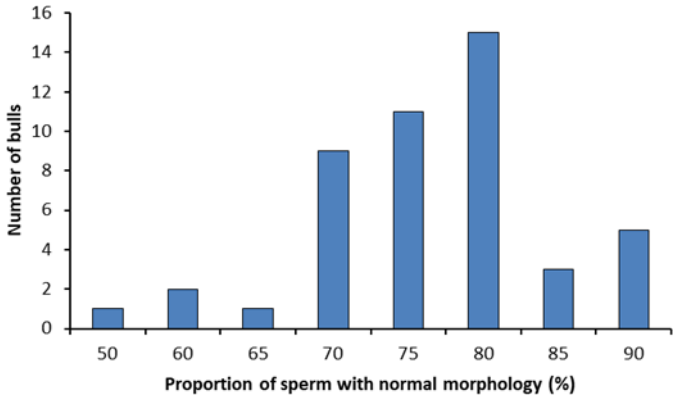


**Figure 10:** Proportions of bulls with sperm of normal morphology

## Overall assessment

Taking into account ejaculate density, progressive motility and normal morphology as the key identifiers of ejaculate quality that is acceptable for use in AI, five bulls met the criteria of ≥800 x$10^6$ sperm/mL, ≥80% progressive motility and ≥70% morphologically normal sperm (Table 2). Three other bulls were adequate in all but one criterion. These eight bulls would meet the

criteria for candidates for inclusion in an AI study. Bulls 14901 and 14808 had scrotal circumferences that were below the minimum recommended for fully grown *Bos indicus* bulls of improved breeds, but were commensurate with the mature body weight that is achieved by Mpwapwa bulls.

**Table 2:** Bulls that meet minimum criteria for use in an AI service

| ID | SC (cm) | Volume (mL) | Mass activity | Individual motility (%) | Morphology (% normal) | Sperm/mL ($x10^6$) |
|---|---|---|---|---|---|---|
| 14967 | 28 | 5.8 | +++ | 75 | 80 | 966 |
| 14983 | 29 | 2.8 | +++ | 80 | 85 | 896 |
| 14901 | 25 | 3 | +++ | 90 | 80 | 873 |
| 13128 | 29 | 8 | ++ | 75 | 75 | 829 |
| 14804 | 26 | 5.5 | +++ | 85 | 75 | 820 |
| 14911 | 29 | 8 | +++ | 90 | 80 | 815 |
| 14841 | 33 | 6 | ++ | 80 | 80 | 746 |
| 14842 | 29 | 8 | +++ | 85 | 80 | 734 |

SC: scrotal circumference

Mass activity: +++ vigorous swirling, + sluggish swirling

*The light green colour: indicate bulls meeting almost all of the minimum criteria for AI service. The light brown colour: indicate bulls meeting some of the minimum criteria for AI service.*

## Discussion

### *Breeding soundness*

This is the first large-scale study of the breeding suitability of Mpwapwa bulls that has been reported since this breed was developed during the 1940s. Although the breed has been maintained for dissemination and conservation, it has only been subject to a relatively low-level selection for production and has not been subjected to selection for breeding. Several studies have been conducted on the female reproductive performance of Tanzania Short-horned Zebu (TSZ) cattle (e.g. Kanuya *et al.*, 2006a, b), but studies on breeding bulls of Tanzanian origin are rare. A parallel study on TSZ breeding bulls was done by Kashoma *et al.* (2010): comparison of that study with present findings shows that Mpwapwa breed bulls had scrotal circumference (SC) of 25 – 33 cm (at 2 – 4.5 years of age), whereas, TSZ bulls of similar age (2.0 – 4.0 years) had SC of 24 – 34 cm. Thus, the SC of Mpwapwa breed bulls in this study is very similar to that reported for TSZ bulls.

There was no direct relationship between the age and SC of the bulls such that the SC of younger bulls was similar to those of older bulls. Additionally, some of the older bulls (i.e. over 36 months old) had SC below the mean (27.1, SD: 1.6 cm), whereas some of the younger bulls (i.e. under 36 months old) had SC above the mean (27.8, SD: 2.0 cm). For *Bos indicus* and derived crosses, the recommended minimum for a 2-year-old bull kept in a herd

under semi-arid agro-ecological conditions, the SC should be ≥28 cm, (McGowan *et al.*, 2002). However, in the case of the Mpwapwa breed bulls in the present study, only 51% (27/53) had SC of ≥28 cm. Of the animals which failed to meet that target, 28% (15/53) had SC of ≤26 cm and 9% (5/53) had SC of ≤25 cm. Thus, whilst only about half of the bulls met the criterion of SC ≥28 cm, the SC of the majority of the bulls were within 1-2 cm of the minimum.

Whether the minimum of 28 cm is relevant to the Mpwapwa breed therefore requires some careful consideration. Firstly, it was clear that semen quality (in terms of total sperm/ejaculate and proportion of sperm with normal morphology) was largely unrelated to SC, and certainly could not be divided into groups that were defined by having SC >28 or <28 cm. Of the bulls that were deemed to have produced semen that was of acceptable quality for AI, 2/8 had SC of <28 cm. Despite failing to score the minimum SC requirement, these bulls managed to produce semen of enough quantity and quality. Further, the correlation between scrotal circumference and ejaculate density was weak (R=0.33) in the present study. This weak correlation is somewhat at variance with the literature at large, in which SC and capacity for sperm production are generally held to have a positive correlation (Palasz *et al.*, 1994; Moura & Erickson, 1997; Bourgon *et al.*, 2018), and, indeed even in the study of a similar breed of low-bodyweight *B. indicus* bulls in Costa Rica, a positive relationship between SC, sperm production, semen quality and age was reported by Chacon *et al.* (1999). Perhaps the generally low overall mean ejaculate density of the bulls in the present study may underlie the weak relationship between SC and ejaculate density: either as a reflection of the inherent characteristics of an unselected breed or as a reflection of the novelty of the electro ejaculation process in the breeding centre.

Secondly, the bulls in the present study were of mixed ages. Most of the bulls were between 2 and 4.5 years of age: a period that is associated in the literature with the maximum growth rate of the testes (Palasz *et al.*, 1994; Moura & Erickson, 1997; Bourgon *et al.*, 2018), whereby the scrotal circumference of bulls continues to increase after puberty, to reach a plateau at ~4 years of age. Thus, the SC of bulls that are 2 years old is less than that for a 3-year-old (and, similarly that of a 3-year-old is less than that of a 4-year-old), so the standards for judging the breeding soundness of bulls of different bulls vary with age over this period of their development (McGowan *et al.*, 2002; Parkinson, 2004; Silva *et al.*, 2014). Hence, variation in the relationship between SC and ejaculate density in the present study might, perhaps, be accounted for in terms of the ages of the bulls. However, it is not clear that this was the case since there was considerable overlap in the SC of bulls of different age groups. Thus, in the present study,

the SC of bulls of ≤36 months of age varied from 25-30 cm, but that of older bulls had a similar range (25-29 cm, excluding 2 outliers of 24 and 32 cm). Hence, there was no evidence that there had been a net increase in SC between the young bulls and the older bulls. Perhaps more extensive SC measurements of bulls between 2 years and 4 years old would show in better details how the SC of Mpwapwa breed bulls' changes with time: for example, as a low bodyweight breed, it might be that SC had already reached its plateau at ~2.5 years. Regardless, there would appear to be an opportunity for selecting animals for SC at somewhere around 3 years of age. The benefit of this can be seen through the hereditary nature of SC which is related to age at puberty and improved fertility and reproductive outcomes in female animals (Quirino & Bergmann, 1998; Parkinson, 2004). Nonetheless, the post-pubertal or 2-year-old SC is regarded as a relatively strong indicator of the SC that the bull will attain at full maturity (Palasz *et al.,* 1994; Moura & Erickson, 1997; Bourgon *et al.,* 2018).

More general studies of *B. indicus* and *B. taurus* bulls have established that the latter have larger SC than the former (Brito *et al.,* 2002). Likewise, studies of different breeds of both *B. taurus* and *B. indicus* show significant variation of SC between breeds and strains within breeds (Brito *et al.,* 2004a, b), which may be intrinsic characteristics of the breeds themselves (Troconiz *et al.,* 1991; Brito *et al.,* 2004a, b), or may be a consequences of differences in mature body weight (Troconiz *et al.,* 1991; Brito *et al.,* 2004a, b). Thus, the relatively high target for SC of *B. indicus* bulls in the Australian BSE recommendations, may be a reflection that they were based on improved breeds of *B. indicus*, which are typically both larger framed and selected for breeding, rather than the relatively unimproved animals that are common in the *B. indicus* strains of East Africa, South Asia and South-East Asia.

The SC may also be affected (probably limited) by nutritional circumstances during the rearing period: Chacon *et al.* (1999) reported that SC was smaller in extensively managed *B. indicus* bulls that had low BCS than in bulls with normal BCS. Likewise, Fordyce *et al.* (1996) found that mature SC of *B. indicus* bulls raised in dry-tropical conditions was limited by adverse climatic and nutritional circumstances under which bulls had been raised (and was also related to the immunity of such bulls to tick infestation). Further, Kashoma *et al.* (2010) examined SC in extensively reared TSZ bulls in the Morogoro and coastal regions of Tanzania, and found that SC was correlated with heart girth (i.e. body weight/size). Both studies showed that most bulls (48/53 in the present study and 169/303 in Kashoma *et al.,* 2010) produced a sperm-rich ejaculate in response to electro-ejaculation with the exception of a few cases, and bulls with larger

SC tended to produce ejaculates with higher density and volume compared to bulls with smaller SC.

As reported by Barth (2000) and Menegassi *et al.* (2011), larger testes (SC) are usually observed in bulls from large maturing breeds compared to bulls from small maturing breeds. Thus, *B. taurus* bulls and bulls of large maturing body size tend to have larger testes than those that are *B. indicus* or are of smaller mature body size. Further, bulls tend to produce greater numbers of sperm (although not necessarily greater volumes of semen) per ejaculate as they mature. Whether this occurs in Mpwapwa breed bulls with advancing age was not clear from the present study, as there were few aged bulls included in it. Similarly, as the youngest bulls in the present study were ~24 months old, the present study does not demonstrate the changes in semen quality that would be expected during the post-pubertal period. Studying these aspects of changes in semen quality of bulls as their age advances would add useful information to understanding of the Mpwapwa breed.

Nonetheless, the present BSE and semen evaluations of Mpwapwa breed bulls reveal their breeding potential and the possibility for at least some of them to be used in an AI breeding programme. There is probably, however, a need for conducting repeated electro-ejaculation tests on each bull prior reaching to a decision of selecting bulls for use in AI or natural breeding programmes (Pace, 1980), given the significant impact on semen volume and ejaculate appearance that electro-ejaculation can have and, hence, the lack of precision of that single electro-ejaculation collections can have. On the other hand, bulls with low semen evaluation outcomes should be culled after repeated electro-ejaculations. Thus, it is important to consider this when producing semen from Mpwapwa breed bulls in an AI programme to be utilised by the smallholder farmers in the Mpwapwa district. Despite being the principal procedure for evaluation of bulls' reproductive potential, BSE faces temporal limitation when assessing bulls' current reproductive performance which can not guarantee their future performance (Ellis *et al.*, 2005). Young bulls of 1-2 years old, as reported by Ellis *et al.* (2005) are more likely to be affected by BSE evaluation, especially when they are meant to be used for natural service, because of their vulnerability to environmental challenges. Conduction of BSE in this age category of bulls might result in changes to their reproductive performance.

Conduction of BSE has a significant impact on the reproductive performance of herds, particularly the conception/ pregnancy rate. Consistently higher pregnancy rates are reported in bulls classified as satisfactory, compared to bulls that are not tested or which failed to score satisfactory standards (Ellis *et al.*, 2005). One of the earlier studies conducted in the

US reported 75%, 52% and 12% pregnancy rates using single-sire bulls classified as satisfactory, questionable and unsatisfactory respectively (Chenoweth, 2000). Similarly, an improvement in pregnancy rate was reported in cows bred to bulls selected for semen quality versus cows bred to unselected bulls (Wiltbank & Parish, 1986). Nonetheless, the effects of environmental factors are known to be the major reported limitations influencing the predictive value of BSE (Ellis *et al.*, 2005).

### *Alternative means of predicting fertility*

Hence, other tests have been introduced to try to improve the relationship between bull testing and fertility outcomes. Potential tests include (Ho & Suarez, 2001):

1) Ca2+ swimming test. This detects a hyperactivated swimming pattern displayed by sperm in the uterine tube during fertilisation at the time of ovulation. In this test, Ca2+ initiates and maintains hyperactivated motility. As a result, hyperactivated motility is considered to be necessary and thus, should be regulated precisely to attain fertilisation. Whilst hyperactivation is described as a subset of capacitation, the two processes are reported to occur separately.

2) Acrosome reaction/function. Several tests involving different techniques have been conducted to assess the functional properties of sperm. As reported by Celeghini *et al.* (2007), the most commonly applied acrosome reaction/function tests are fluorescent probe techniques which mainly evaluate sperm cell integrity and functionality. Celeghini *et al.* (2007) also reported the key role played by plasma membrane integrity in the survivability of sperm within the female reproductive tract and the maintenance of its fertilisation capability. Marquez & Suarez (2004) considered that, in order for sperm to acquire fertilisation capability, they undergo changes to become competent in fertilising an oocyte (acquisition of acrosomal responsiveness). In the process of capacitation, phosphorylation of sperm proteins occurs on tyrosine residue regulated by a CaMP pathway under the activation of a protein kinase A (PKA). Some tyrosine phosphorylated proteins during capacitation become localised to the flagellum and are considered to be responsible for hyperactivation. The performance of oxidative phosphorylation and the production of ATP to provide metabolic energy for motility are basic physiological roles/functions played by the mitochondria in sperm cells (Celeghini *et al.*, 2007).

3) Spontaneous hypotonic-induced acrosome damage. Joseph *et al.* (2010) reported that sperm cells become hypo-osmotic once water enters inside them, and they do so in an attempt to establish an osmotic equilibrium. Consequently, sperm volume and plasma membrane expand due to increased water inflow inside sperm cells. The cause of the latter is that sperm expand

in a form that is characterised by flagellar coiling/angulation at the cytoplasmic droplet. The swollen sperm resulting from failed volume regulation are usually considered to lack fertilisation capability.

### *Conclusion*

This study was undertaken as no assessment of the breeding soundness of the Mpwapwa bull has ever been previously undertaken. The results show that there is a great deal of bull-to-bull variation in BSE and semen quality results; although most bulls produce semen that would be adequate for use in the natural service of small herds, and at least a proportion of the bulls produce semen that is of acceptable quality for use in AI. The relationship between BSE and conception rate is, however, tenuous, once the overtly subfertile/infertile animals have been identified. The results which were obtained in this study are quite similar to the results of other BSE studies done on *B. indicus* bulls globally, particularly the smaller-framed breeds of East Africa and South East Asia.

However, given the limitations of BSE and semen evaluation as a means of predicting the fertility of bulls in an AI program, other tests were sought that were within the capacity of the TALIRI laboratory that might provide additional information. One such test is that of sperm DNA fragmentation. No such studies have been undertaken on the Mpwapwa breed and few, if any, on *B. indicus* cattle anywhere. Further, the opportunities for studying sperm DNA fragmentation in a breed that is wholly unselected for fertility are also very rare. Hence, the next section of this chapter describes the examination of semen from Mpwapwa bulls for DNA fragmentation.

## B. Sperm DNA Fragmentation

### Study Introduction

Although much useful information is obtained from BSE studies (e.g. sperm production, viability, motility, and functionality of the genital tract), the methods are not sufficiently precise to reliably predict bulls' fertility, because of their primary focus on structural, rather than functional, aspects of sperm (Graham, 2001; Rodríguez-Martínez, 2013; Klein *et al.*, 2022). Thus, much literature shows that no simple laboratory test reliably correlates with fertility and, hence, most reviews of bovine semen evaluation report wide variation on the relationship between bull fertility and laboratory semen quality results (see Graham *et al.*, 1980). The association between these semen parameters and fertility is therefore only partial, inasmuch as once low-quality ejaculates have been eliminated, semen quality parameters are not very

predictive of fertility (Kumaresan *et al.*, 2020; Tanga *et al.*, 2021; Klein *et al.*, 2022). Graham *et al.* (1980) reviewed literature which reported this variability between laboratory semen results and bull fertility from different studies as follows; 0.15 – 0.84 for the correlations between sperm motility and fertility; 0.06 – 0.86 for the correlations between morphology and fertility; and 0.33 – 0.66 for the correlations between cell viability and fertility. Similar variability was also found by Januskauskas *et al.* (2003). Pooled indices of semen quality parameters have also been evaluated (Morell *et al.*, 2017; Tanga *et al.*, 2021), with moderate success, but still are not highly predictive of fertility.

The inclusion of emerging molecular technologies in the BSE evaluation programme, has been suggested as a means of improving the efficiency of predicting sperm fertility (Bailey *et al.*, 2003; Klein *et al.*, 2022). Even so, it is not easy to associate fertility and semen laboratory results, partly due to the complex nature of fertility itself and partly due to the variability of the semen laboratory results (Graham, 2001; Moce & Graham, 2008). This, in turn, is due to reasons such as that fertility is directly affected by the number of sperm that are inseminated and the fact that the laboratory tests that are most easily performed do not directly assess most attributes of the sperm that are associated with fertilization. Thus, whilst measures of motility, for example, are generally modestly correlated with fertility, measures of capacitation, acrosome activity and DNA integrity are generally more highly correlated with fertility. The fact that these tests are technically demanding, time-consuming and require specialized equipment means that they are not widely used in the routine evaluation of semen in commercial AI practice.

Recent studies have focussed on DNA integrity as a means of assessing sperm function. Integrity of the DNA in the nucleus of the sperm is necessary for the transmission of genetic information from the sire to the embryo. Damage to that DNA (DNA fragmentation) can therefore result in reduced fertility. Sperm DNA fragmentation is the condition by which the DNA is damaged by either intrinsic, extrinsic or post-ejaculation factors. Major intrinsic causes of fragmentation include the protamination process, in which the level of damage to DNA is related to the protamine: histone ratio within testicular tissues (Elango *et al.*, 2022; Mannucci *et al.*, 2022; Nagaki *et al.*, 2022). It is also a consequence of the action of reactive oxygen species (ROS) and or absorptive apoptosis (Kumaresan *et al.*, 2020; Castleton *et al.*, 2022; Elango *et al.*, 2022; Mannucci *et al.*, 2022; Nagaki *et al.*, 2022). Extrinsic factors that affect fragmentation include animal management, vaccination status, body condition, genital infection, testicular temperature and age, whilst processing factors include semen extension,

cryopreservation and sperm sex sorting can also affect it (Kumaresan *et al.,* 2020; Kumar & Singh, 2022; Szabó *et al.,* 2023). Sperm DNA integrity, as reported from various studies, influences the capability of sperm functionality, even including motility (Kumaresan *et al.,* 2020; Farkouh *et al.,* 2022; Ruiz-Díaz *et al.,* 2023). These functional assessments of sperm are an important outcome of DNA fragmentation tests, which make DNA fragmentation to be an advanced method of evaluating semen quality compared to traditional methods (Bailey *et al.,* 2003; Rodríguez-Martínez, 2013; Klein *et al.,* 2022).

Sperm DNA fragmentation has been identified in recent years as a major cause of male infertility, with published studies on sperm DNA fragmentation increasing rapidly in the literature. Most studies have been conducted in humans: of the 10,490,000 papers on sperm DNA fragmentation that were extant in April 2023, 5,400,000 were about humans, 3,310,000 about livestock and 1,780,000 about cattle. Data from human studies (Zeqiraj *et al.,* 2018; Esteves *et al.,* 2020; Ferrigno *et al.,* 2021; Punjabi *et al.,* 2022), have ascribed causal roles on fragmentation to many environmental insults (Kumar & Singh, 2022; Szabó *et al.,* 2023). Fragmentation damage also increases with the age of the sire, so is particularly identified as a cause of declining fertility in aging men (Evenson *et al.,* 2020; Gill *et al.,* 2020; Ashapkin *et al.,* 2022). Age-related fragmentation has been tentatively identified as a cause of impaired fertility in non-human mammals (Ram: Falchi *et al.,* 2018, Stallion: Neuhauser *et al.,* 2019, Boar: Khezri *et al.,* 2019). Similarly, in bulls, fertility appears to be inversely related to the degree of fragmentation: a process that may be dependent upon age as well as upon intrinsic levels of fragmentation (Elango *et al.,* 2022; Mannucci *et al.,* 2022; Nagaki *et al.,* 2022). Estimation of the degree of DNA fragmentation may therefore be useful for identifying bulls in AI programmes that are at risk of having inherently impaired fertility. Given that, in long-lived species such as humans, fragmentation increases with age, there is also a risk in cattle, that, as most AI sires are relatively aged (i.e. because progeny testing is a lengthy process), they may be at increased risk of sperm DNA fragmentation.

Thus, despite the relatively fewer studies of cattle than other species, the potential for the method as a means of routine analysis of semen quality is clear. Interestingly, studies of *B. indicus* are very infrequent compared with those of *B. taurus*. Further, studies of sperm fragmentation in *B. indicus* cattle in developing countries are conspicuous by their rarity. No information exists to determine whether DNA fragmentation contributes to heterogeneity of fertility in the Mpwapwa breed cattle in Tanzania. However, there are differences in fertility that cannot be attributed to simply-assessed parameters such as semen motility, meaning that

there is a case for determining whether there are underpinning differences between sires in levels of DNA fragmentation which could contribute to fertility outcomes.

The present study aimed to evaluate the level of differences in DNA fragmentation between sires that might affect fertility outcomes in Mpwapwa breed bulls' sperm.

### *Study area*

This study was undertaken partly at the TALIRI Mpwapwa research centre in the Dodoma region and partly at the National Artificial Insemination Centre (NAIC) in Arusha region of Tanzania. Semen samples were collected from Mpwapwa breed bulls kept at TALIRI Mpwapwa research centre, extended and transported to NAIC for sperm DNA fragmentation assessment. This study was undertaken in October 2022. All animal-related manipulations had received Livestock Research Ethical Clearance from the Tanzanian Livestock Research Institute (TALIRI) (12/02/2021) prior to the start of the study.

### *Animal procedures*

Bulls were managed and selected, and semen was collected as described in BSE study.

### *Principle of the Method*

The method is governed by the principle that sperm having DNA fragmentation will fail to produce the halo which is the characteristic of dispersed DNA loops to be observed in sperm having non-fragmented DNA after the acid denaturation and the removal of the nuclear proteins. (Fernandez *et al.*, 2003, 2005). The sperm chromatin dispersion (SCD) (GoldCyto DNA: Microptic, Barcelona, Spain) test was used for this study according to the manufacturer's recommendation.

Intact unfixed sperm (diluted) were immersed in an inert agarose microgel on a pre-treated slide. An initial acid treatment denatured the DNA in those cells with fragmented DNA. Following this, the lysing solution removed most of the nuclear proteins and, in the absence of DNA breakage, produced nucleoids with large halos of spreading DNA loops, which emerged from a central core. Conversely, the nucleoids from sperm with fragmented DNA either did not show a dispersion halo or the halo was minimal. Details for steps involved in sperm DNA fragmentation test are in Appendix 3.

### *Statistical methods*

Results from the fragmentation analysis were that sperm could be:

i. Without halo

ii. With a small halo

iii. With a medium halo

iv. With a large halo

v. Degraded

Categories (i) and (ii) were regarded as normal/undegraded, the others as degraded. Descriptive analysis was based on the sum of (i) and (ii).

## Results

Mean results for the proportion of undegraded sperm are shown in the box plot in Figure 11a. Most bulls clustered around the mean of 94.7% (SD: 6.8, Mode: 100%). The proportion of unfragmented sperm was unrelated to bull age (Figure 11b) or the initial progressive motility of the ejaculate (Figure 11c).

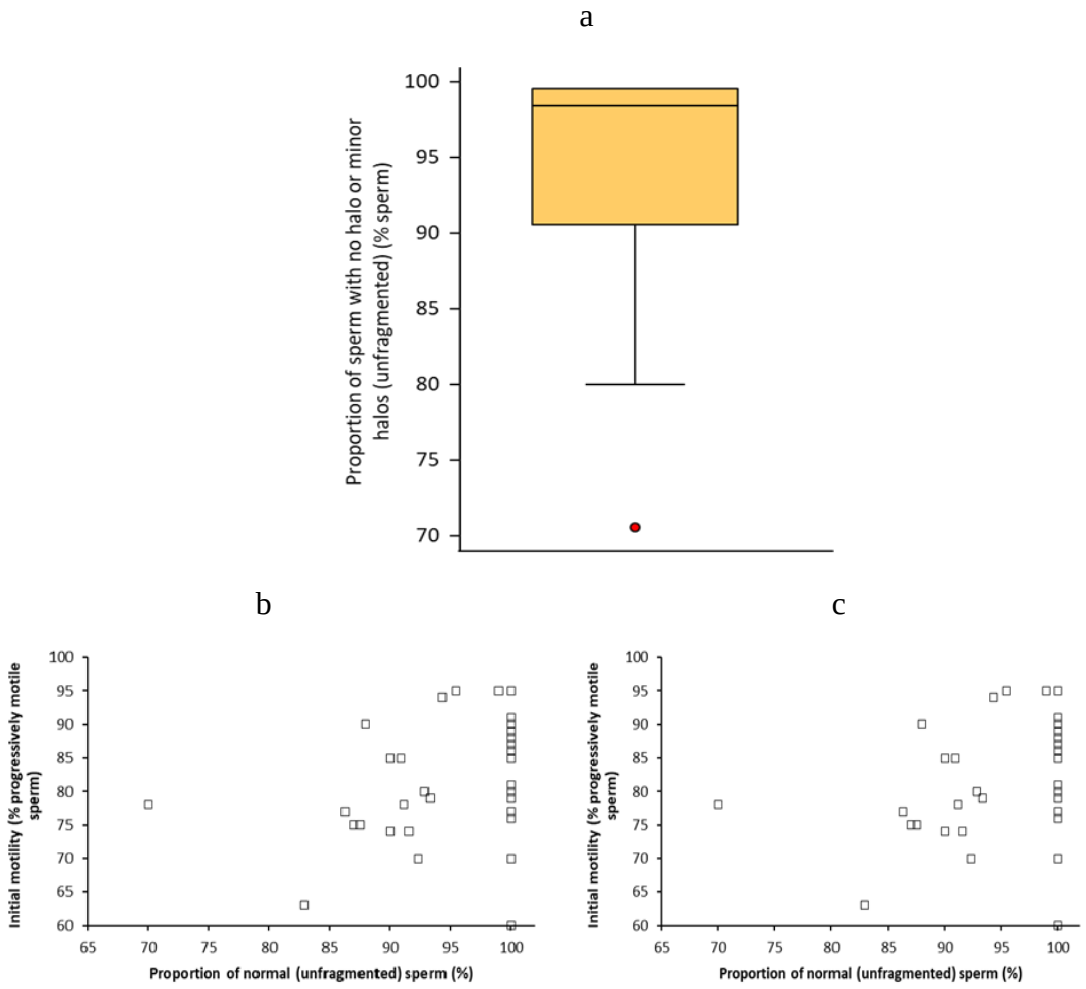


**Figure 11:** Sperm fragmentation in Mpwapwa bulls. (a) Distribution of the proportion of unfragmented sperm in 35 ejaculates (b) relationship between fragmentation and motility and (c) relationship between bull age and fragmentation.

With respect to the 8 bulls identified in Table 2 which would meet criteria for AI, three (14804. 14976 and 14983) had 100%

unfragmented sperm and only 14842 was below 90% (88%).

## Discussion

### *Sperm DNA fragmentation*

There are limited studies on sperm DNA fragmentation in bulls compared to the many human studies (Zeqiraj *et al.,* 2018; Esteves *et al.,* 2020; Ferrigno *et al.,* 2021). This means that even fewer studies have been conducted in *B. indicus* bulls compared to *B. taurus* bulls. Interestingly, of those few studies conducted in *B. indicus* bulls, the majority of them come from large body-size, improved strains of *B. indicus* bulls from Brazil, India and Australia, as compared to the small framed body semi-improved strains of East African Zebu cattle represented by the Mpwapwa breed cattle. The scarcity of such studies is a reflection of the costliness of sperm DNA fragmentation facilities which precludes it from routine use in standard operating procedures for semen production commercial AI centres. Nonetheless, there is increased interest in this area of bull fertility globally (Bailey *et al.*, 2003; Klein *et al.*, 2022). Age and/or genetic factors, as reported from various studies, are the most probable causes of sperm DNA fragmentation (Falchi *et al.,* 2018; Neuhauser *et al.,* 2019; Khezri *et al.,* 2019). Little is known about the significance of other causes of fragmentation to *B. indicus* bulls, although it seems likely that adverse environmental conditions could well contribute.

The results from the current study revealed the lack of a relationship between the proportion of unfragmented sperm and bull age (Figure 11b) or initial progressive motility of the ejaculate (Figure 11c). This was concluded from the proportion of normal sperm which clustered around the mean of 94.7% (SD: 6.8, Mode: 100%). There were, however, two bulls in which the proportion of unfragmented sperm was well below 85%, so these animals might have been worth further investigation. Out of the 8 bulls listed in Table 2, three had 100% unfragmented sperm (i.e. bull ID: 14804, 14976 and 14983) and one (i.e. bull ID: 14842) was below 90% (i.e. 88%). The results obtained from the sperm DNA fragmentation study (i.e. the degree of DNA fragmentation) suggest that there is no clear effect of bulls' age on the level of fragmentation.

Thus, the current findings did not indicate that sperm DNA fragmentation was likely to be of significance in Mpwapwa breed bulls with respect to fertility. However, further research is needed as a follow-up investigation on this area, especially on how reactive oxygen species (ROS), testicular temperature, semen extension and chromosome fragility/stickiness affect the level of sperm DNA fragmentation when other intrinsic and extrinsic factors are kept constant. This was also reported by Prasanthi *et al.* (2004), Kumaresan *et al.* (2020), Elango *et al.*

(2022) and Kumar and Singh (2022): i.e. that the action of reactive oxygen species (ROT) and or absorptive apoptosis, testicular temperature, and semen extension can also be responsible for DNA fragmentation. This will give more insightful details on the effect of the level of sperm DNA fragmentation on fertility in Mpwapwa breed bulls.

More importantly, considering the time involved during progeny testing of beef and dairy bulls, there is a higher likelihood for age-related sperm DNA fragmentation to be observed in dairy bulls than it is in beef bulls. This is because beef bulls usually have a direct performance assessment, which lasts for 400 days, before they can be involved in AI services (usually at 2 years of age), whereas dairy bulls are progeny tested and get involved in AI services when they are about 7 years of age and get used until they are 10 years old. Thus, the utilisation of beef bulls in AI services starts at a younger age than dairy bulls. As such, even though, dairy bulls have shorter life span as AI bulls, they are more prone to age-related sperm DNA fragmentation than beef bulls.

Nonetheless, the low level of DNA fragmentation observed in these suggests that that selecting Mpwapwa bulls based on their semen quality and culling those which do not meet semen targets is likely to be just as successful at identifying using bulls irrespective of whether it is combined with measures of fragmentation.

### General discussion (Breeding Soundness Examination and Sperm DNA fragmentation studies)

Several, yet similar, BSE protocols are available for evaluating bull fertility, of which the most commonly used schemes include the Australian Bull Check and the American Society for Theriogenology (Chenoweth *et al.*, 1992; Fordyce *et al.*, 2006). These two BSE schemes provide simple, repeatable and unambiguous procedures for bull fertility evaluation. The procedures for bovine semen evaluation during a BSE are generally confined to motility, morphology and concentration (Rodriguez-Martinez, 2003; Chenoweth &Mcpherson, 2016; Tanga *et al.*, 2021), for which acceptance criteria for different classes are well-established (e.g. *B. indicus* versus *B. taurus*, age, breed) of bulls. The development of semen evaluation methods (i.e. visual or computer-assisted) principally aimed to predict the fertilisation capability of a bull's ejaculate (Graham, 2001; Moce & Graham, 2008). Nonetheless, meeting these acceptance criteria does not guarantee a correlation with conception (or non-return service) rates in an AI service. However, they help to screen out the use of such bulls in the programme that are unlikely adequately to score the minimum requirements.

Due to its principal focus upon structural rather than functional aspects of sperm, BSE methods are not sufficiently precise to predict bulls' fertility although they do provide much useful

information (e.g. sperm production, viability, motility, and functional of the genital tract) (Graham, 2001; Rodríguez-Martínez, 2013; Klein *et al.,* 2022). The relationship between these semen parameters is only partial, however, inasmuch as once low-quality ejaculates have been eliminated the correlation is limited: hence, semen quality parameters are not very predictive of bull fertility (Kumaresan *et al.,* 2020; Tanga *et al.,* 2021; Klein *et al.,* 2022).

The inclusion of emerging molecular technologies with the fertility evaluation methods as used in BSE programme has been suggested as a means of improving the efficiency of predicting sperm fertility (Bailey *et al.,* 2003; Klein *et al.,* 2022). Be that as it may, it is not easy to relate fertility and semen laboratory results, partly due to the complex nature of fertility itself and partly due to the variability of the semen laboratory results (Graham, 2001; Moce & Graham, 2008). This, in turn, is due to the following reasons: the number of sperm that are inseminated can affect fertility directly, and the laboratory tests that are most easily performed do not directly evaluate most of the attributes of sperm that are most closely related to fertilisation. For example, measures of motility are generally weakly related to fertility as compared to measures of capacitation, acrosome activity and DNA integrity. However, these molecular measures of sperm fertility are technically demanding, time-consuming and require specialised facilities something which make them not to be widely used in the routine semen evaluation in commercial AI practices. As a result, no simple test is reported to correlate with fertility and thus, several reviews of bovine semen evaluation have reported wide variation in the relationship between bull fertility and laboratory semen results (e.g. Graham *et al.,* 1980).

Nonetheless, there is an increasing interest in assessing DNA integrity as a means of assessing sperm functionality. These studies are built on the basis that the integrity of the DNA in the nucleus of the sperm is essential for the transmission of genetic materials from the sire to the embryo. As such, the outcome of DNA damage (DNA fragmentation) can lead to reduced fertility. As reported from different studies (Kumaresan *et al.,* 2020; Farkouh *et al.,* 2022; Ruiz-Díaz *et al.,* 2023), sperm DNA fragmentation can be caused by either intrinsic, extrinsic or post-ejaculation factors. These studies also revealed that the capability of sperm functionality and motility is significantly influenced by sperm DNA integrity. In bulls, fertility is inversely related to the degree of fragmentation, a process that may both be dependent upon age and intrinsic levels of fragmentation. Thus, estimation of the degree of DNA fragmentation may therefore be useful for identifying bulls in AI programmes that are at risk of having inherently impaired fertility. Studies of *B. indicus* cattle are far less frequent than those of *B. taurus* such that, studies of sperm fragmentation in *B. indicus* cattle are conspicuous by their

rarity. Hence, until the present study, no information existed to indicate whether DNA fragmentation contributes to the heterogeneity of fertility in the Mpwapwa breed cattle in Tanzania.

The position of Mpwapwa breed cattle (i.e. as a partially improved *B. indicus* breed of cattle) with regard to the current science of bull fertility globally is by far behind compared to *B. taurus* cattle. This study paves the way for the further evaluation of Mpwapwa breed cattle fertility. Hence, it builds the foundation for future studies which will be essential in improving fertility, reproductive performance and consequently, productivity of Mpwapwa breed cattle. As a result, this will assist in the current work of developing an AI programme using improved Mpwapwa bulls.

The BSE technique (i.e. electro-ejaculation) used in the present study could be replaced by other techniques which could assist in improving the predictive efficiency of bulls' sperm fertility. Some bulls produced low-density ejaculates, but this may be, at least in part, the consequence of using electro-ejaculation as the means of collecting semen. Unfortunately, such techniques are limited by the available facility and the level of expertise. An example of these techniques includes the use of an artificial vagina (AV) for the collection of semen, but this requires bulls to be trained first prior to its application. Similarly, like sperm DNA fragmentation techniques, other advanced techniques (e.g. reactive oxygen species (ROS), chromosome fragility/stickiness and semen extension) could be used, but they have limited availability and little data. However, it is hoped that in the future, AV technique will be included with EJ for conducting BSE in bulls and advanced molecular technique will be introduced to evaluate sperm functionality.

## Conclusion

Taken together, the results of the present study show that there is much variation in BSE results from 2-4 year-old Mpwapwa bulls, but that at least a proportion of them produce semen that would be regarded as suitable for use in an AI programme. Some bulls produced low-density ejaculates, but this may be, at least in part, the consequence of using electro-ejaculation as the means of collecting semen. The variation of semen quality is largely as expected for a breed that has not hitherto been subject to any form of selection for breeding ability. Findings for these Mpwapwa breed bulls largely align with those of similar low body-weight breeds of *B. indicus* bulls that are found in various places in East Africa and South-East Asia, but are significantly less than would be expected from the improved *indicus* breeds of South America, Australia and southern Africa. The absence of significant levels of DNA fragmentation suggests that this is unlikely to affect the fertility of these Mpwapwa bulls. This may be a function of their generally young age, but seems to reflect a relatively low incidence of the

abnormality in cattle (*taurus* and *indicus*) generally. Perhaps the risk of fragmentation may increase with age, although the lifespan of cattle is short compared with (e.g.) humans, in which fragmentation is associated with old age.

For use in an AI service, electro-ejaculation is unlikely to be acceptable as a long-term method of semen collection. Although cattle tolerate the process relatively well, animal welfare concerns suggest that its use is limited primarily to the diagnostic arena, rather than to routine collection. That being so, it seems that the AI centre at TALIRI would probably need to invest in training this cohort of bulls to collection by artificial vagina. Whilst this would increase the infrastructure needed for the service, it would have the dual benefits of (a) being more sustainable in the long term and (b) a likely increase in the harvest of sperm per ejaculate/ collection.

Further study of the bull herd at TALIRI would be beneficial to establish (a) the time course of semen changes after puberty, (b) the time course of development of full, mature sperm production capacity and (c) whether senescent changes occur in aged bulls.

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## Appendix 1

Details of mineral content of the blocks:

| Minerals | Content/mg |
|---|---|
| Calcium | 500 |
| Zinc | 300 |
| Copper | 300 |
| Manganese | 200 |
| Iodine | 200 |
| Potassium | 200 |
| Iron | 160 |
| Cobalt | 50 |
| Selenium | 20 |

## Appendix 2

Semen evaluation results of the 53 Mpwapwa breed bulls involved in the BSE study

**Table 2** Evaluation of fresh semen samples collected from selected Mpwapwa breed bulls in the BSE study during the March-May breeding season 2021

| BULL PARTICULARS | | | MACROSCOPIC EVALUATION | | | MICROSCOPIC EVALUATION | | Concentration |
|---|---|---|---|---|---|---|---|---|
| ID | AGE (months) | SC (cm) | Volume (mL) | Color | Mass activity | Individual motility (%) | Morphology (% normal) | Sperm/mL ($x10^6$) |
| 13117 | 28 | 27 | 4 | Milk | ++ | 60 | 60 | 132 |
| 13119 | 28 | 26 | 2.5 | Light Milk | + | 60 | 60 | 94 |
| 13122 | 27 | 25 | 2 | Light Milk | + | 70 | 70 | 125 |
| 13128 | 27 | 29 | 8 | Light Cream | ++ | 75 | 75 | 829 |
| 13139 | 26 | 25 | 2 | Light Milk | + | 80 | 80 | 86 |
| 13174 | 24 | 26 | 7 | Light Milk | + | 70 | 70 | 117 |
| 14566 | 30 | 29 | 5.5 | Light Milk | + | 70 | 75 | 110 |
| 14574 | 30 | 28 | 5 | Milk | ++ | 55 | 65 | 192 |
| 14590 | 29 | 26 | 3.4 | Light Milk | + | 50 | 50 | 108 |
| 14718 | 49 | 28 | 5 | Milk | ++ | 70 | 75 | 137 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14722 | 49 | 30 | 14.9 | Milk | + | 70 | 70 | 116 |
| 14739 | 48 | 27 | 7.5 | Milk | ++ | 75 | 70 | 223 |
| 14747 | 48 | 29 | 3.3 | Milk | ++ | 85 | 90 | 123 |
| 14750 | 48 | 28 | 5 | Milk | ++ | 80 | 85 | 286 |
| 14762 | 47 | 28 | 4.5 | Milk | +++ | 85 | 90 | 266 |
| 14785 | 44 | 28 | 4 | Milk | ++ | 70 | 80 | 263 |
| 14791 | 44 | 27 | 6.4 | Milk | ++ | 75 | 70 | 325 |
| 14802 | 44 | 28 | 13 | Milk | +++ | 90 | 90 | 240 |
| 14804 | 44 | 26 | 5.5 | Cream | +++ | 85 | 75 | 820 |
| 14817 | 44 | 28 | 3 | Cream | ++ | 80 | 80 | 202 |
| 14835 | 44 | 25 | 8 | Milk | ++ | 80 | 80 | 205 |
| 14836 | 44 | 28 | 4.3 | Light Milk | + | 75 | 75 | 102 |
| 14841 | 43 | 33 | 6 | Milk | ++ | 80 | 80 | 746 |
| 14842 | 43 | 29 | 8 | Milk | +++ | 85 | 80 | 734 |
| 14843 | 43 | 25 | 5 | Milk | ++ | 80 | 80 | 107 |
| 14852 | 43 | 28 | 3 | Milk | +++ | 85 | 87 | 227 |
| 14858 | 43 | 27 | 4 | Milk | ++ | 75 | 75 | 403 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14865 | 43 | 28 | 5 | Milk | ++ | 75 | 85 | 137 |
| 14869 | 43 | 27 | 5.5 | Light Milk | ++ | 75 | 75 | 103 |
| 14887 | 43 | 27 | 7 | Light Milk | + | 70 | 80 | 57 |
| 14889 | 43 | 27 | 7.2 | Milk | ++ | 75 | 80 | 244 |
| 14891 | 43 | 28 | 6.4 | Milk | ++ | 85 | 80 | 253 |
| 14901 | 42 | 25 | 3 | Cream | +++ | 90 | 80 | 873 |
| 14903 | 42 | 27 | 1.9 | Milk | ++ | 70 | 75 | 100 |
| 14911 | 42 | 29 | 8 | Milk | +++ | 90 | 80 | 815 |
| 14922 | 41 | 26 | 3.2 | Milk | ++ | 80 | 75 | 310 |
| 14924 | 41 | 27 | 3 | Milk | ++ | 70 | 70 | 305 |
| 14952 | 37 | 28 | 5 | Milk | +++ | 80 | 80 | 208 |
| 14965 | 37 | 33 | 6.5 | Milk | +++ | 90 | 90 | 190 |
| 14966 | 35 | 28 | 6 | Milk | + | 20 | 70 | 408 |
| 14967 | 32 | 28 | 5.8 | Milk | +++ | 75 | 80 | 966 |
| 14975 | 32 | 26 | 4.1 | Milk | ++ | 70 | 70 | 326 |
| 14976 | 32 | 30 | 8.2 | Milk | ++ | 75 | 75 | 201 |
| 14977 | 32 | 26 | 3.5 | Milk | ++ | 70 | 70 | 284 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14980 | 32 | 26 | 5.5 | Milk | + | 70 | 75 | 315 |
| 14983 | 32 | 29 | 2.8 | Cream | +++ | 80 | 85 | 896 |
| 15946 | 54 | 26 | 10.5 | Milk | + | 65 | 80 | 111 |
| Bulls from which no sperm-rich fraction was collected | | | | | | | | |
| 13161 | 24 | 26 | 2.5 | Watery | | | | 0 |
| 14755 | 47 | 27 | 5 | Watery | | | | 0 |
| 14770 | 44 | 27 | 6 | Watery | | | | 0 |
| 14916 | 42 | 29 | 5 | Watery | | | | 0 |
| 14925 | 41 | 24 | 8 | Watery | | | | 0 |
| 15195 | 122 | 32 | 8 | Watery | | | | 0 |

| | | |
|---|---|---|
| Key to mass motility scores | (0) Poor, no waves and the spermatozoa are immobile | (++) Good, less dark waves with moderate movement |
| | (+) Normal, clear waves with very slight movement | (+++) Very good, lots of dark waves moving rapidly |

## Appendix 3

Steps involved in DNA fragmentation test:

a) The lysis solution was set at ambient temperature of 22°C;

b) Then semen samples were diluted in culture medium to a concentration of 5 million per millilitre;

c) The agarose eppendorf tubes were put through a float in a water bath set at 90°C for 5 minutes;

d) Thereafter, the agarose eppendorf tubes were transferred to a different water bath set at 37°C for 5 minutes;

e) 30 microliters of the semen samples were added to the agarose microgel and mixed. Then, the cell suspension was placed from the agarose eppendorf onto the treated side of the slide and covered with a glass coverslip;

f) The slides were placed on a glass plate pre-cooled at 4°C and put in a fridge at 4°C and samples were left to gel for 5 minutes;

g) Then cover slips were removed by sliding them gently and were immediately immersed into the acid denaturation in a horizontal position and left to incubate for 7 minutes at ambient temperature of 22°C;

h) Using hand gloves, slides were picked, held and placed horizontally into another incubation tray containing 5 ml of tempered lysis solution and incubated for 25 minutes;

i) Then slides were picked and set up horizontally into a tray containing abundant distilled water and left to incubate for 5 minutes to wash the lysis solution;

j) Thereafter, slides were placed horizontally into a tray with 70% ethanol for 2 minutes, followed by 90% ethanol for 2 minutes and finally, 100% ethanol for 2 minutes;

k) Slides were left to dry at ambient temperature;

l) Then slides were placed horizontally on the float inside the petri dishes;

m) Stain solution –TA (0.3 mL) was then applied on the slides fully immersed and incubated for 1 minutes;

n) Stain solution –TB (0.6 mL) was then applied on the slides fully immersed, TA and TB were thoroughly mixed and incubated for 5 minutes;

o) Then slides were washed briefly and smoothly in tape water and allowed to dry at ambient temperature; and

# Chapter Five: Effect of diluent temperature and time on survival of Mpwapwa bull sperm

## Introduction

The ability of sperm to survive and retain its capacity to fertilise the ovum is a key determinant of the success of any artificial insemination (AI) regimen. In the context of AI programmes, sperm survival may be considered as the probability of sperm remaining viable at a given period during storage (Foote & Kaproth, 1997; Vishwanath & Shannon, 2000; De Pauw *et al.*, 2003; Yang *et al.*, 2018). For cattle AI, sperm survival is mainly determined by the success of the preservation/storage segment of the semen production chain (Shannon *et al.*, 1984; Foote & Kaproth, 1997, Vishwanath & Shannon, 2000; De Pauw *et al.*, 2003). Cattle AI centres therefore evaluate the quality of semen after ejaculation, as an indicator of its likely ability to survive the preservation process, and evaluate it after extension/preservation, as an indicator of its actual ability to do so.

Sperm survival is assessed for the following reasons. Firstly, it can be used to determine the total number of sperm to be packed per AI dose. This has to take into consideration not only the number of sperm present at the start of the preservation/storage period but also the proportion of sperm that can survive preservation, compensating for the number of dead sperm, so that sufficient live sperm remain to enable conception after insemination (Shannon *et al.*, 1984; Foote & Kaproth, 1997; Vishwanath & Shannon, 2000; Yang *et al.*, 2018). Secondly, survival can be assessed to determine the ideal preservation conditions for storage. Such conditions include, for example, determining the optimum storage temperature and appropriate diluents/extenders (Vishwanath & Shannon, 2000; De Pauw *et al.*, 2003; Murphy *et al.*, 2016; Yang *et al.*, 2018). Finally, survival can be assessed to determine the duration for which, using the chosen preservation technique, the semen can be stored (i.e. life span/shelf life of semen) (Vishwanath & Shannon, 2000). This assessment aims to ensure that the preserved semen maintains its viability throughout the period of storage. The length of time over which semen can be stored is affected by a range of factors particularly storage conditions (i.e. temperature and diluents/extenders), but also other factors such as freezing and thawing regimens (Vishwanath & Shannon, 2000). All of these conditions must be

considered when determining optimal strategies for semen preservation. Whilst the optimal preservation conditions for the semen of cattle are relatively well documented, in principle, usage varies between AI organisations, with each having its own preferred method. For preservation methods other than cryopreservation (i.e. chilled or ambient temperature), there are a plethora of potentially useable techniques (see Dziekonska & Partyka, 2023), and the optimal technique depends upon the environment in which it is to be used (especially the climate), the AI service that is provided (e.g. technician vs owner-inseminator), and the supply chain logistics between collection and insemination.

Nonetheless, whilst there is significant literature on semen preservation in general, including from *B. indicus* cattle (see Shannon, 1978; Crespilho *et al.*, 2009), in order to provide an effective AI service based on Mpwapwa bulls, specific information is needed on the survival characteristics of Mpwapwa bull semen. The two key questions are i) identifying whether ambient rather than chilled storage is feasible and, depending on that outcome, ii) which diluent is most suitable for use. This present study therefore aimed to evaluate sperm survival in semen produced from Mpwapwa breed bulls to determine the optimum temperature, time, and diluents/extenders for ambient temperature preservation of semen for use in the proposed AI and fertility programme.

## Materials and Methods

### *Animals*

This study was undertaken concurrently with the semen evaluation studies in March 2021 and March 2022 at the TALIRI Mpwapwa research centre. The semen samples from the 35 bulls that were selected, managed and used for that study were also used for this comparative semen preservation study. The experiment was undertaken in two stages. In March 2021, semen from three bulls was used in a pilot study to test the process, and then in March 2022, semen from all 35 bulls was used.

### *Semen collection and evaluation*

Semen was collected and evaluated as described in Chapter Four. Briefly, semen was assessed for volume, sperm density, mass and individual sperm motility and sperm morphology immediately after collection.

### *Survival study*

The study methodology is summarised in Figure 1. Each ejaculate was divided into three aliquots. Aliquots were diluted in: i) Coconut water (Wadood *et al.*, 2022; Odrada *et al.*, 2023); ii) Egg yolk-TRIS diluent (Minitube, Tiefenbach, Germany); and iii) Optixcell diluent (IMV, L'Aigle, France).

Each aliquot was diluted within five minutes after collection at a constant ratio of 1:1, regardless of the sperm concentration of the initial ejaculate. Semen samples were diluted at an ambient temperature of 32°C. A one-step dilution method/protocol was used for dilution (Arif *et al.*, 2020). After dilution, semen was loaded into 0.25 mL French straws (IMV, L'Aigle, France). Straws were then maintained in water baths set to three different temperatures (5 straws per ejaculate per diluent per water bath). In Year 1, straws were incubated at 20°C, 27°C and 33°C. In Year 2, they were incubated at 8°C, 17°C and 33°C. Straws (n=1 per ejaculate) were removed after 6, 24, 48, 72 and 120 hours of incubation for evaluation of sperm motility. Sperm motility was evaluated using computer-assisted sperm analysis (CASA, Microptic Automatic Diagnostic Systems, Barcelona, Spain) (Tanga *et al.*, 2021).

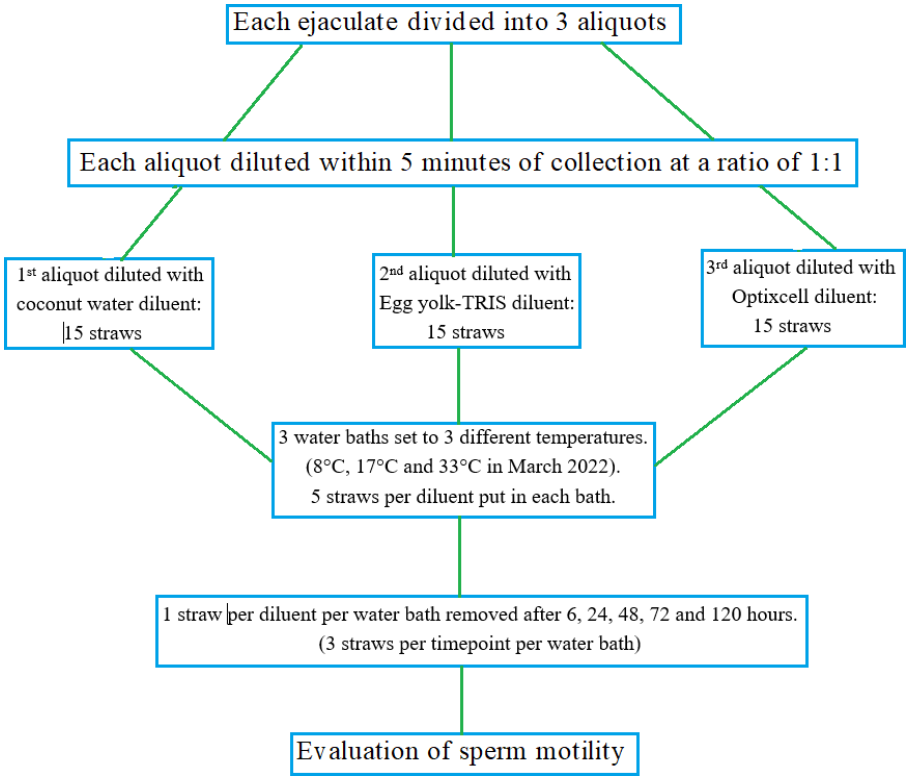


**Figure 1** Straw creation, dilution, incubation and selection process for sperm survival study

### *Statistical Methods*

The sperm motility data were treated and analysed as count data (i.e. number of motile sperm per 100 sperm observed). The presence of high numbers of zeros in the data and a variance that was greater than the mean (over-dispersion) meant that a two-stage zero-inflated negative binomial model was used. In the first component, excess structural zeroes were modelled using a repeated-measures binary logistic model, with the presence/absence of motile sperm as the outcome; with diluent, incubation temperature, time and all two-way interactions as the fixed predictor variables and bull as a random effect. The second component was a repeated measures negative binomial model (i.e. count model), with the percentage of motile sperm as the outcome; and diluent, incubation temperature, time and all two-way interactions as the fixed predictor variables and bull as a random effect. In this component, the outcome (proportion of motile sperm) could still be zero (sample zero) even if the logistic model identified an absence of a structural zero. For both models,

a backward selection procedure was used, with fixed predictors and their two-way interactions removed when $p>0.05$.

## Results

The range of semen scores for the bulls used in the study are shown in Table 1

Table 1. Range of semen scores of the bulls that were selected from the population of 35 for use in the study

| Volume (mL) | Mass activity (0 – +++) | Individual motility (%) | Morphology (% normal) | Sperm $x10^6$/mL |
|---|---|---|---|---|
| 2.8 – 8.0 | ++ – +++ | 75 – 90 | 75 – 80 | 734 – 966 |

### *Summary data*

Summary survival data from Years 1 and 2 are shown in Tables 2 and 3 respectively. The Year 1 study showed that sperm remained viable for up to 48 h (72 h in Optixcell) and that there was a progressive decline of motility with time in all diluents, with this decline being more marked as temperature increased. The reduction in the proportion of motile sperm was markedly worse in coconut water than in other diluents.

**Table 2:** Effect of time, storage temperature and diluent on the proportion of motile sperm (Year 1, pilot study, n = 3 bulls)

| Time (hours) | Diluent | Proportion of motile sperm (%) | | |
|---|---|---|---|---|
| | | Temperatures (°C) | | |
| | | 20 | 27 | 33 |
| 0 | Optixcell | 94.7 | 83.9 | 84.8 |
| | Tris | 93.5 | 83.0 | 80.1 |
| | Coconut | 76.1 | 68.5 | 27.9 |
| 6 | Optixcell | 93.3 | 82.5 | 82.9 |
| | Tris | 92.2 | 81.4 | 90.7 |
| | Coconut | 74.2 | 67.8 | 15.6 |
| 24 | Optixcell | 86.5 | 78.1 | 5.7 |
| | Tris | 61.3 | 61.7 | 9.6 |
| | Coconut | 13.6 | 10.2 | 0.0 |
| 48 | Optixcell | 66.3 | 60.8 | 0.0 |
| | Tris | 32.5 | 18.0 | 0.0 |
| | Coconut | 10.7 | 9.4 | 0.0 |
| 72 | Optixcell | 32.8 | 9.3 | 0.0 |
| | Tris | 27.0 | 6.7 | 0.0 |
| | Coconut | 0.0 | 0.0 | 0.0 |
| 120 | Optixcell | 6.7 | 7.7 | 0.0 |
| | Tris | 12.6 | 0.0 | 0.0 |
| | Coconut | 0.0 | 0.0 | 0.0 |

**Table 3:** Effect of time, storage temperature and diluent on the proportion of motile sperm (Year 2, n = 35 bulls)

| Time (hours) | Diluent | Proportion of motile sperm (%) | | |
|---|---|---|---|---|
| | | Temperatures (°C) | | |
| | | 8 | 17 | 33 |
| 0 | Optixcell | 96.7 | 89.5 | 85.0 |
| | Tris | 94.1 | 87.2 | 81.3 |
| | Coconut | 77.5 | 73.8 | 49.9 |
| 6 | Optixcell | 85.2 | 77.5 | 69.6 |
| | Tris | 81.5 | 73.0 | 66.8 |
| | Coconut | 70.1 | 59.8 | 42.8 |
| 24 | Optixcell | 79.7 | 72.0 | 62.1 |
| | Tris | 65.4 | 56.5 | 44.6 |
| | Coconut | 43.2 | 33.4 | 23.3 |
| 48 | Optixcell | 63.2 | 54.6 | 22.9 |
| | Tris | 46.9 | 37.8 | 21.4 |
| | Coconut | 23.3 | 14.7 | 4.9 |
| 72 | Optixcell | 24.2 | 14.7 | 0.0 |
| | Tris | 24.6 | 17.6 | 0.0 |
| | Coconut | 0.0 | 0.0 | 0.0 |
| 120 | Optixcell | 11.4 | 8.0 | 0.0 |
| | Tris | 14.0 | 0.0 | 0.0 |
| | Coconut | 0.0 | 0.0 | 0.0 |

Year 2 results were consistent with those of Year 1, with sperm survival being increased by decreasing storage temperature (with the data for 17°C in Year 2 being broadly comparable with those from 20°C in Year 1). Similarly, survival in coconut water was poorer than the other diluents at all temperatures, whereas survival rates in Tris and Optixcell were largely similar to each other. Survival results for individual bulls averaged across the three storage temperatures but separated by diluent, are illustrated in Figure 2.

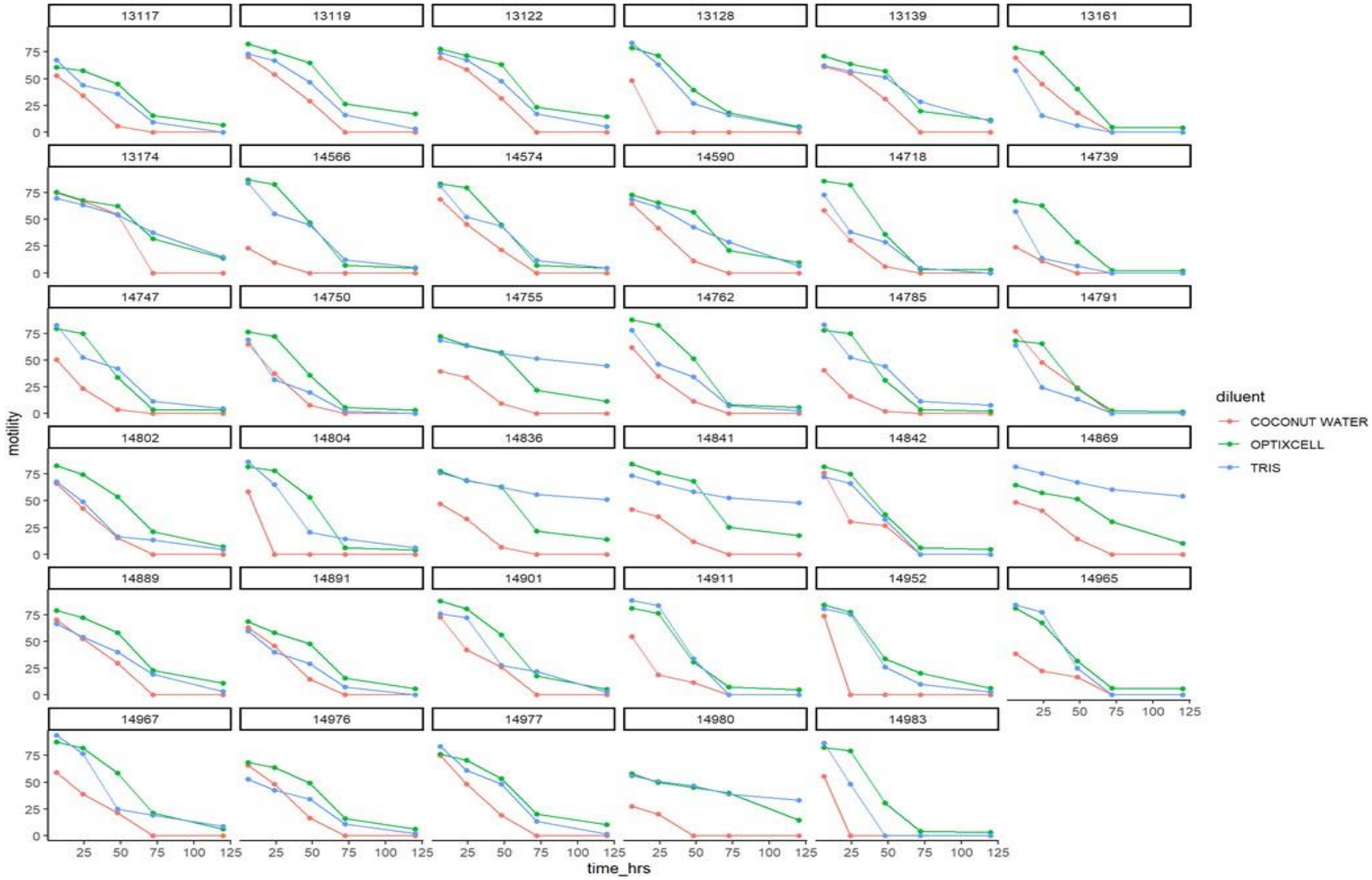


**Figure 2** Effect of time (6-120 h) and diluent on % motile sperm for samples from individual (Data are for 35 bulls with results averaged across three temperatures: 8, 17 and 33 °C).

### *Results from the zero-inflated negative binomial model*

The zero-inflated component of the model identified that increasing temperature and time both increased the odds of motility being 0% (Table 4). The backward selection procedure did not identify diluent or any of the interactions as statistically significant in this component of the model.

**Table 4**: Effect of time and temperature on odds of sperm motility being 0% (structural zero from zero-inflated part of model)

| | Odds Ratio (95% CI) |
|---|---|
| *Reference temperature 8°C* | |
| Temperature 17°C | 1.34 (0.88 – 2.04) |
| Temperature 32°C | 6.33 (4.11 – 9.75) |
| | |
| *Reference time 6 hours* | |
| Time 24 hours | 5.25 (1.87 – 14.75) |
| Time 48 hours | 28.73 (10.79 – 76.5) |
| Time 72 hours | 131.53 (48.65 – 355.6) |
| Time 120 hours | 131.92 (45.88 – 379.33) |

*Example interpretation of odds ratio:* the odds of a semen sample having a structural count of zero) was 6.33 (95%CI 4.11-9.75) times higher for semen stored at 32°C than for semen stored at 8°C.

The model confirmed that the data did not fit the Poisson distribution with the dispersion parameter being affected by both time and temperature ($P<0.001$). For the negative binomial component, the model identified that temperature, time and diluent were all significantly associated with the proportion of motile sperm, and that there was an interaction between diluent and time. This is summarised in Table 5, and illustrated in Figure 3.

**Table 5:** Effect of time and storage temperature on counts of motile sperm

| | Incidence Rate Ratio (95%CI) |
|---|---|
| *Reference temperature: 8°C* | |
| Temperature 17°C | 0.88 (0.85– 0.9) |
| Temperature 32°C | 0.76 (0.73 – 0.79) |
| | |
| *Reference time 6: hours* | |
| Time 24 hours | 0.245 (0.235 - 0.255) |
| Time 48 hours | 0.142 (0.132 - 0.154) |
| Time 72 hours | 0.085 (0.073 - 0.098) |
| Time 120 hours | 0.0049 (0.0034 -0.007) |
| | |
| *Reference diluent: Tris* | |
| Coconut Water | 0.79 (0.76 - 0.82) |
| Optixcell | 1.04 (1.0002 -1.08) |
| | |
| *Reference†: Tris * 6 hours* | |
| Coconut water*48 hours | 0.72 (0.63 - 0.83) |
| Optixcell*24 hours | 1.27 (1.19 - 1.35) |
| Optixcell*48 hours | 1.21 (1.09 - 1.34) |
| Optixcell*72 hours | 0.73 (0.59 - 0.9) |
| Optixcell*120 hours | 4.98 (3.46 - 7.18) |

*Example interpretation of incidence rate ratio (IRR):* the percentage of motile sperm for semen stored at 32°C was 0.76 (95%CI 0.73 -0.79) times that of semen stored at 8°C. †, interactions only included where 95%CI of IRR excluded 1. *Example interpretation of interaction:* For coconut water, the ratio of motile sperm at 48 hours to motile sperm at 6 hours is 0.72* that of Tris for the same comparison; i.e. it is 0.72 * 0.142, i.e. 0.102

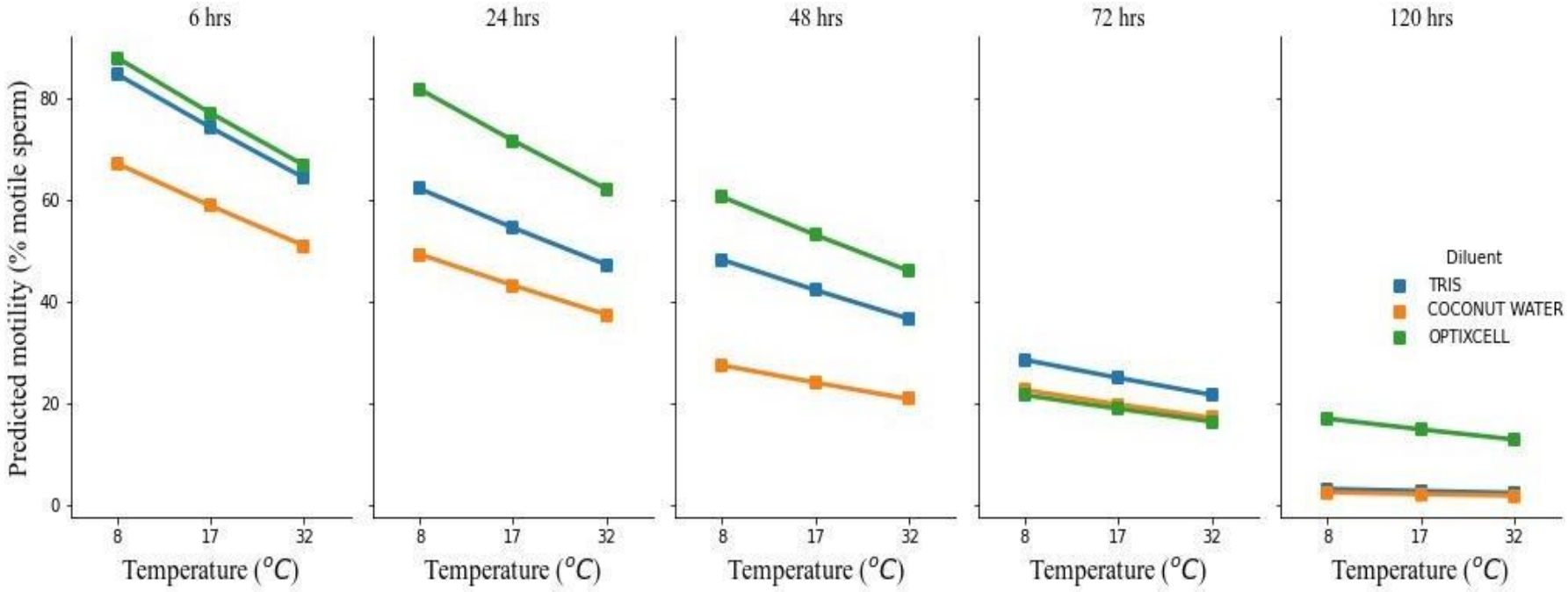


**Figure 3** Predicted survival (linear prediction) of sperm (% motile sperm) at different durations of incubation, with respect to diluent and incubation temperature

### *Evaluating individual bulls for potential use as AI sires*

The target number of sperm per insemination dose at the time of AI for ambient-temperature/chilled semen was chosen to be 2 x $10^6$ total sperm/dose (Shannon *et al.*, 1984). For a 0.25 mL dose, this is equivalent to 8 x $10^6$ sperm/mL. At the 1:1 dilution used in the present experiment, this is equivalent to a minimum starting ejaculate density of 16 x $10^6$ sperm/mL if no sperm are lost before insemination.

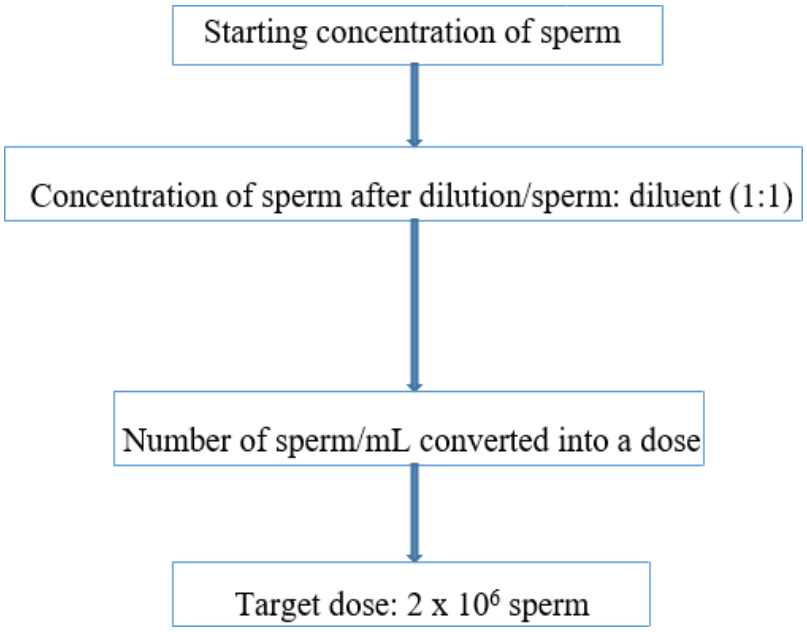


**Figure 4** Flow chart showing the steps involved in the development of a threshold number of sperm in a target dose

In this study, the lowest sperm concentration of a selected bull prior to dilution was 57 x $10^6$ sperm/mL (i.e. 23.5 x $10^6$ sperm/mL after dilution). For this bull, sperm motility of 34% would result in a dose of 2 million sperm. That level of motility was achieved at 48 h after dilution (on average) in samples in Tris and Optixcell (at both 8°C and 17°C), while for coconut water none of the 48 h samples had sufficient average motility (see Figure 3). However, a simple estimate of the number of motile sperm, may overestimate the proportion of fertile straws as the accumulation of dead sperm may result in the straw no longer being fertile even though the number of viable sperm

is >2 million per dose (Shannon & Curson, 1972), while fertility has been reported with straws containing <1 million sperm per dose (Kim & Lee, 1972; Shannon, 1972). As such, it would be best to combine our 2 million sperm per dose target with a target minimum motility, especially as our initial sperm concentrations and dilutions allow for very low motility in straws containing >2 million sperm/dose. One potential minimum motility is 50% (as used by Mandal *et al.*, 2005 in post-thaw frozen semen in Sahiwal bulls). At 48 hours, estimated mean motility was >50% for semen diluted with Optixcell and kept at 17 or 8°C, while for TRIS-diluted semen, estimated mean motility was near 50% (48.3%) only for semen kept at 8°C.

However, using mean results ignores the large variations between individual bulls, with many bulls showing significantly better survival than the mean. Results for bulls with motility >50% more at 72 hours or more after dilution are shown in Table 6.

**Table 6**. Individual bull motility exceeding 50% threshold at different times and temperatures, in Tris and Optixcell diluents.

| Bull ID | Diluent | Temperature (°C) | Time (h) | Motility (%) |
|---|---|---|---|---|
| 13139 | Tris | 8 | 72 | 51 |
| 13174 | Tris | 8 | 72 | 54 |
| | Optixcell | 17 | 72 | 54 |
| 14755 | Tris | 8 | 72 | 59 |
| | | 8 | 120 | 52 |
| | | 17 | 72 | 52 |
| 14802 | Optixcell | 8 | 72 | 50 |
| 14836 | Tris | 8 | 72 | 60 |
| | | 8 | 120 | 56 |
| | | 17 | 72 | 55 |
| | | 17 | 120 | 50 |
| | | 32 | 72 | 53 |
| 14841 | Tris | 8 | 72 | 57 |
| | | 8 | 120 | 53 |
| | | 17 | 72 | 52 |
| | | 32 | 72 | 50 |
| 14869 | Optixcell | 8 | 72 | 53 |
| | Tris | 8 | 72 | 72 |
| | | 8 | 120 | 66 |
| | | 17 | 72 | 57 |
| | | 17 | 120 | 53 |
| | | 32 | 72 | 52 |
| 14967 | Optixcell | 8 | 72 | 50 |
| 14980 | Optixcell | 8 | 72 | 50 |

## Discussion

Sperm survival at temperatures at which their metabolism is not completely inhibited is crucial for an AI service based on methods of semen preservation other than cryopreservation. Sperm not only have to survive the processes of dilution and processing but also have to survive the vagaries of storage conditions through the semen supply chain. Only if the diluents are robust enough to protect the sperm through the supply chain to the point of insemination can the use of the AI service result in the advancement of animal fertility, genetics and health (Verberckmoes *et al.*, 2004). The current study aimed to determine optimum preservation conditions (i.e. time, diluent and temperatures) for ambient storage of liquid/fresh Mpwapwa bull semen to be used in the proposed AI and fertility programme in Tanzania, based upon the premise that an AI service can be successfully based upon the use of ambient-temperature preservation of semen. The main precedent for present-day use of non-cryopreserved semen is in the dairy AI service of New Zealand, which is based on the Caprogen-based ambient-temperature diluent (Shannon, 1978) However, all of the pioneering of bovine AI services prior to the development of cryopreservation were based on semen preserved in relatively simple chilled or ambient temperature diluents, and these were generally very successful (Phillips, 1939; Phillips & Lardy, 1940; Salisbury *et al.*, 1941; Salisbury *et al.*, 1978). Hence, there was a considerable level of confidence in the approach taken in the present experiment.

The intention of this study was to determine whether the diluents could maintain sperm survival at the ambient temperatures that pertain in Tanzania. As discussed above, we believe that the target sperm survival should be a combination of motile sperm/dose and the proportion of motile sperm. Our target number of motile sperm/dose was relatively easy to meet, especially with the low dilution used in this study, but our target proportion of 50% motile sperm was not. Survival in Coconut water was only over 50% up to 24 h after dilution (and then only at a storage temperature of 8$^{o}$C), and, although survival in Tris and Optixcell was better, mean motility remained >50% only up to 48 h after dilution in samples diluted with Optixcell (and then not at 32$^{o}$C) although samples diluted with Tris and stored at 8$^{o}$C had a mean motility close to 50% (48.3%). This target of 50% may appear to be low, but it was used by Mandal *et al.*, (2005) for frozen and thawed semen. If that threshold is satisfactory for the relatively damaged cells that survive freezing and thawing,

cells that have been preserved in chilled/ambient temperature diluents are likely to be less damaged than those that have been cryopreserved, so 50% survival would be a reasonable threshold for ambient temperature semen. Indeed, it may actually be high as 40% survival is often regarded as a threshold for survival in cryopreserved semen (Pon-Rejraji *et al.,* 2009). Furthermore, as Table 6 shows, there remains considerable scope for choosing individual bulls which have motility >50% for significantly longer than 48 h, so we believe that the 50% motility target is both robust and achievable.

Temperature had a substantial effect on survival, such that survival decreased in a linear manner as storage temperature increased from 8$^{o}$C to 32$^{o}$C. Temperature-dependent decreases in survival were, of course, not unexpected, given the well-established effects of temperature on the metabolic rate of cells. Interestingly, there are studies in the literature that have used Optixcell with reasonable success up to 22$^{o}$C (e.g. Verberckmoes *et al.*, 2004; Arif *et al.,* 2020), but no studies were found that had used it at temperatures as high as those found in Tanzania (i.e. 32$^{o}$C, the maximum incubation temperature used in the present study). It was important therefore, in the present study, to note that the predicted survival of sperm in Optixcell at 48 h at 32$^{o}$C was substantially less than its survival at 17$^{o}$C, and had declined to well below the 50% threshold that has been previously discussed. The implications of this effect of temperature on the distribution network of a potential AI service are discussed below. There are, however, other approaches to managing ambient-temperature dilution that might mitigate the high ambient temperatures of Tanzania.

One of these is the temperature at which the initial dilution is made. In the present study, the ambient temperature of 32$^{o}$C was used. However, it is clear that diluents are not devoid of toxicity to sperm (Thacker *et al.,* 1954; Shannon & Curson, 1972); and it is, for example, well-established that cryopreservation diluents that contain glycerol have both temperature-related and concentration-related toxic effects of glycerol (Shannon, 1978; Papa *et al.,* 2014). It may be that performing the initial dilution at a more controlled (i.e. lower) temperature might have mitigated some of the effects of dilution on sperm survival. The decrease in survival at 6 h at 32$^{o}$C might be taken as evidence that this is the case.

The one-step dilution method/protocol itself may also have affected sperm survival. The method used for dilution in this study was based on the work of Arif *et al.* (2020), in which, regardless of the sperm concentration of the initial ejaculate, each aliquot was diluted (within five minutes after collection) at a constant ratio of 1:1 at an ambient temperature of 32°C. Whilst 32ºC might appear to be a relatively high temperature for the extension of semen, it is, however, close to the range of temperatures recommended for the short-term storage of semen (Shannon, 1978) and, indeed, is in the middle of the range of temperatures (18°C-37°C) recommended by Murphy *et al.* (2018a, b). Semen that is to be stored in a chilled format would, of course, be reduced in temperature from 32ºC to ~5ºC (slow cooling from ambient to chilled temperature) rapidly thereafter, but the temperature at which extension is performed does not appear to have an adverse effect upon sperm survival *per se*. Indeed, sudden change in temperature (such as might occur if semen at body temperature were suddenly added to a diluent at ~5ºC) would undoubtedly be harmful (Salisbury *et al.,* 1979), so taken together, there seems to be little evidence that extension at 32ºC would be harmful of itself.

Variations exist in the temperature and protocols of semen extension among AI centres. For example, some AI centres apply a one-step dilution, whereas, some apply a two-step dilution and others a three- or four-step dilution (Arif *et al.,* 2020). There are still ongoing debates concerning the merits of each of these semen dilution techniques, however, a one-step dilution technique is widely recommended for extension for preservation of semen at both ambient and freezing temperatures (Arif *et al.,* 2020), providing that (in the case of cryopreservation media) the toxic effects of glycerol and/or other components are mitigated (Papa *et al.,* 2014).

In New Zealand, the ambient-temperature/liquid semen AI service is based on the Caprogen diluent. That diluent, as well as having the usual properties of an ambient temperature diluent (i.e. carbonation-based suppression of metabolic activity), further suppresses motility by creating very low oxygen tension by saturating the diluent with $N_2$ gas (Vishwanath & Shannon, 1997). In that diluent, the achievable preservation period as reported by Vishwanath & Shannon (1997) is 72-120 hours (i.e. 3-5 days). The storage period for fresh/liquid semen as practised in New Zealand is rather better than the duration of sperm survival recorded in both Tris and Optixcell diluents (i.e. up to 48 h) in the present study. Differences between the present results and those reported elsewhere for Caprogen diluent are worth consideration. As pointed out by Vishwanath & Shannon

(1997), minimising oxidative stress by decreasing the oxygen tension through adding antioxidant and chelating agents is pivotal to extending sperm shelf life and storage period whilst maintaining its motility at ambient environment. However, as used in New Zealand, ambient temperatures rarely exceed mid-20s$^{o}$C during the AI season, so, again, the potential for differences in preservation qualities at higher temperatures remains largely unexplored.

Extending the shelf life for semen in the face of high ambient temperatures therefore remains an important topic for research. It appears from the present study that at 48 hours neither Tris or Optixcell diluents were suitable at high ambient temperatures (32°C) (Figure 3), while at lower temperatures, 48 h seems to be the maximal survival time (based on mean survival) with Optixcell generally performing better than Tris. However, when the survival of sperm of individual bulls (rather than mean survival) is examined, some rather different conclusions might be drawn. Three bulls (14755, 14836, 14841 and 14869) had >50% sperm survival at 120 h at 8$^{o}$C and 8 bulls had >50% survival at 72 h. Interestingly, also, although Optixcell had the best overall performance, based upon the *mean* survival of sperm, most of the bulls that achieved survival at 72 or 120 h did so in the Tris diluent. The number of such bulls in the present study is, of course, small, and generalisation on larger numbers of bulls and more repeats per bull would be necessary to establish this as a trait that could be exploited. Nonetheless, if repeatable, these results might mean that because, unlike dairy bulls in developed countries, these Mpwapwa bulls are entirely unselected for semen quality and/or the ability of sperm to survive extension, there could be a possibility of selecting animals as AI sires on the basis of the ability of their sperm to survive for (e.g.) 72 h at 17$^{o}$C. Such considerations could be important when determining the feasibility of an ambient-temperature AI service.

However, regardless of whether some of the semen of some bulls survives for a little longer than others, it is important to determine whether it is feasible within the infrastructural capabilities of the Mpwapwa region of Tanzania, to introduce some form of temperature control for the diluted semen. Clearly, as reviewed in Chapter 2, the use of cryopreservation is not feasible, but more modest cooling might be. Basic refrigeration in the semen processing centre (i.e. to mitigate temperature effects after dilution) or in the distribution network (which could be as simple as ‘chilly bins’ and ice packs, or as complicated as vehicle-mounted mini-fridges) would probably be

sufficient to maintain temperatures at ~17$^{o}$C, at which temperature mean sperm survival at 48 h is well above a 40% threshold, and, depending on which diluent is used, close to or above a 50% one. Whether a combination of reducing temperatures to 8$^{o}$C and selecting bulls whose semen survives for 72-120 h, would confer additional advantages would be a matter for further exploration. Perhaps also how much of an effect a variable holding temperature has on sperm would be worthwhile to be considered as well for investigation.

From these findings, the possibility of using chilled semen alongside or instead of ambient temperature semen as part of the planned AI and fertility programme is worth considering, and provides some positive expectations due to its complementarity and practicability in the programme. Alternatively, a Caprogen diluent could be tried. Using that diluent, Vishwanath & Shannon (2000) generally maintained adequate fertility performance for between 2.5 and 3 days, with some ejaculates maintaining fertility for up to 96 h (Shannon & Curson, 1984; Vishwanath & Shannon, 1997; Vishwanath & Shannon, 2000; Raseona *et al.,* 2017; Murphy *et al.,* 2018a, b). Thus, either apart from, or as well as, the Optixcell and Tris diluents that were used in the current study, the Caprogen diluent could also be considered in the proposed AI programme (Vishwanath & Shannon, 1997, 2000). Although costlier and/or more difficult to produce than simple diluents such as Tris-egg yolk, it is substantially less expensive than cryopreservation, as it does not require the industrial infrastructure essential for producing liquid nitrogen. These benefits could improve the uptake of cattle AI in situations where cost and supply chain difficulties are presently prohibitive.

The dilution rate is dependent upon the volume and the number of sperm an insemination dose should have and, axiomatically, the volume and density of the initial ejaculate. Whilst, arguably, it is the quality of the sperm that matters the most and not the volume and number of sperm in an AI dose (Oliveira *et al.,* 2013; Yoon *et al.,* 2022), nonetheless, for ambient temperature preservation of fresh/liquid semen using Caprogen diluent, semen can be extended to as low as 5 x10$^{6}$ sperm/dose, whereas, for cryopreservation, semen needs to be diluted to (20-25 x10$^{6}$ sperm/dose) (80-100 x10$^{6}$ sperm/mL) (Shannon *et al.,* 1984). The sperm/insemination dose used in the current study was similar to that used by Shannon *et al.* (1984) for ambient preserved fresh/liquid semen using Caprogen diluent. In practice, of course, the constant dilution rate used in the present experiment would not be repeated for field use. In the early days of cattle AI, there

was much concern that the process of dilution *per se* caused damage to sperm (Salisbury *et al.*, 1978). This notion of a 'dilution effect' was largely discredited in subsequent practice (Cheng *et al.*, 1949; Garner *et al.*, 1997; Vera-Munoz *et al.*, 2009; Patil *et al.*, 2020), but there still seems to be a critical minimum dilution ratio that is needed for sperm to be exposed to adequate quantities for the components of the diluent. By analogy with sheep, in which semen for intra-cervical insemination commonly has very low dilution rates (Salamon *et al.*, 1987), the low dilution rate (1:1) used in the present study might have adversely affected the responses of the sperm at higher temperatures and/or longer durations of study. Alternatively, the sperm densities of the ejaculates used in the present study were relatively low (all <1000 $x10^6$/mL), so the opportunity for high dilution rates (and, hence, exposure to high concentrations of beneficial diluent components) was reduced. Even so, if sperm doses of 5 $x10^6$ were achievable, adequate dilution rates of even low density ejaculates of ~1:20 might be achievable: a figure that is commensurate with that used to good effect for cryopreserved ejaculates.

In order to put all of these together into practice, it needs a well-developed and supportive infrastructure to be in place within Mpwapwa district in the central zone and later on in the other zones in the country for upscaling. Improvements are needed in terms of accessibility and distribution. At present, the cryopreserved AI services of the country are limited to only a few places (Msalya *et al.*, 2017), where distribution and infrastructure are adequate. In addition to better infrastructure, additional appropriate expertise and sufficient AI technicians are also necessary to accommodate even the current demands for AI services. Further equipping of the TALIRI Mpwapwa animal biotechnology laboratory is essential in order to uplift the standard of the laboratory. Examples of such facilities are those related to technologies for performing emerging/advanced molecular technologies such as: tests for Ca2+ swimming, acrosome reaction/function (i.e. fluorescent probe techniques), spontaneous hypotonic-induced acrosome damage, reactive oxygen species (ROS), chromosome fragility/stickiness and semen extension. The inclusion of these tests/techniques in the TALIRI Mpwapwa animal biotechnology laboratory would enhance the results of bull testing and make the bull fertility evaluation process more robust. Enabling the laboratory to carry out a full range of tests on both bull and cow fertility at both structural and functional levels would significantly enhances the value of the support it can provide to the planned AI and fertility programme. Alongside the suggested improvements in infrastructure

and expertise these changes are likely to be key to the successful implementation and delivery of AI services within Mpwapwa district of the Dodoma region in Tanzania. Such changes will require support from the government and from development partners.

One key area which has not been addressed in his study is the high potential risk of transmission of infectious diseases through AI. Indeed, many of the provisions of the WOAH for the management of AI services are designed to prevent the spread of disease. These risks, whilst of critical importance to sustaining an AI service (MLD, 2006; MLFD, 2010), are beyond the scope of the present chapter (see Chapters 2 and 7).

## Conclusion

The present study has shown that Tris-egg yolk and Optixcell diluents, when used at a 1:1 dilution ratio can successfully sustain sperm survival for up to 48 h, and in some individuals for up to 72 or even 120 h. Coconut water, despite the promising positive results as shown in the literature, was not effective. Perhaps there might be some factors affecting the quality of coconut water in this regard. Thus, considering the cost benefit analysis of producing local *vs* imported diluents, it is worthwhile to consider conducting further studies on coconut water extenders as a cheap alternative diluent. Sperm survival was also dependent upon temperature, with survival declining in nearly linear manner from 8$^{o}$C to 32$^{o}$C. To manage these effects in an AI service based upon liquid (or ambient) temperature semen, a number of further possibilities could be considered; namely, (i) higher dilution rates; (ii) use of alternative diluents (notably Caprogen); (iii) making initial dilutions at a lower ambient temperature and (iv) provision of low-cost refrigeration/cooling in the distribution chain. Despite these caveats, the results of the present experiment show that simple diluents are capable of sustaining sperm survival for a sufficient period of time to be usable in a local AI service.

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# Chapter Six: Pregnancy rates from ambient and frozen semen in Mpwapwa cattle after synchronisation

## Introduction

The TALIRI Mpwapwa research farm has bred Mpwapwa cattle using frozen semen after oestrus synchronisation on the Mpwapwa cattle breeding programme over many different breeding seasons during the 1970s to 1990s. Recent outcomes of AI breeding have been less promising than those earlier results, due to the combined effects of poor oestrus management, intermittent availability of cryopreserved semen and inadequate numbers and competence of inseminators. Relatively little of the data about the insemination outcomes over this period has been formally published, but the results are documented in a range of internal reports and compilations (Mejool, 1977; Kyomo & Kifaro, 2005; Michael *et al.*, 2018). More recently, as part of testing the possibility of restarting the AI programme at TALIRI Mpwapwa, Kabuni *et al.* (2022) reported that 39% (39/100) of cows became pregnant after a $PGF_{2\alpha}$-based oestrus synchronisation (OS), with either one AI on observed oestrus or a single fixed-time artificial insemination (FTAI). In contrast, only 49% (49/100) of cows became pregnant after a 12-week natural mating period. This difference was not statistically significant despite the markedly increased opportunities to get pregnant in the naturally-mated group. Given that this was the first relatively large-scale FTAI programme that had been undertaken through TALIRI under its present management regime, the results were considered to be sufficiently successfully to warrant further application.

One of the most difficult problems for the (re)development of an AI programme based at TALIRI has been obtaining liquid nitrogen (LN) for the production and maintenance of cryopreserved semen. Cryopreserved semen not only requires substantial quantities of LN at the production/storage centre, but it also requires that LN is available and transportable to the point of insemination. Whilst this is feasible to some extent in the denser cattle populations of the dairy-farming areas of the country, consistent availability of LN in the beef-rearing areas is less easy to ensure, and therefore presents a significant limit to the redevelopment of an AI service for beef cattle. As a result, preservation of frozen semen becomes not only a key challenge in the TALIRI Mpwapwa breeding programme but also to smallholder farmers using AI services within the Mpwapwa district.

A potential solution to the problems caused by the lack of reliability of LN supply is to use alternative methods of semen extension and preservation. When AI programmes were first developed, they were based upon short-term semen preservation in chilled media, and, a little later, on slightly longer-term preservation in ambient media (Phillips, 1939; Salisbury *et al.*, 1978; Vishwanath & Shannon, 2000; Dziekonska & Partyka, 2023). These were later superseded by cryopreservation as LN became more widely available – particularly for cattle, whose semen is relatively resistant to the damaging effects of cryopreservation (Phillips, 1939; Bustani & Baiee, 2021). Even so, in species that tolerate cryopreservation less well (notably the pig and the horse), chilled/ambient temperature preservation is still the dominant method of AI, whilst, in cattle, countries such as New Zealand that have a very high peak demand for bovine semen during the short breeding season, the ambient temperature diluent, Caprogen (Shannon & Curson, 1984; Shannon *et al.*, 1984), has proved the basis for an effective bovine AI service.

In the previous chapter of this thesis, it was established that Optixcell diluent could maintain the motility of bovine semen at >50%, even at ambient temperatures as high as 32°C, for 48 h for many bulls, and, for some bulls, could do so for 72 h. In principle, such a period of survival at ambient temperature should be adequate for distribution to and insemination of the cows of smallholder farmers in the beef cattle-raising regions of Tanzania. However, survival of the semen *in vitro* is not, of course, evidence that it will achieve adequate pregnancy rates; this can only be done using an *in vivo* insemination trial. Therefore, the aim of this study was to determine whether the pregnancy rates resulting from the use of ambient-temperature semen produced from Mpwapwa bulls in an oestrus-synchronisation programme were high enough to justify the development of an ambient-temperature-semen based programme for local smallholder beef farmers.

## Materials and Methods

Prior to the start of the study, all animal-related measurements and manipulations received Livestock Research Ethical Clearance from the Tanzanian Livestock Research Institute (TALIRI) (12/02/2021). This study was undertaken through the TALIRI Mpwapwa research centre in the Dodoma region of Tanzania. The region is tropical in climate, with two breeding seasons March to May (at the end of the rainy season) and September to November (at the end of the dry season).

The research was undertaken using two cohorts of Mpwapwa breed cattle, with the first cohort being studied in April 2021 and the second cohort in April 2022.

## Animal selection

### *Bull selection*

All bulls came from the TALIRI Mpwapwa research herd (Figure 1). In both years there were 120 mature (≥2 years old) Mpwapwa breed bulls available for selection. In March 2021 and March 2022, the bulls were weighed and body condition scored (1-5 scale; Nicholson & Sayers, 1987) and the heaviest 50 bulls were selected. Four weeks later, the 50 selected bulls underwent a breeding soundness examination. This process evaluated scrotal circumference (SC), mating ability, structural soundness and semen quality (Barth, 2018). In each year, the six bulls with the largest SC (≥30 cm) were then selected from the bulls which passed the breeding soundness evaluation. From those six bulls, the two bulls with the highest ejaculate density and individual sperm motility (i.e. 80% and 896 sperm/mL for bull 14983 and 90% and 873 sperm/mL for bull 14901) were then chosen for semen collection for AI.

**Figure 1.** A group of Mpwapwa breed bulls at TALIRI Mpwapwa farm

### *Cow selection*

All cows came from the TALIRI Mpwapwa research herd (Figure 2). In 2021 and 2022, 200 and 300 mature (≥2 years old) Mpwapwa breed cows were available for selection (i.e. they were not being used for other research programmes), respectively. In March 2021 and March 2022, all available cows were pregnancy tested and weighed. Cows that were not pregnant and had no recorded history of previous reproductive problems were eligible for selection for the study. Study selection was based on body weight; in 2021, the 100 heaviest eligible cows were selected and in 2022, the heaviest 203 eligible cows were selected.

**Figure 2.** A group of Mpwapwa breed cows at TALIRI Mpwapwa farm

## Animal health management and pre-breeding nutrition

After the selection of the animals, cows and bulls were managed as follows:

Immediately after selection, animals were treated for internal parasites using either 0.5 mL/kg of a levamisole/oxyclozanide combination (Nilfarm, Farmers Centre – Tanzania) (lactating cows only) or 50 µg/kg ivermectin (Ivermectin, Anglian Nutrition Product – UK) (dry cows and bulls). For the four-week management period, all animals were given access to unrestricted grazing

(stocking rate of 0.36 livestock unit/ha) in twenty separate 15-ha paddocks (bulls were kept separate from cows) (Figure 3). The principal grass species in these paddocks were *Cenchrus ciliaris, Hyperrhenia rufa, Themeda spp, Cynodon dactylon* and *Chloris gayana*. In addition, animals were trough-fed 0.3 kg/cow/day and 0.6 kg/bull/day of a concentrate made from 600 g/kg maize bran, 390 g/kg sunflower seed cake, 10 g/kg salt, and had access to mineral lick blocks (Farmers Centres Ltd, Tanzania) at an allocation rate of 200 g/cow/week and 400 g/bull/week. Details of the mineral content of the blocks are in appendix 1. No mineral or metabolic profile testing was undertaken at any stage of the study. During this period, all selected animals were dipped once weekly in a bath containing 100 g/L of alphacypermethrin (Paranex, Farm base Ltd, Tanzania) for the control of ecto-parasites. Finally, in March 2021 and 2022, BCS was assessed and weight measured as the cows and bulls left the crush after one month of deworming, supplementation and grazing management.

**Figure 3.** A group of selected Mpwapwa breed bulls grazing in one of the paddock at TALIRI Mpwapwa farm

## Allocation of cows to treatment

In both breeding seasons, selected cows were ranked based on age, parity within age and ear tag number within parity within age. Cows were thereafter divided into four breeding groups (n=25 in 2021 and n=50/51 in 2022), with the first ranked cow going to Group 1, the second to Group 2 and so on. Each breeding group was assigned a separate grazing area (8 cows/ha).

## Oestrus synchronisation and AI protocol

In each breeding season (2021 and 2022), to simplify management, mating started in the four breeding groups at slightly asynchronous times (see Table 1). The same AI protocol was used in all groups in both years. On Day 0 (morning) each cow received 500 µg of cloprostenol (Estroplan, Parnell, Australia) followed by observation of behavioural oestrus for 96 h, aided by the use of one vasectomised teaser bull per group. These teaser bulls had all been successfully used for heat detection at TALIRI Mpwapwa in previous breeding seasons. Cows showing signs of oestrus (i.e. mounting others, mucus discharge or standing to be mounted) were marked with coloured crayons prior to insemination, with the timing based on the AM/PM rule (Trimberger, 1948). Cows that were recorded as not having shown signs of oestrus within 96 hours of the 1$^{st}$ cloprostenol injection, were re-treated with 500 µg of cloprostenol on Day 14. Seventy-two hours after this 2$^{nd}$ injection, all re-treated cows were inseminated (single insemination). The semen used depended on the group, with ambient temperature using (AT) semen being used for all inseminations in cows in Groups 1 and 2, and frozen (F) semen for all inseminations in cows in Groups 3 and 4.

## Semen processing, storage and handling for insemination

In each year, one selected bull was used to produce AT semen (bull 14983) and one to produce F semen (bull 14901).

### *Ambient temperature semen*

Semen was collected from the selected bull (14983) on Day 1 (afternoon) after the 1$^{st}$ cloprostenol injection and on day 15 (afternoon), i.e. ~32 – hours after the 2$^{nd}$ cloprostenol injection. Semen was collected, using electro-ejaculation (Ejakulator, Minitube, Tiefenbach, Germany), into a hand-mounted semen collection cone (Minitube, Tiefenbach, Germany). The semen was then visually checked for volume and colour, with motility and morphology evaluated microscopically

(MBL2000 Kruss Optronic GmbH, Germany) at x100 magnification (motility) and 1000x magnification (morphology) (Barth, 2018). Concentration was determined using a previously calibrated spectrophotometer (Accuread Photometer, Biochrom Ltd, USA). The collected semen was then extended (1:1) using Optixcell diluent (IMV, L'Aigle, France), and packed and sealed into 0.25 mL Minitube 'straws' (Minitube, Tiefenbach, Germany) and placed into a water bath at 20°C. AT semen straws were stored in this water bath at 20°C for up to two days (i.e. 48 h) before use.

### *Frozen semen*

Semen was collected from the selected bull (14901) on Day 1 (afternoon) after the 1$^{st}$ cloprostenol injection only. The collection, evaluation and extension were the same as used for the AT semen, except that the diluent also contained 7%v/v glycerol. Once diluted, semen was loaded and sealed into 0.25 mL straws at a temperature of 4°C checked using a calibrated thermometer. Semen straws were then loaded onto freezing racks at a temperature of 4°C. One third of a 35 L liquid nitrogen (LN) container was then filled with LN and the container was allowed to equilibrate with the added LN. Thereafter the semen was frozen in the LN vapour at a temperature of -125°C for 10 minutes, after which the straws were plunged into LN at a temperature of -196°C. They were then loaded into goblets and stored in a LN container.

### *Insemination of the cows*

Firstly, cows were loaded in a crush and prepared for artificial insemination. For the ambient temperature semen, a straw was picked up from a water bath, then wiped with tissue paper, cut and loaded into an AI catheter, followed immediately by insemination of the cow. For the frozen semen, a straw was picked up from the LN container and thawed in a water bath maintained at 35°C for 2 minutes, then wiped with tissue paper, cut and loaded into an AI gun followed by insemination.

**Table 1.** Mating plan for selected cows for the two breeding seasons (n = 25 per group in 2021 and 50/51 - per group in 2022)

| **Mating groups** | **Type of Semen** | **1st $PGF_{2\alpha}$ injection** | **Oestrus detection/AI** | **2nd $PGF_{2\alpha}$ injection** | **FTAI** | **Transrectal US PD** | **Bull mating** | **Transrectal US PD** |
|---|---|---|---|---|---|---|---|---|
| 1 | AT | 01/04/21*22 | 3-6/04/21*22 | 14/04/21*22 | 17/04/21*22 | 6-10/07/2021 & 6 -15/07/2022 | 28/07/21*22 | 29-31/10/2021 & 29/10-02/11/2022 |
| 2 | AT | 07/04/21*22 | 9-12/04/21*22 | 20/04/21*22 | 23/04/21*22 | | | |
| 3 | F | 13/04/21*22 | 15-18/4/21*22 | 26/04/21*22 | 29/04/21*22 | | | |
| 4 | F | 19/04/21*22 | 21-24/4/21*22 | 02/05/21*22 | 05/05/21*22 | | | |

*AT: ambient temperature semen, F: frozen semen, AI: artificial insemination, FTAI: fixed-time artificial insemination, US: ultrasound PD: pregnancy diagnosis.*

### *Management after AI*

Two months after the second round of artificial insemination, all cows were tested for pregnancy using transrectal ultrasound (Chison Medical Imaging Co, Jiangsu, China). Non-pregnant cows were then grouped together and then separated into different three paddocks (i.e. 24/25 cows/ paddock of 3 ha) with each paddock allocated one bull on a ratio of 1:24 and 1:25 for breeding seasons 1 and 2, respectively. These bulls were kept with the cows for 60 days. Approximately 30 days after end of that period of natural mating, transrectal pregnancy diagnosis was conducted. The number of non-pregnant cows was recorded.

### *Statistical analysis*

For comparison between proportions, relative risk (RR) was calculated with confidence intervals calculated as per Gardner & Altman (1986).

## Results

The overall conception rates achieved by AI over the two years of study were 62% with ambient-temperature (AT) semen (94/151) and 38% with frozen (F)/cryopreserved semen (58/152) (Table 2). The relative risk of conception to AT semen was 1.63 (95%CI 1.27 - 2.1) times that of F semen. At the conclusion of the 60 days of bull mating after the FTAI, the final pregnancy rates (PR) were 96% with AT semen (145/151) and 92% with F semen (140/152). The relative risk of pregnancy over the whole breeding season was 1.04 (95% CI 0.98 -1.1).

**Table 2.** Pregnancy rates to AI in Mpwapwa cows after oestrus synchronisation and FTAI with AT or F semen

| Breeding Season | Semen | | | |
|---|---|---|---|---|
| | AT | | F | |
| | Cows pregnant to AI (%) | Cows pregnant at end of breeding season (%) | Cows pregnant to AI (%) | Cows pregnant at end of breeding season (%) |
| Year 1 (2021) | 30 (60) | 45 (90) | 22 (44) | 41 (82) |
| Year 2 (2022) | 64 (63) | 100 (99) | 36 (35) | 99 (97) |
| Total | 94 (62) | 145 (96) | 58 (38) | 140 (92) |

*AT: ambient temperature semen, F: frozen semen*

Across both years, the proportion of Mpwapwa cows responding to first $PGF_{2\alpha}$ was the same between the two groups (see Table 3), with, in both groups, ~⅓ of cattle responding to first $PGF_{2\alpha}$; RR for responding to first $PGF_{2\alpha}$ in AT cows vs F cows was 1.03 (95%CI 0.75 to 1.41). Over both years, 73% of AT cattle became pregnant following the AI after the first $PGF_{2\alpha}$, while only 20% of cattle given F semen became pregnant (RR 3.7; 95%CI 2.07 to 6.61). The equivalent figures after the second $PGF_{2\alpha}$ injection were 57% and 47%, respectively (RR 1.21; 95%CI 0.92 to 1.59).

**Table 3**: Comparison between AT-semen-inseminated and F-semen-inseminated cattle in: i) proportion responding to 1st $PGF_{2\alpha}$ injection and ii) proportion getting pregnant to inseminations after first and second $PGF_{2\alpha}$ injection.

| | | Year 1 | | Year 2 | | TOTAL | |
|---|---|---|---|---|---|---|---|
| | | AI | Pregnant | AI | Pregnant | AI | Pregnant |
| 1st $PGF_{2\alpha}$ injection | AT | 21 | 15 | 30 | 22 | 51 | 37 |
| | F | 18 | 5 | 32 | 5 | 50 | 10 |
| 2nd $PGF_{2\alpha}$ injection | AT | 29 | 15 | 71 | 42 | 100 | 57 |
| | F | 32 | 17 | 70 | 31 | 102 | 48 |

*AT: ambient temperature semen, F: frozen semen*

## Discussion

Over the two years of the study, the overall conception rate (CR) to the ambient temperature semen was 62% (60% in year 1 and 63% in year 2). If similar results could be obtained as part of an AI service, these would be very good outcomes. As far as the authors are aware there are no similar studies reporting conception rates with AT-semen after prostaglandin injections in zebu-type cattle in East Africa. However, similar outcomes (55 to 60%) have been reported in European cattle being managed under African conditions inseminated with AT-semen to a natural oestrus (El-Wishy *et al.*, 1976), while similar results have been reported in South American *Bos indicus* inseminated with chilled semen after synchronization with progesterone and oestradiol (61%; Cresphilo *et al.*, 2012) and in cycling dairy cattle in New Zealand synchronized using a single $PGF_{2\alpha}$ injection (58%; Agrihealth, 2019). These results suggest that the results achieved using AT-semen in this study may be routinely achievable but larger scale studies are needed to better

characterise the range of conception rates that are likely to be encountered in the Mpwapwa region when using AT-semen.

The overall conception rate in the cattle inseminated using the F-semen was 38% (44% year 1, 35% year 2). This result is consistent with the previous studies conducted at the TALIRI Mpwapwa research centre. In particular, it is consistent with the 39% conception rate achieved by the author after a double $PGF_{2\alpha}$ injection protocol (Kabuni *et al.,* 2022). However, it is lower than the 55% reported by Kabuni *et al.* (2023) after a double $PGF_{2\alpha}$ injection protocol, in 200 mixed breed zebu/zebu cross cattle owned by smallholders in villages close to TALIRI Mpwapwa which were grazed on unrestricted grazing on communally owned grazing land. So as for AT-semen we need more data on the range of conception rates likely to be associated with F-semen in cattle in Mpwapwa, and we need more understanding of the factors that drive differences in conception rates.

Across the two years of the study, oestrus behaviour was observed in 101 of the 303 cows (33%) after the first $PGF_{2\alpha}$ injection and were inseminated. The response in the cows in the AT-semen groups was very similar to that in F-semen groups (34 and 33%, respectively). These results are much lower than the 55% reported by Kabuni *et al.* (2023) in smallholder-owned cattle in the Mpwapwa region but much higher than the 10% reported by Kabuni *et al.* (2022) in Mpwapwa cattle at TALIRI Mpwapwa. The range of these results strongly suggests that we need better information on the factors driving the response (in terms of oestrus behaviour) to the first $PGF_{2\alpha}$ injection in cattle in the Mpwapwa region as these may be related to the success of the program.

For the AT-semen, conception rates across the two years were higher for the cows inseminated after showing an oestrus response to the first $PGF_{2\alpha}$ than for those inseminated using FTAI after the second $PGF_{2\alpha}$ (73 *vs* 57%, respectively), whereas for F-semen this was reversed (20% after first $PGF_{2\alpha}$ *vs* 47% after second $PGF_{2\alpha}$). This apparent contrast is consistent with the published data which has shown that conception rates for timed AI protocols after $PGF_{2\alpha}$ can be better, worse or equal to conception rates after $PGF_{2\alpha}$-induced oestrus (Stevenson *et al.,* 1987). We are not aware of any study directly comparing AT-semen to F-semen after a two-stage $PGF_{2\alpha}$ protocol, but the data from the current study suggest that a high proportion of the better conception rate after AT-semen in this study was due to the better conception rate after the first $PGF_{2\alpha}$ injection.

However, study design means the differences between the results from AT-semen and F-semen may not be solely due to the type of semen used for insemination. Firstly, cows were assigned to four groups and within each group only one semen type was used. Cows were put into groups for ease of management (as had been done for Kabuni *et al.*, 2022, 2023). Staff availability and level of facilities meant that it was not thought practical to randomly assign cows within a group to F- or AT-semen. To simplify the process, AT-semen was used in the first two groups in both years. Secondly, one bull was used for AT-semen and one bull for F-semen, with the higher tag number bull (14983) being used for AT-semen production in both years. This made the process simpler and cheaper. This simplification of the process made the study achievable, but meant that the effect of semen type was confounded by group and bull. Nonetheless, we have data that suggest that the cow groups and bulls were comparable. Firstly, in relation to the cow groups, across both years fertility seemed comparable across all cow groups (similar rates of observed oestrus after first $PGF_{2\alpha}$ and similar final overall pregnancy rates). Secondly, the two bulls chosen were the bulls with the highest ejaculate density (i.e. 896 $x10^6$ sperm/mL for bull 14983 and 873 $x10^6$ sperm/mL for bull 14901), and both had high proportion of actively motile sperm (80% for bull 14983 and 90% for bull 14901). That both the bulls had similar levels of fertility is shown by our finding, across both years, that the conception rate of cows inseminated after the second $PGF_{2\alpha}$ injection was similar for both bulls (57% and 47% for AT-semen and F-semen, respectively). Thus, despite the confounding, we believe that the results of this study do support the conclusion that, in Mpwapwa cattle, the use of AT-semen is likely to result in conception rates that are as good as, if not better than, F-semen.

Comparison between the steps involved in the production, storage and usage of AT and F-semen is also important when determining the choice of semen type to be used in a potential TALIRI Mpwapwa animal AI service for smallholder farmers. The process prior to the development of the AI straws is the same irrespective of semen type requiring a comprehensive BSE which includes confirmation of semen quality. Development of the semen straws is very similar with both AT and F-semen being extended/diluted before being packed into straws. In this study both F- and AT-semen were diluted 1:1 with the key difference being in the diluent which was Optixcell for AT-semen and Optixcell +7%v/v glycerol for F-semen. The principal difference was that AT-semen was stored at ambient temperature in a water bath for 2-3 days, whereas F-semen was frozen using LN and maintained in a LN container. Prior to usage, F-semen needed to be thawed before being

taken to the AI site in an ambient temperature semen kit, while AT-semen could be placed directly into the kit. These differences resulted in estimated costs per dose for AT-semen of approximately 20 USD (i.e. 50,000 TZS) and for F-semen of approximately 40 USD (i.e. 100,000 TZS). These costs are based on the use of equivalent 1:1 dilution for both AT- and F-semen, when one key advantage of AT- over F-semen is the higher dilutions that can be used with AT-semen (Arif *et al.,* 2020), further increasing its cost advantage. Thus overall AT-semen is cheaper and easier to produce and use than F-semen, even before we include the advantage of not having to rely on a consistent supply of LN. In Tanzania, inconsistent LN supply has been a major brake on the use of AI, especially in non-dairy regions (MLFD, 2011b; Katjiuongua & Nelgen, 2014; Ogutu *et al.,* 2014). Thus developing an AT-semen program at TALIRI Mpwapwa may result in the development of a model which is suitable for other centres across Tanzania which do not have access to LN.

The principal issue with AT-semen is that it is not long lasting. Our data from sperm survival studies (chapter 5 in this thesis) suggest that survival for 48 hours is achievable for most bulls, (although some bulls may last 72 hours or more). This means that if AT-semen is to be used it needs to be linked, at least at the start, to mass AI after synchronisation rather than to AI after individual heat detection. Interestingly, in a survey of local smallholder cattle owners, of those owners who expressed a preference, 69% stated that if they were going to use AI they preferred using a mass AI approach. This begs the question: If we are going to use synchronisation, what synchronisation program are we going to use? In this study, as in similar studies in the Mpwapwa region we have used a double $PGF_{2\alpha}$ system. Compared to other protocols which involve more than one hormone (e.g. Lean *et al.,* 2003; Kesler, 2005), the principal benefits of $PGF_{2\alpha}$ synchronization for FTAI are: i) it is effective and cheap (i.e. we get reasonable conception rates to FTAI in *B. indicus* cattle combined with a lower drug cost than for other FTAI synchronisation programs), and ii) it is simple to schedule and implement (as the timing of the second $PGF_{2\alpha}$ injection is flexible). The high conception rates with AT-semen and a double $PGF_{2\alpha}$ protocol, strongly support the use of the double $PGF_{2\alpha}$ protocol within the context of the proposed AI and fertility programme at TALIRI Mpwapwa.

Nevertheless, in order for such a programme to be successful we need to optimise the response to $PGF_{2\alpha}$. The most important factor determining the response to such a program is optimising body

condition score (BCS) and animal health. In this study cows underwent a 4-week preparation period where cows underwent treatment for ecto- and endoparasites and were fed good quality pasture and supplementary feed. This clearly improved BCS and thus is likely to have improved pregnancy rates. For example, Cooke *et al.* (2021) reported in a mixed FTAI/natural mating system better calving rates in B. indicus beef with a BCS ≥5.0 than with BCS <5.0 (83.6% *vs* 73.3%, respectively). In addition, cows calved earlier and weaned more calves. It is crucial that, if an FTAI-based programme is introduced into the Mpwapwa district, it is not used as a substitute for inappropriate nutrition, poor quality herd health programs, or as a support for poor management (Barros *et al.,* 2000; Bó & Baruselli, 2014; Bihon *et al.,* 2021). In fact, introducing nutrition, fertility and herd health plans is likely to be a critical part of any successful AI program in the district.

## Conclusion

The conception rates seen in the current study after AI with AT-semen in cows synchronised using a double $PGF_{2\alpha}$ protocol were consistently high across both years and consistent with results from other studies. If these results can be consistently obtained on Mpwapwa farms, this protocol could be a significant addition to Tanzanian beef cattle breeding programmes. Being relatively cheap and simple to schedule makes the programme accessible and implementable by the relatively poor semi-commercial smallholder farmers of the Mpwapwa district. Nevertheless, in order for such a programme to be truly successful we need to optimise the response to $PGF_{2\alpha}$ particularly by optimising animals' body condition score (BCS) and health status. Last but not least, we need further improvement to bull fertility evaluation procedures and semen production protocols before starting disseminating this service at farm-level to smallholder farmers.

## Appendix 1

Mineral composition per kg

| Minerals | Contents/mg |
|---|---|
| Calcium | 500 |
| Zinc | 300 |
| Copper | 300 |
| Manganese | 200 |
| Iodine | 200 |
| Potassium | 200 |
| Iron | 160 |
| Colbat | 50 |
| Selenium | 20 |

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# Chapter Seven: General Discussion

## *General overview of the research project*

The over-arching aim of this thesis has been to facilitate continued genetic improvement in Mpwapwa cattle so that they become the cattle breed of choice for small and medium-scale farmers in Tanzania who want a dual purpose (i.e. milk and meat) cattle breed. Developing a feasible AI programme is seen as a key measure to achieving this goal; using semen produced from the best genetic merit Mpwapwa bulls, for a low-cost AI service for small-holder farmers. To develop such a service would probably also require the use of oestrus synchronization programmes that are cost-effective for smallholder famers; particularly to circumvent the inefficiencies of oestrus detection in very small herds. Developing such a programme has been the focus of this thesis.

The thesis addressed the following objectives:

i. To understand smallholder farmers' knowledge, attitudes and practices regarding to fertility and AI programmes
ii. To understand the fertility of Mpwapwa breed bulls and the potential for their use in a local AI scheme
iii. To evaluate the survival of sperm from Mpwapwa bulls in simple ambient-temperature diluents, as the basis for a low-cost, ambient-temperature AI service
iv. To evaluate the fertility outcomes of ambient-temperature AI after a prostaglandin-based oestrus synchronisation protocol in Mpwapwa cows on smallholders' farms.

The breeding soundness and semen evaluations (Chapter 4) investigated, for the first time, the breeding potential of Mpwapwa breed bulls. The studies showed that at least some of the bulls had breeding and semen characteristics that would be suitable for use in an AI breeding programme. The work in Chapter 4 requires extension in several areas before definitive judgements can be made regarding the suitability of individual bulls as AI semen donors. Firstly, it would be preferable to conduct repeated electro-ejaculation tests on each bull prior deciding on which bulls to select for use in the AI programme, given the significant impact on semen density, volume and ejaculate appearance that electro-ejaculation can have (i.e. hence, the lack of precision that single electro-ejaculation collections can have).

Further, there is the possibility of extending the numbers of bulls that are evaluated, and the duration over which they are evaluated. For example, the best bulls that were identified from the current regime could be included in with the entire recruited cohort of 50 bulls; after which the entire cohort could be examined for effects of age, semen dilution ratio, storage time, temperature and diluents on sperm motility before utilisation in an AI programme. Moreover, as additional bulls become sexually mature year by year, these animals could be routinely evaluated for breeding soundness and semen quality. Recruiting a cohort of 50 bulls would generate more data to facilitate the selection process and improve fertility outcomes. Repeated semen collections could enable bulls with poor semen evaluation outcomes to be culled from the programme. There are two aspects to be considered in this: firstly, some bulls were identified as having poor to moderate semen quality and/or inadequate scrotal circumference. Bulls with poor semen quality are unlikely to achieve adequate results even as natural-service sires (Chenoweth *et al.,* 1992, Chenoweth, 2000, Chenoweth & McPherson, 2016, Koziol & Armstrong, 2018), so would thus be unsuitable for use as AI sires. Secondly, to be an effective AI sire, semen needs to be collected regularly and often. Bulls have to have adequate sperm-production capacity to enable this. Scrotal circumference is a useful guideline in this regard (as is the maintenance of turgor in the cauda epididymis) (Chenoweth, 2000, Parkinson, 2004), but AI practitioners recognise that the ability to maintain an appropriate semen collection regime can only be fully characterised by the process of repeated collections (Chenoweth, 2000, Graham, 2001, Moce & Graham, 2008). This would be an important practice for consideration when producing semen from Mpwapwa breed bulls in the planned AI programme.

Thus, the findings of Chapter 4 have shown that the majority of the BSE-tested Mpwapwa bulls produced semen that would be of satisfactory quality for natural-service sires, and that a proportion of them produced semen that met established criteria for use in an AI service. It is, however, important to recognise that the minima that are established in the literature for (e.g.) semen quality and scrotal circumference for use in improved/selected strains of *B. indicus* bulls in an AI programme (Troconiz *et al.,* 1991; Brito *et al.,* 2004a, b; Menegassi *et al.,* 2019) would need to be reconsidered for the smaller-framed breeds of East Africa and South East Asia. In other words, the present study on Mpwapwa bulls represents perhaps the first phase in establishing the criteria for judging the suitability of such small-framed breeds in local AI programmes. It should also be noted that most of the bulls in the present study were between 24 and 36 months of age. No data exist as

yet to show when the scrotal circumference of Mpwapwa bulls reaches its plateau: however, by analogy with *B. taurus* beef breeds (Barth & Ominski, 2000), it is unlikely to occur before 48 months of age. For the *B. indicus* breeds and indicus-derived bulls farmed on native vegetation in arid areas, a SC of ≥28 cm is recommended for a 2-year-old bull (McGowan *et al.,* 2002; Menegassi *et al.,* 2019). Nonetheless, these authors also pointed out that < 3% of these bulls fail to meet this target, while the SC for those bulls who met the target continues to increase in size after puberty reaching 34 cm when they are 3+ years of age. Characterising the growth trajectory of the testes of Mpwapwa bulls will therefore, be needed as an extension of the present studies: more importantly, as bulls mature, their capacity for producing sperm (in terms of number per ejaculate, morphological normality and capacity for repeated ejaculation) increases, so (potentially at least) selection of bulls for both semen quality and conception rate might be feasible.

Finally, the method of collection of semen would probably require revision to be sustainable for long-term collection from a few bulls. Electro-ejaculation is satisfactory as a diagnostic tool and even for collections for AI over the short term. However, electro-ejaculation is not suitable for frequent collections over a long period, as it becomes poorly tolerated by the bulls. Most AI centres train their bulls for collection into an artificial vagina (AV). Apart from the welfare of the bulls, AV collections have the advantage of harvesting better ejaculates which are of more consistent quality (Chenoweth *et al.*, 1992, Chenoweth, 2000, Mulu *et al.*, 2018), thereby increasing the number of sperm harvested (Mulu *et al.*, 2018) (and, therefore, the number of insemination doses that can be produced). Training bulls for AV use is best started when animals are yearlings/two year-olds so that they are used to being handled/restrained and are used to the process of AV collection itself. Suitable decoy animals have to be provided and, in the context of an AI centre, there is a consensus that large, halter-trained steers are ideal. Females are not ideal, as (i) they only stand for mounting when they are in oestrus and (ii) there is a risk of spread of venereal disease that cannot occur with a steer (Givens, 2018). Thus, training bulls to the AV method could be considered as a next step when collecting semen from Mpwapwa bulls, as this would improve both the quality of the semen and the welfare of the bulls in the programme.

Thus, the conclusion to Chapter 4 is that some bulls in the present study meet quality standards to achieve expected fertility outcomes in a local AI programme: recognising, of course, that the relationship between BSE and conception rate is intrinsically relatively weak, so that a means of field evaluation of pregnancy rates would have to be developed as the final arbiter of the suitability

of individual sires in the programme. Some evidence for this case is derived from Chapter 6, which showed that ambient-temperature semen from the selected Mpwapwa bulls in the present study gives fertility outcomes that were at least as good as those derived from commercially-produced semen from selected sires. Indeed, the conception rates achieved in Chapter 6 were at least commensurate with some of the global studies of the fertility of *B. indicus* bulls in AI programmes (Bó *et al.,* 2013*;* Bó *et al.,* 2018).

Chapter 5 considered the use of ambient-temperature diluents as a means of preserving sperm viability for the period of time required for a local AI service. Overarching this focus on ambient-temperature diluents is the need to circumvent the constraints imposed by the use of cryopreserved semen. Most critically, cryopreserved semen requires abundant and secure availability of liquid nitrogen. The availability of liquid nitrogen in Tanzania is neither abundant nor secure, nor is it cheap. Further, cryopreserved semen has to be maintained in liquid nitrogen to the point of insemination; which means that as well as the central semen-production facility having access to liquid nitrogen, it also has to be available to any distribution depots and to the vehicles of the inseminators. This, in turn, means that inseminators have to have 4-wheeled vehicles, with a separate storage area for the nitrogen containers, and have to have access (at least daily) to a central nitrogen store. None of these requirements are feasible in the smallholder farming zones of the Mpwapwa district. Consequently, if a low-cost AI programme is to be made available to these farmers, it has to use cheaper technology than that needed for cryopreserved semen. The use of non-frozen (chilled or ambient temperature) semen has, of course, been practised for a very long time and there is extensive literature on its use (e.g. Warnick, 1998; Lonergan, 2018).

Nonetheless, most descriptions of the use of ambient temperature diluents come from temperate climates (Salisbury, 1957; Shannon, 1968; Salisbury *et al.,* 1978; Shannon & Curson, 1984), and reports of their use in the very high ambient temperatures that pertain in Tanzania are limited. Further considerations of cost also dictate that it would be ideal if an ambient temperature diluent such as Coconut water and Caprogen could be locally manufactured from locally available materials. Finally, the use of chilled semen was considered, but the use of ambient temperature diluents was preferred, mainly due to the logistical and infrastructural requirements to maintain a cold chain from production centre to the point of insemination. It was for these reasons that the survival of semen in ambient temperature diluents, in the climatic conditions that pertain in Tanzania, was examined.

The key results from the present experiment were that adequate semen quality was maintained for most bulls for 48 hours in two diluents (Tris-egg yolk and Optixcell, particularly the latter), but not in Coconut water. In both Tris and Optixcell, the semen of a few bulls survived to 72 hours or more, indicating the potential for selection on this basis.

Adequate sperm motility results were therefore maintained until 48 hours of storage. This was somewhat less than the 72 hours that was anticipated and, clearly, maintenance of viability for only 48 hours imposes greater constraints on the distribution system than would its maintenance for 72 hours. When Vishwanath & Shannon (1997) reviewed the use of ambient-temperature semen, they suggested the optimum preservation period for the semen should be between 72-120 hours (i.e. 3-5 days); which, in effect, allows at least one extra day for distribution from the production centre to remote centres.

Optixcell and Tris were used in the current study since they are commonly used by the Tanzania national artificial insemination centre as the primary diluents for production of frozen semen. Coconut water was also used as earlier reports have shown good preservation (Sawitri *et al.,* 2021; Odrada *et al.,* 2023), and it is abundantly locally available. Despite these encouraging results in the literature for coconut water, in the present study it failed to support sperm viability beyond ~24 hours, so it is not discussed further. Optixcell, Tris-egg yolk and similar diluents have been evaluated widely in temperate climates, where they have generally supported viability to ~72 hours. Interestingly, however, Murphy *et al.* (2018a) found very similar survival of *B. taurus* semen diluted in Optixcell to that of the present study, with viability declining from ~60% on Day 0 to ~25% on Day 3. That study maintained the semen at 18$^{o}$C, rather lower than the ambient temperatures in the present study. Thus, even in ambient temperature diluents, the decline in sperm survival is greater at higher than lower temperatures, especially when temperatures exceed 25$^{o}$C (Vishwanath and Shannon, 1997). Whilst Optixcell is well-established as a diluent for bovine semen, including for cryopreservation, ambient or chilled preservation (Ansari *et al.,* 2017; Elamurugan *et al.,* 2023) it has not been established for how long it can maintain sperm viability at the ambient temperatures that pertain in Tanzania.

The main alternative to these ambient temperature diluents that could have been used in the present experiments is Caprogen. Optixcell and similar diluents pose less biosecurity risk than egg yolk-based diluents, as they replace the 'natural' components with defined media that provide similar

protection to the sperm (Luna-Orozco *et al.,* 2018, Murphy *et al.,* 2018b). Many ambient temperature diluents further reduce sperm metabolism by making the medium hypoxic (Bustani & Baiee, 2021). Adding $CO_2$ or its precursors to the medium is a well-tested method for creating ambient-temperature/chilled diluents (as originally described for the IVT: see Vishwanath & Shannon, 2000), but is not used in either the Tris-egg yolk or Optixcell diluents. Caprogen reduces the availability of oxygen by displacing it with nitrogen gas. Studies of Caprogen have shown longer-term maintenance of sperm viability (i.e. 72-120 hours) than was achieved in the present study. The period for optimal insemination with that diluent (i.e. 2.5 and 3 days: Shannon & Curson (1984), Vishwanath & Shannon (1997, 2000), Raseona *et al.* (2017) and Murphy *et al.* (2018a)) would also likely circumvent many of the logistical difficulties that the 48 hours' usable period for semen in the present study would create. Interestingly, Murphy *et al.* (2018a) found the performance of Caprogen at ambient temperatures of ~18$^{o}$C to be better than that of either Optixcell or Tris-egg yolk. For this reason, Caprogen diluent could well be considered in the planned AI and fertility programme due to its good performance in preserving fresh/liquid semen at ambient temperatures (Vishwanath & Shannon, 1997; 2000); although noting that its performance at the high ambient temperatures of Tanzania has not been well established. Nonetheless, it is also less expensive than cryopreservation, as it does not require the industrial infrastructure essential for producing liquid nitrogen; and hence there is potential for simplification and cost reduction in the supply chain between production and insemination. These benefits could improve the uptake of cattle AI in situations where cost and supply chain difficulties are presently prohibitive. However, it is important to undertake a cost benefit analysis of the improved AI outcomes against the extra cost of the more expensive diluents.

Apart from the choice of the diluents to be involved in the planned AI and fertility programme, dilution rate is another factor which has to be considered. The dilution rate is dependent upon the ejaculate volume/density and the number of sperm required for an insemination dose. Dilution rate may also take into account semen-quality assessments (Oliveira *et al.,* 2013; Yoon *et al.,* 2022), although, in practice, most ejaculates that meet minimal density, motility and morphology are used without further modification of dilution rate. For ambient temperature preservation of fresh/liquid semen using the Caprogen diluent, semen can be extended to as low as 5 x$10^6$ sperm/dose, whereas, for cryopreservation, semen can only be diluted to 80-100 x$10^6$ sperm/mL (20-25 x$10^6$ sperm/insemination dose) (Shannon *et al.,* 1984). The number of sperm/ insemination dose used in the current study (and in Chapter 6) was similar to that described by Shannon *et al.* (1984) for

ambient preserved fresh/liquid semen using Caprogen diluent. However, a comparison would have to be drawn between Optixcell *vs* Tris and Caprogen *vs* Optixcell for their suitability in terms of their logistics and availability into the proposed AI and fertility programme. Of particular interest would be to determine how effective these diluents are at temperatures of ≥20ºC. The ambient temperatures investigated in the present study were 8°C, 17°C, 20°C, 27°C and 33°C. Of these, 8°C unsurprisingly produced the best sperm survival performance, followed in descending order by 17°C, 20°C, 27°C and 33°C for the 72 hours, at least in Optixcell and Tris. In other words, optimal sperm survival performance was observed between 8°C-17°C for Optixcell and Tris for the first 48 hours, with performance declining thereafter, or at higher temperatures of 20°C-33°C.

From these findings, the possibility of using chilled semen alongside ambient temperature semen into the proposed AI and fertility programme has to be considered, and this could give some positive expectations due to its complementarity and practicability in the programme: previous studies have shown that ambient temperature semen can be stored between 10°C-25°C and chilled semen at 4°C-5°C and still produce acceptable performance (Vishwanath & Shannon, 1997; Verberckmoes *et al.,* 2004; Arif *et al.,* 2020).

Chapter 3 therefore examined the perceptions, understanding and desires of smallholder farmers towards the planned AI and fertility programme. Briefly, the farmers were interested in using AI, although relatively few of them had done so. Farmers seemed to have greater faith in AI as a part of controlled breeding programmes (i.e. after PGF synchronisation) than for *ad hoc* use with detected oestrus. Further, despite smallholder farmers' interest in adopting and utilising AI technology into their breeding programmes, substantial preparations (e.g. nutrition, health and fertility plans) would need to be done prior to its uptake. Currently, the AI services are partially accessible and mainly constrained by high delivery costs and centralisation of AI services in the northern zone of the country. This could be overcome once fresh/liquid semen AI from the TALIRI Mpwapwa AI facility is introduced into smallholder farmers' breeding programmes. It would, of course, require effective and efficient AI- related infrastructure and expertise to deliver AI services in the region. Nonetheless, management practices appeared to be a challenge to some of the smallholder farmers. Thus, there was little consistency in reproductive management (e.g. breeding/calving seasons), and there was much variation in such routine management practices as feeding, drenching and dipping. Of greater concern, however, was the dearth of effective record-keeping. A majority of the farmers who were

interviewed did not have any useable records of calving dates, breeding dates and breeding weights. Clearly, for effective adoption and efficient performance of the AI technology, improvement is needed in management practices in the smallholder farmers' production systems. This would not only improve both the performance of the AI technology but also the productivity of beef cattle farming in the district and the entire central zone of the country.

Given that the respondents in Chapter 3 had identified mass-breeding as their preferred use of AI, Chapter 6 therefore examined the outcomes of the use of ambient temperature semen in a controlled breeding programme (i.e. after PGF synchronization). The results of this chapter extended the work previously reported by the Author, in which cows were inseminated (frozen semen) to detected oestrus or after PGF synchronization (Kabuni, 2017). The earlier study showed that pregnancy rates were similar after an 11-day PGF synchronization protocol to those achieved after a 12-week mating period of natural mating. The present study showed that the pregnancy rates achieved over the two years by AI with ambient-temperature semen (94/151: 62%) were at least as good as those with cryopreserved semen (58/152: 38%). Taken together, these two studies demonstrate the feasibility of PGF synchronization and fixed time AI with ambient temperature semen. These results, although promising, also underscore the need for general improvements in management processes: of which, breeding at the optimal time(s) of year and overall improvement in nutritional management are likely to be key.

The introduction of the proposed AI and fertility programme to the smallholder farmers in the Mpwapwa district could revive the previous open-nucleus breeding scheme which initially operated using Mpwapwa breed bulls. This would replace smallholder farmers' current breeding practices of using unproved and untested homebred bulls with no specific breeding programme. Regardless of the fact that most smallholder farmers preferred natural service sires over AI, their target breeding goal would be to have one calf per year. However, these expectations are yet to be realised due to the lack of sound breeding programmes. In the present narrative, the outcome of this research project would provide a way forward which would enable smallholder farmers to have a sound breeding programme with well-developed sub-programmes for herd health and fertility, BSE, AI and record keeping. Considering all these and putting them into practice would result in increased genetic gain and consequently higher productivity of Mpwapwa breed cattle and improved livelihoods of smallholder farmers in the Mpwapwa district.

Therefore, this thesis provides useful findings which could be used as the basis for developing an ambient-temperature AI regimen for use in breed improvement of the Mpwapwa cattle of Tanzania. In order to make the proposed programme more robust, efficient and effective, more bulls would need to be recruited so that those sires that are genetically superior, and whose semen is suitable for an ambient-temperature AI regime, can be identified. For the repeated semen collections needed to sustain an AI service, bulls (and staff) would have to be trained to the AV method.

In terms of the present thesis, extending the work by harvesting more ejaculates would allow the examination of more replicates for more definitive assessments of sperm survival *in vitro*. Once consistent results have been achieved for each bull, decisions could be made regarding the culling of unsatisfactory bulls from the programme. Similarly, once consistent results have been achieved in terms of optimal storage conditions (temperature and diluent), the supply chain from the AI processing centre to the point of insemination can be set up and managed accordingly.

It is important to recognise that, the bulls that were involved in the present study had not been previously selected for semen production, so the observed wide range of semen scores and sperm survivability were largely as expected. Bulls that are widely used in AI programmes (e.g. dairy breeds of *B. taurus*) are genuinely generally expected to produce good quality semen that survives cryopreservation well. However, it is worth noting that, when AI was first developed in the 1940s-60s, some of the donor AI sires/bulls produced semen of adequate quality and freezing ability per the requirement of the AI services, as (at that time) they were from a population that was hitherto unselected for semen characteristics. Thus, the spread of bulls used in the present study was exactly what would be expected from an unselected population. Clearly, therefore, the bulls in the present study that had the poorest semen characteristics would be removed from the AI stud. They might be usable as natural-service sires in small herds (i.e. as they are probably genetically superior to farm-bred bulls), but would not be suitable for widespread use in AI. Although there has not been a declared selection for semen quality for the bulls used in the present study, there has been a *de facto* selection based on semen quality. In other words, the high quality of semen that is routinely collected from *B. taurus* bulls in 2020 is the result of 60 years of selection in semen quality. In order to achieve adequate semen scores results in the proposed AI and fertility programme as it is in *B. taurus* AI sires/bulls, it is necessary to recruit substantial cohorts of bulls that would pave the

way for semen quality evaluation and selection for improved reproduction, production and genetic gain in Mpwapwa breed cattle.

The decision to use a "1½" $PGF_2\alpha$ synchronisation program and FTAI was taken on the basis of it being more likely to produce satisfactory outcome to AI than would insemination after detected oestrus. FTAI is not, of course, without its drawbacks, but it is affordable within the economies for smallholder farmers and, so long as the body condition of the cows is maintained at an adequate level, should produce acceptable fertility outcomes. Finding a method of recording fertility outcomes amongst small, dispersed herds will be challenging, but will be essential if the goal of fertility improvement is to be achieved. Perhaps, if the proposed fertility management program can be implemented, one of its outcomes will be accurate data on pregnancy rates to FTAI.

All of the foregoing factors should be considered in developing an AI programme for the Mpwapwa breed, as collectively they would improve bulls' and cows' fertility outcomes compared to what is currently being achieved. Even though the study was limited in time and to a research facility to accommodate other alternative techniques and methods for the assessment of bull fertility, it nonetheless managed to reveal the potential of Mpwapwa breed bulls which could be tapped into the proposed AI and fertility programme.

On the other hand, some potential gaps were identified that need to be filled in the future, one being the limitations of BSE and semen evaluation as a means of predicting fertility of bulls in an AI programme. Various techniques exist to improve the predictive ability of semen examination, for example, the $Ca^{2+}$-induced swimming test (Ho & Suarez, 2001) which detects a hyperactivated swimming pattern displayed by sperm in the uterine tube during fertilisation at a time of ovulation (this would provide additional information on the relationship between BSE and conception rates in Mpwapwa cattle). Another example is acrosome reaction/function (Marquez & Suarez, 2004; Celeghini *et al.,* 2007; Joseph *et al.,* 2010), which is another gap where several tests involving different techniques have been conducted to assess the functional properties of sperm (e.g. fluorescent probe techniques which mainly evaluate sperm cells' integrity and functionality), and other tests targeted to address spontaneous hypotonic-induced acrosome damage.

The proposed AI and fertility programme would address the present challenges on AI utilisation in the Mpwapwa district particularly on:

a) Fertility and herd health

The weak fertility and herd health programmes that are currently used by most smallholder farmers would be improved and strengthened through the proposed AI and fertility programme, chiefly by introducing appropriate programmes for fertility and herd health into the smallholder farmers' local breeding schemes. By comparison to the current situation, the introduction of these programmes into the smallholders' farmers and their wise application would thus improve their herds' fertility and health and consequently increases the success of the programme.

b) Oestrus detection

The presence of weak, inefficient and ineffective methods of smallholder farmers in oestrus detection would be improved and strengthened through the planned AI and fertility programme by introducing simple, but efficient and effective methods for detecting oestrus into their breeding programmes. It is clear from the Author's experience, both in the MSc thesis and based on day-to-day experience at TALIRI on these issues, oestrus detection is difficult, so perhaps using single- or double-PGF synchronisation protocols would improve reproductive outcomes. Perhaps the way forward would be for TALIRI to work alongside a cohort of local farmers to identify the most successful oestrus detection methods, and then to disseminate those methods more widely. If farmers found that these were effective, it would likely be better than using pharmacological methods of controlling the time of oestrus. If they do not, a timed AI would be an alternative. These simple oestrus detection methods could be used alongside teaser bulls in herds to improve the efficiency of these methods.

c) Nutrition regimen

The weak, inefficient and ineffective nutrition regimen of the smallholder farmers would be improved and strengthened through the planned AI and fertility programme by introducing efficient and effective nutrition regimens into their breeding programmes. Notably, the introduced nutrition regimen would account for the effects of pre and post AI nutritional management plans.

d) AI services

The absence of effective and efficient AI infrastructure and AI delivery systems has resulted into poor AI service delivery. The proposed AI and fertility programme would reintroduce these infrastructures to deliver effective and efficient AI services. This could also address the situation of high costs of AI services and the dearth of skilled AI technicians.

### The proposed AI and fertility programme would increase the likelihood of smallholder farmers adopting and utilising AI into their breeding programmes

The fact that the proposed AI and fertility programme would have all the necessary practices needed by any AI programme, would account for issues concerning to oestrus detection, herds' fertility and health, pre and post AI nutritional management plans and AI delivery services and charges. The programme would provide quality Mpwapwa bull semen which would yield better conception rates as opposed to their homebred and untested bulls. As a result, this would therefore increase the likelihoods of smallholder farmers' adoption and utilisation of the AI technology into their local breeding schemes.

### Government intervention to support the application and utilisation of the proposed AI and fertility programme in Mpwapwa district and its upscaling to other zones in the country

The Government should further support the re-equipped TALIRI Mpwapwa animal biotechnology laboratory as a facility to enhance the production and distribution of fresh/liquid semen of Mpwapwa breed cattle to the smallholder farmers at the on-farm level. Smallholder farmers' access to fresh/liquid semen services from the TALIRI Mpwapwa research centre would play a key role in the reproduction and production performance of Mpwapwa breed cattle at the on-farm level. The TALIRI Mpwapwa animal biotechnology laboratory would thus play a crucial role in the proposed AI and fertility programme as it would provide the AI facility/infrastructure needed to deliver the intended AI services. Thus, further re-equipping of the laboratory and training of more AI technicians would build the capability of TALIRI Mpwapwa research centre to effectively and efficiently deliver the proposed AI and fertility programme.

## Conclusion

Under the conditions that predominate on smallholder beef farms, the Mpwapwa breed of cattle is well adapted to the many different agro-ecological zones across Tanzania. The lack of significant genetic progress using bulls of Mpwapwa breed cattle in open nucleus breeding schemes over decades has resulted in the necessity of changing the breeding strategy in order to increase the genetic improvement of smallholder cattle. Fresh/liquid semen produced from improved Mpwapwa bulls seems to offer an effective alternative breeding strategy that could be introduced to smallholder farmers at the on-farm level to increase beef cattle genetic gain and productivity. As such, understanding the perceptions and preferences of smallholder farmers in relation to the proposed AI and fertility programme could result in increased rate of adoption and utilisation of fresh/liquid semen AI at the on-farm level. Such understanding would earn smallholder farmers' trust and support and facilitate the easy and smooth provision of AI service education/training. Both farmers and AI providers need to improve in order for effective and efficient fresh/liquid semen AI outcomes to be achieved within the Mpwapwa district: smallholder farmers need to improve their management practices while TALIRI Mpwapwa Research Centre and the Ministry of Livestock need to improve AI facilities/infrastructure.

As observed from the current study, most bulls produced semen that would be adequate for use in natural service of small herds, and a reasonable proportion of the bulls produced semen that is of acceptable quality for use in AI. The obtained results were quite similar to the results on other BSE studies done on *B. indicus* bulls globally, particularly the smaller-framed breeds of East Africa and South East Asia. For use in an AI service, electro-ejaculation is unlikely to be acceptable as a long-term method of semen collection. Although cattle tolerate the process relatively well in the short term, animal welfare concerns suggest that its use is best limited primarily to the diagnostic arena, rather than to routine semen collection. That being so, it seems that the AI centre at TALIRI would probably need to invest in training its bulls to collection by artificial vagina. Whilst this would increase the infrastructure needed for the service, it would have the dual benefits of (a) being more sustainable in the long term and (b) a likely increase in the harvest of sperm per ejaculate/collection.

Further study of the bull herd at TALIRI would be beneficial to establish: (a) the time-course of semen changes after puberty, (b) the time-course of development of full, mature sperm production

capacity and (c) the time-course of senescent changes in aged bulls. Therefore, the inclusiveness of the suggested infrastructure and expertise aligned with the proposed AI and fertility programme is key for the successful implementation and delivery of AI services within Mpwapwa district of the Dodoma region in Tanzania. Thus, this will largely require support from the government and partly from development partners in order to largely produce, distribute and utilise chilled/ambient Mpwapwa bull semen in the region and the whole country as a whole.

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