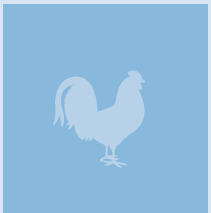
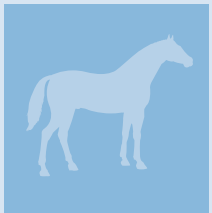
SECTION 8

Collection of germplasm and tissues





Collection of germplasm and tissues

Whether germplasm is collected on farm or at collection facilities will depend on the conditions within country, the availability of resources and the accessibility of the targeted animal populations. Collection and processing procedures will differ widely depending upon the type of germplasm being collected and the donor species. This section presents an overview of collection procedures for the various types of germplasm, including information particular to each of the major livestock species. Further details regarding germplasm processing and cryopreservation are presented in the appendices.

SEMEN

Bull, ram, buck goat, buck rabbit and stallion semen is typically collected using an artificial vagina (AV), whereas boar semen is usually collected by using the hand-glove method. These methods are usually performed on animals that have been trained for semen collection and are thus habituated to contact and interaction with humans and the collection equipment. Electro-ejaculation may be considered for animals that have not been properly trained, although it should be avoided in the case of boars and stallions. If a male has died before sufficient semen has been collected or if a male can be sacrificed because its genetic material is considered more valuable than the live animal, epididymal semen can be collected directly from the testes. In poultry, the abdominal stroking technique can be used for semen collection. For a scientific review of semen collection methods for various mammals, see Watson (1978). For the basic supplies and equipment needed for semen collection and freezing, see Appendix B.

Collection with an artificial vagina

Different types and sizes of AV are available for use in different species and breeds within species (bull, ram, buck goat, stallion and buck rabbit). Before collection, the AV should be prepared with a large-enough volume of warm water to ensure sufficient physical pressure to stimulate the glans penis of the male. The inner wall of the AV should be between 42 and 48 °C, depending on the body temperature of the animal, and should remain within the same temperature range throughout the semen collection process. The collection liner of the AV should then be lubricated with a non-spermicidal sterile gynaecological lubricant.

The use of a "teaser" animal of the same species is recommended. The teaser animal facilitates collection by allowing the donor male to mount and ejaculate in a way similar to natural mating. A live teaser may also be used to increase arousal, by allowing the donor to follow the teaser while it is led around the collection area immediately prior to collection.

For stallions, collection via AV is often performed by using a "phantom mare" rather than a live teaser animal. A phantom mare is an object constructed to mimic the size and shape of a real horse. For rabbits, the teaser is typically a female and the AV is affixed at the vulva.

Once the teaser is readied, the donor male is allowed to mount one to two times for false collections. The false mounting procedure will allow the male to become aroused and increase the final volume of the ejaculate. During these false collections the penis of the male is diverted to protect the teaser animal and does not enter the AV.

Once aroused, the male is allowed to mount and the penis is guided so that it enters the AV. The AV should be held at a height and angle that allow for easy and comfortable entry by the penis of the donor male. The male is allowed to thrust and remain on the teaser until ejaculation is complete. The AV is then taken into the laboratory and the semen is processed for insemination or cryopreservation.

Safety of the personnel, donor and teaser animals should be a primary concern during semen collection with an AV, especially in the case of large species. The teaser animal should be securely restrained. The collection area must provide secure footing for both the semen donor and the teaser. For stallions in particular, protective head gear, such as a helmet, should be worn by all persons handling the animals prior to, during and following collection.

Electro-ejaculation

In general, the AV method is preferred for semen collection, as it tends to yield the highest-quality semen and cause the least stress in the animal. However, in some situations where the donor male cannot be trained for conventional collection, such as at remote sites in the field, collection via electro-ejaculation is the most practical option (bull, ram, buck goat, not stallions).

Prior to the electro-ejaculation procedure, the collection tubes (or cones) will need to be prepared. In the case of bulls, the conical glass tubes need to be insulated using a 37 °C water jacket. Ram- and buck-semen collection tubes can be handled similarly or simply kept insulated by the hand of the collecting technician.

The probes of electro-ejaculators differ in size according to species, as do the specific collection methods. Operating instructions differ according to the manufacturer, and must therefore be carefully followed. Training and experience are needed in order to be proficient in semen collection using this methodology.

For cattle, electro-ejaculation is performed with the bull restrained in a standing position. The probe is lubricated and inserted into the rectum with the metal electrodes facing ventrally (downward). The electro-ejaculator is turned on and the voltage increased (manually or automatically) in small increments until the bull maintains an erection. The oscillating voltage peaks are then continued until semen is ejaculated and collected in a clean vessel.

In the case of bucks and rams, the animal is placed on his side and the penis is extended from the sheath by stretching the sigmoid flexure. The penis is grasped with sterile gauze and the glans penis (with its urethral process) is diverted into a 50 ml disposable tube. The lubricated electro-ejaculator is then inserted into the rectum of the animal and used to gently massage the accessory glands by exerting a downward pressure on the bottom of the rectum. This pressure should be applied for 10 to 15 seconds prior to turning on the

electro-ejaculator. After massaging, the electro-ejaculator is turned on for three to eight seconds and then the animal is allowed to rest for 15 to 20 seconds. Massaging the accessory glands in between stimulation will cause the male to ejaculate. Stimulation is usually not performed more than three times during the collection process and at least one hour is allowed between collections. After ejaculation, the semen is covered to maintain its temperature and taken to the laboratory for processing.

Gloved-hand collection technique

For collection of pig semen, the boar is first allowed to mount a teaser animal or mounting dummy. The penis needs to be fully extended prior to semen collection. The protruding penis is then grasped so that the ridges of the penis are between the collector's fingers and pressure can be applied to the glans penis with the smallest finger of the collector's hand. After the initial fractions of the semen are ejaculated, the sperm-rich portion (which has a milky appearance) should be collected into a 37 °C insulated container covered with two layers of sterile gauze to remove the gel fraction. The remaining fraction is then ready for further processing. For an overview of methods for evaluating the quality of boar semen, see Woelders (1991) or Colembrander *et al.* (2000).

Abdominal stroking

In poultry, semen collection is performed by the abdominal massage method described by Burrows and Quinn (1935). This procedure works best when done by two persons working together. One person carefully restrains the bird between his or her arms and body, while the second person collects the semen. This person massages the abdomen of the bird with firm rapid strokes from behind the wings towards the tail. The animal's readiness to ejaculate is indicated by the tumescence (erection) of the phallus. At this point, the handler gently squeezes the cloaca with two or three fingers, expressing semen through the external papillae of the ducti deferentis and into a pre-warmed tube held underneath the cloaca. The person collecting the semen must be careful, because the close proximity of the penis and cloaca increases the likelihood of the semen being contaminated with faeces, urates and bacteria, which have a detrimental effect on semen quality.

Semen can be collected from mature males twice or three times per week. Either a graduated glass funnel-shaped tube or a standard graduated glass tube can be used. Preferably, the semen extender (e.g. Lake extender – Lake, 1960) and the collection tubes should be placed in a 25 °C incubator for processing.

Epididymal sperm collection

Over the years, attempts have been made to harvest epididymal sperm from live intact males (mammalian species) either by catheterization or by flushing the lumen of the cauda (tail) of the epididymis with a hypodermic needle and a plastic syringe. Of these two approaches, catheterization of the cauda epididymis in the mature male is reported to be the most successful. In most males, the catheterization procedure is successful, but frequent post-surgical problems with the indwelling catheter have meant that this approach has remained relatively unpopular.

The most often-used approach to harvesting epididymal sperm is to surgically remove the testes from the male before, or shortly after, death. With this approach, the excised testes are placed in a sterile resealable (zip-lock) plastic bag, then into an empty Styrofoam cooler to maintain them at body temperature, and then transported to the laboratory for processing. For longer-distance transport, the testes can be chilled. The temperature during transport is usually adjusted by placing ice or ice packs in the bottom of the cooler. However, the temperature of livestock testes upon arrival at the laboratory (i.e. before processing) should not be below 5 °C.

The most common method of harvesting sperm is by slicing (with a scalpel) across the cauda portion of excised epididymis that has been placed into a sterile Petri dish containing a sperm medium (Guerrero *et al.,* 2008, 2009). A second approach is to make two incisions in the cauda and use a 30 ml syringe attached to a plastic tube to flush the sperm from the lumen into a dish with a retrograde flow of sperm medium (Saenz *et al.,* 2008). The cauda epididymis of goats can be sliced and suspended in Salomon's one-step freezing medium at a one to four dilution rate. After two filtrations (75 and 50 µm) through a nylon grid at room temperature, sperm suspension is free from any somatic cells and ready for further processing (Ehling *et al*., 2006). In some species (e.g. dogs) it is possible to collect viable sperm from intact testes that have been frozen (-20 °C) in a plastic zip-lock bag shortly after collection and then thawed (Graff *et al.,* 2000).

Shipping and processing of collected semen

Following collection, the samples can be immediately cryopreserved or transported to a laboratory (up to 24 hours holding time) for cryopreservation. The protocols for processing are species-specific, and, thus, specific media are required. Detailed information on processing and freezing samples is presented (by species) in Appendix C. Evaluation of semen is an essential aspect of processing. Proper evaluation prevents the freezing of semen that is not viable, and allows possible problems with semen donors to be identified quickly. General guidelines for semen evaluation (valid for all livestock species) are presented in Appendix D.

When semen is collected on farm or in the field, the samples must be maintained in a temperature-controlled environment during transport to the processing centre. A shipping container should, therefore, be prepared immediately before the collection of the semen. A standard shipping container has both an inner and an outer Styrofoam box. The outer box will hold numerous sealed reusable frozen ice packs as well as the inner Styrofoam box. The ice packs are placed in the outer box prior to the collection of the semen samples. After the samples are collected, the ice packs are placed into the inner box. For bulls, rams and buck goats, the number of ice packs must be sufficient to cool the samples to 5 °C. For stallions and boars, the samples need to be cooled to 15 °C. The number of ice packs needed in order to reach these temperatures has to be determined on a case-by-case basis, as different types of commercial ice pack have different cooling capacities because of their differing sizes and volumes.

EMBRYOS

Production, collection, processing and freezing of embryos are more demanding than the equivalent procedures for semen, and a greater level of training and experience is required.

The following subsections address major issues in the cryoconservation of embryos. FAO has previously produced manuals on ET in several species including cattle (FAO, 1991a), buffalo (FAO, 1991b) and sheep and goats (FAO, 1993). In addition, commercial manuals are available for purchase, for example from the International Embryo Transfer Society[5].

Superovulation of donor females

Ideally, to maximize N_e in a reconstituted breed, each female embryo donor will produce at least one male and one female offspring. To reach this goal, each female will obviously need to produce multiple embryos. To increase the number of embryos per collection, donor females are administered various hormone agents (gonadotropin-like) to stimulate the ovaries to produce multiple ova for fertilization and embryos for collection. Early reports in cattle, sheep, goats and pigs described the use of pregnant mare serum gonadotropin (often abbreviated PMSG) at various dose levels to superovulate donor females. Pregnant mare serum gonadotrophin is extracted from the serum of pregnant mares after 40 days of gestation. A single intramuscular injection on pregnant mare serum gonadotrophin has potent follicle stimulatory activity. For information on the use of this agent in donor cattle, see reviews by Elsden *et al.* (1978) and Saumande *et al.* (1978). This agent (now termed equine chorionic gonadotropin or eCG) is still the agent of choice in pigs and often in sheep and goats. However, equine chorionic gonadotropin has a long half life, and often over-stimulates the ovaries of donor cattle. Therefore, equine chorionic gonadotropin is no longer the agent of choice for cattle in North America, although it is still used in countries where other gonadotropic agents are not commercially available.

Today, follicle stimulating hormone (FSH) has become the agent of choice for superovulating donor cattle and is also used in sheep and goats. FSH has a much shorter half life in the circulation and is, therefore, usually administered by twice-daily injections for three to five days (see Monniaux *et al.,* 1983; Armstrong, 1993; Mapletoft *et al.,* 2002). However, success using once-daily injections in cattle has also been reported (e.g. Looney *et al.,* 1981; Bo *et al.,* 1994). *Bos indicus* cattle appear to be more sensitive than *Bos taurus* cattle to FSH. Various modifications to techniques for superovulating *Bos indicus* cattle have been developed and are now in use (see Baruselli *et al.,* 2006, 2008; Bo *et al.,* 2008).

Information on various superovulation procedures for cattle and buffalo is presented in FAO training manuals (FAO, 1991a, b). Some of the more commonly used superovulation schemes for cattle donors today are presented in Appendix E of these guidelines. Current recommendations for optimum fertilization and successful embryo transfer are that one or two inseminations per donor cow with one or two units of good-quality semen per insemination are needed (see Schiewe *et al.,* 1987).

It is very important to select the appropriate number of embryo-donor females to match each sire in the breeding schedule and thereby to improve rates of genetic variability in the cryoconservation programme (e.g. Woolliams, 1989). The data presented in Table 3 (Section 4) give an indication of the number of transferable embryos that can be obtained after a single superovulation treatment and embryo recovery, as well as estimates of the number

[5] http://www.iets.org

of embryos obtainable from one donor female during one year. However, the figures vary quite considerably from animal to animal. Some females simply do not respond to the stimulatory agents, or stop responding. In addition, they may develop physiological conditions that make it difficult to retrieve the embryos. Thus, although 25 donor females and 25 donor males is the recommended minimum, a larger number of candidate females may be needed, because of the likely failure to obtain embryos from some donors.

The expected rates of success in both collection and transfer must be considered when determining the number of embryos to collect and store. Experienced ET professionals can be expected to achieve cattle embryo recovery rates greater than 75 percent, with four to eight good-quality bovine embryos per donor collection. Using good-quality embryos for transfer, 65 to 80 percent pregnancy rates can now be expected in well-managed cattle operations. Expected pregnancy rates from ET in a variety of livestock species are presented in Table 3.

Stages of embryo development

Embryos develop through various morphological stages after *in vivo* fertilization. As the embryos divide, the number of embryonic cells (blastomeres) per embryo increases as they migrate through the reproductive tract of the female (Table 13). It is important to know when the embryos can be expected to be in the uterus of the superovulated female, so that the embryos can be obtained from the uterine horns through non-surgical recovery.

An embryo technician must be able to recognize not only the stage of embryo morphological development, but also to assess embryo quality before selecting and transplanting embryos. The ability to make this judgment can be developed only with experience gained in assessing and grading embryos in the laboratory. For reviews on assessing embryo quality and classifying embryos, see the classic training publications by Lindner and Wright (1983) and Robertson and Nelson (1998).

TABLE 13
Location of the embryo following oestrus

Number of days from standing oestrus	Morphological stage	Location
1	Fertilized ovum	Oviduct
2–5	2- to 16-cell stage	Oviduct
3–4	Early morula	Oviduct
4–5	Compact morula	Distal uterine horn
5–6	Early blastocyst	Distal uterine horn
6–7	Blastocyst	Distal uterine horn
7–8	Expanded blastocyst	Distal uterine horn
8–9	Expanding hatched blastocyst	Distal uterine horn
9–10	Hatched blastocyst	Uterine horn*

*After hatching the embryo begins to migrate towards the middle portion of the uterine horn.

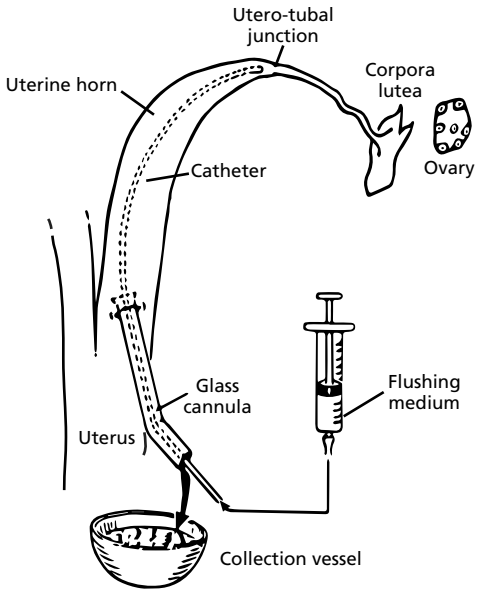
Embryo collection

Livestock embryos are collected from donor females by flushing the reproductive tract using a physiological flushing medium. The most often-used flushing medium for cattle is phosphate-buffered saline (PBS), which can be obtained by mixing commercially available dry packets with water or purchased as a ready-prepared solution. Various other media are also commercially available. In some species (e.g. cattle, horses and buffaloes) harvesting donor embryos is most often done using a non-surgical standing method, but in other species (e.g. pigs, sheep and goats) a surgical approach is usually required (see review by Betteridge, 1977).

Surgical embryo collection. Today, surgical embryo collections in pigs, sheep and goats are usually done at commercial ET units. Information on surgical procedures available for sheep and goats is provided in FAO (1993). In addition, see Kraemer (1989) or Baldassarre and Karatzas (2004). Over the years, research reports have described various non-surgical approaches to embryo collection and transfer in these species (see reviews by Foote and Onuma, 1970; Betteridge, 1977). However, in most cases, the number of embryos recovered per collection and the pregnancy rates per embryo transferred are lower than those achieved using the standard surgical approaches.

Figure 12 is a simple diagram of the surgical collection procedure for sheep and goats. The procedure is typically performed laparoscopically via the insertion of a glass cannula and catheter into the uterus. The uterus is flushed by injecting an appropriate medium using a syringe

FIGURE 12
Commonly used approach for collecting embryos (morulae and blastocysts) from sheep and goats



Drawing courtesy of R. A. Godke.

attached to the catheter. The injected liquid causes the embryos to flow out of the uterus into the collection vessel. The embryos can then be identified, processed and cryopreserved.

Embryo collection in pigs is also usually done surgically. Given that pigs naturally produce multiple offspring, superovulation is usually not practised, as embryo viability may be reduced. Collection is performed five to eight days after insemination, via laparotomy at a mid-ventral position. The animal is maintained under general anaesthesia while the procedure is performed. At this point, embryos will be in the blastocyst stage. Collection can be done earlier, at the four- to eight-cell stage, but in this case the embryos will require further culture before transfer and additional manipulation (e.g. delipidation).

Non-surgical embryo collection in cattle. Today, virtually all cattle embryos collected in the field and in-clinic by commercial ET companies are collected by a simple, non-invasive non-surgical procedure (see FAO, 1991a). Non-surgical embryo collection and transfer pose little risk to the cow, and greatly reduce the time needed for harvesting embryos. The drawback of non-surgical embryo collection is that embryo recovery rates may be a little lower if collection is done by a less-experienced technician.

There are two basic approaches to non-surgical recovery of embryos from cattle (see review by Chapman and Godke, 2004).The body of the uterus and uterine horns can be flushed simultaneously using a single flushing procedure, often referred to as "uterine body flushing" or "body flushing". Alternatively, each uterine horn can be flushed separately using two flushing procedures, a process known as "uterine horn flushing" or "horn flushing". Flushing with either of these approaches usually recovers 50 to 90 percent of available ova/embryos, depending on the experience of the technician. The potential number of embryos available for collection can be determined through palpation or ultrasonic examination based on the number of corpora lutea present on the ovaries of the donor animal. However, rectal palpation of donors with a large number of ovulations yields a rather imprecise estimate. It is therefore recommended that, if possible, ultrasonography be used to evaluate the ovaries of the donor prior to the embryo collection procedure. A list of equipment used in non-surgical embryo collection and transfer procedures in cattle is provided in Appendix F.

When using the body flush procedure, a Foley catheter is inserted through the cervix and into the uterine body. The cuff is then inflated and pulled back against the internal os of the cervix. The catheter is connected to a "Y" connector that allows medium to flow through the catheter, out of the cow and into an embryo filter apparatus where the embryos are captured. During flushing, the uterus and the horns are allowed to fill with medium until turgid. They are then manually massaged, and embryos are recovered as the uterus is drained. This filling and draining process is repeated until the volume of flushing medium fluid allotted to the female is depleted. The body flushing procedure uses about 1 000 ml of flushing medium for each donor animal

With independent horn flushing, the catheter is passed through the cervix and into the uterine horn. The tip of the catheter should be placed anterior to the external bifurcation of the uterus, half to three-quarters of the distance through the lumen towards the distal tip of the uterine horn. The cuff of the catheter is then inflated and 750 ml of flushing medium is allowed to flow into the horn. Manual manipulation is then used to recover the embryos

and the medium from the horn. When one horn has been flushed, the cuff is deflated and the catheter removed. It is then placed into the contralateral horn and the same flushing procedure is repeated. This approach uses about 1 500 ml of medium per donor animal.

Hay *et al.* (1990) conducted a comparative study of body and horn flushing for the recovery of embryos. On average, a greater number of embryos were obtained through horn flushing, but the difference was not significant. Given that the difference between the two approaches was not significant, the conclusion was that the technician should choose which approach to use based on his or her preference and proficiency. A modified approach to uterine flushing in cattle – termed the "shallow uterine horn flushing technique" – has recently been reported successful in dairy heifers (Sartori *et al.,* 2003).

The potential advantages of a single-embryo collection procedure (i.e. without superovulation) for on-farm use in cattle should not be overlooked. With such an approach, the donor female can remain on the farm, thus reducing the risk of disease transmission. Moreover, no ovarian stimulating agents are needed. The approach can be expected to cause less stress in the donor animals and, therefore, allow them to maintain a constant level of productivity. The collection method is the same as that for superovulated donor females. However, there is usually less uterine endometrial swelling (caused by the hormone stimulating agents), and this means that a less-experienced technician has a better chance of harvesting the seven or eight day-old embryo. A single-embryo approach may be particularly useful when reconstituting populations using a combination of stored semen and embryos or as part of *in vivo* conservation programmes. In such circumstances, large numbers of offspring per living female are not necessarily desired, as they would increase the genetic relationships among the animals in the live population. Sexed semen can be used to increase the probability of obtaining offspring of the desired sex.

Many factors can adversely affect recovery rates, including poor nutritional status of the donor, improper (over- or under-) hormonal stimulation of the donor, failure of the fimbria of the oviduct to pick up the ova, use of poor-quality semen to inseminate the donor cow, failure of embryos to enter the uterus after fertilization, and failure to collect the embryos during the flushing procedure. Many of these factors may be associated with inexperience on the part of the technicians.

Non-surgical embryo collection in horses. Successful production of live offspring following ET in horses was first reported in the early 1970s in the United Kingdom and Japan. The non-surgical embryo collection and transfer procedures used today in the mare are easier to perform than those used in the cow. The basic non-surgical collection and transfer procedures used in the mare were reported by Colorado State University (Imel *et al.,* 1981). Several modifications have subsequently been introduced to improve the procedure (see Wilcher and Allen, 2004). The latter-stage horse embryo is large enough to see even without a microscope.

In some countries, embryo collection and transfer technologies have been held back by the rules and regulations of various breed associations, whereas in other countries the use of these technologies is increasing rapidly. The use of ET in horses has become particularly common in Brazil.

Although the mare can be given hormones to superovulate her ovaries, donor mares tend to produce fewer oocytes for fertilization (range of two to four) post-treatment than donor cows. Usually, no more than one embryo is produced from a donor mare per cycle for potential embryo collection. To obtain early-stage embryos (single ovulated, < 300 µm in diameter) for cryobanking would require more than 130 mare cycles to harvest 100 embryos. By superstimulating the mares the number of cycles needed to produce the same number of embryos can probably be reduced to some extent.

General recommendations on embryo collection. To maximize efficiency, the collection, processing and storage of embryos must be carried out by trained professionals. In fact, many countries will have specific regulations on who can perform embryo collection. Technicians will need to undergo special training on sanitation and specific techniques.

Donor animals, if possible, should be subject to quarantine and/or health testing prior to collection. At the time of collection, the donor animals must also be kept as clean as possible. Body parts that will be accessed and manipulated during the procedure (e.g. tail and vulval area) should be washed and dried. Before surgical collection, hair should be clipped from incision sites and the area must be washed, rinsed and disinfected. Animals must be well restrained and treated in a manner that avoids stress and does not compromise their welfare.

The embryo-collection team (usually two or three technical people) needs to have access either to well-maintained, clean and sanitary permanent facilities, or to a mobile laboratory, where embryos can be collected, evaluated, processed and packed. The processing laboratory needs to be clean and equipped with an appropriate working space, electricity, temperature-controlled incubator(s), microscope(s) and other technical equipment and supplies (see Appendix F). Small equipment must be sterilized between collections and single-use disposable materials are recommended for sanitation purposes, when possible.

The direct disease risk associated with embryos, depends very much on the handling of the embryos by the collection team. This places great responsibility on the team, who must be competent in collecting, processing and storing embryos according to the relevant defined protocols. Given this major responsibility, and to ensure that the work is always done to high standards, it is recommended that a procedure for approving and officially recognizing members of embryo collection teams be introduced.

The potential health risk can be large if the recommended procedures regarding collection and handling are not followed precisely. It is very important to review the International Embryo Transfer Society recommendations for the sanitary handling of *in vivo*-produced embryos before beginning embryo collection (see Stringfellow, 1998) as well as the OIE standards (see Section 9 and http://www.oie.int). If gene banking involves transboundary movement of embryos, particular attention should be paid to the OIE standards.

Abundant results from worldwide research on the risks of disease transmission via embryos are available for cattle. Less information is available for sheep, goats and pigs, and information is almost non-existent for other species. Any embryo collection should be preceded by an extensive clinical examination of the donor animal for the presence of diseases. Its herd or flock mates should also be checked, as should the general environment in which the animals are kept. The clinical examination may eliminate a potential donor from

consideration or indicate that a treatment needs to be applied. The results of the examination may also influence the precise protocol applied for superovulation and recovery, as good results can only be expected from perfectly healthy animals.

The disease risk may be lower in some species than others, but this should not influence the level of attention to the animals' health status. For more information on disease control in embryos see FAO (1985).

Conventional embryo freezing

Embryos are usually frozen when they are at the morula or blastocyst stage, which, depending on the species, is reached by five to nine days after fertilization. After collection, embryos are placed into a hypertonic solution containing a cryoprotective agent, such as glycerol or ethylene glycol (see Leibo, 1992). These agents mildly dehydrate the embryo before and during the cooling process. Today, most livestock embryos are frozen in 0.25 ml or 0.50 ml plastic straws, similar to those used for freezing bull semen.

Because embryos are a collection of many interacting individual cells, whereas each sperm consists of a single cell, the freezing protocols for embryos are generally more sophisticated than those for semen. For more details on the procedures for cryopreserving livestock embryos, see Appendix G. Cellular properties often vary between species and between the stages of embryonic development. Thus, to minimize damage to the embryo and optimize survival rates, it is important to ensure that the cryopreservation procedure accounts for the particular characteristics of the targeted species (Rall *et al.,* 2000) (see Section 7 for basic principles of cryopreservation). The most often-used embryo freezing method is slow freezing, which is based upon a reversible dehydration of the cells that prevents the most damaging effects of intracellular ice crystallization. Most technicians who use the slow-freezing technique use an automated embryo-freezing machine. These machines can be adapted to work under field conditions.

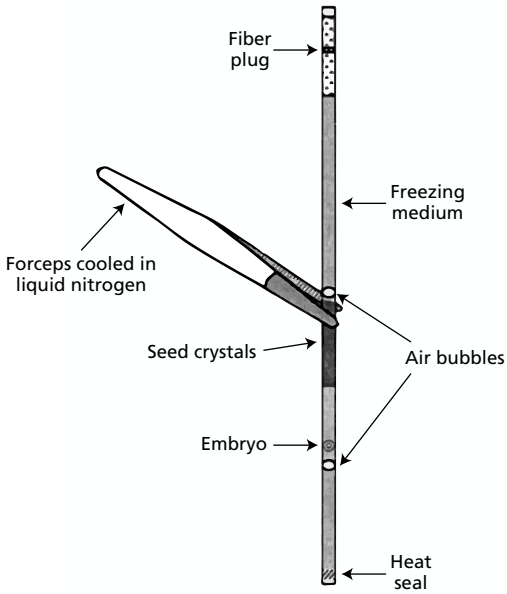
After the embryo and cryoprotectant are placed in the plastic straw, one critical step in the freezing process is "seeding", which is the act of purposefully inducing ice-crystal formation in the cryoprotectant solution surrounding the embryo (Figure 13). After embryos are cooled to approximately -35 °C, they are plunged into liquid nitrogen for storage at -196 °C. A summary of the methods and their applications is presented in Leibo (1992) and Rall (1992).

Several factors have been shown to be critical in determining the success or failure of cryopreservation:

1. the quality of the embryo – as estimated from its morphology examined under a stereomicroscope;
2. the time from embryo collection to the onset of freezing, which should be no longer than three to four hours; and
3. the appropriateness of the freezing and thawing procedure for the type of embryo being cryopreserved.

Field reports indicate that *Bos indicus* cattle embryos do not survive the freezing process as well as *Bos taurus* embryos. The greater lipid content found in most *Bos indicus* embryos at freezing may explain the lower post-thaw survival rates (see Ballard *et al.,* 2007; Looney *et al.,* 2008).

FIGURE 13
Using a forceps to induce ice-crystal formation (seeding) in a plastic straw



Drawing courtesy of R. A. Godke.

Cryopreservation of embryos using vitrification

As explained in Section 7, vitrification is a process that uses the rapid increase in the viscosity of solutions during freezing to obtain a glassy solid phase, both inside and outside the cells, without the formation of ice crystals (see Rall and Fahy, 1985; Rall, 1992). Vitrification involves the use of a high-concentration mixture of cryoprotective agent. Embryos placed into vitrification solutions are plunged directly into liquid nitrogen, saving valuable time and eliminating the need to purchase an embryo freezing machine (Vajta and Kuwayama, 2006; Vajta and Nagy, 2006). For further details on vitrification procedures, see Vajta *et al.* (2005) and Vajta and Kuwayama (2006). Although vitrification is a quick procedure and does not require special equipment, it can be technically more demanding and typically yields pregnancy rates that are 10 to 15 percent lower than those obtained using slow freezing until the technicians have gained enough experience to master the technique.

Success rates with vitrification in cattle are now approaching the rates achieved with conventional embryo freezing (Seidel and Walker, 2006). Commercial kits for vitrification of livestock embryos are now available. As vitrification methodologies improve, there are some indications that the approach may have some advantages over standard slow-freezing procedures in cattle (Vajta *et al.,* 1997; Visintin *et al.,* 2002). The future for vitrification technology appears promising, especially for embryos that have lower viability following conventional cryopreservation, such as pig embryos and embryos produced via IVF. At present, good success is being reported using vitrification to cryopreserve horse oocytes.

Embryo sexing and genetic diagnosis technology

Sexing and selecting embryos prior to cryoconservation may decrease the costs of storage, and particularly of subsequent thawing, transfer and production of offspring, especially if a greater proportion of animals of a particular sex is desired in the future.

One simple approach to gender determination is to bisect the embryo and identify the sex of one of the halves. Once the sex is established, the remaining half of the embryo can be transferred to a recipient female (e.g. Nakagawa *et al.,* 1985; Herr and Reed, 1991). Using another approach, White *et al.* (1987) bisected bovine embryos and then sexed one of the half-embryos of each pair by using an H-Y antibody procedure. Then both the half-embryos of the pairs were transferred to a different recipient animal. The success rate for embryo sexing was 90 percent, and there was no significant difference in pregnancy rates between the sexed half-embryos and control half-embryos (47 percent vs. 44 percent).

Studies using polymerase chain reaction (PCR) technology on fresh and frozen–thawed animal embryos clearly indicate that embryo biopsy techniques can be used for embryo sexing (Peura *et al.,* 2001; Kirkpatrick and Monson, 1993) without reducing post-biopsy transfer pregnancy rates. With today's embryo-sexing technology, only a few cells from the trophoblast of the embryo are needed for the *in vitro* procedures. In fact, the equipment and the supplies needed to sex bovine embryos are commercially available for use by veterinarians and livestock producers worldwide. If the instructions of these commercial embryo sexing kits are carefully followed, reported success rates approach 100 percent for cattle embryos.

At present, research efforts are directed towards minimally invasive embryo biopsy approaches for harvesting cells to be used in identifying potential genetic abnormalities and diseases prior to transferring the embryo. In the near future, cells from embryonic biopsy will be used by breeders and AI companies to identify genotypic and/or phenotypic traits of the embryo by using quantitative trait loci (commonly abbreviated QTL) and genomic selection technology. The potential for using genomic information to select the appropriate embryo to transfer would provide significant benefits to commercial breeders. Various companies have recently started making genome-based technology available on a commercial basis to livestock producers. With regard to cryoconservation, technologies for genomic selection of embryos will be particularly useful for cryobanking undertaken with the objective of gene introgression (see Section 6). For general cryoconservation programmes, these technologies may also be useful for selecting animals or embryos with the aim of maximizing the amount of genetic variability conserved in the gene bank (de Cara *et al*., 2011).

OOCYTES

Two approaches can be used for collection of oocytes. Conventional oocyte collection consists of harvesting oocytes from ovaries that have been removed from a donor female. Transvaginal ultrasound-guided oocyte collections (TUGA), on the other hand, consists of removing oocytes from the ovary of a living animal. The choice between the two approaches will depend on a number of factors, including technical capacity, financial resources and the value of the donor female.

Conventional oocyte collection

Oocytes are often harvested from slaughterhouse ovaries for research purposes. This can also be an option for cryoconservation, especially if the genetic characteristics of the donor animals (i.e. beyond their breed) are not important and the germplasm is expected to remain within the country (in which case strict adherence to OIE sanitary standards for export is not necessary). The ovaries of the donor females are collected immediately after slaughter, placed in reclosable plastic zip-lock bags and kept warm *en route* to the laboratory. In general, livestock ovaries should not be cooled, as this dramatically reduces the success rate of embryo production from IVF. Individual bovine oocytes are generally aspirated from small, medium and large follicles (see Appendix H), subsequently matured, fertilized and cultured, and then either frozen or transferred fresh to a recipient. Usually, four to twelve oocytes per ovary can be harvested from cows (using a sterile needle and plastic syringe). Once collected, the oocytes are evaluated for quality and placed in oocyte maturation medium overnight (e.g. 20 to 23 hours for cattle oocytes) in preparation for *in vitro* maturation and IVF procedures (see examples in Appendices I and J). This methodology can be used to collect oocytes from nearly all species of livestock and is relatively simple, inexpensive, and highly recommended for training students and laboratory personnel.

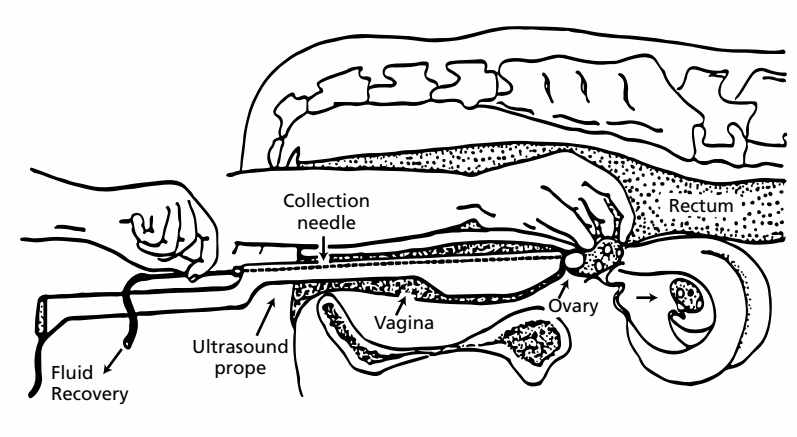
This oocyte harvesting procedure could be used to produce offspring from females that unexpectedly die or suffer incapacitating injuries or from old or clinically subfertile animals. A novel conservation strategy might be systematically to recover the ovaries from all the females of an at-risk breed when they die or go for slaughter. These oocytes could then be fertilized in the laboratory and the subsequent embryos frozen for transfer to recipient females at a later date. In the event of the compulsory slaughter of a herd because of a non-viral disease outbreak, it is still possible to produce clean embryos (using the International Embryo Transfer Society and OIE animal-health and embryo-handling procedures) that can later be used to re-establish the herd.

Transvaginal ultrasound-guided oocyte collection

Oocytes can be collected from live donors via follicle aspiration by using one of three basic surgical procedures. The first is the standard laparotomy technique to expose the ovaries, which can be performed using various anatomical approaches in all large livestock species. Although this method has been successful in cattle and horses, today it is most often used in sheep, goats and pigs. The second approach is the endoscopic technique, which is also used most commonly in sheep and goats, but has also been used for follicle aspiration in cattle and horses. The third approach uses transvaginal ultrasound-guided oocyte recovery (TUGA), which is now most commonly used in cattle, buffalo and horses. TUGA is often referred to by its common name, "ovum pick-up" (OVU). All three of these approaches to oocyte collection require the services of a skilled technician; a descriptive overview of the TUGA procedure is presented below.

TUGA was originally developed in humans to retrieve oocytes by using ultrasonography to evaluate the ovary and guide a hollow needle transvaginally into each visible ovarian follicle (e.g. Wikland and Hamburger, 1984; Dellenbach *et al.,* 1985). The needle is attached to a source of vacuum and is used to aspirate fresh *in vivo* oocytes from the

FIGURE 14
Transvaginal ultrasound-guided oocyte aspiration (TUGA) in the cow



Drawing courtesy of Dr. A. Meinjtes.

follicle, which are then subjected to *in vitro* maturation, IVF and then *in vitro* culture procedures. The procedures for humans were modified for harvesting oocytes from live cattle (e.g. Callesen *et al.,* 1987; Pieterse *et al.,* 1988, 1991) and other species. TUGA is now routinely used in cows, doe goats, mares and more recently in sows and large, hoofed, wild animal species.

TUGA can expand the time that animals can be reproductively active. For example, both pregnant cows and mares continue follicle wave development during early to mid-gestation. A novel approach is to take advantage of these developing ovarian follicles to produce IVF offspring from oocytes during the early stages of pregnancy. This procedure does not compromise the pregnancy, and oocyte yield actually tends to be greater than from non-pregnant animals (Meintjes *et al.,* 1995b; Cochran *et al.,* 1998a,b). This procedure can be especially useful for larger animals, because they tend to have only one offspring per pregnancy and their relatively long gestation periods mean that potential donors pass through long periods when they cannot be used for embryo production. In addition, TUGA can be used on animals before they reach sexual maturity. Oocytes from prepubertal sheep and cattle have produced IVF offspring (see Looney *et al.,* 1995; Bols *et al.,* 1999). Oocytes from near-term bovine foetuses and new-born calves are being harvested for IVF procedures, but at the time of writing these guidelines no offspring have been produced through this approach.

To retrieve the oocytes for IVF, a trained professional inserts an ultrasound-guided stainless steel needle through the wall of the vagina near the cervix to extract the oocytes from the follicles visible on the ovaries. The procedure is conducted on the small, medium and large follicles on both ovaries of the donor female.

TUGA in cattle. The donor cow is restrained in a suitable holding chute and administered an epidural block. A convex ultrasound 5 megahertz (MHz) sector transducer is fitted onto the distal end of a specially designed plastic handle and used to visualize the ovaries on the screen of the ultrasound monitor. The plastic handle (with a latex protective covering) is inserted into the vaginal canal, and then the ovary is grasped *per rectum* and placed against the transducer in the vagina (Figure 14).

Follicles are identified as black (hypoechoic) circular shapes on the monitor screen. An 18-gauge, 55 or 60 cm-long needle is inserted through the needle guide in the plastic handle. This needle is connected to a suction pump by means of polyethylene tubing that passes through an embryo filter or into a 50 ml conical-shaped test tube for collection of the follicular fluid containing the oocytes. The basic solution used for this procedure is PBS, with 10 percent bovine serum, antibiotics and heparin added. Using this aspiration method, oocytes are recovered from 60 to 70 percent of the medium to large-size follicles punctured, with an average of three to ten oocytes per non-stimulated donor female. A significant training period is required in order to become proficient in using this procedure in cattle.

Aspirations are usually performed once a week, but have been performed twice a week for up to three months in cows (Gibbons *et al.,* 1994; Broadbent *et al.,* 1997) with no overt effects reported in the donor females. With TUGA and IVF, more embryos can be produced in a shorter period of time than is possible using conventional ET. The procedure can realistically be repeated on the same cow three to four times a month. In addition, using TUGA for oocyte collection does not require any hormone treatment of the donor. The frequency of recovery can be much greater than that for embryo collection after superovulation (up to 80 recoveries during one year in cattle compared with no more than six collections when embryos are collected non-surgically). Oocytes can be harvested from donor cows at any time of the oestrous cycle, including at standing oestrus and the growth phase of the first follicular wave (Paul *et al.,* 1995). Although superovulation is not necessary, the number of oocytes collected per female can be increased by treating the cow with gonadotropic hormones prior to the aspiration procedure. *In vitro* production of embryos generally results in one to three embryos for transfer per oocyte collection procedure in non-stimulated donors.

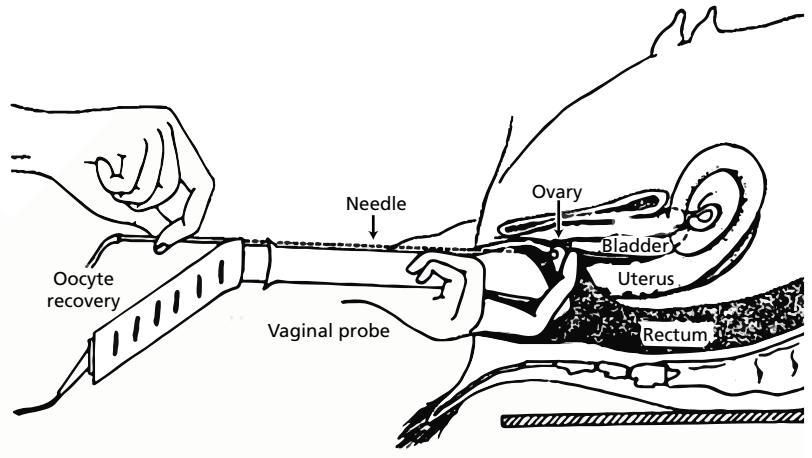
TUGA in buffalo. Similar oocyte collection procedures to those used in cattle are now being developed for buffaloes in various parts of the world. Again, the primary objective is to fertilize the collected oocytes *in vitro* for fresh transfers or for cryopreservation. The successful use of TUGA for harvesting oocytes has been reported in the swamp buffalo (Pavasuthipaisit *et al.,* 1995; Techakumphu *et al.,* 2004; Promdireg *et al.,* 2005) the Italian Mediterranean buffalo (Boni *et al.,* 1996) and the Murrah buffalo (Gupta *et al.,* 2006). The basic bovine IVF procedures are being fine-tuned for embryo production from buffalo oocytes.

TUGA in the horse. The horse has presented a unique problem for researchers working on assisted reproductive technology. As noted above, although embryo collection and transfer are relatively simple in the mare, superovulation is generally ineffective. Due to the unique anatomical structure of the horse ovary, usually only a single oocyte matures to an ovum and ovulates at the appropriate time during each oestrous cycle. Also, for some as yet unknown reason, typical IVF procedures have not worked consistently in the horse.

Even though only one follicle normally matures and ovulates during an oestrous cycle, mares are thought also to have one or two waves of multiple follicles during the cycle. This developing follicle population makes it possible to use TUGA to collect oocytes from live mares for the production of embryos for transfer (see Brück *et al.,* 1992; Cook *et al.,* 1992). The first foals obtained from oocytes aspirated from live mares were produced using ICSI (see Section 4). After ICSI, embryos are surgically transferred at the two- to four-cell stage into the oviducts of suitable recipients, because the culture of IVF-derived equine embryos has not yet been perfected.

The aspiration set-up for mares is similar to that used in cattle, but with some modifications. Briefly, mares require sedation instead of an epidural block, and a 12-gauge needle is used to puncture the follicles. Extra rinsing of the follicle is necessary in the horse, as the oocyte is usually well-embedded in the follicle wall. In such cases, the needle recommended is a double-lumen needle. This allows the follicular fluid to be aspirated and the medium used again to rinse the follicle (two to four times per follicle). The follicular fluid is collected into a 500 ml bottle, and later passed through the standard embryo filter. Using this modified method, oocytes have been successfully recovered from mixed-breed cyclic horses and ponies (Meintjes *et al.,* 1995a), pregnant mares (Meintjes *et al.,* 1994, 1996) and from free-ranging zebras in South Africa (Meintjes *et al.,* 1997). Oocyte recovery rate usually ranges between 50 and 75 percent of follicles punctured per mare. After IVF or sperm injection procedures, developing two- to four-cell stage embryos are transferred surgically into the oviducts of recipient mares. The ICSI procedure (Section 4) appears to be the method of choice, at present, for producing horse embryos in the laboratory.

FIGURE 15
Ultrasound-guided oocyte aspiration in goats



Source: drawing courtesy of R.A. Godke; reprinted from *Theriogenology*, 51, Graff, K.J., Meintjes, M., Dyer, V.W., Paul, J.B, Denniston, R.S., Ziomek, C. & Godke, R.A. Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats, 1099–111, Copyright (1999) with permission from Elsevier.

TUGA in small ruminants. *In vitro* production has also proven successful in goats. Transvaginal aspirations have been performed on cyclic and non-cyclic adult does with good success (Graff *et al.,* 1999). Although the oocyte recovery rates usually range between 60 and 80 percent of the follicles punctured per donor female, there are some problems with the aspiration of ovarian follicles from goats using TUGA. First, the ovaries cannot be grasped *per rectum* for optimum visualization with ultrasonography. Second, as the ovaries cannot be easily grasped, it is more difficult to puncture follicles and aspirate the oocytes. Although the methodology for puncturing the follicle is similar to that in the cow and the mare, the doe must be sedated, put under anaesthesia and then placed in dorsal recumbency (Figure 15).

Manual pressure is placed on the abdomen in an effort to stabilize the ovaries for aspiration. The ultrasound probe, which is smaller than that used for cattle, buffaloes and horses, is inserted into the vagina with the convex transducer at the distal end of the handle. The aspiration proceeds with no need for extra rinsing of the follicles to recover the oocytes.

Oocyte recovery is usually a little slower than desired because not all follicles can be visualized, and not all follicles visualized can be adequately punctured due to the difficulty involved in securing the ovary. Offspring have been obtained from frozen–thawed goat embryos produced using the transvaginal aspiration procedure together with IVF methods (Han *et al.,* 2001). Although this non-invasive procedure requires expertise and patience, it is an important technology that involves less risk of ovarian adhesions or death than the standard surgical method for harvesting oocytes from goats. An efficient TUGA method for harvesting oocytes has not, as yet, been developed for sheep.

TUGA in other species. TUGA has also been used successfully in other animals, with modifications made primarily to account for anatomical differences among species. For example, TUGA been used successfully in adult pigs (Bellow *et al.,* 2001), llamas (Brogliatti *et al.,* 2000) and various other hoofstock, such as red deer (Berg *et al.,* 2000), sika deer (Locatelli *et al.,* 2006), the rare bongo antelope (Pope *et al.,* 1998; Wirtu *et al.,* 2009) and the African eland (Wirtu *et al.,* 2009). Therefore, this technique may be particularly useful for managers of gene banks that preserve both domestic and wild animal genetic resources.

SOMATIC CELLS

As discussed in Section 4, collection of tissues other than germ cells and embryos can be useful for gene banking, either for the production of new animals (through SCNT) or to obtain genetic and health-related information about the animals sampled (DNA isolated from cells).

Tissue

Somatic cells for subsequent use in DNA analyses or SCNT can be sampled from the tissue of live animals or from animals shortly after death (Silvestre *et al.,* 2004). Because the requirements for the two objectives (DNA or SCNT) differ, separate protocols have to be used for each. For DNA, one approach is to use a sterile scalpel blade to aseptically remove thin strips of skin from the body surface (e.g. shoulder area) of an animal and then place them in a pre-labelled sterile screw-top vial for transport to the processing laboratory. Tissue can also be easily obtained from the peripheral border of the ear of a live animal (or immediately after the animal's death) using a sterile hole punch. Prior to freezing (or

vitrification), the tissue samples should be wrapped in blotting paper moistened with PBS and maintained at 4° C to minimize degradation of the samples. As an alternative to cryo-preservation, tissue samples can be preserved through dehydration by storing each sample in a glass vial containing a grain of silica gel. In addition, for DNA analyses in the relatively short term (less than two years) hair samples may be an option. Hairs must be plucked from the roots and then desiccated, placed in separate labelled containers for each animal and stored in a dry environment. Various protocols for DNA extraction exist, but the simplest approach is to use a commercial kit designed for use with the specific tissue.

Collection of tissue for SCNT needs to follow a more stringent protocol than that used for samples intended for DNA analyses. Appendix K presents two protocols, the first for simple and low-cost field collection and freezing (Groeneveld *et al.,* 2008) and the second for situations in which samples will be processed in a laboratory. In both cases, the same procedures are generally applicable to all species, particularly to mammalian species.

Blood

Blood samples collected from live animals or from animals shortly after death can be used for DNA analyses or for cloning via SCNT. The DNA from the blood of mammals comes from the white cells only, because the red cells do not have nuclei. Birds have DNA in both red and white cells, and thus a smaller volume of blood is required from birds than from mammals in order to obtain ampoule quantities of DNA for analyses. Blood is relatively simple to obtain in quantities that provide sufficient DNA for genetic analyses. Collection of blood from the jugular or caudal vein with a needle and vacutainer tube is the common procedure in mammals. Blood from poultry is usually sampled from veins in the wing.

It is recommended that two vials of whole blood (total of 10 to 14 ml) be collected at the time of collecting any other germplasm from animals selected for the gene bank; this will reduce the risk of accidental loss of all samples from an individual animal. White blood cells can be harvested from fresh whole blood following centrifugation. The buffy coat (the thin middle layer that forms when blood is centrifuged) is carefully pipetted from the sample and divided into at least two small pre-labelled sterile vials for use in nuclear transfer. The vials are then frozen in nitrogen vapour and stored in liquid nitrogen.

If only DNA is needed, it can be extracted using commercial kits, following their specific procedures. For long-term storage, the extracted DNA should be put into labelled aliquots of 50 µl, with a concentration of 200 µg/ml. Division into aliquots will provide repeated access while avoiding freezing and thawing of the entire sample. The DNA may be safely stored at 4 °C for two months before being divided into aliquots, provided the preparation is pure enough. Otherwise rapid transfer to long-term storage is necessary. For long-term storage, DNA can be maintained at -20 °C or lower (-80 °C or in liquid nitrogen), but the latter is not necessary.

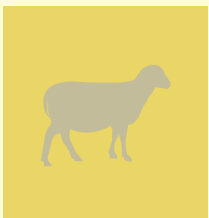
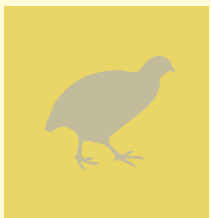
Gonadal tissue (poultry)

As discussed in Section 4, recent studies have reported that ovarian tissue can be harvested from immature female chicks, frozen, thawed and transferred back to other young females (see Song and Silversides, 2006). Subsequently, newly hatched chick testicular tissue has

been harvested and transplanted successfully to host chicks, resulting in live offspring born from sperm derived from the donor testicular tissue (see Song and Silversides, 2007b). The procedures used for cryopreservation of ovarian and gonadal tissue are presented in Appendix L.

SECTION 9

Sanitary recommendations


Sanitary recommendations

Collection and banking of AnGR can present various complications. Animals to be sampled will likely originate from different farms, and this presents the possibility of disease transmission when the animals or their germplasm come in contact with each other in the central gene bank. At-risk breeds will likely be found in only a few locations, leaving little opportunity for selection of donor farms on the basis of sanitary conditions. Disease outbreaks may present both an urgent need to collect germplasm from animals in the affected area and a health and sanitation risk for the gene bank. Each country will need to balance its breed conservation needs and its compliance with national and international health regulations. Decisions will be based, in part, on the types of diseases that are present and how contagious, virulent and damaging to animal production they are. Certainly, animals that have a highly contagious and possibly fatal disease, such as foot-and-mouth disease, should not be collected except in the most dire of circumstances (i.e. if no non-infected animals exist).

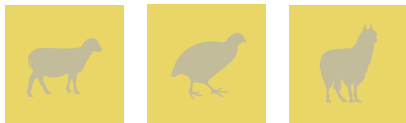
The diseases of concern to gene bank managers will vary from country to country, as will the health regulations that gene banks have to follow in developing their collections. However, if the gene bank is interested in distributing germplasm to other countries, especially those with widely divergent health concerns, attention must be paid to OIE health regulations (http://www.oie.int). International transfer of germplasm that does not conform to these regulations could put a country at risk of losing its OIE export status.

The primary issue for a gene bank collecting germplasm in the field is to minimize the risk of spreading diseases from farm to farm while collecting germplasm from animals belonging to different owners. Additionally, efforts must be made to reduce the risk of spreading diseases during the utilization of germplasm that the repository has collected and cryopreserved.

A high standard of health testing and of sample preparation is easier to achieve if germplasm is collected in an AI centre or other controlled environment. Maintaining the required protocols is more difficult when collecting is done in the field. In certain instances it may be necessary to obtain a waiver from national or international veterinary agencies so that germplasm from breeds at risk can be collected and cryoconserved. Obviously, conditions in the field may mean that maintaining animals in quarantine for any length of time is impractical. Even if there are no such problems, quarantining animals in the field increases collection costs. A potential means of addressing the risk of collecting germplasm from infected animals in the field is to draw blood samples from the donor animals and then have the blood samples tested for the relevant disease(s).

The following sources serve as excellent reference material on sanitary requirements for the collection, testing and processing of germplasm:

- OIE recommendations and regulations, set out in its Terrestrial Animal Health Code, covering the collection, testing and processing of germplasm samples for international exchange (http://www.oie.int/index.php?id=169&L=0&htmfile=titre_1.4.htm);



- AI centre and semen import and export protocols of the United States of America's National Association of Animal Breeders (http://www.naab-css.org/guidelines/ and http://www.aphis.usda.gov/regulations/vs/iregs/animals); and
- European Union regulations for AI centres and international exchange of germplasm (http://ec.europa.eu/food/animal/index_en.htm).

Although the above-listed regulations and protocols may not apply directly to all countries, applying the procedures described will improve sanitary safety and thus reduce the risk of disease transmission.

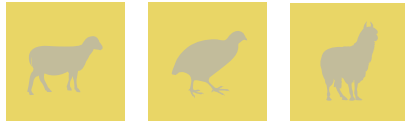
COLLECTION AND PROCESSING FACILITY

It is likely that the animals brought into a collection centre will remain there for only a relatively short period of time. Quarantine and health testing procedures should be as cost-effective as possible. In order to minimize the risk of disease transmission, three components of the collection facility have to be addressed:

1. As animals enter the collection facility they should be maintained in isolation buildings or pens for a specified period of time during which they can be tested for various diseases, placed on selected rations and trained for collection. Ideally, personnel that handle quarantined animals should not be involved in the care or collection of animals that have already been tested. As noted in Section 5, for ease of management, the quarantined area should be subject to an all-in all-out policy, i.e. if any animal fails to meet the predetermined health criteria, none of the animals in the group should be accepted.
2. Once animals have passed the health tests they should be moved into the main facility, where they will be maintained and their germplasm collected. At this stage in the collection process, the main health concerns relate to the potential for introducing a disease from outside the facility. Such risks can be minimized by requiring animal handlers to follow specific sanitation protocols and by keeping rodents and wild birds out of the facility. The risk of contamination from bedding can be addressed by cleaning animals prior to collection. Even though the animals in the collection facility will have passed through quarantine, equipment for germplasm collection such as AV and collection tubes should be kept clean and changed for each animal.
3. After collection, samples of semen and other genetic materials are transferred to a laboratory. Protocols for biosafety of at least Level 2 (see http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf) need to be in place in order to maintain minimal sanitation standards and prevent any cross-contamination between samples.

FIELD COLLECTION

Although germplasm collection in a closed facility is desirable for control of health and sanitation, in some instances it may be more practical and cost effective, or even necessary, for gene banks to perform collections in the field. During field collection, collectors must respect the sanitation of each collection site, and minimize the risk of spreading diseases. Shipping boxes and other supplies used at one collection site should never be used at other sites. The boxes and supplies may be reused after they have been sanitized, but only at the same collection site. If frozen materials arrive at the gene bank, the liquid nitrogen tank or



dry shipper should be sanitized with a 10 percent bleach solution after it has been warmed to room temperature.

If personnel travel from collection site to collection site, then very specific sanitation practices should be implemented. The undercarriage and tires of vehicles should be washed, preferably with a disinfectant, after visiting each site. Likewise, the boots of the personnel should be disinfected, or covered with disposable boot covers that are discarded after leaving each site. Clothing should also be laundered or changed between collection sites or disposable suits should be worn. Polyvinyl or nitrile gloves should be worn, and changed between handling different animals.

Equipment such as syringes and needles should never be used on more than one animal, and must be disposed of properly, according to local regulations, following use. Non-disposable equipment, such as electro-ejaculators, must be sanitized and rinsed between animals in order to avoid spreading pathogens.

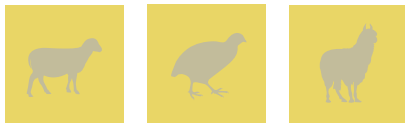
DISEASE TESTING

As mentioned above, germplasm should not, if at all possible, be collected from animals that are clearly infected with a highly contagious disease. In general, it is preferable to avoid sampling animals that are affected by any kind of disease. Hence, quarantine of all animals entering a collection centre is recommended. Infected animals may not show any outward symptoms of disease even if they spend time in quarantine. For this reason, it is advisable to collect samples of blood or other tissues (e.g. nasal smears) that can be used in more comprehensive tests for the presence of disease agents.

STORING SAMPLES

Cross-contamination of samples in liquid nitrogen is possible, but this has only been found to be of consequence in studies where the contaminant was placed in the tank (Grout and Morris, 2009). Otherwise, the probability of contamination seems to be very low. Nevertheless, because pathogen transmission is possible, care should be taken to minimize any risk of cross-contamination. It is critically important to consider how the material will be handled and stored. For example, semen straws can be sealed with a variety of substances, including polyvinyl alcohol (PVA) powder, clay and metal ball bearings, or by heat sealing. While PVA powder, clay and ball bearings are inexpensive, the quality of the seal they provide is inferior to that achieved by heat sealing, and consequently the potential for contamination through leakage or rupturing of straws is greater.

If the sanitary status of previously stored samples is questionable, then the semen straws or other storage devices can be cleaned with ethanol and allowed to dry following thawing. However, a more important question may be how to deal with newly collected and frozen samples from sources where biosecurity standards may be lower than desirable, such as those collected in the field rather than at an approved collection centre. In such circumstances, separate storage of samples that have uncertain sanitary status should be considered. For maximum security, distinct storage sites can be used. At minimum, samples of unknown status should be stored in separate tanks. In addition, specific liquid nitrogen tanks should be designated for quarantine purposes. Then, for example, samples



not received from facilities that are known to be sanitary, such as designated collection facilities or AI centres, can easily be segregated and held in a dedicated liquid nitrogen tank until their sanitation status is determined. If samples collected in the field are considered to be suspect in sanitation and health terms, they can be quarantined until the results of blood tests from the sampled animals demonstrate a clean bill of health. In some countries, local regulations may stipulate that germplasm from different species have to be stored separately, regardless of the samples' health status and the site of collection.

Segregating germplasm according to its health and sanitary status is of particular importance when there is a possibility that germplasm may be transferred internationally and adherence to OIE standards is critical. Even if possible international transfer is not a factor, given the potentially high value of stored germplasm and the fact that it may in the future be the only means by which to recover a lost breed or support the maintenance of a highly endangered breed, the risks involved in storing samples with known and unknown levels of sanitation together must be given serious consideration.

Regardless of the system used, information on the sanitary status of each individual sample must be included in the gene bank database and information system (see Section 10). In this way, all samples can be tracked and managed properly regardless of their storage site.