University of Veterinary Medicine Budapest

Department of Obstetrics and Farm Animal Medicine

Deep Freezing of Mangalica Boar Semen

Mangalica Kan Ondójának Mélyfagyasztása

By
Rebecca Anne Grainger

Supervisor:

Professor Rátky József, DVM, PhD, DSc

Budapest, Hungary

2022

Abstract

Artificial insemination (AI) is used globally throughout the swine industry, at present the most used method is with liquid semen, which has very successful results. In recent years, the cryopreservation of swine semen has been researched due to its success in species like cattle. Cryopreservation has many benefits including gene preservation and for use in emergency situations. However due to the poor survival of boar sperm post freezing and thawing it is not used widely throughout the industry. Its use in the in vitro gene preservation of the endangered Mangalica pig breed shows promise and should be considered as a viable option for saving the breed in the future- once further investigations have been completed to further improve the viability of sperm post cryopreservation. The use of cryopreserved boar semen can be important especially for use in case of emergency situations, such as African Swine Fever Virus (ASFV) outbreaks. Again, more investigation is required to further improve the post-thaw fertility.

Absztrakt

Mesterséges megtermékenyítés a sertés iparban világszerte használt módszer, manapság a leggyakoribb eljárás folyékony ondóval történik, mely nagyon sikeresnek bizonyul. Marha tenyésztésbeni sikerein alapulva, az utóbbi években a sertés ondó mélyfagyasztását is vizsgáljak. Mélyfagyasztásnak számos előnye van, köztuk a gének megőrzése, valamint surgősségi esetekben törtenő használat. Azonban a sertés kan ondójának fagyasztás és olvasztás utáni alacsony tulélése miatt ez a módszer nem széles körűen használt a sertés iparban. A veszélyeztetett Mangalica sertés lombikban történő megtermékenyítése sikereket mutat és járható útnak tekinthető a fajta megmentése céljából a jövőben, miután további kutatásokkal az ondó mélyfagyasztás utáni túlélési esélye javul. Mélyfagyasztott sertés kan ondó különösen hasznos lehet szükséghelyzetekben, például az afrikai sertés pestis (ASFV) járványok esetén. Azonban meg kell ismételni, hogy további kutatásokra van szükség az olvasztás utáni termekenyítő képesség emelésére.

Contents

1. Introduction ... 4

2. Literature Review ... 6

2.1 Importance and History of Artificial Insemination ... 6

2.2 Different Methods of Semen Usage ... 8

2.2.1 Deep Freezing ... 8

2.2.2 Cooling ... 13

2.2.3 Fresh Semen ... 14

2.3 Swine Semen ... 16

2.3.1 18°C ... 16

2.3.2 Dilution ... 16

2.3.3 Deep Freezing ... 17

2.4 Semen Preservation in Mangalica Boars ... 21

2.4.1 History and Uses of Mangalica Pigs ... 21

2.4.2 Use in Normal Farm Practice ... 23

2.4.3 Importance of Deep Freezing ... 24

3. Conclusions ... 30

4. Acknowledgements ... 32

5. References ... 33

6. Electronic License Agreement and Copyright Declaration ... 37

7. Thesis Supervisors Acceptance Letter ... 39

1. Introduction

In this thesis I will be using current literature to investigate the cryopreservation of boar semen and its use in the in vitro gene preservation of endangered Hungarian pig breeds. The aim of this thesis is to analyse the current information available and discuss the uses, benefits and problems associated with cryopreservation in boars. As well as to discuss how this technique can be used to further increase the numbers of the endangered Mangalica breed. Furthermore, I will discuss the benefits of using frozen semen in regard to animal health prevention, using the current African Swine Fever Virus (ASFV) situation in Hungary as an example. Fundamentally this research is important not only to preserve an endangered breed, but also for the swine industry as a whole, which could benefit from higher sperm survival rates post freezing and thawing.

Cryopreservation of semen is the process by which semen is frozen in liquid nitrogen to be stored [13]. This method has been highly successful in cattle, however similar success has not been achieved in swine [3]. Frozen boar sperm have lower survival rate and lower function within the oviduct when compared to liquid semen [9].

It has been shown that this results in lower pregnancy rates and smaller litter sizes [3], which is uneconomical. Studies have been underway to determine the optimal cooling and thawing rate of the semen, which can result in higher survival rates [17,34]. In addition to this, studies have found that the freezability of semen depends on the boar and may be linked to genetics [17, 34, 36, 37]. Genetically determining which boars have ‘good’ freezability could be very beneficial in achieving higher survival rate of sperm post freezing and thawing.

Benefits of cryopreservation include preserving diversity within domestic breeds, treating infertility, storing genetically valuable lines of various breeds [5]. Cryopreservation can also be used in the preservation of endangered breeds [13] and in animal health prevention [35]. These last 2 benefits will be discussed in detail.

Mangalica pigs are a breed native to Hungary [38]. After the Second World War their numbers dropped [40] and due to their low reproductive capacity, they struggled to increase again in number [41]. They have many desirable traits including resistance to stress and disease, and good adaptability [39]. They are a fat-type breed and the meat they produce is

of a very high quality [38]. The market for ‘traditional products’ has increased, and thus it has become increasingly popular to raise native breeds such as the Mangalica [43].

The African Swine Fever Virus (ASFV) is present in Europe [45]. It can be spread from wild boars to domestic swine [46]. The disease has devastating effects on the swine industry [45] and in a breed like the Mangalica which already has low population numbers it could prove to be detrimental. A gene bank containing Mangalica sperm is a possible way to increase the numbers of the breed [13] as well as to prevent the spread of disease during emergency situations such as an ASFV outbreak [35].

2. Literature Review

2.1 The Importance and History of Artificial Insemination

Exploration and investigation into artificial insemination (AI), has spanned over 200 years and nearly half of this time it has been used commercially [1].

Artificial insemination has several fields of application, these include production of hybrids, research into fertilisation and hereditary traits, as well as preservation of species close to extinction [2]. Further benefits of AI include a decrease in the transmission of venereal diseases and lethal genes [3]. Its foundations are built on the fact that it is 'simple, economical and successful' [1].

In order to understand the use, as well as the success of AI in the current day, I will take a look back at the history surrounding it. In 1678 Leeuwenhoek was the first person to visualise sperm: he termed them 'animalcules' [3]. He was able to see the sperm through the use of a single lens microscope which he designed and made himself. It was in 1827 that the word 'spermatozoa' was termed by Karl von Baër. Research in 'sperm biology' has spanned centuries and progressed in leaps and bounds. The first International Spermatology Congress was held in 1969. The congress involves experts from multiple fields and discusses a variety of topics including sperm motility and fertilisation [4].

The first successful AI was achieved by Lazzaro Spallanzani [5]. He has been termed 'the forefather of modern in vitro fertilisation' [2]. In the year 1780 he injected semen into the uterus of a bitch via a syringe, sixty-two days after the insemination three puppies were born. After Spallanzani's success, other researchers began to replicate his work on animals and in 1799 John Hunter even performed this method on a woman resulting in a pregnancy [5].

From this point on a number of academics in the late eighteenth century became interested in AI. However, their interest was not related to the agricultural uses of AI, rather it was in the creation of hybrids [2]. As Walters et al [5] described; it wasn't until almost actually 100 years later (in 1880s) that AI began to be used for increasing fertility. It was also used to attain a higher number of offspring produced by a single sire throughout his lifetime. A prominent figure in the development of the AI process was Walter Heape. Heape was widely known as an excellent biologist in the field of reproduction and he discovered the foundation of the correlation between reproduction and seasonality. Heape and researchers in other

countries began publishing studies involving the use of AI in various species such as dogs, horses and rabbits [3].

However, it was Russian scientists who were among the first to attempt to introduce the use of AI as a practical technique. Ivanow began this quest in 1899, and by 1907 he had studied AI in multiple species. While working in this field he taught workers how to choose the most desirable stallions, as well as the techniques to produce their offspring via AI. One of his most notable achievements was the creation of a semen extender. Later, towards the end of the 1930s, his work was continued by another scientist; Milovanov. He was a pioneer in his own right: creating an artificial vagina, a model similar to this is still used currently [3]. By the end of 1938 in Russia, it was said that over '40,000 mares, 1.2 million cows, and 15 million sheep' had undergone AI [5].

Word spread of the successful use of AI in Russia and subsequently the practice became more common throughout the Western world. In Denmark veterinarians developed a special technique in cows which enables semen to be deposited in the cervix or uterine body. This method meant that less sperm was required per cow for AI. A second noteworthy idea to originate in Denmark was the use of straws to store semen, later these straws have been used in commercial settings all over the world [3].

Robert Foote [6] said that it was in 1939 that the American Society of Animal Production began creating protocol in regard to semen collection, evaluation and preservation. Shortly after this in Milan in 1948 the first meeting of the International Congress on AI and Animal Reproduction was held. Since then it has taken place every 4 years [3]. It focuses on various areas of reproduction including physiology, pathology and current reproductive technologies and involves different papers, workshops and exhibitions [7].

Pork is in high demand all over the world and this trend is expected to increase in the coming years, as a result of this the swine industry is constantly trying to improve the genetics within the industry to keep up with the increasing requirements. The ultimate focus is producing a higher amount of good quality meat from a small number of breeding animals [8]. Presently, AI is used globally in the swine industry, and it plays a large role in increasing the production of swine [9]. It is the best method for 'disseminating genetic progress' within the industry [8].

2.2 Different Methods of Semen Usage

2.2.1 Deep Freezing

The word 'Cryobiology' has Greek origins- 'kryo' meaning cold and 'bios' meaning life [10]. The quantitative biochemical and biophysical changes that happen during cryobiology are known as fundamental cryobiology. Examples of these include alterations in the transmembrane in correlation to the addition and removal of cryoprotective agents (CPAs), the formation of ice both intra- and extracellularly, storage temperatures and the rates of freezing and warming up. The use of this information can result in further improvement of cryopreservation [5]. Cryopreservation is the method of storing cells and tissues at below freezing temperatures [11]. In this chapter I will discuss the various advantages and reasons for using cryopreservation as well as the methods used in this process and then finally, I will address the challenges involved.

There are multiple reasons as to why the cryopreservation of sperm is so important. These include preserving diversity within the genetics of both wild and domesticated animals, treating infertility, storing, and distributing 'genetically superior' lines of various domestic animals. Finally, it can also be used to store animal models of human diseases [5]. The aforementioned reasons are extremely important in today's world of assisted reproduction and high intensity farming to achieve optimal reproduction goals and output.

Dating back to the late 1780s, the effects of cold temperatures on cells have been observed, and this created the basis of the causes of the outcomes of unsuccessful or successful freezing [5]. Human, frog, and stallion spermatozoa were the first species observed regarding the preservation of semen [12]. But it was after the successful use of AI within the dairy industry, that they required a way to store the bull semen for longer periods of time, and thus studies into the cryopreservation of semen became a popular area of research [5]. Currently this area of science is still prominent within the reproduction field.

At present there are two methods used for the cryopreservation of semen: vitrification and slow freezing [13]. Vitrification involves the transformation of living cells containing liquid into 'a glass like amorphous solid' [14]. In the process of vitrification there is no ice formed [15]. It is a fast process which lessens the effects of cold shock but comes with the risk of crystallisation [13]. Slow freezing results in the freezing of cells containing liquid via the use of low cooling rates [14]. Slow freezing can lead to dehydration and shrinkage of the cells, there is intracellular formation and re-crystallization of ice [15]. In general, the process

of cryopreservation includes the following four components; decrease in temperature, dehydration of the cell, freezing and finally thawing [13].

To begin the process ejaculates are collected, in this case of swine this is usually via the gloved hand method. Next the ejaculate is filtered to get rid of the gelatinous fraction, and then an extender is added to the semen, most often it is Beltsville thawing solution. Semen is evaluated and ejaculates are selected for freezing. Usually the sperm 'pellets' are frozen within straws and an extender is added, these often contain glycerol. The semen is kept in a refrigerator until reaching the desired temperature of 5°C, then it is packed in 0.5ml straws, and placed in frozen liquid nitrogen vapours and it gradually cools. At the final step the semen straws are placed into liquid nitrogen [13].

There are multiple factors involved in the survival of spermatozoa during cryopreservation and these include: hygiene, cooling rate, extender used and concentration of CPAs [13].

A pivotal discovery in the development of cryopreservation was in 1949 when Polge et al found that glycerol has cryoprotective properties and could be used as a CPA. This discovery allowed cryopreservation of semen to reach its optimal use [5]. It also allowed for the preservation of different types of cells and tissues [12]. As mentioned, this discovery was fundamental in the progression of the successful use of cryopreservation, and it still plays a major role in the process even today.

It was a chance discovery by Polge et al [16] when they found that glycerol could be used in order to preserve sperm cells at lower temperatures. Their study found that a larger number of sperm survived after vitrification when glycerol was a component of the diluent. They found that 5% glycerol concentration was the optimal. Since this discovery further research has been conducted to determine the optimal concentration of glycerol as a CPA. Hernández et al [17] concluded the optimal glycerol concentration is between 2-4%. Furthermore, Bamba et al [18] additionally found that at concentrations below 5% there were minimal negative effects of glycerol. Meaning that since the original findings in 1949, the concentration of glycerol used has largely remained the same.

The original findings of Polge et al [16] show that lower concentrations produced less prominent results while higher concentrations can have detrimental effects. Further research since this time has agreed with this, finding that the use of glycerol in higher concentrations can result in cell death. It can also add to the 'disorganisation of the plasma membrane,' as

well as decreasing the sperm's fertility and motility [19]. These findings show that it is vital to use the correct concentration of glycerol in order to achieve desirable results.

In addition to glycerol there are other substances which can be used as CPAs. CPAs can be divided into different groups depending on their mode of action. For example, glycerol, DMSO and methanol are all classed as permeating CPAs [20]. Permeating CPAs can enter through the plasma membrane and here they create hydrogen bonds with the water molecules, and this results in stopping the formation of ice both intra and extracellularly. Combining two permeating CPAs can help to lower the concentration of each and thus decrease the cytotoxic nature of each. Non-permeating CPAs are too big to enter the plasma membrane, instead they stay in the extracellular compartment, they are less cytotoxic. Examples include sugars like sucrose and high molecular weight polymers like polyvinyl pyrrolidone. They can be used in combination with permeating CPAs, meaning the concentration of the permeating CPA can be lower which results in a decrease in cytotoxicity [14].

In order to maximise the success of fertilisation after cryopreservation, we must understand what is required for fertilisation and ensure these aspects are not damaged during the process. Sperm cells are haploid and contain very little cytoplasm [13]. They have a head which has an acrosome [21, Figure 1], this is for penetrating the oocyte at fertilisation [13]. The sperm cells also have a tail which makes them mobile [21].

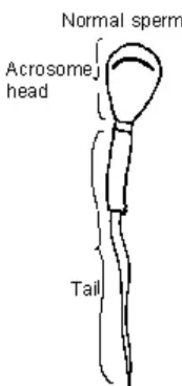

Figure 1:

Schematic drawing of a normal swine sperm cell

In swine, once deposited, the semen moves through the reproductive tract via contraction [22]. To achieve successful fertilisation, the sperm must penetrate the oocyte. Several aspects are required to achieve this including: a working flagellum to move the sperm forward, as well as special chemical environment of the membrane in order to prevent the sperm being phagocytosed or binding to cells within the reproductive tract. A signalling pathway is activated when the sperm binds to the zona pellucida leading to the acrosome reaction, and there is fusion of the sperm and the oolemma. Next, to activate the egg, a second signalling pathway is triggered. Disruption of the signalling pathways will again result in infertility. The physiological structure of the plasma, acrosomal membranes and chromatin must not be damaged and should stay unimpaired during freezing and thawing- disturbance of these will result in the infertility of the sperm [20]. As described above there are multiple factors which must remain intact for fertilisation to be successful.

Damage of the sperm occurs due to the process of freezing and then thawing. The phenomenon of ice crystallisation causes stress to the cells. The cells shrink due to the removal of intracellular water as a consequence of their exposure to hyperosmotic conditions [20]. ‘Chilling injury’ can lead to disturbances in the sperm. In severe cases it can cause damage to the cytoskeleton or even components involved in the genome [13]. The plasma membrane, acrosome and nucleus of the sperm cell can be damaged during freezing. In addition, sperm motility and function of the mitochondria can be altered. Furthermore, cryopreservation can lead to the breakdown of proteins and mRNA as a result of alterations to homeostasis [23, Figure 2]. Upon thawing the influx of water intracellularly can result in damage to the membrane. Some seemingly small damages can result in the individual spermatozoa losing its ability to fertilise [20].

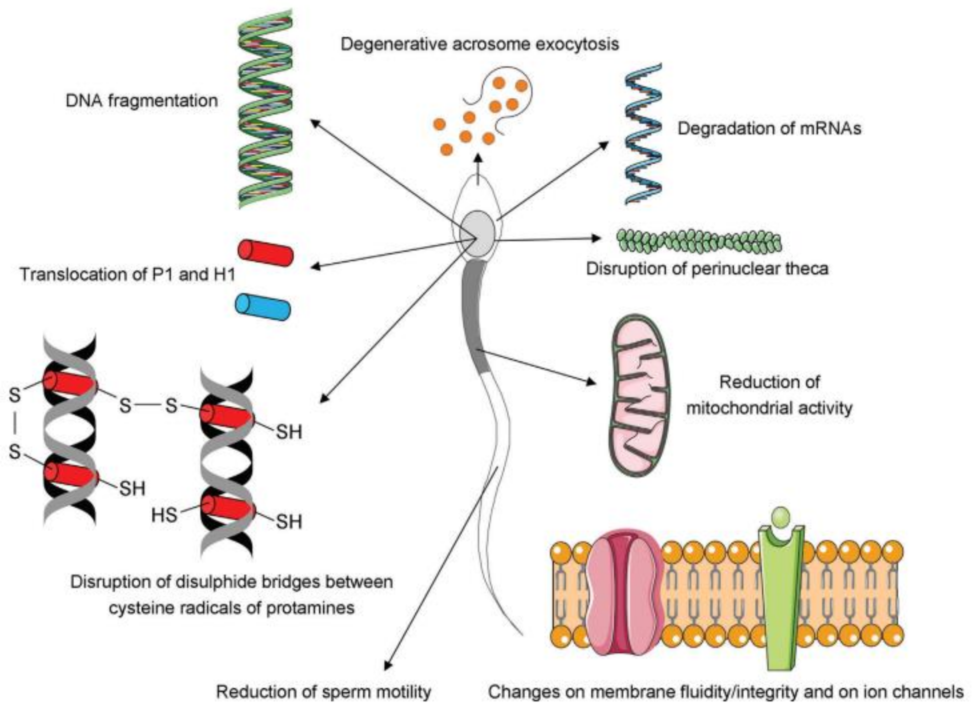

Figure 2:

Schematic diagram of the main injuries resulting from boar sperm cryopreservation

Thawing is also an important part of the cryopreservation process. The rate at which thawing should occur is based on how high or low the cooling rate was. If it was high cooling rate a fast thawing rate is required. This is because at a high cooling rate freezing occurs intracellularly and thus a fast thawing rate is required to prevent recrystallisation [13].

As discussed in the above chapter cryopreservation has various components which must all occur within the optimal conditions to have successful cryopreservation and thus successful fertilisation.

2.2.2 Cooling

For years it has been widely known that the lethality to sperm during cooling is proportional to the rate of cooling and range of temperature. The death of sperm occurring during freezing, and thawing is known as ‘cold shock’. Between species there are vast differences in sensitivity of sperm to cold shock, with boar spermatozoa being some of the most sensitive [24]. At cooler temperatures there is an increase in the loss of motility of the sperm [22]. If fresh sperm is cooled quickly the cold shock can result in a decrease in the viability of the sperm, it has been found if fresh semen is left to acclimatise at room temperature for several hours the sperm can obtain protective capacity to the cold shock. In addition, resistance against cold shock can be enhanced via slow cooling [25].

The optimal rate of freezing should be slow enough to stop ice crystals being made intracellularly but also fast enough to stop the cell becoming severely dehydrated [24]. In the study by Kumar et al [26] they cooled the semen slowly from 22°C to 5°C over 90 minutes. Next, they were cooled to -5°C at -5°C/minute. Finally, the samples were cooled at various rates- either -1, -30 or -50°C/minute and then they were plunged into liquid nitrogen. After thawing and analysis it was found that the rate of -30°C/minute was the optimal for decreasing damages caused as a result of cryopreservation. However, Medrano et al [27] found that the optimal rate was dependent on the ejaculate itself and thus questioned the idea of an optimal cooling rate. Despite these findings it is still widely agreed that the cryosurvival of sperm is increased when cooling rates are modified.

Cholesterol content of the sperm can also influence the effect that cooling has on the sperm membranes. In comparison to other domestic animals the ratio of cholesterol to phospholipids is low in boars and again this makes them more susceptible to cold shock, storing at higher temperatures can help in the prevention of this. However, bacterial growth can be more easily facilitated at these higher temperatures, especially in combination with the nutrients used in the media. All semen ejaculates contain a certain number of bacteria both from the animal and the environment, but it is when the bacteria are allowed to grow without inhibition that this has a negative impact on the quality of the sperm. In some cases, it can result in pathogen transfer to the sow leading to loss in production values. This can result in a shorter time in which the semen can be used for AI, however, certain extenders can also facilitate longer storage- up to 7-10 days [25]. Studies have shown that when semen

is stored for 2-3 days there is no decrease in the fertility or litter size when this semen is used [22].

Furthermore, the sensitivity of the cooling can be decreased to a certain degree by cutting down the volume of seminal plasma. This can be achieved by working with the sperm-rich part of the semen [22].

2.2.3 Fresh Semen

Globally liquid semen is used as the predominant form of semen used for AI. The basics of liquid semen are built on the addition of various substances such as metabolic buffers, nutrients, extenders, and antibiotics in order to stop bacterial growth [25].

In the swine industry semen is most often collected with the gloved hand method. The boar either mounts a dummy or a sow in oestrus, the boar's penis is extended, and pressure is applied via the hand of the collector. There are multiple advantages to using a dummy as opposed to a sow, for example it prevents injury occurring to the female during mounting and saves time [28].

Usually, the semen is collected into a thermos covered with gauze [28]. Boars have several phases of ejaculation- the first phase is the pre-sperm faction and is heavily contaminated with bacteria, it should not be collected. The next phase is the sperm-rich fraction, and it should be collected. Finally, the last fraction is seminal plasma and a gel plug which is excreted from the accessory glands [29], the gauze layer on the thermos will filter this [28]. Typically, the process of ejaculation takes 5-10 minutes [29].

Once the semen is collected it is evaluated [29]. The volume can be measured in a volumetric cylinder [28]. The average volume is between 100-500 mL [29]. The concentration is usually based on visual assessment of its opacity [28]. The motility of the sperm is assessed via microscope on a warm slide, it can be the best way to evaluate semen quality. To be termed as good quality the sperm should have individual movement and the semen should have a 'wave' motion, in a poorer quality sample the sperm clump together [29]. Non-motile sperm are unable to fertilise the egg [21]. Another factor to be evaluated is the presence of abnormalities [29]. Abnormalities to the head or tail can decrease the ability and likelihood of the sperm penetrating the egg [21, Figure 3]. Broken, bent and twin tails, as well as detached or twin heads are all abnormalities of note. When evaluating semen less than 25% abnormalities are acceptable [29].

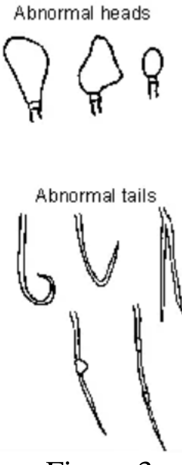

Figure 3:

Schematic drawing of abnormalities in morphology of the head and tail of swine sperm

Once the semen is deemed to be of satisfactory quality it is processed and stored. The semen can be used as undiluted fresh semen or diluted and chilled [29].

Undiluted fresh semen is usually used for small in-herd AI programmes. The semen should be used within a few hours of collection. Prior to use the semen must be kept at 34°C- the temperature at which it was collected. The semen is then divided into doses and then used to inseminate sows and gilts [29].

2.3 Swine Semen

Within the swine industry successful reproduction is measured based on the farrowing rate, meaning the total number of animals which farrow after AI, and the litter size- meaning the number of piglets born alive [22]. The use of semen in various forms such as cryopreserved semen must maintain these factors to a high level otherwise it is not economical for the farm.

2.3.1 18°C

The length and conditions of storage influence the structural and functional changes which occur to the spermatozoa [28]. The longer liquid semen is stored in vitro the more the fertility of the sperm decreases [30]. Boar semen is usually stored at temperatures between 15 and 18°C, this is higher than in other species as boar semen is more sensitive to cooler temperatures [25].

2.3.2 Dilution

An integral part of the preservation of boar semen is the dilution of the ejaculate and this has been used in practice for years. The process of dilution is performed in order to extend the lifespan of the sperm within an ejaculate and as a consequence of this, the semen of boars with high genetic value can have increased usage [31].

There are several criteria which dictate what makes a good extender, these include optimal pH- usually between 6.75 and 7, good buffering capacity and the ability to protect sperm at low temperatures. Usually, extenders contain a permeating and a non-permeating CPA, antibiotics, salt, sugar(s) and a buffer [13]. There are various extenders available on the market. Some are short-term extenders which preserve the semen for around 3 days, usually these are cheaper, for example Beltsville Thawing Solution (BTS), Merck III or Kiev. Long-term extenders which preserve the semen for 5 or more days have a higher cost, these include Androhep, MR-A and Modena [32]. Physiological saline, or other isotonic solutions can also be used in small volumes to dilute sperm [33].

Dilution can activate or even excite the motility. However, if semen is excessively diluted it can result in a decrease in the viability of the sperm, as well as a reduction or even loss in the motility [33]. Furthermore, it can be damaging to the integrity of the membrane, which can result in the membrane becoming destabilised [31]. This phenomenon is known as the 'dilution effect' [33]. There are several reasons as to why dilution may have detrimental consequences, some of these include: the physical stress involved in the process of dilution,

the speed at which dilution occurs and the elimination of factors which are involved in the stability of the membranes of the sperm [31]. The 'dilution effect' can be diminished via the use of isotonic media which have a buffering capacity, salts and energy [33].

Usually, the extender is added to the ejaculate and mixed, as it was previously believed that using this method resulted in few alterations and thus meaning it was less damaging to the spermatozoa. However, the study by Schulze et al [31] found that the reverse of this practice – which is adding the semen to the extender – did not alter the quality of the semen. In fact, they found that this method allows for a higher 'homogeneity' of the semen, as if a long period of time is left between collection and dilution, the sperm can be dispersed unequally. Which ends up in a differing number of sperm within AI doses, which in turn can have an effect on the success of the insemination. Foam can be produced upon the addition of extenders to semen, and this proves to be a possible hazard in regard to hygiene. Upon using the 'reverse' method there is less production of foam meaning the process has a higher level of hygiene

Bamba et al [18] found that if spermatozoa are diluted rapidly at both 37°C and at 5°C it can cause acrosomal damage. They also found that acrosomal damage is more prominent at higher dilution levels.

2.3.3 Deep Freezing

After the success of using frozen bull semen in cattle AI, extensive efforts were made to attempt to have similar success with boar semen [3]. As a result of several studies, viable sperm were seen in the frozen-thawed boar semen [12] and it was in 1975 that cryopreserved boar semen began to be used commercially. However, it has not been used within the swine industry to the same extent as liquid semen [34].

Developing technologies in regard to deep freezing boar semen will mean that it can be used in many breeding programmes as it can be transported and used in places far away from the collection site and it can be used at any time [12]. Currently the use of cryopreserved boar semen is the exchange on an international level between 'nucleus farms', in order to uphold genetic diversity [35]. These so-called 'special breeding programmes' are the main area of use for cryopreserved boar semen. There is the possibility for both short-term and long-term banking of cryopreserved semen. Short-term it can be stored while boars are undergoing the assessment of breeding value and during times when there is less demand. Long-term the semen can be used for preserving breeding lines as well as being used to evaluate the genetic

progress of various breeds. Finally, it can also be stored and used in emergencies, an example of this could be if the farm was unable to receive an adequate amount of liquid semen to reach breeding targets due to weather or disease [35].

However, unfortunately in swine, frozen semen has limitations in its use as it has been found that pregnancy rates and litter size are decreased [3]. In boars the sperm of cryopreserved semen is weaker than liquid stored semen and this can mean that during its journey through the female's reproductive tract it is more at risk. Some studies have found that cryopreserved semen is fertile for a shorter time once deposited in the female reproductive tract and it has been reported that the numbers of spermatozoa in the oviduct which are functional is often 10-fold lower in comparison to fresh semen. In addition to this, cryopreserved sperm are required in 4-6 times higher amounts per oestrus than fresh semen- thus making it less economical [9]. When we compare AI in cattle to that of swine it has been found that a single bull can produce a sufficient amount of semen to serve more than 40,000 cows per year via AI, while boars can only serve less than 2000 sows per year [35]. Again, when compared to a cow, which only requires thousands of spermatozoa for successful AI, the pig requires millions in order to gain similar success [20]. Furthermore, most cattle only require a single AI service while most swine require a double service [35]. Looking at these figures we can see that AI in swine requires far larger quantities of sperm/semen than cattle and this can be a potential area of difficulty- especially when it comes to cryopreservation when the numbers of surviving sperm are usually lower. In order for cryopreserved semen to be used for AI on a large scale in commercial farms there must be a massive decrease in the total number of sperm required in order to reach optimal litter size [9].

Today efforts are still underway to cryopreserve boar semen via changing the freezing medium composition, an example of this is the addition of antioxidants to the extender before the freezing takes place. Another method being trialled in order to improve the use of frozen boar semen is the way the semen is stored- often a farmer will have to defrost multiple straws; between 10 and 15 in order to have a single AI dose. Researchers are trying to reduce this number as this will encourage farmers to use this method [5]. Presently there is also research into multiple methods in order to improve the quality and number of fertile boar spermatozoa available after cryopreservation. It has been determined that the rates at which the semen is cooled and then thawed are extremely important factors involved in the survival of the sperm [5].

In order for cryopreservation to be used commercially the temperature and timing of thawing seem to be of great importance [17]. Research suggests that if the cooling rate of -30°C per minute is used, the complementary optimal thawing rate is 1200°C per minute. When these rates are used, the post-thawing survival rates of the sperm are good in large numbers of boars [34]. Another study by Medrano et al [17] found that sperm tolerated a freezing rate of -10, -40 and -60°C per minute without significant differences however a warming rate of 1800°C per minute was the optimal rate. They found that a water bath of 70°C for 8 seconds was the best. Commercially thawing machines which control such variables are now available.

Thurston et al [36] found that between individual animals' spermatozoa have varying sensitivity in regard to rapid cooling. Thanks to developments in science, the ability to study individual sperm within an ejaculate have become much more advanced and thus it has come to light that 'considerable heterogeneity exists'. In their study Thurston et al collected 5 ejaculates from each of the 15 boars during a 5-week period. 3 different breeds of pigs were used: Landrace, Duroc and Large White. Prior to freezing they analysed the quality of the semen and it was found that there were no significant changes prior to freezing. The sperm was centrifuged, and the supernatant removed, next it was diluted with 5mL of commercial freezing buffer and then cooled from 15°C to 5°C at 0.2°C/min, it was then further diluted and packaged in straws and finally it was frozen. Upon thawing the straws were placed in a 40°C water bath for 1 minute. They assessed the acrosome integrity, plasma membrane integrity and evaluated the motility, it was found that for all 3 of these factors there was variation between the breeds. Their study concludes that there is variation in the freezability of sperm between boars which could be genetically inherited and that there is the possibility to be able to identify such boars within the breed. Being able to identify these boars would have a huge impact on the use of cryopreservation within the swine industry.

A further study by Hernández et al [17] was also able to classify the boars into 'good', 'moderate' and 'bad' based on the post-thaw semen quality. Meaning it is extremely important within the swine industry to eliminate those boars with 'bad' freezability from breeding.

In another study the semen from Large White, Landrace and Duroc breeds were examined. It was found that Landrace had more 'good' freezers when compared to the other 2 breeds. Next, they used Amplified Fragment Length Polymorphism (AFLP) in order to compare the

‘good’ and ‘bad’ freezers within the Landrace breed. Using this they can find differences in sequences of DNA that correlate with ‘phenotypic variability’ such as semen freeze-ability. Differences found in the AFLP profile mean that there are markers present for semen freezer-ability. In the future this could result in a gene test becoming available to identify ‘good’ boars [34].

Roca et al [37] performed several experiments and found that in total over 70% of variability in quality of the sperm between the ejaculates can be ‘explained by boar’. Thus, meaning that the major factor influencing the variability in the survival of the sperm is the boar. It was found that interboar variability (variance between boars) was significant in both the viability and the motility of the sperm post-thaw. Intraboar variability (variance between ejaculates of the same boar) were also found to be significant in some boars. Overall interboar variability was a lot higher.

Other studies echo the aforementioned findings and also agree that boar effects could account for 70% of the variability in survival of boar semen after and during cryopreservation. Hence it can be suggested that boars should undergo ‘test-freezing’. Under standard protocols they would be able to identify boars whose semen can be frozen and then thawed with the resulting semen being of suitable quality [34].

Roca et al [37] also discovered that there is a positive correlation between the quality of the sperm post thaw and the measure of the ejaculate prior to freezing. This means that boars with ejaculates containing a higher concentration of sperm and with a higher number of sperm with normal motility and morphology prior to freezing have better recovery of the sperm after freezing. This data can be applied in order to decrease the variability in the sperm quality post thaw between ejaculates, but it cannot anticipate the number of sperm to survive freezing.

2.4 Semen Preservation in Mangalica Boars

2.4.1 The History of Mangalica Pigs

The Mangalica pig is a breed native to Hungary, it was a very common breed in Hungary until around the middle of the 20th century [38]. The Mangalica originated approximately in the 1830s from the age-old race of the Carpathian basin and imported Serbian breeds [39].

This breed is a 'fat-type' breed with curly-hair [38]. There are only a few fatty pig breeds worldwide. The Mangalica carcass has around 65-70% adipose tissue, its lean meat content is less than 35% [40]. The first descriptions recall 2 types of Mangalica- the white and black type. Later, five colour types were described; Blond, Black, Swallow-Belly, Brown, and Red. However, today only three remain: Blonde, Red and Swallow-Belly Mangalica [38, Figure 4,5,6]. Recent molecular genetic analysis shows that these are three different breeds [40].

Figure 4:

Blonde Colour Type

Figure 5:

Sallow- Belly Colour Type

Figure 6:

Red Colour Type

The Mangalica have many desirable characteristics; they have great adaptability to various extensive housing and severe climate conditions. Furthermore, they have good resistance to stress and disease and their meat is of excellent quality [39]. However, they have a lower reproductive capacity, the reasons behind this are not fully known, but one theory which has been observed in slaughterhouses is the 'modest ovulation rate' in comparison to other breeds [41].

Throughout history this breed was kept on small farms, it was at the end of the 19th and beginning of the 20th century that bigger farming units housing thousands of animals were built [38]. Fat products such as bacon or lard were a staple in the diet of Hungarian people. The Mangalica products were forerunners in exports from Hungary, products including sausage, salami and lard were well renowned throughout Europe [40].

Historically, there were several setbacks in the increase of the population including the swine fever outbreak in the late 1890s and the First World War. However, in 1927, the National Association of Mangalica Breeders (NAMB) was created. The organisations goal was to create and decide the breed standards. It also gave advice and guidance on the successful breeding of the Mangalica [38].

The population increased rapidly up until the Second World War. After this time, the consumers' desire for lean pork increased [40]. White pig breeds outcompeted the Mangalica in terms of productivity [38] and despite the Research Institute for Animal Breeding implementing the cross breeding of the Mangalica with commercial breeds their numbers decreased quickly throughout the 1950s. By the 1970s the breed was almost extinct [40]. By 1975, a mere 34 sows were registered [41]. Luckily in the 1990s several organisations and programmes, both private and governmental, were established in order to 'preserve, propagate and exploit unique Mangalica characteristics' [38]. In 1999 the Mangalica won breed of the year from the German Safeguard Society of Ancient and Endangered Domestic Animal Breeds [38].

However due to the poor reproductive abilities of the breed it meant that their numbers increased at a slow rate [39]. In the early 2000s, male Mangalica pigs began to be studied, this research included new artificial insemination methods as well as developing the deep freezing of their semen [42].

2.4.2 Use in Normal Farm Practice

Currently there is increasing interest in breeding endangered animals [38]. Within the farming industry in Hungary the Mangalica Pig breed has 'special significance.' As the want for 'traditional products' has begun to increase again, it has become more and more popular to raise native breeds such as the Mangalica [43].

As previously mentioned, the Mangalica is one of the fattiest pig breeds in the world with 65-70% of the carcass being lard. Only around 30-35% is lean meat compared to commercial pig breeds which have over 50% lean meat. The quality of the meat is considered to be of the highest standards and there is now a renewed demand for Mangalica products on the European market [38]. The breed also has many desirable traits like resistance to stress and disease. [39]

Outside of Hungary there are other countries which breed the Mangalica pig. Austria and Switzerland each have around 300 sows registered. Serbia, Romania and Slovakia have in total 1000 breeding animals. The breed extends outside of Europe to America, where again they have around 1000 breeding animals. Here they breed the Mangalica to be served in high class restaurants. Japan is currently the biggest importer of Mangalica products [43].

As mentioned, the Mangalica is bred not just in Hungary but in various countries and its unique and high-quality meat are sought after.

2.4.3 Importance of Deep Freezing

There are several reasons as to why the successful cryopreservation of Mangalica boar semen is so important and these will be discussed in detail in the following chapter.

Cryopreservation can be applied in multiple fields in order to preserve endangered breeds or species. Its uses span across agriculture, aquaculture and within biotechnology. In the agriculture industry, cryopreservation can be used in domestic species in order to improve genetics and conserve rare or endangered breeds. The cryopreserved semen can be used as a source of genetic material to be used in genetic management programmes. At present semen banks exist for rare domestic breeds in species such as cattle, pigs, and sheep [13]. Semen banks can be instrumental in the protection of rare breeds and their use in the Mangalica pig breed could help to conserve the breed.

The use of cryopreserved boar semen within the swine industry could also decrease the risk of spreading disease which can be involved with liquid semen and boars. When using liquid semen, it is usually a short period of time between collection and use on the farm which does not leave enough time for diseases which have longer incubation periods [35]. For example, the incubation period of the African Swine Fever Virus (ASFV) is on average 15 days [44]. Freezing semen can allow for further testing and also for observation of the boar prior to using the semen and thus can rule out potential diseases [35].

A current example of a disease outbreak which causes major problems within the swine industry is ASFV. As stated by the World Organisation for Animal Health (OIE) [44], ASFV is a DNA virus, which is a member of the Asfarviridae family, in the genus Asfivirus. Both wild and domesticated swine are susceptible. Globally since January of 2020 ASFV has affected more than 1,131,000 pigs and 37,000 wild boars [45, Figure 7]

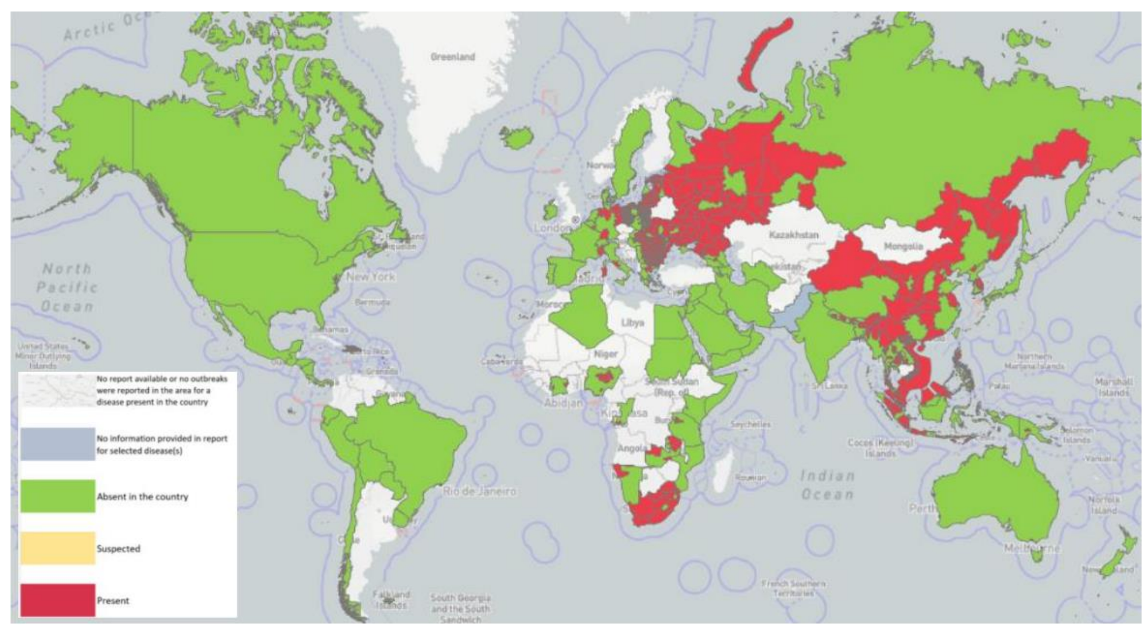

Figure 7:

World Map Showing the Presence of ASFV

In several parts of Europe ASFV is now endemic within the wild boar populations. The National Food Chain Safety Office (NÉBIH) in Hungary documents the new and old cases of ASFV in wild boars throughout the country [46, Figure 8].

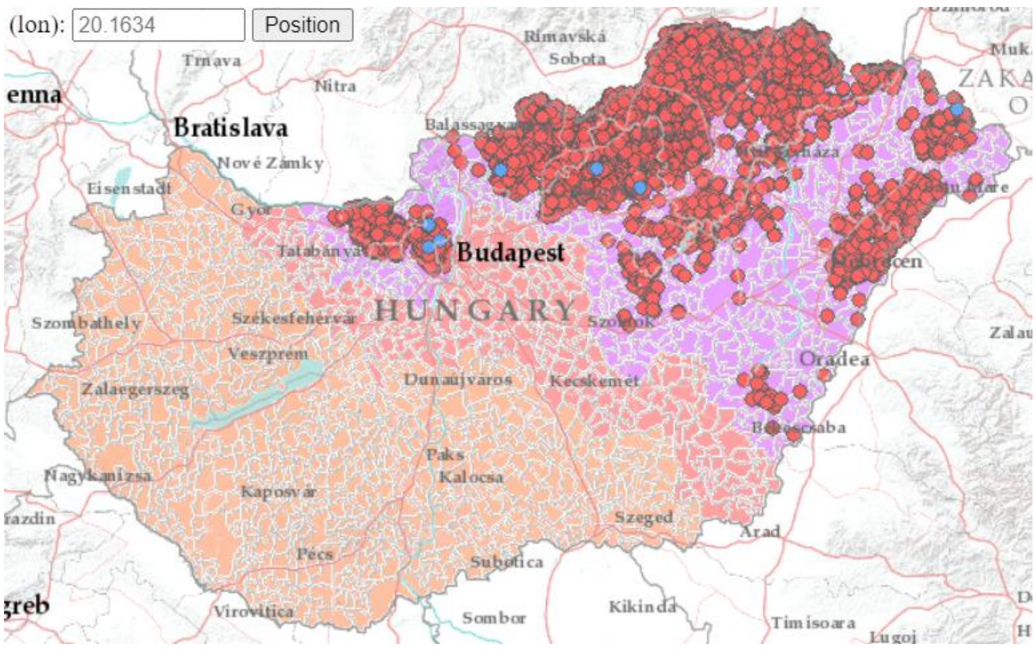

Figure 8:

Map of Hungary showing the new (blue) and previous (red) cases of ASFV in wild boars

There is a wild boar-habitat cycle which has developed within Europe, and it helps to spread the disease over wide geographical areas. Human activity also plays a huge role in transmission of the disease within domestic swine [47, Figure 9]. The virus is spread via direct contact; between sick and healthy animals and indirect contact; through biological vectors (ticks), fomites and through feeding on garbage containing infected meat [44]. It is spread via semen [32].

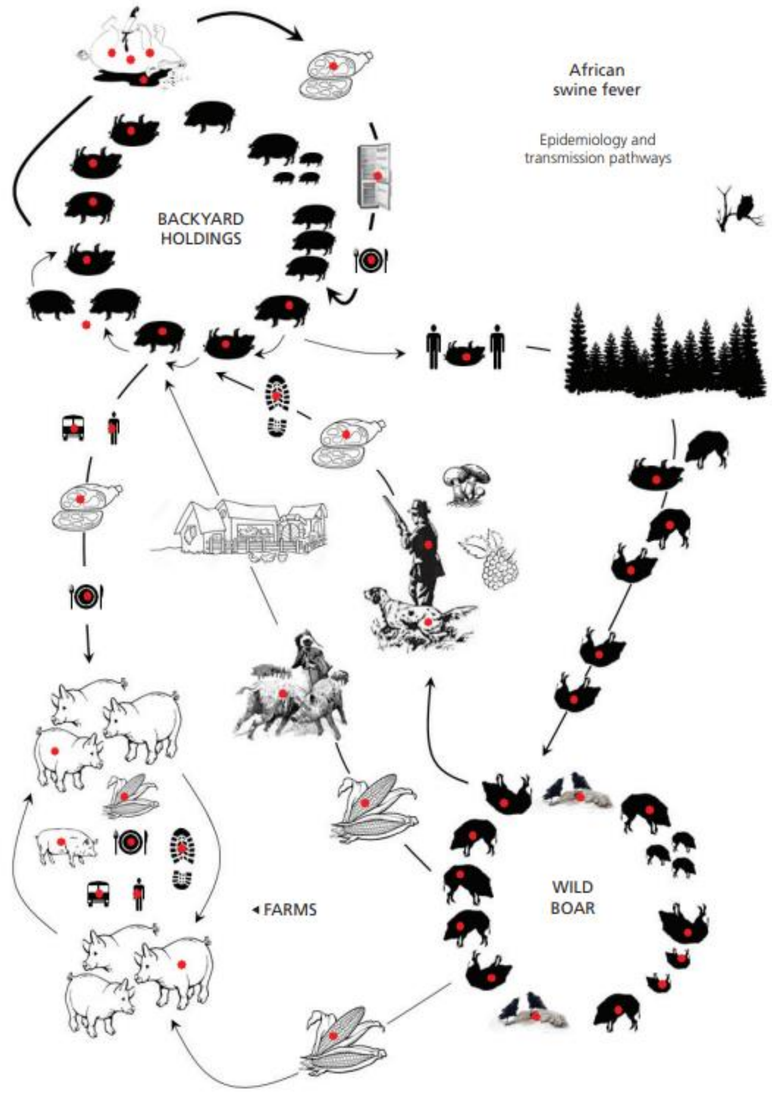

Figure 9:

Diagram of the factors and transmission pathways involved in sustaining endemicity and facilitating geographical expansion of African swine fever virus in eastern Europe

ASFV is very resistant to low temperatures, and it persists for a long time in blood, faeces, tissues and in infected uncooked pork products. It also multiplies within the vector which acts as a reservoir for the disease [44].

ASFV has low, moderate, and high virulence variants and these result in several different clinical presentations. The peracute form is caused by the highly virulent variant and leads to sudden death, often without clinical signs. The acute form is also caused by the highly virulent strain in the domestic pig and can have a mortality rate of up to 100%. Clinical signs include fever, reddening of the skin, cyanosis, vomiting, diarrhoea, abortion and finally death. Some pathological lesions that can be seen are haemorrhages in lymph nodes and kidneys, splenomegaly and ecchymosis on the legs and abdomen. The moderate virulence variant can cause the subacute form which has a lower mortality rate and less extreme clinical signs. Finally, the chronic form of the disease is a result of the low virulence variant. This form has a low mortality rate and clinical signs such as weight loss, necrosis of the skin and respiratory signs [44].

Various tests are available for diagnosis of ASFV such as; cell culture inoculation, hemadsorption test, PCR, ELIZA and indirect fluorescent antibody test. These tests are not only important for diagnosis of ASFV, but also for differentiation from Classical Swine Fever (CSF). As it is not possible to differentiate between the two via the clinical signs or pathological lesions- laboratory examination is the only way [44].

Presently there are no treatments and no vaccines available against ASFV. If outbreaks do occur in domestic swine all pigs must be slaughtered and their carcasses disposed of properly. There should be thorough cleaning and disinfection of the holding as well as implementation of infection zones and controls on movements of swine and swine products. An in-depth epidemiological investigation must be launched involving the pursuit of the possible sources of infection, as well as possible spread. The infection zone along with the surrounding area should be kept under close surveillance [44].

As described in the above information provided by the OIE [44], ASFV can have devastating effects on domestic swine if it becomes present on a holding. Undiagnosed outbreaks can result in huge production losses leading to financial losses and upon diagnosis of ASFV the slaughter of all the pigs will again lead to financial loss. In situation reports from the OIE [45, Table 1] they calculated that in total there were 1,965,343 domestic pigs lost due to ASFV from January 2020 to October of this year, with Europe having the highest loss at 1,460,936.

	Outbreaks		Cases		Losses*
	Domestic pigs	Wild boar	Domestic pigs	Wild boar	Domestic pigs
Africa	206		16,291		23,316
Americas	255		9,594		17,798
Asia	1,303	2,105	101,526	2,765	462,896
Europe	3,729	20,665	1,003,978	34,377	1,460,936
Oceania	4		500		397
Total	5,497	22,770	1,131,885	37,142	1,965,343

Table 1:

ASFV Outbreaks, Cases and Associated Losses

These figures only include animals which died or were killed and disposed of on infected establishments and does not include animals culled in surrounding areas to control the disease [45]. If these figures were included the losses would be far greater. As we can see from this data ASFV causes massive losses throughout Europe and the rest of the world.

In breeds like the Mangalica pig which already have low population numbers, an outbreak of ASFV could devastate the breed even further. In emergency situations like this, it is important to have a genetic reserve, including semen, for the purpose of re-establishing breed numbers. Cryopreserving semen of Mangalica boars can be a method of preserving genetic material to use for the creation of future generations of the breed.

Thus, cryopreservation can be used in order to decrease and stop the spread of diseases like ASFV which can have devastating effects once present on a swine farm. Furthermore, the semen banks can be used in emergency situations when the movement of swine is forbidden or restricted.

3. Conclusions

Cryopreservation of boar semen still has many limitations. The post freezing and thawing survival rate of the sperm is not high enough to provide adequate pregnancy rates and litter sizes. Thus, it is not an economical option for farmers.

However, as research progresses in this field, and the optimal conditions for freezing boar semen are further investigated, there is the possibility that the survival rate will improve. This will result in higher reproduction values and may lead to the use of frozen boar semen being used in everyday swine practice.

Furthermore, if the identification of boars with 'good' freezability becomes easily available via genetic tests, this could have a huge impact on the use of cryopreserved semen within the swine industry. As it could lead to only boars with sperm which have a higher survival rate post freezing and thawing being used. This again will increase reproduction values.

An important swine breed in Hungary is the Mangalica. It is a native breed which has low population numbers. Their numbers began to decrease post-World War Two when eating habits changed and this trend continued into the 1970s. After efforts were made to save the breed in the nineties their numbers slowly began to rise, however due to its low reproductive capacity, progress in population increase is slow. This breed has multiple desirable traits including resistance and adaptability, which we want to preserve. An outbreak of disease could very easily decrease the breed's numbers even and in an extreme case the extinction of the breed.

The current ASFV situation in Europe, and in particular within Hungary, is an excellent example. ASFV is a highly resistant disease which has dire consequences when introduced into swine farms. In this situation it is important to have genetic reserves- like cryopreserved semen, for the purpose of re-establishing the numbers of the breed. It can also act as a reserve when there are restrictions on the movement of swine and swine products.

Furthermore, the cryopreservation of semen allows further health checks to be performed on the semen and can rule out the semen being infected with diseases like ASFV. Again, this stops the spread of the disease and prevents a further decrease in breed numbers.

The use of cryopreservation is hugely important in the agricultural industry. Two of its most crucial applications beings its usage in the preservation of endangered breeds such as the

Mangalica and as a measure for the prevention of animal health crises. If the survival rate of boar semen is improved these uses could prove invaluable.

4. Acknowledgements

I would like to thank my supervisor Professor Rátky József for his continuous guidance, advice, and support throughout this process. I would also like to thank the University of Veterinary Medicine for the last 5.5 years, studying here has been a pleasure. Finally, I would also like to thank my Mum and Dad; Angela and Martin Grainger, without whom the pursuit of this career would not have been possible.

5. References

1. Vishwanath R (2003) Artificial Insemination: The State of The Art. Theriogenology 59: 571-584. https://doi.org/10.1016/S0093-691X(02)01241-4

2. Orland B (2017) The Invention of Artificial Fertilization in the Eighteenth and Nineteenth Century. HPLS 39:11. https://doi.org/10.1007/s40656-017-0136-3

3. Foote RH (2002) The History of Artificial Insemination: Selected Notes and Notables. Journal of Animal Science 80:1–10. https://doi.org/10.2527/animalsci2002.80E-Suppl_21a

4. Birkhead TR, Hosken DJ, Pitnick S (eds) (2009) Sperm Biology: An Evolutionary Perspective. Elseiver, USA

5. Walters EM, Benson JD, Woods EJ, Critser JK (2009) The History of Sperm Cryopreservation. In: Pacey AA, Tomlinson MJ (eds) Sperm Banking, 1st ed. Cambridge University Press, pp 1–17

6. Foote RH (1998) Artificial Insemination to Cloning: Tracing 50 Years of Research. Cornell Univ, New York

7. (2022) About ICAR. ICAR - International Congress On Animal Reproduction. Accessed 24th September 2022

8. Roca J, Perez-Patiño C, Barranco I, Padilla LC, Martínez EA, Rodriguez-Martinez H, Parrilla I (2020) Proteomics in Fresh and Preserved Pig Semen: Recent Achievements and Future Challenges. Theriogenology 150:41–47. https://doi.org/10.1016/j.theriogenology.2020.01.066

9. Roca J, Parrilla I, Rodriguez-Martinez H, Gil M, Cuello C, Vazquez J, Martinez E (2011) Approaches Towards Efficient Use of Boar Semen in the Pig Industry: Swine Artificial Insemination. Reproduction in Domestic Animals 46:79–83. https://doi.org/10.1111/j.1439-0531.2011.01828.x

10. Borini A, Coticchio G (eds) (2009) Preservation of Human Oocytes: From Cryobiology Science to Clinical Applications. Infroma Health care, London, UK

11. Yawn David H (2020) Cryopreservation https://www.britannica.com/technology/cryopreservation Accessed 26th September 2022

12. Bwanga CO (1991) Cryopreservation of Boar Semen. Acta Vet Scand 32:431–453. https://doi.org/10.1186/BF03546944

13. Barbas JP, Mascarenhas RD (2009) Cryopreservation of Domestic Animal Sperm Cells. Cell Tissue Bank 10:49–62. https://doi.org/10.1007/s10561-008-9081-4

14. Yong KW, Laouar L, Elliott JAW, Jomha NM (2020) Review of Non-Permeating Cryoprotectants as Supplements for Vitrification of Mammalian Tissues. Cryobiology 96:1–11. https://doi.org/10.1016/j.cryobiol.2020.08.012

15. Vingua CE (2020) Cryopreservation of Boar Semen by Vitrification. Int J Sc and Tech Res 9: ISSN 2277-8616

16. Polge C, Smith AU, Parkes AS (1949) Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures. Nature 164: 666. https://doi.org/10.1038/164666a0

17. Hernández M, Roca J, Gil MA, Vázquez JM, Martínez EA (2007) Adjustments on the Cryopreservation Conditions Reduce the Incidence of Boar Ejaculates with Poor Sperm freezability. Theriogenology 67:1436–1445. https://doi.org/10.1016/j.theriogenology.2007.02.012

18. Bamba K, Cran DG (1988) Further Studies on Rapid Dilution and Warming Of Boar Semen. Reproduction 82:509–518. https://doi.org/10.1530/jrf.0.0820509

19. Buranaamnuay K, Grossfeld R, Struckmann C, Rath D (2011) Influence of Cryoprotectants Glycerol and Amides, Combined With Antioxidants on Quality of Frozen-Thawed Boar Sperm. Animal Reproduction Science 127:56–61. https://doi.org/10.1016/j.anireprosci.2011.07.007

20. Holt WV (2000) Basic Aspects of Frozen Storage of Semen. Animal Reproduction Science 62:3–22. https://doi.org/10.1016/S0378-4320(00)00152-4

21. (2018) Facts About Semen https://www.thepigsite.com/genetics-and-reproduction/insemination/facts-about-semen Accessed 1st October 2022

22. Polge C (1978) Fertilization in the Pig and Horse. J Reprod. Fert 54(2): 461-470 https://doi.org/10.1530/jrf0.0540461

23. Yeste M, Rodríguez-Gil JE, Bonet S (2017) Artificial Insemination with Frozen-Thawed Boar Sperm. Mol Reprod Dev 84:802–813. https://doi.org/10.1002/mrd.22840

24. Dalal J, Kumar A, Dutt R, Singh G, Chandolia KR (2018) Different Cooling Rates for Cryopreservation of Semen in Various Livestock Species: A Review. Int J Curr Microbiol App Sci 7(08): 1903-1911 https://doi.org/10.20546/ijcmas.2018.708.219

25. Wiebke M, Hensel B, Nitsche-Melkus E, Jung M, Schulze M (2021) Cooled Storage of Semen from Livestock Animals (Part I): Boar, Bull, And Stallion. Animal Reproduction Science 106822. https://doi.org/10.1016/j.anireprosci.2021.106822

26. Kumar S, Millar JD, Watson PF (2003) The Effect of Cooling Rate on the Survival of Cryopreserved Bull, Ram, and Boar Spermatozoa: A Comparison of Two Controlled-Rate Cooling Machines. Cryobiology 46:246–253. https://doi.org/10.1016/S0011-2240(03)00040-3

27. Medrano A, Watson PF, Holt WV (2002) Importance of Cooling Rate and Animal Variability for Boar Sperm Cryopreservation: Insights from The Cryomicroscope. Reproduction 123: 315-322 PMID: 11866699.

28. Crabo BG, Dial GD (1992) Artificial Insemination in Swine. Veterinary Clinics of North America: Food Animal Practice 8:533–544. https://doi.org/10.1016/S0749-0720(15)30702-7

29. (2013) Collecting Semen From Boars Collecting semen from boars | Department of Agriculture and Fisheries, Queensland (daf.qld.gov.au) Accessed 29th October 2022

30. Waberski D, Henning H, Petrunkina A (2011) Assessment of Storage Effects In Liquid Preserved Boar Semen: Storage Effects in Liquid Preserved Boar Semen. Reproduction in Domestic Animals 46:45–48. https://doi.org/10.1111/j.1439-0531.2011.01836.x

31. Schulze M, Ammon C, Schaefer J, Luther A-M, Jung M, Waberski D (2017) Impact of Different Dilution Techniques on Boar Sperm Quality and Sperm Distribution of the Extended Ejaculate. Animal Reproduction Science 182:138–145. https://doi.org/10.1016/j.anireprosci.2017.05.013

32. Glossop C (1998) Al in Pigs the Production of Quality-Assured, Healthy Semen. In Practice 20(4): 182-188. https://doi.org/10.1136/inpract.20.4.182

33. Maxwell WMC, Johnson LA (1999) Physiology of Spermatozoa at High Dilution Rates: the Influence of Seminal Plasma. Theriogenology 52:1353–1362. https://doi.org/10.1016/S0093-691X(99)00222-8

34. Großfeld R, Sieg B, Struckmann C, Frenzel A, Maxwell WMC, Rath D (2008) New Aspects of Boar Semen Freezing Strategies. Theriogenology 70:1225–1233. https://doi.org/10.1016/j.theriogenology.2008.07.017

35. Knox RV (2011) The Current Value of Frozen-Thawed Boar Semen for Commercial Companies: Commercial Uses for Frozen-Thawed Boar Semen. Reproduction in Domestic Animals 46:4–6. https://doi.org/10.1111/j.1439-0531.2011.01822.x

36. Thurston LM, Watson PF, Mileham AJ, Holt WV (2001) Morphologically Distinct Sperm Subpopulations Defined by Fourier Shape Descriptors in Fresh Ejaculates Correlate with Variation in Boar Semen Quality Following Cryopreservation. Journal of Andrology 22(3): 382-394 https://doi.org/10.1002/j.1939-4640.2001.tb02194.x

37. Roca J, Hernández M, Carvajal G, Vázquez JM, Martínez EA (2006) Factors Influencing Boar Sperm Cryosurvival. Journal of Animal Science 84:2692–2699. https://doi.org/10.2527/jas.2006-094

38. Egerszegi I, Rátky J, Solti L, Brüssow K-P (2003) Mangalica – an Indigenous Swine Breed from Hungary (Review). Arch Anim Breed 46:245–256. https://doi.org/10.5194/aab-46-245-2003

39. Rátky J, Manabe N, Jaskowski J, Egerszegi I, Cseh S, Brüssow KP Biotechnology in Mangalica pig breeding. Accessed 28th October 2022

40. Rátky J, Egerszegi I, Toth P, Keonuchan S, Nagai T, Kikuchi K, Manabe N, Brüssow K-P (2013) Saving Genetic Resources of Native Pigs in Occidental and Oriental Countries- Practical Examples of the Characterization and Utilization of Native Pigs in Hungary and Laos. J Reprod Dev 59:437–441. https://doi.org/10.1262/jrd.2013-055

41. Egerszegi, I, Brüssow, K-P, Sarlós, P., Manabe, N, Rátky, J (2018) Reproductive

Capacity of Mangalica pigs – What is the Reality? Arch Zootec 68: 81–86. https://www.uco.es/.../1917

42. Rátky J, Egerszegi I, Páble T, Balogh E, Manabe N, Keonouchan S, Brüssow K-P (2017) Invited Review: Reproductive Physiology in Commercial and Premium Pig Breeds – History of 30-Year-Long Cooperation. Arch Anim Breed 60:253–257. https://doi.org/10.5194/aab-60-253-2017

43. Pocsai K, Szabó P, Balogh P (2012) Concentration Analysis of the Hungarian Mangalica Pig Stock. CBR 11 (3): 274-282. https://doi.org/10.17265/1537-1506/2012.03.003

44. OIE (2019) African Swine Fever African swine fever - WOAH - World Organisation for Animal Health Accessed 2nd November

.

45. OIE (2022) African Swine Fever (ASF) – Situation Report 22 African swine fever (ASF) – Situation report 22 - WOAH - World Organisation for Animal Health Accessed 6th November 2022

46. (2022) African Swine Fever Measures http://airterkep.nebih.gov.hu/aaijo/asp/asp.htm Accessed 6th November 2022

47. Guberti V, Khomenko S, Masiulis M, Kerba S (2022) African Swine Fever in Wild Boar - Ecology and Biosecurity. Second Edition. FAO Animal Production and Health Manual No. 28.https://doi.org/10.4060/cc0785

6. Electronic License Agreement and Copyright Declaration

HuVetA
ELECTRONIC LICENSE AGREEMENT AND COPYRIGHT DECLARATION*

Name: Rebecca Anne Grainger

Contact information (e-mail): beccagrainger2005@gmail.com

Title of document (to be uploaded: Deep Freezing of Mangalica Boar Semen

Publication data of document: November 2022, Budapest, Hungary

Number of files submitted: 1

By accepting the present agreement the author or copyright owner grants non-exclusive license to HuVetA over the above mentioned document (including its abstract) to be converted to copy protected PDF format without changing its content, in order to archive, reproduce, and make accessible under the conditions specified below.

The author agrees that HuVetA may store more than one copy (accessible only to HuVetA administrators) of the licensed document exclusively for purposes of secure storage and backup, if necessary.

You state that the submission is your original work, and that you have the right to grant the rights contained in this license. You also state that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the document has parts which you are not the copyright owner of, you have to indicate that you have obtained unrestricted permission from the copyright owner to grant the rights required by this Agreement, and that any such third-party owned material is clearly identified and acknowledged within the text of the licensed document.

The copyright owner defines the scope of access to the document stored in HuVetA as follows (**mark the appropriate box with an X**):

[ ] I grant unlimited online access,

[X] I grant access only through the intranet (IP range) of the University of Veterinary Medicine,

[ ] I grant access only on one dedicated computer at the Ferenc Hutÿra Library,

[ ] I grant unlimited online access only to the bibliographic data and abstract of the document.

Please, define the **in-house accessibility of the document** by marking the below box with an **X**:

X I grant in-house access (namely, reading the hard copy version of the document) at the Library.

If the preparation of the document to be uploaded was supported or sponsored by a firm or an organization, you also declare that you are entitled to sign the present Agreement concerning the document.

The operators of HuVetA do not assume any legal liability or responsibility towards the author/copyright holder/organizations in case somebody uses the material legally uploaded to HuVetA in a way that is unlawful.

Date: Budapest, ..…14….day .…11…..month……..…2022…..year

Author/copyright owner
signature

—

***HuVetA Magyar Állatorvos-tudományi Archívum – Hungarian Veterinary Archive** is an online veterinary repository operated by the Ferenc Hutÿra Library, Archives and Museum. It is an electronic knowledge base which aims to collect, organize, store documents regarding Hungarian veterinary science and history, and make them searchable and accessible in line with current legal requirements and regulations.*

HuVetA relies on the latest technology in order to provide easy searchability (by search engines, as well) and access to the full text document, whenever possible.
Based on the above, HuVetA aims to:

- *increase awareness of Hungarian veterinary science not only in Hungary, but also internationally;*
- *increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals;*
- *present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question;*
- *facilitate professional relations and collaboration;*
- *support open access.*

7. Thesis Supervisors Acceptance Letter

I hereby confirm that I am familiar with the content of the thesis entitled *Deep Freezing of Mangalica Boar Semen*

written by *Rebecca Anne Grainger* which I deem suitable for submission and defence.

Date: Budapest: 14th November 2022

UNIVERSITY OF VETERINARY MEDICINE,
BUDAPEST

INTERNATIONAL STUDY PROGRAMS

secretary, student@univet.hu

founded in 1787, EU-accredited since 1995

Thesis progress report for veterinary students

Name of Student: *Rebecca Anne Grainger*

Neptun code of the student: *3532/E*

Name and title of the supervisor: *Professor Rátky József, DVM, PhD, DSc*

Department: *Department of Obstetrics and Farm Animal Medicine*

Thesis title: *Deep Freezing of Mangalica Boar Semen*

Consultation – 1st semester

	year	month	day	Topic / Remarks of the supervisor	Signature of the supervisor
1.	2021.	02.	15.	topic introduction	[signature]
2.	2021.	03.	10.	1st draft, content	[signature]
3.	2021.	03.	30.	literature	[signature]
4.	2021.	04.	15.	next topics	[signature]
5.	2021.	05.	02.	progress discussion	[signature]

Grade achieved at the end of the first semester: excellent (5)

Consultation – 2nd semester

	year	month	day	Topic / Remarks of the supervisor	Signature of the supervisor

1.	2022.	03.	05.	2nd draft correction	
2.	2022.	04.	20.	Discussion to finalize	
3.	2022.	09.	30.	Figures, tables	
4.	2022.	10.	15.	Final form	

5.					

Grade achieved at the end of the second semester: excellent (5)

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

....

signature of the supervisor

Signature of the student: Rebecca Grainger

Signature of the secretary of the department: ....

Date of handing the thesis in: 14/11/2022