

## Semen donation and establishment of an open canine semen bank: a novel tool to prevent inbreeding in pedigree dogs

*Spermadonatie en de start van een open spermabank voor honden: een nieuw hulpmiddel om inteelt bij rashonden te voorkomen*

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### A BSTRACT

At present, only 5% of pedigree dogs is being used for breeding. To increase the number of breeding dogs, one solution could be to start a canine semen bank based on the principle of semen donation, like in humans. Many dog owners have no desire to become dog breeders but are willing to preserve the genetic material of their dog, if offered this possibility. However, not all canine ejaculates are suitable for cryopreservation as the initial quality may differ and the resistance of sperm cells to survive the freezing procedure is highly variable. In order to freeze the semen of as many male dogs as possible, it is important to optimize and individualize the cryopreservation protocol per ejaculate. Practically, frozen semen can be stored in the CanIFreeze-semen bank or in veterinary practices adjacent to the owner of the bitch and can be used for insemination at a later time.

### SAMENVATTING

Slechts 5 % van de rashonden wordt momenteel gebruikt voor de fok. Om dit aantal te verhogen, zou het opstarten van een spermabank voor honden, die gebaseerd is op donatie van sperma zoals bij de mens, een mogelijke oplossing kunnen bieden. Veel eigenaars willen niet per se met hun hond fokken, maar willen wel het genetisch materiaal van hun reu bewaren, indien deze mogelijkheid aangeboden wordt. Niet alle ejaculaten zijn geschikt om ingevroren te worden, omdat de kwaliteit van het staal kan verschillen en omdat de weerstand van het sperma om het invriesproces te overleven sterk variabel is tussen honden onderling. Om sperma van zoveel mogelijk verschillende reuen te kunnen invriezen, is het belangrijk om het invriesproces te verbeteren en te individualiseren. Het ingevroren sperma kan bewaard worden in de CanIFreeze-spermabank of in dierenartspraktijken dichtbij bij de eigenaar van de teef om op een later tijdstip gebruikt te kunnen worden voor inseminatie.

### INTRODUCTION

Dogs are popular pets in Belgium. With a current Belgian canine population of 1.34 million, they are considered as a family member in one out of four Belgian households (<https://www.fediaf.org>). Unfortunately, dogs exhibit a high number of recognized genetic diseases (Mellersh, 2008). At present, more than

700 different inherited conditions are reported in that species (<https://omia.org/home/>). Although the estimates tend to vary a little bit, for about 50% of these diseases, the mode of inheritance is known, a third being monogenic with a majority being autosomal recessive (Patterson, 2000; Leroy et al., 2011; <https://omia.org/home/>). The dissemination of these genetic disorders is therefore highly associated with a loss of

genetic variation within a breed and is a major concern, especially in purebred dogs. Important contributors to the loss of genetic diversity are two major population bottlenecks events: domestication and breed formation. Dog breeds have been generated over several centuries by selecting and mating a (sometimes very) small number of dogs with particular physical and/or behavioral characteristics, which were different from the large canine population. The propagation of breed-specific phenotypic traits has contributed to the high level of homogeneity within a breed and the high level of heterogeneity between breeds (Farrell et al., 2015). Nowadays, the Fédération Cynologique Internationale (FCI) recognizes more than 400 dog breeds (Wijnrocx et al., 2016) but the 20 most popular breeds represent 72% of the total number of registrations, whereas the rarest 100 represent only 2% (Farrell et al., 2015). As such, most breeds are represented by a small and closed population with no or at best a very limited gene flow. For example, in Belgium, genetic diversity in purebred dogs is very low in about half of the 23 studied breeds (Wijnrocx et al., 2016). Aside from the two bottlenecks mentioned earlier, this poor genetic variation can also be explained by breeding practices that are commonly used. These breeding practices are the so-called popular sire effect, line breeding and close inbreeding (Leroy et al., 2010; Leroy et al., 2011). The popular sire effect is used to describe the intense use of some sires (mainly champion dogs) and is considered to be the main reason of dissemination of genetic disorders. In an attempt to counteract this, the FCI recommends that the number of offspring per sire should not exceed 5% of the number of puppies born in this breed during a five-year period. Line breeding represents the mating of two individuals not too closely related but having a common ancestor. In a study by Leroy et al. (2007), it has been shown that half of the French dog breeders commonly used line breeding. Finally, close inbreeding represents the mating of even more closely related relatives (sibling or sire-daughter) in order to fix a trait present in an individual. Overall, (partially) as a resultant of these breeding practices, only 5% of the dog population is used for reproduction on average (KMSH, personal communication, 2019).

Due to the combination of the aforementioned breeding practices and because of the incorrect interpretation of genetic tests, e.g. exclusion of carriers of mutations responsible for autosomal recessive mutations, many potential breeding dogs have been excluded from breeding and unintentionally, the effective population size has decreased. The inadvertent decrease of genetic diversity and the negative impact this has on canine health have recently become one of the top priorities of the Belgian Kennel Club (KMSH). To tackle this problem, the KMSH has adopted a novel approach, based on the combination of limiting the popular mating practices, increasing the number of breeding dogs as much as possible and an

increase of the international exchange of genetic material. The number of reproducers can be increased by registration of dogs without pedigree but with the appropriate phenotype (or even genotype) (Wang et al., 2017) or by semen donation. If owners of purebred dogs who have no breeding plans, donate the semen of their dog, these dogs can also contribute to the next generations and increase the effective population size. In both cases, the goal is to include as many dogs as possible in the breeding program because every dog is valuable. The exchange of genetic material between canine populations of different countries should also be increased, since it has proven to be beneficial, especially for breeds with small or medium (effective) population sizes. An important tool to achieve this is the use of artificial insemination (AI) of frozen semen. Unfortunately, the resistance of semen to withstand the freezing procedure is highly variable between dogs and sometimes even between ejaculates (Rota et al., 2005; Farstad, 2009). This process must therefore be optimized and individualized to allow successful freezing of the semen of as many breeding sires as possible. Individualization of semen freezing refers to the testing of different freezing protocols and the subsequent selection of the optimal freezing protocol for each individual dog.

To follow this novel vision on breeding, a large collaboration involving the departments of Small Animal Reproduction and the laboratory of Animal Genetics of Ghent University, together with specialist veterinarians and the KMSH has been initiated. The goal of this project is the development of a semen bank as a tool to increase the number of dogs used in reproduction. Furthermore, to maximize the number of stud dogs, it aims to improve the cryopreservation protocol of canine semen. In this article, the current cryopreservation process will shortly be described before detailing how semen donation and the improvement of cryopreservation can help to produce healthier dogs in the future.

## CRYOPRESERVATION

Semen cryopreservation is a process, in which spermatozoa are preserved by freezing at very low temperature (either at  $-196^{\circ}\text{C}$  in liquid nitrogen or in dry ice at  $-78.5^{\circ}\text{C}$ ). As in other domestic animals, this is the best technique to preserve the semen for a longer period of time (Alamo et al., 2005). Since Seager reported the first pregnancy in dogs following AI with frozen-thawed semen in 1969, cryopreservation of dog semen has experienced an increased interest and became more and more requested by breeders, owners and institutions, e.g. police, military, guiding dogs (Futino et al., 2010; Hollinshead et al., 2017). This interest has led to the establishment of private canine semen banks (Farstad, 2000; Kim et al., 2010). Long-term storage of semen from valuable males and

easier exchange of genetic material between populations at distant locations are the main advantages of semen cryopreservation (Axner et al., 2016). The preserved genetic material can be used even when the sire is no longer fertile, unavailable or deceased (Kutzler, 2005). Moreover, as in other types of artificial inseminations in dogs, the cryopreserved semen can be used to overcome behavioral incompatibility between the male and the female and to protect them against venereal diseases (Farstad, 2000; Schäfer-Somi et al., 2006).

The main downside of cryopreservation is that it affects the fertility of the semen as it leads to detrimental structural and functional changes in spermatozoa (Kim et al., 2010; Brito et al., 2017; Khan et al., 2017). These changes are triggered by stress and may affect the sperm cells at any step of the procedure: the technique of semen collection, the dilution rate, the type and composition of the extender, the cooling rate, the time and temperature of equilibration, the packing method and the rate and method of freezing and thawing (Alamo et al., 2005; Schäfer-Somi et al., 2006; Barbas et al., 2009). All together, these factors are the reason why only 50% of spermatozoa survive the cryopreservation process (Alhaider et al., 2008; Amidi et al., 2016). Understanding the causes of cryoinjury is a key factor to develop new cryopreservation techniques with the goal to improve the survival of sperm cells (Alhaider et al. 2008; Barbas et al., 2009).

### Semen collection and evaluation

The most common method to collect semen from a dog is by digital manipulation. Before collection, it is important to eliminate or minimize any distraction and/or anxiety. Indeed, in case of fear or pain, a complete erection of the penis and ejaculation may not be reached. Ideally, a bitch in oestrus (called teaser bitch) is present to increase the libido of the male and the likelihood of a successful collection. If no teaser bitch is available at the time of collection, vaginal swabs from bitches in oestrus presented for cycle follow-up and preserved in the freezer (-20°C) can be used. The pheromones present on the swabs can help to stimulate the dog. The digital manipulation of the penis starts with a vigorous massage at the level of the bulbus glandis until a partial erection develops (light engorgement of the bulbus glandis). Then, the prepuce is retracted caudally behind the bulbus glandis and a constant pressure behind the bulbus glandis is applied. Dog ejaculates consist of three fractions and only the second and third fractions are collected in plastic vials. The first and the third fractions originate from the prostate (the sole secondary gland in dogs) while the second fraction, also called the sperm-rich fraction, originates from the epididymis and contains the spermatozoa (Kutzler, 2005; Rijsselaere et al., 2011).

Immediately after collection, the second fraction is placed in a 37°C environment and analyzed macro-

scopically (volume, color, admixtures and homogeneity) and microscopically (motility, concentration, morphology and membrane integrity). The motility (both total and progressive) can be assessed subjectively under light microscope, on a pre-warmed glass slide, on a scale ranging from 0 to 100% or objectively by computer-assisted sperm analysis (CASA). The concentration can be evaluated after a 1:40 dilution and fixation in tap water or diluted formol, with the use of a counting chamber, e.g. Bürker or Thoma chamber, or with an automated sperm cell counter. The morphology and membrane integrity can be assessed using eosin-nigrosin staining. Alternatively, morphology can also be determined with a Diff-Quick staining (Rijsselaere et al., 2011).

### Semen extenders

Spermatozoa are the endpoint of the spermatogenesis and have particular anatomic and metabolic characteristics. They have little biosynthetic activity and depend mostly on their extracellular environment to stay alive (Barbas et al., 2009). The seminal plasma, consisting of the epididymal fluid and the prostatic fluid, plays essential roles in the survival of the sperm cells. It provides metabolic support, energy and serves as a vehicle for the spermatozoa (Korochkina et al., 2014). However, canine seminal plasma does not appear to have the ideal composition to support cryopreservation of spermatozoa (Sirivaidyapong et al., 2001). The sperm-rich fraction should therefore be centrifuged (at 720g for 5 minutes) to separate the spermatozoa from the seminal plasma (Rijsselaere et al., 2002). Thereafter, sperm cells should be diluted in a proper extender to promote semen survival during the cryopreservation process. Several extenders for dog semen are available and commercialized. Even though their formulations differ slightly from each other, they all contain substrates to provide energy, e.g. glucose, lactose, raffinose, saccharose or trehalose, salts, e.g. sodium citrate or citric acid, buffers to maintain an adequate pH and suitable osmolality, e.g. TRIS, a non-permeating cryoprotectant, e.g. egg yolk, skim milk, a permeating cryoprotectant, e.g. glycerol, ethylene glycol, to protect the spermatozoa from cryogenic injury and antibiotics to prevent the growth of bacteria, e.g. penicillin or streptomycin. Cryoprotectants reduce the physical and chemical stress sperm cells undergo during cooling, freezing and thawing (Barbas et al., 2009; Rijsselaere et al., 2011). Egg yolk is a common compound present in extenders due to its cryoprotectant and buffering properties associated with the presence of phospholipids (lecithin) and low density lipoproteins. Its concentration varies from 10 to 20% (Bencharif et al., 2008; Barbas et al., 2009; Abe et al., 2008). Egg yolk is however a biologically hazardous compound with a risk of bacterial contamination. Moreover, its composition is not constant and may vary between batches making it dif-

difficult to standardize (Axner et al., 2016). These disadvantages have motivated researchers to find alternatives, e.g. vegetal lecithin, egg yolk-derived phospholipids, milk-based diluters, but egg yolk remains so far superior to its replacements (Abe et al., 2008; Farstad, 2009; Axner et al., 2016). In recent studies, a positive effect of adding a detergent (Equex STM paste) and antioxidative compounds, e.g. melatonin, glutathione peroxidase or superoxide dismutase, to the extender has been demonstrated on canine semen survival (Amidi et al., 2016; Axner et al., 2016). The detergent Equex STM paste is supposed to exert its positive effect by modifying the egg yolk and should therefore be exclusively used in extenders containing egg yolk.

In some protocols, spermatozoa are diluted with the extender in one step but in recent studies, a beneficial effect of a two-step dilution has been shown in extenders only differing by their concentration of glycerol. The second extender contains a higher glycerol concentration and is added just before freezing, to reach a final glycerol concentration of 5%, in order to avoid the detrimental toxic effect of glycerol on the spermatozoa during the equilibration period (Peña et al., 2000; Nugraha Setyawan et al., 2015). This method is however more tedious, as it requires more handling and carries more risk of error or contamination during manipulation (Brito et al., 2017).

### Cooling of the semen

After dilution, a leak proof container with the extended semen is closed and placed in a water bowl (at 37°C) and cooled in the fridge ('au bain marie') for 1 to 2 hours at 4°C (Peña et al., 2000; Rijsselaere et al., 2011). This cooling period, called equilibration, is an essential step during the cryopreservation procedure. It allows the different components of the extender to carry out several modifications on the spermatozoa in order to prepare them to survive at low temperatures (Barbas et al., 2009). The plasma membrane suffers, however, from different stressors and some harmful cellular modifications may lead to the death of the spermatozoa, especially because of the toxic effect of glycerol (Barbas et al., 2009; Belala et al., 2016; Alcantar-Rodriguez et al., 2017).

### Freezing-thawing of the semen

After cooling, the extended semen is deposited in straws of 0.5 ml, which have been shown to be the best storage device for dogs, and properly identified with date, name of the dog, breed, chip number and freezing centre (Nöthling et al., 2005; Rijsselaere et al., 2011). Freezing can be obtained with a static method consisting of a rack, in which the straws are placed, and located at about 4 cm above the liquid nitrogen (temperature of -130°C). By varying the distance between the straws and the liquid nitrogen and the time of exposure before plunging into liquid nitrogen, dif-

ferent freezing rates can be obtained. However, the repeatability of these freezing rates is not optimal. To improve this technique, an automated programmable computerized freezer, e.g. IceCube®, can be used. The semen is cooled from 4°C to -130°C and then plunged into the liquid nitrogen (-196°C) and stored until further use (Schäfer-Somi et al. 2006). When the semen is needed, straws can be thawed in a water bath at 37°C for 30-60 seconds or at 70°C for 6-7 seconds (Rijsselaere et al., 2011).

### Insemination of frozen semen

Intrauterine insemination is the method of choice for frozen-thawed canine semen insemination (Mason, 2017). Transcervical insemination or TCI is preferable over surgical insemination, which is subject to ethical issues and even illegal in many countries (Kim et al., 2007; England et al., 2008). Transcervical insemination can be performed under visual guidance with fibre optic endoscopy, or can be performed blindly (by palpation of the cervix) with a Norwegian catheter (Scandinavian method). However, TCI performed by endoscopy allows visualization of the cervix and minimizes the risk of trauma during the procedure. A semen concentration of minimum 150 million live, morphologically normal spermatozoa has been shown to maximize the pregnancy rate and is therefore the recommended dose for artificial insemination (Mason, 2018). Higher whelping rates and litter sizes are obtained when the bitch is inseminated twice, respectively two and three days after ovulation (Thomassen et al., 2006; Hollinshead et al., 2017). In many studies, a whelping rate has been described varying between 60% and 70% after intrauterine insemination with frozen-thawed semen, whereas it reaches 85% when fresh semen is used (Alamo et al. 2005; Thomassen et al., 2006; Hollinshead et al., 2017; Mason, 2017). If the frozen-thawed semen cannot be deposited inside the uterus for any reason but intravaginally, the whelping rates drop dramatically to 10% (Thomassen et al., 2006).

### SEMEN DONATION

To broaden the genetic diversity in purebred dogs, the availability of stud males in the dog population needs to be increased. As only 5% of the entire Belgian canine population is used to produce puppies for the next generations, the idea of semen donation and the start of a public semen bank for dogs has emerged to counteract this unavoidable loss of genetic diversity. Since most owners of purebred dogs do not have the intention to breed with their dogs, persuading them to let their dog do a semen donation could be a tool to keep this valuable genetic material in the pool of reproducers. Semen donation can be somehow compared to blood donation in dogs. In semen donation however, the gift has an effect on the entire population

and not only on the individual.

After collection, the donated semen is analyzed and if of sufficient quality, is cryopreserved to be part of the first open canine semen bank (CanIFreeze). Information concerning the semen donors present in the bank is then shared publicly in a new software program developed by the KMSH, which is called the Mate Select Program. This program lists all registered dogs available for breeding and allows owners to simulate mating of their dog with another one. The inbreeding and kinship coefficients resulting from this mating are calculated based on the input of pedigrees and based on the results, the owner receives an advice to use this combination for breeding or not. Advice to breed is given for all combinations resulting in low levels of inbreeding and kinship coefficients.

The concept of an open canine semen bank is very simple: as for blood donation, specific criteria need to be fulfilled to become a canine semen donor. The dog needs to be a purebred dog or should have the breed-specific phenotype. For the start-up of the semen bank, the weight of the dog needs to be minimum 15 kg because the total sperm output is correlated to the size of the dog and small dogs will not give enough semen that can be cryopreserved for several insemination doses. At later stages, when cryopreservation techniques will have been optimized, smaller dogs might be included too. Finally, the dog needs to be minimum one year old because semen in younger dogs can be of lesser quality. The semen collection needs to be performed on different occasions, until enough semen has been collected for a minimum of two and a maximum of ten insemination doses. The lower limit is set at two to include at least one insemination dose in the semen bank, the second one being reserved for the owner of the donor dog. The upper limit is set at ten to avoid overuse of the sire.

To motivate the owner to come with his dog for semen donation, a free health check-up is performed and advice is given to the owner in case of any abnormality. Also, a free semen evaluation is performed and if the semen is suitable for cryopreservation, the owner of the stud dog is entitled to a free intrauterine insemination with a bitch of his choice in agreement with the owner of the bitch. This free insemination has to be done with the semen of their own stud and within 5 years following the collection. After this time, they can either remain the owner of this insemination dose but will have to pay a fee for the storage and the further insemination(s) or they can donate the insemination dose to the semen bank.

## CANIFREEZE

CanIFreeze is the name of the first public semen bank for dogs established worldwide and based primarily at the Faculty of Veterinary Medicine of Ghent University. However, every practicing veterinarian interested in canine reproduction can become a part-

ner in this semen banking project, after following a training on freezing, identification and storage of dog semen, either at the European Veterinary Society for Small Animal Reproduction (EVSSAR) congress or at the Faculty. By initiating this project and making it open for collaboration, it should achieve its primary goal, which is an increase in the number of pedigree dogs that will be successfully cryopreserved in Belgium and the Netherlands, resulting in an increased genetic diversity, if properly used. However, CanIFreeze is also a scientific project aiming to increase and improve the cryopreservation of dog semen. As previously described, there is a high variability in the resistance of sperm cells to withstand the freezing procedure (Rota et al., 2005; Schäfer-Somi et al., 2006; Farstad, 2009). Unsuitable semen leads to the elimination of a part of the canine gene pool that could be used in reproduction. Although an optimized standard protocol for the cryopreservation of dog semen would be ideal, this is not achievable due to variations in the cellular response to cryopreservation (Eilts, 2005). At present, Ghent University is using the 'Uppsala method' for freezing of dog semen. It consists of a two-step protocol with a Tris-citric acid-egg yolk-glucose-based extender. The first extender contains 3% of glycerol and is equilibrated with the semen until 4°C for 1-2 hours before the second extender, containing 7% of glycerol and Equex STM paste, is added. The semen is then packaged in straws of 0.5 ml and frozen with the automated programmable computerized freezer (IceCube) using the freezing curve described by Schafer-Somi in 2006 (Schäfer-Somi et al., 2006; Rijsselaere et al., 2011). With selected ejaculates of sufficient quality (at least 70 % progressive motility), this method is yielding about 50 % sperm survival and equal pregnancy rates. Therefore, in order to increase sperm survival and subsequent pregnancy rates, efforts will be made to develop new cryopreservation techniques and protocols to adapt the cryopreservation procedure on the individual. As such, during the period of this project, a part of the donated semen will be used for research.

## CONCLUSION

Semen donation can be an important tool to improve the poor genetic diversity currently observed in pedigree dogs. Cryopreservation of a fixed amount of semen doses from a large donor population increases the number of reproducers and helps to produce healthier generations of dogs. Furthermore, an open semen bank might increase the international gene flow. Involvement of many veterinary practices in the development of CanIFreeze is imperative in this respect. However, in order to successfully preserve the semen of as many dogs as possible, the cryopreservation procedure has to be optimized and individualized. Overall, the goal is to increase the genetic diversity in the Belgian (and other dog) population(s) worldwide.

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## Oproep



**CANIFREEZE**  
**SPERMABANK BIJ DE HOND**

Het aantal fokreuen moet toenemen om het aantal genetische ziekten bij honden te verminderen.

**CRITERIA:**

- Reu (niet gecastreerd)
- Rashond
- Minimum 15 kg
- Minimum 1 jaar oud

**VOORDELEN:**

- Gratis gezondheidscontrole
- Gratis sperma-onderzoek
- Eén gratis inseminatie binnen 5 jaar na collectie

**WIL JIJ ONS HELPEN OM DE GEZONDHEID VAN RASHONDEN TE VERBETEREN? JOUW HOND KAN HIERBIJ HELPEN**

De Universiteit van Gent zoekt intacte reuen voor  
**SPERMADONATIE**

**VOOR INLICHTINGEN, CONTACTEER ONS OP 09/264.75.64**

Jij houdt van je hond maar je wilt er zelf niet mee fokken? Jij kan ons helpen om een spermabank op te starten voor toekomstige generaties.

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