
# APPROVED STANDARD PROTOCOLS

Animal Ethics Sub-committee
of the
University of KwaZulu-Natal
Ethics Committee

# 1. STANDARD PROTOCOLS FOR FROGS

## 1.1 HANDLING (Code: AH)

Frogs can be transferred by hand and net. Because of their aquatic nature, some frogs, Xenopus for example, have a slippery protective coating of mucous, making them difficult to hold. It is advisable to hold your hand like a cage, not a vice. Place your index finger between the hind legs and the remainder of your hand around the body.

Alternatively, you may gently dry the skin with a paper towel for easy handling. Aquatic frogs should not remain out of the water for more than 15 minutes.

## 1.2 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. Gases and anaesthetic agents are readily transferred across the skin of amphibians to the circulation. A list of agents and dosages is provided in Appendix I. Recovery can be enhanced by rinsing off the animal and increasing the ambient temperature. The frog should be recovered in the cage without water. A damp piece of gauze may protect the skin and not endanger the animal when it is recovering.

### 1.2.1 Inhalation Anaesthesia (Code: AAIH)

Inhalation anaesthesia is instituted in an inhalation jar or chamber constructed so that the agent does not directly contact the animal. The amounts of inhalation anaesthesia used are sufficient to maintain the frog unresponsive to painful stimuli yet insufficient to induce respiratory depression. Several forms of respiration are present in amphibians - pulmonary and buccopharyngeal. Respiratory depression will be difficult to determine, so it is best to use the withdrawal reflex to assess depth of anaesthesia.

### 1.2.2 Injection Anaesthesia (Code: AAIN)

Injection anaesthesia, using tribromoethanol, tricaine methane sulfonate or other agents, can also be used. The route of administration and frequency of additional doses will be determined by the length of the procedure. Injection techniques are described in a separate section

### 1.2.3 Immersion Anaesthesia (Code: AAIM)

Frogs can be immersed in a 1:10 000 solution of tricaine methane sulfate. The depth of anaesthesia is dependent upon time immersed and temperature of the water. The animals are removed and respiration observed to insure adequacy.

### 1.2.4 Hypothermia Anaesthesia (Code: AAHY)

Frogs can also be anaesthetised by subjection to cold temperature. The animal becomes torpid and unresponsive to painful stimuli. The frog is immersed in water or subjected to 5°C for 1-2 hours. This method may be used in conjunction with infiltration of local anaesthesia.

### 1.2.5 Local Anaesthesia (Code: AALO)

Occasionally, supplemental long-acting local anaesthesia is needed. Long-acting local anaesthesia can also reduce the need for post-operative analgesia. 1 ml of xylocaine or procaine can be infiltrated around the excision site. Too much local anaesthesia can be toxic, also local anaesthesia with vasoconstrictors can cause necrosis and sloughing of tissue and must be used with caution.

## 1.3 SAMPLING OF BODY FLUIDS

The skin and other sites should be properly cleaned and prepared to insure maximum cleanliness.

### 1.3.1 Bleeding

Frogs do not have peripheral vessels that are easy to sample. The majority of blood samples are obtained from the heart under anaesthesia. A maximum of 1-2 ml per 100 g of mass may be withdrawn. There is a fair amount of inherent risk with cardiac blood sampling and should be performed as a terminal procedure when possible.

#### 1.3.1.1 Bleeding by Cardiac Puncture (Code: ABCP)

Anaesthetise the frog and place it on its back with its body length perpendicular to you. The apical pulse is observed and/or palpated. Insert a 21 gauge needle and syringe under the xyphoid cartilage with the needles at a 30 degree angle to the skin. Push slowly forward with gentle negative pressure. Care must be taken not to move the needles as this may lacerate the heart resulting in death.

#### 1.3.1.2 Bleeding by Terminal Procedures (Code: ABTB)

Posterior Vena Cava: Anaesthetise the frog and place it on its back. Make a V-shaped incision through the skin and abdominal wall at the base of the abdomen and proceed diagonally across each side ending dorsolaterally at the thorax. Straddle the vessel with the fingers of your free hand and insert a 19-21 gauge needle with gentle negative pressure. As the vein collapses, wait for it to refill and continue. When completed, proceed with euthanasia.

Dorsal Aorta: Similar to the posterior vena cava technique but enter aorta anterior to the distal bifurcation.

## 1.4 INJECTIONS

The skin is prepared to assure maximum cleanliness.

Phosphate buffered saline (PBS) or other isotonic solutions are better than distilled water or a solvent/vehicle for injections. Distilled water can cause hemolysis of administered intravenously, and pain if administered subcutaneously. Oils are suitable for administration of lipid soluble substances or in adjuvants, but absorption is delayed and they cannot be injected IV.

Maximum quantities depend upon route of administration:

IM: 0.25 ml/site
SQ: 1.0 ml/site
IV: 1.0 ml/100 grams
DLS: 1.0 ml/100 grams
IP: 1.0 ml/site

### 1.4.1 Subcutaneous (Code: AISO)

The injection site is under the skin of the animal's back and side. Clean the area and insert the needle through the skin at a shallow angle to the surface.

### 1.4.2 Intramuscular (Code: AIIM)

The usual injection site is the large muscles of the rear limb. Clean the skin and insert the needle in a posterior direction away from the femur and sciatic nerve. Aspiration is necessary to assure the needle tip is not intravascular.

### 1.4.3 Dorsal Lymph Sac (Code: AIDL)

Using a sterile syringe equipped with a 12.5 mm, 26 gauge needle, insert the needle through the skin directly into the dorsal lymph sac at a point 62.5 - 12.5 mm to the side of the midline. Allow the needle to penetrate just under the skin. Lift the hind limbs of the frog and inject. If fluid flows easily, the location is correct. Avoid wiggling the needle as this may cause unnecessary trauma to the tissue.

### 1.4.4 Intraperitoneal (Code: AIIP)

The injection site is the lower left quadrant of the abdomen and away from the midline. Clean the skin and insert the needle tip through the abdominal wall. Inject immediately to push viscera away. A large gauge needle is less likely to penetrate the viscera. The frog can be tilted, head down, which allows the viscera to fall forward with gravity, decreasing the chances of perforating the viscera.

## 1.5 SPECIAL PROCEDURES

### 1.5.1 Superovulation (Code: AISO)

During injection, it is important that the frog be held in an immobile position. Wrap the frog in a paper towel or drape with hind limbs outstretched. Tear a hole in the paper towel or drape to expose the injection site (dorsal lymph sac) and hormone release area. After the injection, it is important that all of the hormone remain in the dorsal lymph sac, and not seep through the needle puncture of the skin. To insure this, use a 1 ml syringe with a 26 gauge needle. Insert the needle through skin directly into the dorsal lymph sac at a point 6.25 - 12.5 mm to the side of the midline. Allow the needle to penetrate just under the skin. Never wiggle the syringe after it is inserted, as this will cause unnecessary trauma to the tissue. You will know you are in the lymph sac by observing the flow of the hormone. Raise the hind limbs of the frog slightly while beginning the injection and release a small amount of fluid. If it flows easily from the syringe and continues anteriorally, you are in the dorsal lymph sac.

## 1.6 SURGICAL PROCEDURE

Surgical procedures are classified according to whether the animal survives the procedure or is terminated prior to being awakened. Surgical procedures require clean, but not necessarily sterile, techniques and should be carried out in a designated approved area. All survival surgeries require consideration of post-surgical analgesia.

### 1.6.1 Abdominal Surgery to Harvest Oocytes (Code: ASHO)

Anaesthetise the animal and place on a moist gauze. Drape the animal with sterile gauze moistened in sterile saline. With a scalpel blade, make an incision on the dorsolateral aspect of the animal over the site where the ovaries are anticipated. Withdraw the string of oocytes. A ligature of nonabsorbable sutures, usually silk, is placed at the base and the string of oocytes transected distal to the ligature. The ligated string of tissue is returned to the abdominal cavity. The incision is closed in one layer with interrupted absorbable or nonabsorbable suture, or with skin staples which are removed in 10-14 days.

## 1.7 PHYSIOLOGICAL MEASUREMENTS

The animal is placed in a metabolic chamber and various measurements are done using specialist analysers connected to the chamber.

### 1.7.1 Respirometry (Code: APRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline). Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 1.7.2 Measurement of food consumption (APF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 1.8 and quote reference number of relevant euthanasia procedure).

### 1.7.3 Measurement of total body water by tritium dilution (Code: APW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April**

**1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste*"- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 1.4 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 1.2 and 1.3 and quote reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 1.7.4 Urine analysis (Code: APU)

Frogs are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 1.7.5 Activity measurements (Code: APA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The frog is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 1.7.6 X-Ray techniques (Code: APX)

The digestive tract of frogs can be studied by X-rays following introduction of a suitable contrast agent such as barium sulfate or gastrografin. The frog is trained before the experiment to be used to the position required for the x-ray measurement. The animal is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the animal is let into the room and all metals are removed form the area. The animal is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The animal is allowed to ingest appropriate feed coated with barium sulfate. It is important that the animal does not move during the experiment. If it was not possible to train the animal to maintain the appropriate position, soft materials and sandbags are used to keep the animal in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 1.8 EUTHANASIA

In any type of euthanasia, care must be taken to assure swift, humane death. The AVMA issued a report in 1986 (see Appendix II) describing the acceptable methods of euthanasia for the different species of laboratory animals.

### 1.8.1 Overdose of Inhalant Anaesthesia (Code: AEOD)

Place cotton or gauze soaked in anaesthetic in the bottom of a bell jar or container and cover it with wire mesh so the animal does not come in direct contact with the anaesthetic. Place the frog in the container and apply the cover. After the animal's respiration cease, check for loss of heartbeat. Thoracotomy, cervical dislocation and exsanguination all assure death.

### 1.8.2 Injectable anaesthetic (Code: AEIN)

An overdose of pentobarbital (50 -250 mg), T-61, or tribromoethanol may be given directly into the heart.

### 1.8.3 Euthanasia in Which Drugs Cannot be Used (Code: AECO)

Euthanasia with carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket, or plastic bag. Place frog in the container and close. After frog no longer moves, check for loss of heartbeat. Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel on Euthanasia (see Appendix II). Explain you special need for these alternative methods under item 8.3 of the Application Form.

# 2. STANDARD PROTOCOLS FOR ELEPHANT SHREWS

## 2.1 CAPTURE (Code: EC)

Elephant shrews are captured in pitfall traps with metal sheets. Should the trapping area get extremely cold, insulation such as paper towel or industrial waste wool should be used. Food and water should be provided for captured animals. Well ventilated transit containers of suitable size should be used. These containers should be lined with strips of paper towel for absorbing urine and for nest material.

## 2.2 HANDLING (CODE: EH)

An elephant shrew should be picked up by its tail close to the body or by gently closing your hand around the body. To restrain an elephant shrew for examination and injections, grasp the loose skin at the nape of its neck between your thumb and index finger and place its tail between the fourth and fifth fingers. Be careful not to grab the tail anywhere but close to the base; remember that the tail can be damaged if excess pressure is applied.

## 2.3 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly used agents and dosages is provided in Appendix I. The veterinarian(s) will assist in selections that are adequate and yet convenient for the investigator. Analgesia requiring controlled substances will be the responsibility of the veterinarian. In completing the Application Form, indicate the method and code (inhalation/injection, etc.) to be used and list the specific agents in your response to item 8.2. Remember that ether is both flammable and explosive. It may not be used unless an explosion-proof hood or room is available and must be disposed of in explosion-proof containers. Proper ventilation is needed with all inhaled anaesthetics for user safety.

### 2.3.1 Inhalation Anaesthesia (Code: EAIH)

A vaporising anaesthetic machine, calibrated for isoflurane, is used with pure medical oxygen to supply a gas mixture of 0-5% isoflurane in oxygen, as required. During initiation of anaesthesia, place the animal in a clear Perspex respirometer and vent an initiation gas mixture of 2-4% isoflurane, depending on the size, into the respirometer at flow rates of 1-2 litres/min, depending upon the size of the animal. Place a cloth over the respirometer to quieten the animal, but remove it periodically to monitor the progress of anaesthesia. Once anaesthetised (usually after ca. five minutes), remove the animal quickly from the respirometer and place it on the preparation table. Place a gas mask appropriate for the shape of the animal over the facial region covering all respiratory surfaces. Lower the maintenance gas mixture to 1-2% isoflurane in oxygen, depending upon the species. Once pre-operative preparation of the animal is complete, move it to the surgery table. Generally the gas mixture can be lowered on commencement of suturing in order to minimise the total time under anaesthesia, and hence hypothermia. At all times during anaesthesia, monitor the rectal or cloacal temperature with a digital thermometer and rectal or cloacal probe. If the body temperature decreases significantly during lengthy procedures, the animal must be heated with a heating pad or infra-red lamp until normothermia is restored.

### 2.3.2 Injection Anaesthesia (Code: EAIN)

Injection anaesthesia, using barbiturates or other agents such as ketamine, can also be used (see Appendix I). The route of administration and frequency of additional doses will be determined by the length of the procedure. In procedures requiring assessment of vascular physiologic responses, the choice of anaesthetic must be carefully considered to avoid

unwanted vascular effects. Injection techniques (IV, IM. IP, etc.) are described in a separate section.

## 2.4 SAMPLING OF BODY FLUIDS

The skin, or other sites of the sampling, should be properly prepped with alcohol to ensure visibility and cleanliness.

### 2.4.1 Bleeding

The maximum amount of blood to be withdrawn to insure survival is around 300 Tl per adult elephant shrew body weight once a week. If bleeding extends over 2-3 weeks, anaemia and abnormalities in serum protein may result.

#### 2.4.1.1 Bleeding from Peripheral Vessels (Code: EBPV)

Orbital sinus: Hold the anaesthetised animal's head firmly against a work surface with your thumb and press down just behind its eye; pull the skin back to open the internal angle of the lid. Gently slide in the microhaematocrit tube or Pasteur pipette through the conjuntiva of the medial canthus. When you reach the sphenoid region, slide the tube down to the orbital sinus, then rotate the tube until the blood is collected. Remove the tube, release pressure and clean blood from the area.

Tail clipping: Using a scalpel blade, transect the tail completely about 3 mm from the tip; blood flow can be increased by placing the tail in warm water for 1-2 minutes before transection. Finger pressure stops bleeding.

Toe clipping: The technique is similar to tail clipping. Blood can be drawn once from each toe.

#### 2.4.1.2 Bleeding by Cardiac Puncture (Code EBCP)

Anaesthetise the animal and place on its back with its length perpendicular to you. With your left thumb and forefinger placed on each side of the thorax, compress slightly and insert a 21 gauge needle and a 3 ml syringe under the xyphoid cartilage. Hold the needle at a 30 degree angle and push forward slowly while aspirating; your left fingers may hold the barrel as your right hand pulls the plunger slowly and steadily. To be sure of its correct placement, feel for the heartbeat against the needle. If no blood flows, withdraw the needle slowly with continued aspiration. Although blood may safely be withdrawn from each elephant shrew once per week, all the blood constituents may not have returned to normal values. This is a risky procedure and should be reserved for terminal procedures.

#### 2.4.1.3 Bleeding by Surgical Approaches (See surgical section)

Blood samples can also be obtained by direct puncture or catheter placement in major vessels such as the carotid artery, jugular vein or femoral artery. these techniques are considered survival strategy and are addressed in the surgical section.

#### 2.4.1.4 Bleeding by Terminal Procedures (Code: EBTP)

Posterior vena cava: Anaesthetise the elephant shrew and place it on its back. Make a V-shaped incision through the skin and abdominal wall at the base of the abdomen and proceed diagonally across each side ending dorsolaterally at the thorax. Lay the skin over the thorax and deflect the gut to the elephant shrew's left, then push the liver forward and enter the vena cava at the level of the kidneys. Straddle the vessel with he fingers of your free hand and insert an appropriately sized needle (27). Withdraw the plunger slowly until the vein collapses; wait for it to refill, then continue. Turning the needle's bevel away from

the wall and tenting the vessel will increase flow. When completed, proceed with euthanasia.

Dorsal aorta: Perform similarly to the vena cava technique, but enter the aorta just anterior to the distal bifurcation. When completed, proceed with euthanasia.

Axillary vessels: Place the anaesthetised elephant shrew on its back with the tail towards you. Stretch one forelimb and hold it in place with a pin through the foot. Make a skin incision in the axillary region; the bottom skin edge can be held up and used as a bowl to collect blood. Cut the axillary vessels with scissors; as the blood wells up , collect it with a Pasteur pipette or syringe. Note: This blood will be contaminated with tissue fluids. When completed, proceed with euthanasia.

### 2.4.2 Ascites Fluid Collection and Production (Code: EAP)

Inject each animal IP with 0.5 ml of pristane using a 27 gauge needle. After 5-10 days, inject each elephant shrew IP with hybridoma or tumour cells in saline (0.1-0.5 cc total volume) using a 27 gauge needle. Ascites or tumours should appear after 7-28 days. Tumours or cell lines can be contaminated with passenger viruses of murine origin which can affect the health of your animals. If you think this is a possibility, please consult the veterinary staff to work out a plan to test your biologics.

To drain ascites, immobilise the elephant shrew by holding its neck, back and tail. Prepare the skin with alcohol, then enter the peritoneal cavity in the lower left quadrant with an 18 gauge needle and collect the fluid into a sterile tube containing heparin. Repeat this draining process every 2 days until the yield of ascites fluid falls off. Do not continue if the animal appears sickly (hunched position, ruffled hair) or the volume is depleted. The animals must be observed daily to assure they are not distressed by large amounts of ascites fluid.

### 2.4.3 Collection of Peritoneal Cells

#### 2.4.3.1 Macrophage Collection (Code: EBMC)

To stimulate yields of activated macrophages, animals are injected with thioglycolate solution or sterile mineral oil (dose 1 ml) IP. The macrophages are collected at the time of euthanasia (3-4 days later).

#### 2.4.3.2 Polymorphonuclear Leukocyte Collection (Code: EPLC)

To stimulate exudation of polymorphonuclear leukocytes into the abdominal cavity, inject a 0.1% glycogen solution IP and collect cells within 4 hours at the time of euthanasia.

## 2.5 INJECTIONS

The skin is prepped to assure maximum visibility and cleanliness. Injections can be made directly into the major vessels by needle or catheter placement. The carotid artery, jugular vein or the femoral vessels can be used. These techniques are considered surgical procedures and are addressed in the surgery section. Preparing the skin may include shaving, cleaning with alcohol or a full surgical prep.

Phosphate buffered saline (PBS) or other isotonic solutions are better than distilled water as a solvent/vehicle for injections. Distilled water causes some hemolysis when given IV and pain when given SQ. Oils are suitable for administration of lipid-soluble substances or in adjuvants, but absorption is delayed and this vehicle cannot be injected IV.

In general, the maximum quantity for an IV injection is 1 ml/100 gm body weight, but the dose really depends on the route of administration. The pH should be physiologic (~7.4).

IM: Maximum 0.1 ml at any site in adult

SQ: Maximum 0.25 ml at any site in adult
IV: More than 1ml/100 gm may cause pulmonary oedema

### 2.5.1 Intravenous (IV) (Code: EIIV)

Insertion of the needle (25-30 gauge) in the lateral tail veins should start distally and work proximally in relation to the heart (because of direction of blood flow) for subsequent injections. After removing the needle, blood flow stops with pressure.

### 2.5.2 Subcutaneous (SQ) (Code: EISQ)

Injections are under the skin of the back or sides. Clean the site and pass the needle through the elephant shrew's skin in an anterior direction. To assure the needle is positioned correctly, move up and down in position to assure that it is SQ; if tenting of skin is not discernible, withdraw slightly until SQ.

### 2.5.3 Intramuscular (IM) (Code: EIIM)

This site should be avoided if possible because of the small muscle mass in elephant shrews. A usual intramuscular site is the large muscles of the rear limb. Insert the needle in a posterior direction away from the femur and sciatic nerve. If the needle penetrates too deeply, you may encounter bone or miss the muscle mass and fall into the fascial plane. Aspirate before injecting to assure needle placement is not intravascular.

### 2.5.4 Intraperitoneal (IP) (Code: EIIP)

Immobilise the animal by holding the skin of its neck, back and tail. Tilt the animal's head down to allow gravity to push the viscera cranially. After cleaning the skin, insert the tip of the needle into the lower quadrant(s) of the abdomen, away from the midline. Inject immediately to push away the viscera and withdraw the needle. A large gauge needle is less likely to penetrate the viscera.

### 2.5.5 Footpad (FP) (Code: EIFP)

Materials can be injected into the rear footpads only, using a small gauge needle inserted under the skin from above the heel.

### 2.5.6 Intragastric (oral) (Code: EIIG)

Use a 15-16 gauge blunt-ended needle cannula about 11 cm long (preferably bulbed). measure the like distance to the stomach on the outside of the elephant shrew and note the amount of the cannula that would still protrude from the mouth. Hold the elephant shrew firmly by the skin of the neck and back so the head is immobile and in line with the back. Pass a needle into the mouth as far to the side as possible. After locating the oesophagus, push the needle gently into the stomach. The elephant shrew may swallow and help with passage. You may feel an obstruction at the back of the mouth and at the sphincter to the stomach; move the syringe back and forth gently to overcome this. Slow the initial discharge to check if the cannula is correctly placed; if placed properly, inject rapidly. The elephant shrew may squeak while being injected and may make swallowing movements. Be careful to avoid injections into the trachea.

Intragastric injections may also use intubation. A mouth gag with a central; hole (5 mm) prevents biting through the catheter (plastic or rubber). Place the gag behind the incisors. Measure the catheter on the outside and pass through the hole of the gag and into the stomach; a conscious elephant shrew will swallow and help with passage. The usual maximum dosage is 3 ml/100 gm body weight. Clear the catheter with a small amount of air before withdrawal to reduce tracheal contamination.

## 2.6 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. Procedures requiring prolonged anaesthesia (more than 2 hours) will require special approval and should be explained in item 5 of the Standard Protocol Form. Surgical procedures require aseptic techniques, but not a formal operating room. They should be carried out in approved surroundings (please check with the Division of Animal Resources). Autopsy with sampling of organs after euthanasia is not considered a surgical technique. All survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 4 of the Standard Protocol Form.

Body heat is rapidly lost during surgical procedures and a heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 2.6.1 Anterior Neck (Code: ESAN)

Anaesthesia is induced appropriate to the duration of the planned procedure. Injection anaesthesia is preferable since inhalation anaesthesia is difficult to maintain while working on the neck or trachea. The elephant shrew is restrained, exposing the throat. The neck is shaved and the skin washed with 70% alcohol and betadine solution. Additional local anaesthetic can be given if needed. A midline incision is made and blunt dissection used to move the incision down to the site of interest.

The carotid vessels, jugular veins and vagus nerve can be located adjacent to the tracheal area. These vessels are located by palpation of the carotid pulse and by feeling the tracheal rings. The vessels are isolated by blunt dissection and length of suture are placed around them for control of haemorrhage during injection or catheter placement. Needles or catheters (P-10 or P-50 tubing) are inserted under direct visualisation. The direction of placement depends on the purpose. Removal of needles or catheters is followed by securing haemeostasis.

The trachea is located by palpation of the tracheal rings. Direct intratracheal injection is accomplished by inserting a 25 gauge needle between two tracheal rings. A small catheter can be inserted if desired. Tracheal intubation is accomplished in a similar manner except that a larger diameter tube is inserted. After the catheter or larger tube is removed, reconstruction of the tracheal structure is accomplished by using one or two 5-0 silk sutures to reappose the divided tracheal rings.

The thyroid is approached in the same manner as the trachea. The two lobes of the thyroid are adjacent to the larynx. For thyroidectomy, the lobes are dissected bluntly and removed with haemeostatic control.

After completion of the desired procedure(s), the incision is closed with interrupted 2-0 silk of absorbable sutures. The skin is cleaned and the elephant shrew is returned to its cage to recover from anaesthesia.

Post-operative analgesia is not normally required but analgesia needs should be decided in conjunction with the veterinarian. The animal should be watched carefully to insure that it is up and moving around in the cage.

### 2.6.2 Thymectomy in Adults (Code: ESTH)

For this procedure, young adult elephant shrews are anaesthetised. Tribromoethanol IP (dose 0.47 mg/gm body weight) works well. Place the animal in dorsal recumbency (on back), then shave and clean the skin of its neck and upper chest with 70% alcohol. make a midline longitudinal incision from the angle of the mandible to the level of the 4th rib and remove the thymus. Close the incision with skin clips which are removed with forceps after 7 days. Post-operative analgesia may not be necessary but should be decided in conjunction with the veterinarian.

### 2.6.3 Intrathymic Injection (Code: ESIT)

Anaesthetise the elephant shrews as above and place in dorsal recumbency. Shave the neck and clean the skin with 70% alcohol. Make a midline incision over the lover cervical and upper thoracic region. Bisect the upper third of the sternum longitudinally with fine scissors to expose the thymus. Injections (10 µl/site) are made in the anterior superior portion of each thymic lobe using a 1 ml syringe and a 28 gauge needle mounted on a Tridak Stepper. Close the incision with skin clips (nothing is done to the sternum) and remove the clips with forceps after 7 days. Post-operative analgesia may not be necessary but should be decided in conjunction with the veterinarian.

### 2.6.4 Abdominal Surgery (Code: ESA)

The techniques for abdominal surgery can be applied to the biopsy and/or removal of various abdominal organs as well as for other manipulations in the abdominal cavity, including vascular injection, body fluid sampling and implantation of minimitters. Anaesthesia appropriate for the surgical approach and duration of the procedure is administered. Consult Appendix I and the veterinarian.

After anaesthesia, the elephant shrew is restrained on an operating surface in an optimal position for the planned incision. For prolonged procedures, heated operating surfaces are used to maintain body heat. Body temperature is monitored.

Midline incision or flank approaches are most common for abdominal surgery. The area of the incision is shaved and the skin surface rendered aseptic with 70% alcohol and betadine solution. If additional anaesthesia is desired, intracutaneous administration of a local anaesthetic such as 1-2% procaine or lidocaine at the site of the skin incision is administered via a 25 gauge needle. The volume of local anaesthetic is to be kept below 1.5 ml. The area of the incision is then isolated in a clean but not necessarily sterile manner and the surgery proceeds using a clean technique.

A scalpel is used to make an incision of the length and position desired for the procedure. Midline incisions have less bleeding and less need for subsequent haemeostasis than incisions through muscle layers such as in the flank. The abdominal incision is divided into 2 layers (1. skin; 2. muscle/peritoneum) to avoid unintentional laceration of the abdominal contents. When entering the abdominal cavity, the second layer is tented and a very small incision made to allow the abdominal contents to fall away from the site before the incision is extended. Haemostasis is established with small clamps or sutures as needed. The abdominal contents are moved aside using saline-dampened gauze sponges and appropriate retractors to isolate and expose the organ, vessel or duct of interest.

If organ biopsy procedures are carried out, the organ of interest is exposed and tissue is obtained using an appropriate biopsy needle or wedge-shaped incision. Bleeding is controlled by suturing the incision with 5-0 silk or absorbable suture. Gelfoam or other haemostatic agent may also be used to advantage if bleeding is difficult to control in a friable organ such as the liver.

If organ removal is desired, the ducts are isolated and ligated with suture material. Either absorbable or nonabsorbable suture may be used as appropriate for the procedure and subsequent course of the experiment. Silk suture of 3-0 to 5-0 is usually satisfactory and may be left in the abdominal cavity without subsequent infection. The organ, once isolated, is then removed by transecting the ligated vessels and ducts and any remaining connective tissue.

If vascular or duct perfusion procedures are being done, the vessel or duct is isolated and a needle or catheter appropriate for the procedure is inserted. After infusion or collection, the needle is removed and haemostasis re-established. In the case of vessels, this is accomplished with gentle pressure and/or the application of Gelfoam. The latter can be left in place and will be reabsorbed by the animal.

If a chronic in-dwelling catheter has been placed, it is tunneled through the abdominal wall and under the skin to an appropriate external site. This is usually over the scapula where it is secured in a way that the animal cannot damage it. A variety of connections can be used as appropriate for the subsequent experimentation. Chronis in-dwelling bladder catheters are usually placed in such a way that they exit to the lower abdominal wall.

After completion of the procedure, care is taken to assure that haemostasis is satisfactory and all gauze sponges and other restraining devices are removed. The abdominal incision(s) is closed in two layers. The muscle and peritoneal layer is closed with a non-locking running stitch of absorbable or nonabsorbable suture, usually of 2-0 silk.

Care is taken to avoid inclusion of abdominal contents in the suture line. A running stitch is used to provide a continuous closure to avoid subsequent herniation of abdominal contents through the suture line. The skin layer is closed with a line of running or interrupted sutures. Alternatively, the skin may be closed with skin staples which are removed 10-14 days later. Care is taken to include the deep fascial layers of the skin in the superficial suture line. The skin surface is then cleaned, restraints are removed and the animal is allowed to recover from anaesthesia under frequent observation.

Post-operative analgesia is not normally required, particularly if methoxyflurane and a long-acting local anaesthetic have been injected into the site of the skin incision. The animal should be watched carefully to ensure that it is up and moving in the cage. Additional post-operative analgesia should be decided upon in consultation with the veterinarian.

### 2.6.5 Skin Grafting (Code: ESSG)

With the animal anaesthetised, use a biopsy punch to remove approximately 1 cm of skin from the trunk of each donor and recipient animal. The donor's skin sample is placed on the recipient at the site of the skin biopsy and sutured in place with absorbable sutures in a simple interrupted fashion. Cover the graft with a plaster bandage. Recipients are observed daily for signs of rejection. There should be minimal pain, but analgesia should be decided upon in conjunction with the veterinary staff.

### 2.6.6 Castration (Code: ESCR)

Anaesthetise male elephant shrews and prepare the scrotum well with 70% alcohol and betadine solution. Make a small (ca. 1cm) incision through the skin at the tip of the scrotum between the two bulges of the testicles. Clear the subcutaneous connective tissue and gently squeeze a testicle out of the scrotum. Make a small 5 mm incision into the tip of the testicle sac and pull out the cauda epididymis, the testis, the caput epididymis, the vas deferens and the spermatic blood vessels. Place a single ligature of 2-0 silk around the vas deferens and the spermatic blood vessels and severe these distal to the ligature. Remove the testis and the epididymis. Repeat the procedure on the remaining testis. Suture the scrotum with one or two silk sutures and clean the wound with betadine solution. Post-operative analgesia is as described for abdominal surgery above.

## 2.7 PHYSIOLOGICAL MEASUREMENTS

The animal is placed in a metabolism chamber and various measurements are done using specialist analysers connected to the chamber.

### 2.7.1 Respirometry (Code: EPRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. For rodents, burrow tube type chambers seem best, as the animals settle down more quickly. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline). Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 2.7.2 Measurement of food consumption (EPF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some animals, particularly fossorial animals are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 2.8 and quote reference number of relevant euthanasia

procedure).

### 2.7.3 Measurement of total body water by tritium dilution (Code: EPW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April 1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste"*- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 2.5 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 2.3 and 2.4 and quote reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 2.7.4 Urine analysis (Code: EPU)

Elephant shrews are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 2.7.5 Non- shivering thermogenesis (Code: EPS)

Non-shivering thermogenesis (NST) or "chemical thermoregulation" is induced by norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the animal. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 2.7.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 2.7.1) and injected with saline (for controls) or NE solutions (1.5mg/kg) subcutaneously (see Sections 2.3 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of animals should be avoided, since high $T_b$s above $40^o$ C may inhibit thermogenesis.

### 2.7.6 Activity measurements (Code: EPA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The elephant shrew is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 2.7.7 X-Ray techniques (Code: EPX)

The digestive tract of elephant shrews can be studied by X-rays following introduction of a suitable contrast agent such as barium sulfate or gastrografin. The elephant shrew is trained before the experiment to be used to the position required for the x-ray measurement. The animal is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the animal is let into the room and all metals are removed form the area. The animal is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The animal is allowed to ingest appropriate feed coated with barium sulfate. It is important that the animal does not move during the experiment. If it was not possible to train the animal to maintain the appropriate position, soft materials and sandbags are used to keep the animal in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 2.8 EUTHANASIA

In any type of euthanasia, care must be taken to assure death has occurred.

### 2.8.1 Overdose of Inhalant Anaesthetic (Code: EEOD)

Under a hood, place cotton or gauze soaked in anaesthetic in a bottom of a bell jar and cover with wire mesh or grate so the animal does not come into direct contact with anaesthetic; place the elephant shrew into the jar and apply the cover. After the animal's respiration stops, check it for complete loss of heart beat. Please clean the bell jar after the procedure is completed.

### 2.8.2 Injectable Anaesthetic or Euthanasia Agent (Code: EEIN)

An overdose of an anaesthetic such as pentobarbital or a euthanasia agent such as T-61 may be given intravenously or directly into the heart.

### 2.8.3 Euthanasia in an Anaesthetised Animal by Physical Means (Code: EEPM)

Cervical dislocation may be accomplished by placing the investigator's fingers, a stout scissors or an iron rod across the neck and rapidly pulling on the tail to break the neck. This procedure is best when followed by a thorocotomy/exsanguination Alternatively, the anaesthetised elephant shrew is sacrificed by guillotine.

### 2.8.4 Brain Fixation by Intracardiac Perfusion (Code: EEBP)

After anaesthesia, the elephant shrew is placed supine on a support table with a down-draft hood. Animals whose tissues are to be examined by ultrastructure are maintained on

artificial respirators through an acutely placed tracheostomy, or an intratracheal cannula. With a midline incision and bilateral transection of the rib cage in the mid-axillary line, expose the heart. The sternum should be reflected dorsally and fractured above the manubrium. Reflect the left lobe of the lung forward to expose the descending aorta and clamp with a haemostat. Puncture the left ventricle with a 12-16 gauge needle to which is connected an airtight pressurised chamber containing the fixative (recently depolymerised paraformaldehyde in phosphate buffer, 4 gm/100 cc). Inject the perfusate under a pressure of 120-140 mm Hg and puncture the right atrium to provide outflow. Artificial respiration is terminated. The perfusion will continue for 5-15 minutes, then excise the brain whole, dissect and process for cytological and cytochemical analysis.

### 2.8.5 Euthanasia in Which Drugs Cannot be Used (Code: EECO)

Euthanasia with carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket or plastic bag. Place the elephant shrew in the container and close. If more than one animal is euthanised, make sure the animals are not overcrowded in the container. After the elephant shrew no longer moves, check for loss of heart rate. The container should be clear so that the animal can be observed to assure that the animal is not distressed.

Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel on Euthanasia (see Appendix II). Explain your special need for these alternative methods under item 5 of the Review Form.

### 2.8.6 Euthanasia by Decapitation (Code: EEDE)

Decapitation should only be performed by trained personnel who have been certified by the veterinarian. Handle the animals carefully and gently, taking care not to frighten or antagonise them unnecessarily. The animals should be brought to the laboratory or a secluded, quiet work station. Since this is aesthetically displeasing, privacy should be guaranteed.

The decapitator should be of good quality and manufactured commercially. Animals waiting to be decapitated should be separated from the decapitator. Gently restrain the animal and accomplish the procedure as quickly as possible without placing the operator at risk.

The lever should be lifted all the way up. The hole in which to place the animal's head should be as large as possible. The animal is gently grasped around the abdomen with the right hand, the animal's head is placed through the hole and with one firm motion the left hand brings down the lever, severing the animal's head. The body is held firmly over a container or sink and the blood allowed to drain. The grasp is tightened around the animal to prevent movement. Blood should be cleaned up between each animal.

# 3. STANDARD PROTOCOLS FOR MICE

## 3.1 HANDLING (CODE: MH)

A mouse should be picked up by its tail close to the body or by gently closing your hand around the body. To restrain a mouse for examination and injections, grasp the loose skin at the nape of its neck between your thumb and index finger and place its tail between the fourth and fifth fingers. Be careful not to grab the tail anywhere but close to the base; remember that the tail can be damaged if excess pressure is applied.

## 3.2 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly used agents and dosages is provided in Appendix I. The veterinarian(s) will assist in selections that are adequate and yet convenient for the investigator. Analgesia requiring controlled substances will be the responsibility of the veterinarian. In completing the Application Form, indicate the method and code (inhalation/injection, etc.) to be used and list the specific agents in your response to item 8.2. Remember that ether is both flammable and explosive. It may not be used unless an explosion-proof hood or room is available and must be disposed of in explosion-proof containers. Proper ventilation is needed with all inhaled anaesthetics for user safety.

### 3.2.1 Inhalation Anaesthesia (Code: MAIH)

A vaporising anaesthetic machine, calibrated for isoflurane, is used with pure medical oxygen to supply a gas mixture of 0-5% isoflurane in oxygen, as required. During initiation of anaesthesia, place the animal in a clear Perspex respirometer and vent an initiation gas mixture of 2-4% isoflurane, depending on the species, into the respirometer at flow rates of 1-2 litres/min, depending upon the size of the animal. Place a cloth over the respirometer to quieten the animal, but remove it periodically to monitor the progress of anaesthesia. Once anaesthetised (usually after ca. five minutes), remove the animal quickly from the respirometer and place it on the preparation table. Place a gas mask appropriate for the shape of the animal over the facial region covering all respiratory surfaces. Lower the maintenance gas mixture to 1-2% isoflurane in oxygen, depending upon the species. Once pre-operative preparation of the animal is complete, move it to the surgery table. Generally the gas mixture can be lowered on commencement of suturing in order to minimise the total time under anaesthesia, and hence hypothermia. At all times during anaesthesia, monitor the rectal or cloacal temperature with a digital thermometer and rectal or cloacal probe. If the body temperature decreases significantly during lengthy procedures, the animal must be heated with a heating pad or infra-red lamp until normothermia is restored.

### 3.2.2 Injection Anaesthesia (Code: MAIN)

Injection anaesthesia, using barbiturates or other agents such as ketamine, can also be used (see Appendix I). The route of administration and frequency of additional doses will be determined by the length of the procedure. In procedures requiring assessment of vascular physiologic responses, the choice of anaesthetic must be carefully considered to avoid unwanted vascular effects. Injection techniques (IV, IM. IP, etc.) are described in a separate section.

## 3.3 SAMPLING OF BODY FLUIDS

The skin, or other sites of the sampling, should be properly prepped with alcohol to ensure visibility and cleanliness.

### 3.3.1 Bleeding

The maximum amount of blood to be withdrawn to insure survival is around 300 Tl per adult mouse body weight once a week. If bleeding extends over 2-3 weeks, anaemia and abnormalities in serum protein may result.

#### 3.3.1.1 Bleeding from Peripheral Vessels (Code: MBPV)

Orbital sinus: Hold the anaesthetised animal's head firmly against a work surface with your thumb and press down just behind its eye; pull the skin back to open the internal angle of the lid. Gently slide in the microhaematocrit tube or Pasteur pipette through the conjuntiva of the medial canthus. When you reach the sphenoid region, slide the tube down to the orbital sinus, then rotate the tube until the blood is collected. Remove the tube, release pressure and clean blood from the area.

Tail clipping: Using a scalpel blade, transect the tail completely about 3 mm from the tip; blood flow can be increased by placing the tail in warm water for 1-2 minutes before transection. Finger pressure stops bleeding.

Toe clipping: The technique is similar to tail clipping. Blood can be drawn once from each toe.

#### 3.3.1.2 Bleeding by Cardiac Puncture (Code MBCP)

Anaesthetise the animal and place on its back with its length perpendicular to you. With your left thumb and forefinger placed on each side of the thorax, compress slightly and insert a 21 gauge needle and a 3 cc syringe under the xyphoid cartilage. Hold the needle at a 30 degree angle and push forward slowly while aspirating; your left fingers may hold the barrel as your right hand pulls the plunger slowly and steadily. To be sure of its correct placement, feel for the heartbeat against the needle. If no blood flows, withdraw the needle slowly with continued aspiration. Although blood may safely be withdrawn from each mouse once per week, all the blood constituents may not have returned to normal values. This is a risky procedure and should be reserved for terminal procedures.

#### 3.3.1.3 Bleeding by Surgical Approaches (See surgical section)

Blood samples can also be obtained by direct puncture or catheter placement in major vessels such as the carotid artery, jugular vein or femoral artery. these techniques are considered survival strategy and are addressed in the surgical section.

#### 3.3.1.4 Bleeding by Terminal Procedures (Code: MBTP)

Posterior vena cava: Anaesthetise the mouse and place it on its back. Make a V-shaped incision through the skin and abdominal wall at the base of the abdomen and proceed diagonally across each side ending dorsolaterally at the thorax. Lay the skin over the thorax and deflect the gut to the mouse's left, then push the liver forward and enter the vena cava at the level of the kidneys. Straddle the vessel with he fingers of your free hand and insert an appropriately sized needle (27). Withdraw the plunger slowly until the vein collapses; wait for it to refill, then continue. Turning the needle's bevel away from the wall and tenting the vessel will increase flow. When completed, proceed with euthanasia.

Dorsal aorta: Perform similarly to the vena cava technique, but enter the aorta just anterior to the distal bifurcation. When completed, proceed with euthanasia.

Axillary vessels: Place the anaesthetised mouse on its back with the tail towards you. Stretch one forelimb and hold it in place with a pin through the foot. Make a skin incision in the axillary region; the bottom skin edge can be held up and used as a bowl to collect blood. Cut the axillary vessels with scissors; as the blood wells up , collect it with a Pasteur pipette or syringe. Note: This blood will be contaminated with tissue fluids. When completed, proceed with euthanasia.

### 3.3.2 Ascites Fluid Collection and Production

Inject each animal IP with 0.5 ml of pristane using a 27 gauge needle. After 5-10 days, inject each mouse IP with hybridoma or tumour cells in saline (0.1-0.5 cc total volume) using a 27 gauge needle. Ascites or tumours should appear after 7-28 days. Tumours or cell lines can be contaminated with passenger viruses of murine origin which can affect the health of your animals. If you think this is a possibility, please consult the veterinary staff to work out a plan to test your biologics.

To drain ascites, immobilise the mouse by holding its neck, back and tail. Prepare the skin with alcohol, then enter the peritoneal cavity in the lower left quadrant with an 18 gauge needle and collect the fluid into a sterile tube containing heparin. Repeat this draining process every 2 days until the yield of ascites fluid falls off. Do not continue if the animal appears sickly (hunched position, ruffled hair) or the volume is depleted. The animals must be observed daily to assure they are not distressed by large amounts of ascites fluid.

### 3.3.3 Collection of Peritoneal Cells

#### 3.3.3.1 Macrophage Collection (Code: MBMC)

To stimulate yields of activated macrophages, animals are injected with thioglycolate solution or sterile mineral oil (dose 1 ml) IP. The macrophages are collected at the time of euthanasia (3-4 days later).

#### 3.3.3.2 Polymorphonuclear Leukocyte Collection (Code: MPLC)

To stimulate exudation of polymorphonuclear leukocytes into the abdominal cavity, inject a 0.1% glycogen solution IP and collect cells within 4 hours at the time of euthanasia.

## 3.4 INJECTIONS

The skin is prepped to assure maximum visibility and cleanliness. Injections can be made directly into the major vessels by needle or catheter placement. The carotid artery, jugular vein or the femoral vessels can be used. These techniques are considered surgical procedures and are addressed in the surgery section. Preparing the skin may include shaving, cleaning with alcohol or a full surgical prep.

Phosphate buffered saline (PBS) or other isotonic solutions are better than distilled water as a solvent/vehicle for injections. Distilled water causes some hemolysis when given IV and pain when given SQ. Oils are suitable for administration of lipid-soluble substances or in adjuvants, but absorption is delayed and this vehicle cannot be injected IV.

In general, the maximum quantity for an IV injection is 1 ml/100 gm body weight, but the dose really depends on the route of administration. The pH should be physiologic (~7.4).

IM: Maximum 0.1 ml at any site in adult
SQ: Maximum 0.25 ml at any site in adult
IV: More than 1ml/100 gm may cause pulmonary oedema

### 3.4.1 Intravenous (IV) (Code: MIIV)

Insertion of the needle (25-30 gauge) in the lateral tail veins should start distally and work proximally in relation to the heart (because of direction of blood flow) for subsequent injections. After removing the needle, blood flow stops with pressure.

### 3.4.2 Subcutaneous (SQ) (Code: MISQ)

Injections are under the skin of the back or sides. Clean the site and pass the needle through the mouse's skin in an anterior direction. To assure the needle is positioned correctly, move up and down in position to assure that it is SQ; if tenting of skin is not discernible, withdraw slightly until SQ.

### 3.4.3 Intramuscular (IM) (Code: MIIM)

This site should be avoided if possible because of the small muscle mass in mice. A usual intramuscular site is the large muscles of the rear limb. Insert the needle in a posterior direction away from the femur and sciatic nerve. If the needle penetrates too deeply, you may encounter bone or miss the muscle mass and fall into the fascial plane. Aspirate before injecting to assure needle placement is not intravascular.

### 3.4.4 Intraperitoneal (IP) (Code: MIIP)

Immobilise the animal by holding the skin of its neck, back and tail. Tilt the animal's head down to allow gravity to push the viscera cranially. After cleaning the skin, insert the tip of the needle into the lower quadrant(s) of the abdomen, away from the midline. Inject immediately to push away the viscera and withdraw the needle. A large gauge needle is less likely to penetrate the viscera.

### 3.4.5 Footpad (FP) (Code: MIFP)

Materials can be injected into the rear footpads only, using a small gauge needle inserted under the skin from above the heel.

### 3.4.6 Intragastric (oral) (Code: MIIG)

Use a 15-16 gauge blunt-ended needle cannula about 11 cm long (preferably bulbed). measure the like distance to the stomach on the outside of the mouse and note the amount of the cannula that would still protrude from the mouth. Hold the mouse firmly by the skin of the neck and back so the head is immobile and in line with the back. Pass a needle into the mouth as far to the side as possible. After locating the oesophagus, push the needle gently into the stomach. The mouse may swallow and help with passage. You may feel an obstruction at the back of the mouth and at the sphincter to the stomach; move the syringe back and forth gently to overcome this. Slow the initial discharge to check if the cannula is correctly placed; if placed properly, inject rapidly. The mouse may squeak while being injected and may make swallowing movements. Be careful to avoid injections into the trachea.

Intragastric injections may also use intubation. A mouth gag with a central; hole (5 mm) prevents biting through the catheter (plastic or rubber). Place the gag behind the incisors. Measure the catheter on the outside and pass through the hole of the gag and into the stomach; a conscious mouse will swallow and help with passage. The usual maximum dosage is 3 ml/100 g body weight. Clear the catheter with a small amount of air before withdrawal to reduce tracheal contamination.

## 3.5 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. Procedures requiring prolonged anaesthesia (more than 2 hours) will require special approval and should be explained in item 5 of the Standard Protocol Form. Surgical procedures require aseptic techniques, but not a formal operating room. They should be carried out in approved surroundings (please check with the Division of Animal Resources). Autopsy with sampling of organs after euthanasia is not considered a surgical technique. All

survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 8.3 of the Application Form.

Body heat is rapidly lost during surgical procedures and a heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 3.5.1 Anterior Neck (Code: MSAN)

Anaesthesia is induced appropriate to the duration of the planned procedure. Injection anaesthesia is preferable since inhalation anaesthesia is difficult to maintain while working on the neck or trachea. The mouse is restrained, exposing the throat. The neck is shaved and the skin washed with 70% alcohol and betadine solution. Additional local anaesthetic can be given if needed. A midline incision is made and blunt dissection used to move the incision down to the site of interest.

The carotid vessels, jugular veins and vagus nerve can be located adjacent to the tracheal area. These vessels are located by palpation of the carotid pulse and by feeling the tracheal rings. The vessels are isolated by blunt dissection and length of suture are placed around them for control of haemorrhage during injection or catheter placement. Needles or catheters (P-10 or P-50 tubing) are inserted under direct visualisation. The direction of placement depends on the purpose. Removal of needles or catheters is followed by securing haomeostasis.

The trachea is located by palpation of the tracheal rings. Direct intratracheal injection is accomplished by inserting a 25 gauge needle between two tracheal rings. A small catheter can be inserted if desired. Tracheal intubation is accomplished in a similar manner except that a larger diameter tube is inserted. After the catheter or larger tube is removed, reconstruction of the tracheal structure is accomplished by using one or two 5-0 silk sutures to reappose the divided tracheal rings.

The thyroid is approached in the same manner as the trachea. The two lobes of the thyroid are adjacent to the larynx. For thyroidectomy, the lobes are dissected bluntly and removed with haemeostatic control.

After completion of the desired procedure(s), the incision is closed with interrupted 2-0 silk of absorbable sutures. The skin is cleaned and the mouse is returned to its cage to recover from anaesthesia.

Post-operative analgesia is not normally required but analgesia needs should be decided in conjunction with the veterinarian. The animal should be watched carefully to insure that it is up and moving around in the cage.

### 3.5.2 Thymectomy in Adults (Code: MSTH)

For this procedure, young adult mice are anaesthetised. Tribromoethanol IP (dose 0.47 mg/gm body weight) works well. Place the animal in dorsal recumbency (on back), then shave and clean the skin of its neck and upper chest with 70% alcohol. make a midline longitudinal incision from the angle of the mandible to the level of the 4th rib and remove the thymus. Close the incision with skin clips which are removed with forceps after 7 days. Post-operative analgesia may not be necessary but should be decided in conjunction with the veterinarian.

### 3.5.3 Intrathymic Injection (Code: MSIT)

Anaesthetise the mice as above and place in dorsal recumbency. Shave the neck and clean the skin with 70% alcohol. Make a midline incision over the lover cervical and upper thoracic region. Bisect the upper third of the sternum longitudinally with fine scissors to expose the thymus. Injections (10 µl/site) are made in the anterior superior portion of each thymic lobe using a 1 ml syringe and a 28 gauge needle mounted on a Tridak Stepper. Close the incision with skin clips (nothing is done to the sternum) and remove the clips with forceps after 7 days. Post-operative analgesia may not be necessary but should be decided in conjunction with the veterinarian.

### 3.5.4 Abdominal Surgery (Code: MSA)

The techniques for abdominal surgery can be applied to the biopsy and/or removal of various abdominal organs as well as for other manipulations in the abdominal cavity, including vascular injection and body fluid sampling. Anaesthesia appropriate for the surgical approach and duration of the procedure is administered. Consult Appendix I and the veterinarian.

After anaesthesia, the mouse is restrained on an operating surface in an optimal position for the planned incision. For prolonged procedures, heated operating surfaces are used to maintain body heat. Body temperature is monitored.

Midline incision or flank approaches are most common for abdominal surgery. The area of the incision is shaved and the skin surface rendered aseptic with 70% alcohol and betadine solution. If additional anaesthesia is desired, intracutaneous administration of a local anaesthetic such as 1-2% procaine or lidocaine at the site of the skin incision is administered via a 25 gauge needle. The volume of local anaesthetic is to be kept below 1.5 ml. The area of the incision is then isolated in a clean but not necessarily sterile manner and the surgery proceeds using a clean technique.

A scalpel is used to make an incision of the length and position desired for the procedure. Midline incisions have less bleeding and less need for subsequent haemeostasis than incisions through muscle layers such as in the flank. The abdominal incision is divided into 2 layers (1. skin; 2. muscle/peritoneum) to avoid unintentional laceration of the abdominal contents. When entering the abdominal cavity, the second layer is tented and a very small incision made to allow the abdominal contents to fall away from the site before the incision is extended. Haemostasis is established with small clamps or sutures as needed. The abdominal contents are moved aside using saline-dampened gauze sponges and appropriate retractors to isolate and expose the organ, vessel or duct of interest.

If organ biopsy procedures are carried out, the organ of interest is exposed and tissue is obtained using an appropriate biopsy needle or wedge-shaped incision. Bleeding is controlled by suturing the incision with 5-0 silk or absorbable suture. Gelfoam or other haemostatic agent may also be used to advantage if bleeding is difficult to control in a friable organ such as the liver.

If organ removal is desired, the ducts are isolated and ligated with suture material. Either absorbable or nonabsorbable suture may be used as appropriate for the procedure and subsequent course of the experiment. Silk suture of 3-0 to 5-0 is usually satisfactory and may be left in the abdominal cavity without subsequent infection. The organ, once isolated, is then removed by transecting the ligated vessels and ducts and any remaining connective tissue.

If vascular or duct perfusion procedures are being done, the vessel or duct is isolated and a needle or catheter appropriate for the procedure is inserted. After infusion or collection, the needle is removed and haemostasis re-established. In the case of vessels, this is

accomplished with gentle pressure and/or the application of Gelfoam. The latter can be left in place and will be reabsorbed by the animal.

If a chronic in-dwelling catheter has been placed, it is tunneled through the abdominal wall and under the skin to an appropriate external site. This is usually over the scapula where it is secured in a way that the animal cannot damage it. A variety of connections can be used as appropriate for the subsequent experimentation. Chronis in-dwelling bladder catheters are usually placed in such a way that they exit to the lower abdominal wall.

After completion of the procedure, care is taken to assure that haemostasis is satisfactory and all gauze sponges and other restraining devices are removed. The abdominal incision(s) is closed in two layers. The muscle and peritoneal layer is closed with a non-locking running stitch of absorbable or nonabsorbable suture, usually of 2-0 silk.

Care is taken to avoid inclusion of abdominal contents in the suture line. A running stitch is used to provide a continuous closure to avoid subsequent herniation of abdominal contents through the suture line. The skin layer is closed with a line of running or interrupted sutures. Alternatively, the skin may be closed with skin staples which are removed 10-14 days later. Care is taken to include the deep fascial layers of the skin in the superficial suture line. The skin surface is then cleaned, restraints are removed and the animal is allowed to recover from anaesthesia under frequent observation.

Post-operative analgesia is not normally required, particularly if methoxyflurane and a long-acting local anaesthetic have been injected into the site of the skin incision. The animal should be watched carefully to ensure that it is up and moving in the cage. Additional post-operative analgesia should be decided upon in consultation with the veterinarian.

### 3.5.5 Skin Grafting (Code: MSSG)

With the animal anaesthetised, use a biopsy punch to remove approximately 1 cm of skin from the trunk of each donor and recipient animal. The donor's skin sample is placed on the recipient at the site of the skin biopsy and sutured in place with absorbable sutures in a simple interrupted fashion. Cover the graft with a plaster bandage. Recipients are observed daily for signs of rejection. There should be minimal pain, but analgesia should be decided upon in conjunction with the veterinary staff.

### 3.5.6 Castration (Code: MSCR)

Anaesthetise male mice and prepare the scrotum well with 70% alcohol and betadine solution. Make a small (ca. 1cm) incision through the skin at the tip of the scrotum between the two bulges of the testicles. Clear the subcutaneous connective tissue and gently squeeze a testicle out of the scrotum. Make a small 5mm incision into the tip of the testicle sac and pull out the cauda epididymis, the testis, the caput epididymis, the vas deferens and the spermatic blood vessels. Place a single ligature of 2-0 silk around the vas deferens and the spermatic blood vessels and severe these distal to the ligature. Remove the testis and the epididymis. Repeat the procedure on the remaining testis. Suture the scrotum with one or two silk sutures and clean the wound with betadine solution. Post-operative analgesia is as described for abdominal surgery above.

### 3.5.7 Transgenic Mice Procedures (Code: MSTP)

Vasectomy: The technique and precautions described above for abdominal surgery apply. Anaesthetise male mice, wipe the abdomen with 70% alcohol and betadine, and make a longitudinal incision (approx. 2 cm) in the skin and body wall at a point even with the top of the legs. Locate the testes and vas deferens and bring them out of the incision. Tie off the vas deferens with 2-0 silk sutures at least 4-5 mm apart and surgically remove the small length between the sutures. Replace the testes and suture the body wall. The skin is then closed with surgical staples which are removed in 10 days. Post-operative analgesia is as described for abdominal surgery above.

Reimplantation: The techniques and precautions above for abdominal surgery apply. Anaesthetise the recipient females, wipe their lower backs with 70% alcohol and betadine, and make a small longitudinal incision (approx. 1 cm) in the skin and body wall approximately even with the last rib. The ovary and oviduct are located, brought out of the incision, and held in place. Working under a dissecting microscope, inject the eggs into the oviduct through the infundibulum with a micropipette. Replace the ovary and oviduct and close the body wall with silk sutures. Close the skin with surgical staples and clean the site. Post-operative analgesia is as described for abdominal surgery above.

Tail removal: After new-born mice are weaned, 1/2 inch of the tail is clipped off each mouse and used to analyse their DNA. For this procedure, the mice are anaesthetised with an inhalant anaesthetic. Cauterisation is usually not necessary.

## 3.6 PHYSIOLOGICAL MEASUREMENTS

The mouse is placed in a metabolism chamber and various measurements are done using specialist analysers connected to the chamber.

### 3.6.1 Respirometry (Code: MPRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. For rodents, burrow tube type chambers seem best, as the animals settle down more quickly. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline). Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 3.6.2 Measurement of food consumption (MPF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves

measurement of food eaten, some animals, particularly fossorial animals are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 3.7 and quote reference number of relevant euthanasia procedure).

### 3.6.3 Measurement of total body water by tritium dilution (Code: MPW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April 1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste"*- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 3.5 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 3.3 and 3.4 and quote reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 3.6.4 Urine analysis (Code: MPU)

Elephant shrews are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 3.6.5 Non- shivering thermogenesis (Code: MPS)

Non-shivering thermogenesis (NST) or "chemical thermoregulation" is induced by norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the animal. Animals are first acclimated to a temperature at the lower end of their thermoneutral

zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 3.6.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 3.6.1) and injected with saline (for controls) or NE solutions (1.5mg/kg) subcutaneously (see Sections 2.3 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of animals should be avoided, since high $T_b$s above $40^o$ C may inhibit thermogenesis.

### 3.6.6 Activity measurements (Code: MPA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The mouse is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 3.6.7 X-Ray techniques (Code: MPX)

The digestive tract of mice can be studied by X-rays following introduction of a suitable contrast agent such as barium sulfate or gastrografin. The mouse is trained before the experiment to be used to the position required for the x-ray measurement. The animal is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the animal is let into the room and all metals are removed form the area. The animal is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The animal is allowed to ingest appropriate feed coated with barium sulfate. It is important that the animal does not move during the experiment. If it was not possible to train the animal to maintain the appropriate position, soft materials and sandbags are used to keep the animal in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 3.7 EUTHANASIA

In any type of euthanasia, care must be taken to assure death has occurred.

### 3.7.1 Overdose of Inhalant Anaesthetic (Code: MEOD)

Under a hood, place cotton or gauze soaked in anaesthetic in a bottom of a bell jar and cover with wire mesh or grate so the animal does not come into direct contact with anaesthetic; place the mouse into the jar and apply the cover. After the animal's respiration stops, check it for complete loss of heart beat. Please clean the bell jar after the procedure is completed.

### 3.7.2 Injectable Anaesthetic or Euthanasia Agent (Code: MEIN)

An overdose of an anaesthetic such as pentobarbital or a euthanasia agent such as T-61 may be given intravenously or directly into the heart.

### 3.7.3 Euthanasia in an Anaesthetised Animal by Physical Means (Code: MEPM)

Cervical dislocation may be accomplished by placing the investigator's fingers, a stout

scissors or an iron rod across the neck and rapidly pulling on the tail to break the neck. This procedure is best when followed by a thorocotomy/exsanguination Alternatively, the anaesthetised mouse is sacrificed by guillotine.

### 3.7.4 Brain Fixation by Intracardiac Perfusion (Code: MEBP)

After anaesthesia, the mouse is placed supine on a support table with a down-draft hood. Animals whose tissues are to be examined by ultrastructure are maintained on artificial respirators through an acutely placed tracheostomy, or an intratracheal cannula. With a midline incision and bilateral transection of the rib cage in the mid-axillary line, expose the heart. The sternum should be reflected dorsally and fractured above the manubrium. Reflect the left lobe of the lung forward to expose the descending aorta and clamp with a haemostat. Puncture the left ventricle with a 12-16 gauge needle to which is connected an airtight pressurised chamber containing the fixative (recently depolymerised paraformaldehyde in phosphate buffer, 4 g/100 ml). Inject the perfusate under a pressure of 120-140 mm Hg and puncture the right atrium to provide outflow. Artificial respiration is terminated. The perfusion will continue for 5-15 minutes, then excise the brain whole, dissect and process for cytological and cytochemical analysis.

### 3.7.5 Euthanasia in Which Drugs Cannot be Used (Code: MECO)

Euthanasia with carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket or plastic bag. Place the mouse in the container and close. If more than one animal is euthanised, make sure the animals are not overcrowded in the container. After the mouse no longer moves, check for loss of heart rate. The container should be clear so that the animal can be observed to assure that the animal is not distressed.

Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel on Euthanasia (see Appendix II). Explain your special need for these alternative methods under item 8.3 of the Application Form.

### 3.7.6 Euthanasia by Decapitation (Code: MEDE)

Decapitation should only be performed by trained personnel who have been certified by the veterinarian. Handle the animals carefully and gently, taking care not to frighten or antagonise them unnecessarily. The animals should be brought to the laboratory or a secluded, quiet work station. Since this is aesthetically displeasing, privacy should be guaranteed.

The decapitator should be of good quality and manufactured commercially. Animals waiting to be decapitated should be separated from the decapitator. Gently restrain the animal and accomplish the procedure as quickly as possible without placing the operator at risk.

The lever should be lifted all the way up. The hole in which to place the animal's head should be as large as possible. The animal is gently grasped around the abdomen with the right hand, the animal's head is placed through the hole and with one firm motion the left hand brings down the lever, severing the animal's head. The body is held firmly over a container or sink and the blood allowed to drain. The grasp is tightened around the animal to prevent movement. Blood should be cleaned up between each animal.

# 4. STANDARD PROTOCOLS FOR RATS

## 4.1 HANDLING (Code: RH)

One must be gentle but deliberate when picking up a rat; a hesitant hand is quickly sensed by these animals. Place the palm of your hand over the rats back and simultaneously grasp the upper thoracic and neck region between you thumb and index finger, with your thumb under the animal's chin. Gentle pressure with the thumb can be used to resist any efforts of the rat to bite. Use your opposite hand to support the animal or grasp him near the base of the tail. A particularly belligerent rat may be picked up by the base of the tail and placed on a rough surface so he pulls forward, then grasp as described above.

## 4.2 ANAESTHESIA

Anaesthesia, including tranquilisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly used agents and doses in provided in Appendix I. The veterinarian(s) will assist in selections that are adequate and yet convenient for the investigator. Analgesia requiring controlled substances will be the responsibility of the veterinarian. In completing the Application Form, simply indicate the method and code (inhalation/injection) to be used and list the specific agents in your response to item 8.2. Remember that ether is both flammable and explosive. It may not be used unless an explosion-proof hood or room is available and must be disposed of in explosion-proof containers. Proper ventilation is needed with all inhaled anaesthetics for user safety.

### 4.2.1 Inhalation anaesthesia (Code: RAIH)

A vaporising anaesthetic machine, calibrated for isoflurane, is used with pure medical oxygen to supply a gas mixture of 0-5% isoflurane in oxygen, as required. During initiation of anaesthesia, place the animal in a clear Perspex respirometer and vent an initiation gas mixture of 2-4% isoflurane, depending on the species, into the respirometer at flow rates of 1-2 litres/min, depending upon the size of the animal. Place a cloth over the respirometer to quieten the animal, but remove it periodically to monitor the progress of anaesthesia. Once anaesthetised (usually after ca. five minutes), remove the animal quickly from the respirometer and place it on the preparation table. Place a gas mask appropriate for the shape of the animal over the facial region covering all respiratory surfaces. Lower the maintenance gas mixture to 1-2% isoflurane in oxygen, depending upon the species. Once pre-operative preparation of the animal is complete, move it to the surgery table. Generally the gas mixture can be lowered on commencement of suturing in order to minimise the total time under anaesthesia, and hence hypothermia. At all times during anaesthesia, monitor the rectal or cloacal temperature with a digital thermometer and rectal or cloacal probe. If the body temperature decreases significantly during lengthy procedures, the animal must be heated with a heating pad or infra-red lamp until normothermia is restored.

### 4.2.2 Injection Anaesthesia (Code: RAIN)

Injection anaesthesia, using barbiturates or other agents such as ketamine, can also be used (see Appendix I). The route of administration and frequency of additional doses will be determined by the length of the procedure. In procedures requiring assessment of vascular physiologic responses, the choice of anaesthetic must be carefully considered to avoid unwanted vascular effects. Injection techniques (IV, IM, IP, etc.) are described in a separate section.

## 4.3 SAMPLING OF BODY FLUIDS

The skin or other sites of sampling should be properly cleaned with alcohol to assure maximum visibility and cleanliness.

### 4.3.1 Bleeding

The maximum amount of blood that should be withdrawn to insure survival is 1-2 ml/100 gm body weight once a week. Prolonged bleeding may result in anemia and abnormalities in serum proteins.

#### 4.3.1.1 Bleeding from Peripheral Vessels (Code: RBPV)

Orbital sinus: Hold the anaesthetised rat with its head pressed firmly against a work surface; press down with your thumb just behind its eye and pull back to open the internal angle of the lid. Using a microhaemocrit tube or Pasteur pipette, gently push in at the internal angle of the eye through the conjunctiva of the medial canthus until you reach the bone in the sphenoid region, then slide down into the orbital sinus beneath. Rotate the tube until the blood is collected; remove the tube and release pressure. Clean blood from the area.

Tail clipping: Using a scalpel blade, transect tail completely about 5 mm from the tip; blood flow can be increased by placing tail in warm water for 1-2 minutes beforehand. Finger pressure stops bleeding.

Toe clipping: Similar to tail clipping. Can use each toe once.

Vacuum assisted tail bleeding: Prepare a Liebig condenser jacket with a test tube attached. Connect the side arm of the jacket to the vacuum line in the restrainer with the rat's tail on the outside. Apply petrolatum to the incision area, 1 cm from the tail tip, then incise the lateral vein longitudinally for 1 cm. Slip the vacuum jacket over the rat's tail to fit snugly against its rump (tail tip will be in test tube). Start the suction and control its intensity as well as the rate of bleeding by intermittent finger occlusion of the connector.

#### 4.3.1.2 Bleeding by Cardiac Puncture (Code: RBCP)

Anaesthetise the rat and place it on its back with its body length perpendicular to you. With your left thumb and forefinger placed at each side of the thorax , compress slightly. Clean the injection site with alcohol. Insert a 21 gauge needle and syringe under the xyphonid cartilage with the needle at a 30 degree angle to the skin, then push forward and aspirate. Your left fingers may hold the barrel as the right hand pulls the plunger. Feeling a heartbeat against the needle ensures proper location. If you neither feel a beat nor see blood, withdraw the needle slightly while still aspirating, withdraw the plunger slowly and steadily. Although blood may be drawn once per week, not all the blood constituents may have returned to normal values. A maximum of 1-2 ml/100 gm may be withdrawn if the animal is to survive. Although this is a survival technique, it should be reserved for terminal procedures because of the risk involved.

#### 4.3.1.3 Bleeding by Terminal Procedures (Code RBTB)

Posterior vena cava: Anaesthetise the rat and place on its back. Make a V-shaped incision through the skin and abdominal wall at the base of the abdomen and proceed diagonally across each side ending dorsolaterally at the thorax. Lay the skin over the thorax and deflect the gut to the rat's left, then push the liver forward and enter the vena cava at the level of the kidneys. Straddle the vessel with the fingers of your free hand and insert a 19 to 21 gauge needle. Withdraw the plunger slowly until the vein collapses; wait for it to refill, then continue. Assure euthanasia is complete.

Dorsal aorta: Perform similarly to the vena cava technique, but enter the aorta just anterior to the distal bifurcation. Assure that euthanasia is complete.

Axillary vessels: Place the anaesthetised rat on its back with the tail toward you. Clean with

70% alcohol and shave operative site. Stretch one forelimb and hold it in place with a pin through the foot. Make a skin incision in the axillary region; bottom skin edge can be held up and used as a bowl to collect blood. Cut the axillary vessels with scissors; as the blood wells up, collect it with a Pasteur pipette or syringe. Note: This blood will be contaminated with tissue fluids. Assure that euthanasia is complete.

### 4.3.2 Ascites Fluid Collection and Production (Code: RAP)

Inject each animal IP with 0.5 ml of pristane using a 27 gauge needle. After 5-10 days, inject each rat IP with hybridoma or tumor cells in saline (0.1-0.5 cc total volume) using a 27 gauge needle. Ascites or tumors should appear after 7-28 days. Tumors or cell lines can be contaminated with passenger viruses of murine origin which can affect the health of your animals. If you think this is a possibility, please consult the veterinary staff to work out a plan to test your biologics.

The drain ascites, immobilise the rat by holding its neck, back and tail. Prepare the skin with alcohol, then puncture the peritoneal cavity in the lower left quadrant with an 18 gauge needle and collect the fluid into a sterile tube containing heparin. Repeat this draining process every 2 days until the yield of ascites falls off. DO not continue if the animal appears sickly (hunched position, ruffled hair) or the volume is depleted. The animals must be observed daily when they have started to produce ascites to assure they are not distressed by large amounts of ascites fluid.

### 4.3.3 Bladder Catheterisation (Code: RBC)

Anaesthesia is administered appropriate to the duration of the planned urine collection. The urethral orifice and surrounding area are washed with an antiseptic solution to reduce the chances of introducing infection to the bladder. Gently insert a small P-50 catheter moistened with saline into the bladder through the urethral orifice. Long-term or repeated catheterization may promote bladder infection (which is screened by routing urinalysis) and may require antibiotic therapy.

### 4.3.4 Collection of Peritoneal Cells

#### 4.3.4.1 Macrophage Collection (Code: RBMC)

To stimulate yields of activated macrophages, animals are injected with thioglycolate solution or sterile mineral oil (dose 1 ml) IP. The macrophages are collected at the time of euthanasia (3-4 days later).

#### 4.3.4.2 Polymorphonuclear Leukocyte Collection (Code: RPLC)

To stimulate exudation of polymorphonuclear leukocytes into the abdominal cavity, inject a 0.1% glycogen solution IP and collect cells within 4 hours at the time of euthanasia.

## 4.4 INJECTIONS

The skin is cleaned with alcohol to assure maximum visibility and cleanliness.

In addition tot he routes listed below, injections can be made directly into major vessels by needle or catheter placement. The carotid artery, jugular vein or the femoral vessels can be used. These techniques are considered surgical procedures and are outlined in the surgical section.

Phosphate buffered saline (PBS) or other isotonic solutions are better than distilled water as a solvent/vehicle for injections. Distilled water causes some haemolysis IV and pain SQ.

Oils are suitable for administration of lipid-soluble substances or in adjuvants, but absorption is delayed and those vehicles cannot be injected IV.

In general, the maximum quantity for injection is 1.0 ml/100 g body mass but the dose really depends on the route of administration. The pH should be 4.0-8.0 (widest range IV, then IM, then SQ).

IM: Maximum 0.25 ml at any site in adult
SQ: Maximum 0.25 ml at any site in adult
IV: More than 1 ml/100 g will cause pulmonary oedema

### 4.4.1 Intravenous (IV) (Code: RIIV)

Insertion of the needle (25 to 30 gauge) should start distally and work proximally in relation to the heart (because to direction of blood flow) for subsequent injections. After removing the needle, blood flow stops with pressure.

Caudal (tail) vein: Vessels are lateral from tail base to tip. Place rat in restrainer and dilate vessels by heat lamp or warm water (1-2 minutes in water bearable to the hand). Using a 25 gauge needle, enter vein at a shallow angle. If needle is in the vein, fluid is injected easily without bleb. Start distally; then, if first attempt fails, you can progress to more anterior sites.

Lateral marginal vein: This procedure requires two people. Remove hair from the animal's inner thigh by wet-shave as assistant holds the rat with the limb extended. Put sufficient pressure on groin to cause dilation of vein. Hold the leg, swab with alcohol, and enter the vein at a shallow angle with a 25 gauge needle. After you have completed the injection, the assistant releases pressure on groin. Withdraw the needle and apply pressure to stop bleeding.

Dorsal metatarsal vein: This procedure requires two people. The assistant holds the rat and extends its leg at the ankle. The dorsal aspect of its foot is wet-shaved and wiped with alcohol. The assistant dilates the vessel by pressure at the ankle and the investigator flicks the vein with fingers to further dilate. With the animal's toes curved over the operator's finger and its skin stretched tightly, the operator inserts a 25 gauge needle at a shallow angle into the vein just where it starts to travel up the animals foot after crossing from its toes.

Sublingual veins: The veins are located on the ventrolateral aspect of the tongue. Place anaesthetised rat on its back with its head toward you. Pull the animal's tongue out and lay over your finger with a slight convex curvature. Dry the tongue and insert the needle (23 gauge for weanlings and 30 gauge for smaller rats). After injection, remove the needle and apply pressure until bleeding stops.

Penile vein: Anaesthetise the rat or, with the aid of an assistant, hold a conscious rat with the back legs and tail out of the way, resting hands to movement is prohibited. The glans penis is extruded and held t tip. Enter the vein using 25 to 30 gauge needle.

### 4.4.2 Subcutaneous (SQ) (Code: RISO)

This injection site is under the skin of the animal's back and sides. Clean the skin and pass the needle through the skin in an anterior direction, at a shallow angle to the surface. Move tip of needle up and down when in position to assure that it is SQ; if not discernable, withdraw the needle slightly until SQ. A successful SQ injection of 0.25 ml will result in a bleb.

### 4.4.3 Intradermal (ID) (Code: RIID)

The usual sites are skin over the rat's back and abdomen. Remove hair with clipper and/or depilatory and clean skin. A needle with a short bevel usually used. Injection of small amounts (50-100 Tl) will raise a bleb.

### 4.4.5 Intramuscular (IM) (Code: RIIM)

The usual skin injection site is muscle of the rear limb. Clean the skin and insert the needle in a posterior direction away from the femur and sciatic nerve. Entering to deeply may cause you to encounter bone or miss the muscle mass and fall into the fascial plane. Aspirate before injection to assure the needle tip is not intravascular.

### 4.4.6 Intraperitoneal (IP) (Code: RIIP)

The injection site is the lower left quadrant of the abdomen away from the midline. Clean the skin and insert the needle tip through the abdominal muscle. Inject immediately to push away the viscera and withdraw the needle. A large-gauge needle is less likely to penetrate the viscera.

### 4.4.7 Intragastric (oral) (Code: RIIG)

Use a 15 to 16 gauge blunt-ended needle cannula about 11 cm in long (preferably bulbed). Measure the like distance to the stomach on the outside of the rat and note the amount of the cannula that would still protrude from the mouth. Hold the rat firmly by the skin of the neck and back so the head is immobile and in line with the back. Pass a needle into the mouth as far to the side as possible. After locating the oesophagus, push the needle gently into the stomach. You may feel an obstruction at the back of the mouth and at the sphincter to the stomach; move the syringe back and forth gently to overcome this. Slow the initial discharge to check if the cannula is correctly placed; if placed properly, inject rapidly. The rat may squeak while being injected and may make swallowing movements. Be careful to avoid injections into the trachea, a bulb ended cannula will usually prevent this.

Intragastric injections may also use intubation. A mouth gag with a central hole (5 mm) prevents biting through the catheter plastic or rubber). Place the gag behind the incisors. Measure the catheter on the outside and pass through the hole of the gag and into the stomach; a conscious rat will swallow and help with the passage. The usual maximum dosage is 3 ml/100 gm body weight. The placement can be checked by stethoscope and injection of air. Clear the catheter with a small amount of air before withdrawal to reduce tracheal contamination.

## 4.5 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. Procedures requiring prolonged anaesthesia (more than 3 hours) will require special approval and should be explained in item 8.3 of the Application Form. Surgical procedures require aseptic technique but not operating rooms, and should be carried out in approved surroundings (please check with the Animal Ethics sub-committee). Autopsy with sampling of organs after euthanasia is not considered a surgical technique. All survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 8.2 of the Application Form.

Body heat is rapidly lost during surgical procedures; a heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 4.5.1 Anterior Neck (Code: RSAN)

Anaesthesia is induced appropriate to the duration of the planned resource. Injection anaesthesia is preferable since inhalation anaesthesia is difficult to maintain while working on the neck or trachea. The rat is restrained, exposing the throat. The neck is shaved and the skin washed with 70% ethyl alcohol and betadine solution. Additional local anaesthetic can be given if needed. A midline incision is made and blunt dissection is used to move the incision down to the site of interest.

The carotid vessels, jugular veins, and vagus nerve can be located adjacent to the tracheal area. These vessels are located by palpitation of the carotid pulse and by feeling the tracheal rings. The vessels are isolated by blunt dissection and lengths of suture are placed around them for control of haemorrhage during injection or catheter placement. Needles or catheters (P-10 or P-50 tubing) are inserted under direct vision. The direction of placement depends on the purpose. Removal of needles or catheters is followed by securing haemostasis.

The trachea is located by palpitation of the tracheal rings. Direct intratracheal injection is accomplished in a similar manner except that a larger diameter tube is inserted. After the catheter or larger tube is removed, reconstruction of the tracheal structure is accomplished by using one or two 5-0 silk or absorbable sutures to reappose the divided tracheal rings.

The thyroid is approached in the same manner as the trachea. The two lobes of the thyroid are adjacent to the larynx. For thyroidectomy, the lobes are dissected bluntly and removed with hemostatic control.

After completion of the desired procedure(s), the incision is closed with interrupted 2-0 silk or absorbable sutures. The skin is cleaned and the rat is returned to its cage for recovery from anaesthesia.

Post-operative analgesia is not normally required, particularly if methoxyflurane and/or long-acting local anaesthetic is used. The animal should be watched carefully to insure that it is up and moving around in the cage. The veterinarian should determine the need and type of any additional post-operative analgesia.

### 4.5.2 Cannulation of Femoral Vessels (Code: RSFV)

Anaesthetise the rat and restrain in a supine position. Shave the femoral area and wash the area of incision with 70% ethyl alcohol and betadine solution. Infiltrate the area with 1-2% procaine, xylocaine or long-acting local anaesthetic if desired. Make a small 1 cm incision and isolate the femoral vessels by blunt dissection. The vessels should be surrounded with 3-0 silk or absorbable sutures to control hemostasis and secure catheters or needles. Individual blood samples or simple injections may be accomplished with straight or hooked needles. For serial sampling, injection, or pressure monitoring, thread-bevelled P-10 or P-50 catheters into the femoral artery and/or vein in a retrograde fashion to the desired level in the aorta or vena cava, respectively. Remove catheters at the conclusion of the experiment. After hemostasis is established, close the skin with 3-0 silk or absorbable sutures and clean the site. The rat is allowed to recover from anaesthesia under frequent observation.

### 4.5.3 Abdominal Surgery (Code: RSA)

The technique for abdominal surgery can be applied to the biopsy and/or removal of various organs as well as for other manipulations in the abdominal cavity, including vascular injection and body fluid sampling. Anaesthesia appropriate for the surgical approach and duration of the procedure is administered. Consult Appendix I and the veterinarian.

After anaesthesia, the rat is restrained on an operating surface in an optimal position for the planned incision. For prolonged procedures, heated operating surfaces are used to

maintain body heat. Body temperature is monitored as needed.

Midline incision or flank approaches are most common for abdominal surgery. The area of the incision is shaved and the skin surface rendered antiseptic with 70% alcohol and betadine solution. If additional anaesthesia is desired, intracutaneous administration of a local anaesthetic such as 1-2% procaine or lidocaine at the site of the skin incision is administered via a 25 gauge needle. A long-acting anaesthetic may obviate the need for post-operative analgesia. The volume of local anaesthetic is to be kept below 1.5 ml. The area of incision is then isolated in a clean, but not necessarily sterile manner, and the surgery proceeds using a clean technique.

A scalpel is used to make an incision of the length and position desired for the procedure. Midline incisions have less bleeding and less need for subsequent hemostasis than incisions through muscle layers such as in the flank. The abdominal incision is divided into two layers (1. skin; 2. muscle/peritoneum) to avoid unintentional laceration of the abdominal contents. When entering the abdominal cavity, the second layer is tented and a very small initial incision is made to allow the abdominal contents to fall away from the site before the incision is extended. Haemostasis is established with small clamps or sutures as needed. The abdominal contents are moved aside using saline-dampened gauze sponges and appropriate retractors to isolate and expose the organ, vessel or duct of interest.

If organ biopsy procedures are carried out, the organ of interest is exposed and tissue is obtained using an appropriate biopsy needle or a wedge-shaped incision. Bleeding is controlled by closing the incision with 5-0 silk or absorbable sutures. Gelfoam may also be used to advantage if bleeding is difficult to control in a friable organ such as the liver.

If organ removal is desired, the ducts and vessels are isolated and ligated with suture material. Either absorbable or nonabsorbable suture may be used as appropriate for the procedure and subsequent course of experiment. Silk suture of 3-0 to 5-0 is usually satisfactory and may be left in the abdominal cavity without subsequent infection. The organ, once isolated, is then removed by transection of the ligated vessels and ducts and any remaining connective tissue.

If vascular or duct perfusion procedures are being done, the vessel or duct is isolated and a needle or catheter appropriate for the procedure is inserted. After infusion or collection, the needle or catheter is removed and hemostasis reestablished. In the case of vessels, this is accomplished with gentle pressure and/or the application of Gelfoam. The latter can be left in place and will be reabsorbed by the animal.

If a chronic in-dwelling catheter has been placed, it is tunnelled through the abdominal wall and under the skin to an appropriate external site. This is usually over the scapula where it is secured in a way that the animal cannot damage it.

A variety of connections can be used, as appropriate, for the subsequent experimentation. Chronic in-dwelling bladder catheters are usually placed in such a way that they exit to the lower abdominal wall.

After completion of the procedure, care is taken to assure that hemostasis is satisfactory and all gauze sponges and other restraining devices are removed. The abdominal incisions(s) is closed in two layers. The muscle and peritoneal layer is closed with a non-locking running stitch of absorbable or nonabsorbable suture, usually of 2-0 silk. Care is taken to avoid inclusion of abdominal contents in the suture line. A running stich is used to provide a continuous closure to avoid subsequent herniation of abdominal contents through the suture line. The skin layer is closed with a line of running or interrupted sutures, or it may be closed with skin staples which are removed 10-14 days later. Care is taken to include the deep

fascial layers of skin in the superficial suture line. The skin surface is the cleaned, restraints are removed, and the animal is allowed to recover from anaesthesia under frequent observation.

Post-operative analgesia is not normally required, particularly if methoxyflurane and/or long-acting local anaesthetic has been injected into the site of the skin incision. The animal should be watched carefully to insure it is up and moving in the cage. Use of post-operative analgesia should be decided upon in consultation with the veterinarian.

### 4.5.4 Skin Grafting (Code: RSSG)

With the animals anaesthetised, use of biopsy punch or scissors to remove approximately 1 cm of skin from the truck of each donor and recipient rat. The donor's skin sample is placed on the recipient at the site of the skin biopsy and sutured in place with absorbable sutures in a simple uninterrupted pattern. Cover the graft with plaster bandage. Recipients are observed daily for signs of rejection. There is no pain.

### 4.5.5 Castration (Code: RSCR)

Anaesthetise male rat and prepare the scrotum well with 70% alcohol and betadine solution. Make a small (ca. 1cm) incision through the skin at the tip of the scrotum between the two bulges of the testicles. Clear the subcutaneous connective tissue and gently squeeze a testicle out of the scrotum. Make a small 5mm incision into the tip of the testicle sac and pull out the cauda epididymis, the testis, the caput epididymis, the vas deferens and the spermatic blood vessels. Place a single ligature of 2-0 silk around the vas deferens and the spermatic blood vessels and severe these distal to the ligature. Remove the testis and the epididymis. Repeat the procedure on the remaining testis. Suture the scrotum with one or two silk sutures and clean the wound with betadine solution. Post-operative analgesia is as described for abdominal surgery above.

## 4.6 PHYSIOLOGICAL MEASUREMENTS

The mouse is placed in a metabolism chamber and various measurements are done using specialist analysers connected to the chamber.

### 4.6.1 Respirometry (Code: RPRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. For rodents, burrow tube type chambers seem best, as the animals settle down more quickly. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline).

Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 4.6.2 Measurement of food consumption (RMF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some animals, particularly fossorial animals are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 4.7 and quote reference number of relevant euthanasia procedure).

### 4.6.3 Measurement of total body water by tritium dilution (Code: RMW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April 1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste"*- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 4.4 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 4.2 and 4.3 and quote reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 4.6.4 Urine analysis (Code: RMU)

Elephant shrews are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 4.6.5 Non- shivering thermogenesis (Code: RMS)

Non-shivering thermogenesis (NST) or "chemical thermoregulation" is induced by norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the animal. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 4.6.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 4.6.1) and injected with saline (for controls) or NE solutions (1.5 mg/kg) subcutaneously (see Sections 4.4 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of animals should be avoided, since high $T_b$s above $40^o$ C may inhibit thermogenesis.

### 4.6.6 Activity measurements (Code: RMA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The mouse is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 4.6.7 X-Ray techniques (Code: RMX)

The digestive tract of mice can be studied by X-rays following introduction of a suitable contrast agent such as barium sulfate or gastrografin. The mouse is trained before the experiment to be used to the position required for the x-ray measurement. The animal is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the animal is let into the room and all metals are removed form the area. The animal is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The animal is allowed to ingest appropriate feed coated with barium sulfate. It is important that the animal does not move during the experiment. If it was not possible to train the animal to maintain the appropriate position, soft materials and sandbags are used to keep the animal in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 4.7 EUTHANASIA

In any type of euthanasia care must be taken to assure death has occurred.

### 4.7.1 Overdose of Inhalant Anaesthetic (Code: REOD)

Under a hood, place cotton gauze soaked in anaesthetic in the bottom of a bell jar and cover with wire mesh or grate so the animal does not come into direct contact with the anaesthetic' place the rat into the jar and apply the cover. After the animal's respiration stops, check it for complete loss of heartbeat. Thoracotomy and exsanguination assure death.

### 4.7.2 Injectable Anaesthetic or Euthanasia Agent (Code: REIN)

An overdose of an injectable anaesthetic such as pentobarbital or an euthanasia agent such as T-61 may be given intravenously or directly into the heart.

### 4.7.3 Euthanasia in an Anaesthetised Animal by Physical Means (Code: REPM)

Cervical dislocation may be accomplished by placing the investigators fingers, a stout scissors or an iron rod across the neck and pulling on the tail to break the neck. This procedure is best when followed with a thoracotomy/exsangunation. This procedure is only acceptable in rats weighing less than 200 grams. Alternately, the anaesthetised rat is sacrificed in a guillotine. If anaesthesia is not used, the technician must be experienced and proficient as determined by veterinary staff.

### 4.7.4 Euthanasia in Which Drugs Cannot be Used (Code: RECO)

Euthanasia with carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket or plastic bag. Place the rats in the container and close. After rats no longer move, check for loss of heartbeat. Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel of Euthanasia (see Appendix II). Explain your special need for these alternative methods under item 8.3 of the Standard Animal Protocol Review form.

### 4.7.5 Brain Fixation by Intracardiac Perfusion (Code: REBP)

After anaesthesia, the rat is placed supine on a support table with a down-draft hood. Animals whose tissues are to be examined by ultrastructure are maintained on artificial respirators through an acutely placed tracheostomy or an intratracheal cannula. With a midline incision and bilateral transection of the rib cage in the mid-axillary line, expose the heart. The sternum should be reflected dorsally and fractured above the manubrium. Reflect the left lobe of the lung forward to expose the descending aorta and clamp with a hemostat. Puncture the left ventricle with a 12-16 gauge needle to which is connected an airtight pressurized chamber containing the fixative (recently depolymerised paraformaldehyde in phosphate buffer, 4 g/100 ml). Inject the perfusate under a pressure of 120-140 mm Hg and puncture the right atrium to provide outflow. Artificial respiration is terminated. The perfusion will continue for 5-5 minutes, then excise the brain whole, dissect and process for cytological and cytochemical analysis.

### 4.7.6 Euthanasia by Decapitation (Code: REDE)

Decapitation should only be performed by trained personnel who have been certified by the veterinarian. Handle the animals carefully and gently taking care not to frighten or antagonize then unnecessarily. The animals should be brought to the laboratory or a secluded, quiet work station. Since this is aesthetically displeasing, privacy should be guaranteed.

The decapitator must be of good quality and be manufactured commercially. Animals waiting

to be decapitated should be separated from the decapitator. Gently restrain the animals and accomplish the procedure as quickly as possible without placing the operator at risk. The lever should be lifted all the way up. The hole in which to place the animal's head should be as large as possible. The animal is gently grasped around the abdomen with the right hand, the animal's head is placed through the hole, and with one firm motion, the left hand brings down the lever severing the animals head. The body is held firmly over a container or sink and the blood is allowed to drain. The grasp is tightened around the animal to prevent movement. Blood should be cleaned up between each animal.

# 5. STANDARD PROTOCOLS FOR HAMSTERS

## 5.1 HANDLING (Code: HH)

Startled or suddenly awakened hamsters often bite. Hamsters may be picked up by placing both hands, palms up, under the animal and scooping it. Alternately, the animal can be picked up and restrained by the loose skin at the neck or over the back. A very firm grip is needed to prevent the animal from twisting around and biting the hand.

## 5.2 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly used agents and dosages is provided in Appendix I. The veterinarian (s) will assist in selections that are adequate and yet convenient for the investigator. Analgesia requiring controlled substances will be the responsibility of the veterinarian. In completing the Application Form, indicate the method and code (inhalation/injection) to be used and list the specific agents in your response to item 8.2. Remember that ether is both flammable and explosive. It may not be used unless a explosion-proof hood or room is available and must be disposed of in explosion-proof containers. Proper ventilation is needed with all inhaled anaesthetics for user safety.

### 5.2.1 Inhalation Anaesthesia (Code: HAIH)

A vaporising anaesthetic machine, calibrated for isoflurane, is used with pure medical oxygen to supply a gas mixture of 0-5% isoflurane in oxygen, as required. During initiation of anaesthesia, place the animal in a clear Perspex respirometer and vent an initiation gas mixture of 2-4% isoflurane, depending on the species, into the respirometer at flow rates of 1-2 litres/min, depending upon the size of the animal. Place a cloth over the respirometer to quieten the animal, but remove it periodically to monitor the progress of anaesthesia. Once anaesthetised (usually after ca. five minutes), remove the animal quickly from the respirometer and place it on the preparation table. Place a gas mask appropriate for the shape of the animal over the facial region covering all respiratory surfaces. Lower the maintenance gas mixture to 1-2% isoflurane in oxygen, depending upon the species. Once pre-operative preparation of the animal is complete, move it to the surgery table. Generally the gas mixture can be lowered on commencement of suturing in order to minimise the total time under anaesthesia, and hence hypothermia. At all times during anaesthesia, monitor the rectal or cloacal temperature with a digital thermometer and rectal or cloacal probe. If the body temperature decreases significantly during lengthy procedures, the animal must be heated with a heating pad or infra-red lamp until normothermia is restored.

### 5.2.2 Injection Anaesthesia (Code: HAIN)

Injection anaesthesia, using barbiturates or other agents such as ketamine, can also be used (see Appendix I). The route of administration and frequency of additional doses will be determined by the length of the procedure. In procedures requiring assessment of vascular physiologic responses, the choice of anaesthetic must be carefully considered to avoid unwanted vascular effects. Injection techniques (IV, IM, IP, etc.) are described in a separate section.

## 5.3 SAMPLING OF BODY FLUIDS

The skin, or other sites of the sampling, should be properly prepped with alcohol to assure visibility and cleanliness.

### 5.3.1 Bleeding

The maximum amount of blood to be withdrawn to insure survival is 1 ml/ 100 gm body weight once a week. If bleeding extends over 2-3 weeks, anaemia and abnormalities in serum protein may result.

#### 5.3.1.1 Bleeding from Peripheral vessels (Code: HHPV)

Orbital sinus: Hold the anaesthetised animal's head firmly against a work surface and press down. Use your thumb to apply pressure to the external jugular vein. Pull the skin back with your forefinger to open the internal angle of the lid. Gently push in the microhematocrit or Pasteur pipette through the conjunctiva of the medial canthus alongside the orbit. When you reach the sphenoid region, slide the tube down to the depression, then rotate the tube until blood is collected. Remove the tube, release pressure and clean blood from the area.

Toe clipping: Blood can be drawn once from each toe. The tip of the toe is transected with a scalpel blade. Blood flow can be increased by placing the tail in warm water for 1-2 minutes before transection. Finger pressure stops the bleeding.

#### 5.3.1.2 Bleeding by Cardiac Puncture (Code: HBCP)

Anaesthetise the animal and place on its back with its length perpendicular to you. With your left thumb and forefinger placed on each side of its thorax, compress slightly and insert a 21 gauge needle and syringe under the xyphoid cartilage. Hold the needle at a 30 degree angle and push forward slowly while aspirating; your left fingers may hold the barrel as your right hand pulls the plunger slowly and steadily. To be sure of its correct placement, feel for the heartbeat against the needle. If no blood flows, withdraw the needle slowly with continued aspiration. This should be reserved as a terminal procedure.

**Bleeding by Surgical Approaches (See surgical section)**

Blood samples can also be obtained by direct puncture or catheter placement in major vessels such as the carotid artery, jugular vein and femoral artery. These techniques are considered survival surgery and are addressed in the surgical section.

#### 5.3.1.3 Bleeding by Terminal Procedures (Code: HBTP)

Posterior vena cava: Anaesthetise the hamster and place it on its back. Make a V-shaped incision through the skin and abdominal wall at the base of the abdomen and proceed diagonally across each side ending dorsolaterally at the thorax. Lay the skin over the thorax and deflect the gut to the hamster's left, the push the liver forward and enter the vena cava at the level of the kidneys. Straddle the vessel with the fingers of your free hand and insert a 23 gauge needle. Withdraw the plunger slowly until the vein collapses; wait for it to refill, then continue. Turning the needle's bevel away from the wall and tenting the vessel will increase the flow. When completed, proceed with euthanasia.

Dorsal aorta: Perform similarly to the vena cava technique, but enter the aorta just anterior to the distal bifurcation. When completed, proceed with euthanasia.

Axillary vessels: Place the anaesthetised hamster on its back with the tail towards you. Stretch one forelimb and hold it in place with a pin through the foot. Make a skin incision in the axillary region; the bottom skin edge can be held up and used as a bowl to collect blood. Cut the axillary vessel with scissors; as the blood wells up, collect it with a Pasteur pipette or syringe. Note: This blood will be contaminated with tissue fluids. When completed, proceed with euthanasia.

### 5.3.2 Ascites Fluid Collection and Production (Code: HAP)

Inject each animal IP with 0.5 ml of pristane using a 27 gauge needle. After 5-10 days, inject each hamster IP with hybridoma or tumour cells in saline (0.1-0.5 ml total volume) using a 27 gauge needle. Ascites or tumours should appear after 7-28 days.

To drain ascites, immobilise the hamster by holding its neck, back and tail. Prepare the skin with alcohol and gauze sponge, then puncture the peritoneal cavity in the lower left quadrant with an 18 gauge needle and collect the fluid into a sterile tube containing heparin. Repeat this draining process every 2 days until the yield of ascites fluid falls off. Do not continue if the animal appears sickly (hunched position, ruffled hair) or the volume is depleted. The animal should be observed daily to ensure it is not distressed from the increased fluid.

### 5.3.3 Collection of Peritoneal Cells

#### 5.3.3.1 Macrophage collection (Code: HMC)

To stimulate yield of activated macrophages, animals are injected with thioglycolate solution or sterile mineral oil (dose 1 ml) IP. The macrophages are collected at the time of euthanasia (3-4 days later).

#### 5.3.3.2 Polymorphonuclear Leukocyte Collection (Code HPLC)

To stimulate exudation of polymorphonuclear leukocytes into the abdominal cavity, inject a 0.1 % glycogen solution IP and collect cells within 4 hours at the time of euthanasia.

## 5.4 INJECTIONS

The skin is prepped to ensure maximum visibility and cleanliness. Injections can be made directly into the major vessels by needle or catheter placement. The carotid artery, jugular vein or the femoral vessels can be used. These techniques are considered surgical procedures and are addressed in the surgery section.

Phosphate buffered saline (PBS) or other isotonic solutions are better than distilled water as a solvent/vehicle for injections. Distilled water causes some hemolysis when given IV and pain when given SQ. Oils are suitable for administration of lipid-soluble substances or adjuvants, but absorption is delayed and this vehicle cannot be injected IV.

In general, the maximum quantity for an IV injection is 0.1 ml/100 gm body weight, but the dose depends on the route of administration. The pH should be physiologic.

IM: Maximum 0.1 ml at any site in adult
SQ: Maximum 0.25 ml at any site in adult
IV: More than 1 ml/100 gm will cause pulmonary oedema.

### 5.4.1 Intravenous Injections (Code: HIIV)

The femoral, saphenous or jugular vein can be used for intravenous injections. The animals are restrained usually with inhalation anaesthesia (methoxyflurane). When the vessel is located, the area is cleaned with alcohol and injection is made with a 25-27 gauge needle. The total volume in an adult hamster should not exceed 1 ml/100 gm or pulmonary oedema may result.

### 5.4.2 Subcutaneous Administration (Code: HISQ)

Subcutaneous administration is best accomplished using the loose skin at the nape of the neck. The area is tented and held firm with one hand and a 23-25 gauge needle is inserted into the subcutaneous space. The total volume should not exceed 1 ml.

### 5.4.3 Intramuscular Injection (Code: HIIM)

The best site for intramuscular injections is the caudal thigh muscle. The animal should be rolled to either side. A 25 gauge needle is used to enter the muscle. Pull back on the plunger to create negative pressure in order to assure that you are not in a blood vessel. The total volume should not exceed 1 ml or the muscle fibres can tear from the excessive pressure.

### 5.4.4 Intraperitoneal (Code: HIIP)

Immobilise the animal by holding the skin of its neck and back. After cleaning the skin, insert the tip of the needle into the lower quadrant (s) of the abdomen, away from the midline. Inject immediately to push away the viscera and withdraw the needle. A large gauge needle is less likely to penetrate the viscera.

## 5.5 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. Procedures requiring prolonged anaesthesia (more than 2 hours) will require special approval and should be explained in item 5. Surgical procedures requires aseptic technique but not an operating room. Techniques should be carried out in approved surroundings (please check with the Animal Ethics Sub-committee). Autopsy with sampling of organs after euthanasia is not considered a survival surgical technique. All survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 8.3.

Body heat is rapidly lost during surgical procedures and a heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 5.5.1 Anterior Neck (Code: HSAN)

Anaesthesia is induced appropriate to the duration of the planned procedure. Injection anaesthesia is preferable since inhalation anaesthesia is difficult to maintain while working on the neck or trachea. The hamster is restrained, exposing the throat. The neck is shaved and the skin washed with 70% ethyl alcohol and betadine solution. Additional local anaesthetic can be given if needed. A midline incision is made and blunt dissection is used to move the incision down to the site of interest.

The carotid vessels, jugular veins, and vagus nerves can be located adjacent to the tracheal area. These vessels are located by palpation of the carotid pulse and by feeling the tracheal rings. The vessels are isolated by blunt dissection and lengths of suture are placed around them for control of haemorrhage during the injection or catheter placement. Needles or catheters (P-10 or P-50 tubing) are inserted under direct vision. The direction of placement depends on the purpose. Removal of needles or catheters is followed by securing haemostasis.

The trachea is located by palpation of the tracheal rings. Direct intratracheal injection is accomplished by inserting a 25 gauge needle between two tracheal rings. A small catheter can be inserted if desired. Tracheal intubation is accomplished in a similar manner except that a larger diameter tube is inserted. After the catheter or larger tube is removed, reconstruction of the tracheal structure is accomplished by using one or two 5-0 silk sutures to reappose the divided tracheal rings.

The thyroid is approached in the same manner as the trachea. The two lobes of the thyroid are adjacent to the larynx. For thyroidectomy, the lobes are dissected bluntly and removed with haemostatic control.

After completion of the desired procedure (s), the incision is closed with interrupted 2-0 silk or absorbable sutures. The skin is cleaned and the hamster is returned to its cage for recovery from anaesthesia.

Post-operative analgesia is not normally required but analgesia should be decided in conjunction with the veterinarian. The animal should be watched carefully to ensure that it is up and moving around the cage.

### 5.5.2 Thymectomy in Adults (Code: HSTH)

Anaesthetise the hamster as above and place in dorsal recumbency (on back), then shave and clean the skin of its neck and upper chest with 70% alcohol. Make a midline longitudinal incision from the angle of the mandible to the level of the 4th rib and remove the thymus. Close the incision with skin clips which are removed with forceps after 7 days. post-operative analgesia may not be necessary but should be decided in conjunction with the veterinarian.

### 5.5.3 Intrathymic Injection (Code: HSIT)

Anaesthetise the hamster as above and place in dorsal recumbency. Shave the neck and clean the skin with 70% alcohol. Make a midline incision over the lower cervical and upper thoracic region. Bisect the upper third of the sternum longitudinally with fine scissors to expose the thymus. Injections (10 (l/site) are made in the anterior superior portion of each thymic lobe using a 1 cc syringe and 28 gauge needle mounted on a Tridak Stepper. Close the incision with skin clips (nothing is done to the sternum) and remove the clips with forceps after 7 days. Post-operative analgesia may not be necessary but should be decided in conjunction with the veterinarian.

### 5.5.4 Abdominal Surgery (Code: HSA)

The technique for abdominal surgery can be applied to the biopsy and/or removal of various organs as well as for other manipulations in the abdominal cavity, including vascular injection and body fluid sampling. Anaesthesia appropriate for the surgical approach and duration of the procedure is administered. Consult Appendix I and the veterinarian.

After anaesthesia, the hamster is restrained on an operating surface in an optimal position for the planned incision. For prolonged procedures, heated operating surfaces are used to maintain body heat. Body temperature is monitored.

Midline incision or flank approaches are most common for abdominal surgery. The area of the incision is shaved and the skin surface rendered aseptic with 70% alcohol and betadine solution. If additional anaesthesia is desired, intracutaneous administration of a local anaesthetic such as 1-2% procaine or lidocaine at the site of the skin incision is administered via a 25 gauge needle. The volume of local anaesthetic is to be kept below 1.5 ml. The area

of the incision is then isolated in a clean, but not sterile manner, and the surgery proceeds using a clean technique.

A scalpel is use to make an incision of the length and position desired for the procedure. Midline incisions have less bleeding and less need for subsequent haemostasis than incisions through muscle layers such as in the flank. The abdominal incision is divided into two layers (1. skin; 2. muscle/peritoneum) to avoid unintentional laceration of the abdominal contents. When entering the abdominal cavity, the second layer is tented and a very small initial incision is made to allow the abdominal contents to fall away from the site before the incision is extended. Haemostats is established with small clamps or sutures as needed. The abdominal contents are moved aside using saline-dampened gauze sponges and appropriate retractors to isolate and expose the organ, vessel or duct of interest.

If organ biopsy procedures are carried out, the organ of interest is exposed and tissue obtained using an appropriate biopsy needle or wedge-shaped incision. Bleeding is controlled by suturing the incision with 5-0 silk or absorbable suture. Gelfoam may also be used to advantage if bleeding is difficult to control in a friable organ such as the liver.

If organ removal is desired, the ducts are isolated and ligated with suture material. Either absorbable or nonabsorbable suture may be used as appropriate for the procedure and subsequent course of the experiment. Silk suture of 3-0 to 5-0 is usually satisfactory and may be left in the abdominal cavity without subsequent infection. The organ, once isolated, is then removed by transecting the ligated vessels and ducts and any remaining connective tissue.

If vascular or duct perfusion procedures are being done, the vessel or duct is isolated and a needle or catheter appropriate for the procedure is inserted. After infusion or collection, the needle or catheter is removed and haemostasis is re-established. In the case of vessels, this is accomplished with gentle pressure and/or the application of Gelfoam. The latter can be left in place and will be reabsorbed by the animal.

If a chronic in-dwelling catheter has been placed, it is tunnelled through the abdominal wall and under the skin to an appropriate external site. This is usually over the scapula where it is secured in a way that the animal cannot damage it. A variety of connections can be used as appropriate for the subsequent experimentation. Chronic, in-dwelling bladder catheters are usually placed in such a way that they exit to the lower abdominal wall.

After completion of the procedure, care is taken to assure that haemostasis is satisfactory and all gauze sponges and other restraining devices are removed. The abdominal incision (s) is closed in 2 layers. The muscle and peritoneal layer is closed with non-locking running stitch of absorbable or nonabsorbable suture, usually of 2-0 silk. Care is taken to avoid inclusion of abdominal contents in the suture line. A running stitch is used to provide a continuous closure to avoid subsequent herniation of abdominal contents through the suture line. The skin layer is closed with a line of running or interrupted sutures.

Skin staples may also be used and are removed 10-14 days later. Care is taken to include the deep fascial layers of the skin in the superficial suture line. The skin surface is then cleaned, restraints are removed, and the animal is allowed to recover from anaesthesia under frequent observation.

Post-operative analgesia is not normally required, particularly if methoxyflurane and a long-acting local anaesthetic have been injected into the site of the skin incision. The animal should be watched carefully to ensure that it is up and moving in the cage. Additional post-operative analgesia should be decided upon in consultation with the veterinarian.

### 5.5.5 Skin Grafting (Code: HSSG)

With the animal anaesthetised, use a biopsy or scissors to remove approximately 1 cm of skin from the trunk of each donor and recipient animal. The donor's skin sample is placed on the recipient at the site of the skin biopsy and sutured in place with absorbable sutures in a sample interrupted pattern. Cover the graft with a plaster bandage. Recipients are observed daily for signs of rejection. There is no pain.

### 5.5.6 Castration (Code: HSCR)

Anaesthetise male hamster and prepare the scrotum well with 70% alcohol and betadine solution. Make a small (ca. 1cm) incision through the skin at the tip of the scrotum between the two bulges of the testicles. Clear the subcutaneous connective tissue and gently squeeze a testicle out of the scrotum. Make a small 5mm incision into the tip of the testicle sac and pull out the cauda epididymis, the testis, the caput epididymis, the vas deferens and the spermatic blood vessels. Place a single ligature of 2-0 silk around the vas deferens and the spermatic blood vessels and severe these distal to the ligature. Remove the testis and the epididymis. Repeat the procedure on the remaining testis. Suture the scrotum with one or two silk sutures and clean the wound with betadine solution. Post-operative analgesia is as described for abdominal surgery above.

### 5.5.7 Transgenic hamsters procedures (Code: HSTP)

Vasectomy: The techniques and precautions described above for abdominal surgery apply. Anaesthetise male hamsters, wipe the abdomen with 70% alcohol and betadine and make a longitudinal incision (approx. 2 cm) in the skin and body wall at a point even with the top of the legs. Locate the testes and vas deferens and bring them out of the incision. Tie off the vas deferens with 2-0 silk sutures at least 4-5 mm apart and surgically remove the small length between the sutures. Replace the testes and suture the body wall. The skin is then closed with surgical staples which are removed in 10 days. Post-operative analgesia is as described for abdominal surgery above.

Reimplantation: The techniques and precautions described above for abdominal surgery apply. Anaesthetise the recipient females, wipe their lower backs with 70% alcohol and betadine and make a small longitudinal incision (approx. 1 cm) in the skin and body wall approximately even with the last rib. The ovary and oviduct are located, brought out of the incision, and held in place. Working under a dissecting microscope, inject the eggs into the oviduct through the infundibulum with a micropipette. Replace the ovary and oviduct and close the body wall with silk sutures. Close the skin with surgical staples and clean the site. Post-operative analgesia is as described for abdominal surgery above.

## 5.6 PHYSIOLOGICAL MEASUREMENTS

The hamster is placed in a metabolism chamber and various measurements are done using specialist analysers connected to the chamber.

### 5.6.1 Respirometry (Code: HPRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber)

design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. For rodents, burrow tube type chambers seem best, as the animals settle down more quickly. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline). Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 3.6.2 Measurement of food consumption (HPF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some animals, particularly fossorial animals are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 5.7 and quote reference number of relevant euthanasia procedure).

### 5.6.3 Measurement of total body water by tritium dilution (Code: HPW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April 1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste"*- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 5.4 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 5.2 and 5.3 and quote

reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 5.6.4 Urine analysis (Code: HPU)

Hamsters are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 5.6.5 Non- shivering thermogenesis (Code: HPS)

Non-shivering thermogenesis (NST) or "chemical thermoregulation" is induced by norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the animal. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 5.6.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 5.6.1) and injected with saline (for controls) or NE solutions (1.5 mg/kg) subcutaneously (see Sections 2.4 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of animals should be avoided, since high $T_b$s above 40$^o$C may inhibit thermogenesis.

### 5.6.6 Activity measurements (Code: HPA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The hamster is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 5.6.7 X-Ray techniques (Code: HPX)

The digestive tract of hamsters can be studied by X-rays following introduction of a suitable contrast agent such as barium sulfate or gastrografin. The hamster is trained before the experiment to be used to the position required for the x-ray measurement. The animal is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the animal is let into the room and all metals are removed form the area. The animal is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The animal is allowed to ingest appropriate feed coated with barium sulfate. It is important that the animal does not move during the experiment. If it was not possible to train the animal to maintain the appropriate position, soft materials and sandbags are used to keep the animal in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 5.7 EUTHANASIA

In any type of euthanasia, care must be taken to ensure that death has occurred.

### 5.7.1 Overdose of Inhalant Anaesthesia (Code: HEOD)

Under a hood, place cotton or gauze soaked in anaesthetic in the bottom of a bell jar and cover with wire mesh or grate so the animal does not come into direct contact with anaesthetic; place the hamster into the jar and apply the cover. After the animal's respiration stops, check it for complete loss of heartbeat.

### 5.7.2 Injectable Anaesthetic or Euthanasia Agent (Code: HEIN)

An overdose of an anaesthetic such as pentobarbital or an euthanasia agent such as T-61 may be given intravenously or directly into the heart.

### 5.7.3 Euthanasia in an Anaesthetised Animal by Physical Means (Code: HEPM)

Cervical dislocation may be accomplished by placing the investigator's fingers, a stout scissors or an iron rod across the neck and pulling the tail to break the neck. This procedure is best when followed with a thoractomy/exsanguination. Alternately, the anaesthetised hamster is sacrificed in the guillotine.

### 5.7.4 Euthanasia in Which Drugs Cannot be Used (Code: HECO)

Carbon dioxide suffocation may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket, or plastic bag. Place the hamster in the container and close. After the hamster no longer moves, check for loss of heartbeat.

Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel on Euthanasia (see Appendix II). Explain your special need for these alternative measures under item 8.3 of the Application Form.

# 6. STANDARD PROTOCOLS FOR GUINEA PIGS

## 6.1 HANDLING (Code: GH)

Lift the guinea pig and support it in cradled hands. Gloves may be used as guinea pigs sometimes bit. The guinea pig may also be restrained using leg ties and a suitable board. The period of restraint without tranquillisation should not exceed a few minutes without specific approval (see item 5 of the Standard Animal Protocol Review Form).

## 6.2 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly used agents and doses in provided in Appendix I. The veterinarian(s) will assist in selections that are adequate and yet convenient for the investigator. Analgesia requiring controlled substances will be the responsibility of the veterinarian. In completing the Application Form, simply indicate the method and code (inhalation/injection) to be used and list the specific agents in your response to item 8.2. Remember that ether is both flammable and explosive. It may not be used unless an explosion-proof hood or room is available and must be disposed of in explosion-proof containers. Proper ventilation is needed with all inhaled anaesthetics for user safety.

### 6.2.1 Inhalation Anaesthesia (Code: GAIH)

A vaporising anaesthetic machine, calibrated for isoflurane, is used with pure medical oxygen to supply a gas mixture of 0-5% isoflurane in oxygen, as required. During initiation of anaesthesia, place the animal in a clear Perspex respirometer and vent an initiation gas mixture of 2-4% isoflurane, depending on the species, into the respirometer at flow rates of 1-2 litres/min, depending upon the size of the animal. Place a cloth over the respirometer to quieten the animal, but remove it periodically to monitor the progress of anaesthesia. Once anaesthetised (usually after ca. five minutes), remove the animal quickly from the respirometer and place it on the preparation table. Place a gas mask appropriate for the shape of the animal over the facial region covering all respiratory surfaces. Lower the maintenance gas mixture to 1-2% isoflurane in oxygen, depending upon the species. Once pre-operative preparation of the animal is complete, move it to the surgery table. Generally the gas mixture can be lowered on commencement of suturing in order to minimise the total time under anaesthesia, and hence hypothermia. At all times during anaesthesia, monitor the rectal or cloacal temperature with a digital thermometer and rectal or cloacal probe. If the body temperature decreases significantly during lengthy procedures, the animal must be heated with a heating pad or infra-red lamp until normothermia is restored.

### 6.2.2 Injection Anaesthesia (Code: GAIN)

Injection anaesthesia, using barbiturates or other agents such as ketamine, can also be used (see Appendix I). The route of administration and frequency of additional doses will be determined by the length of the procedure. In procedures requiring assessment of vascular physiologic responses, the choice of anaesthetic must be carefully considered to avoid unwanted vascular effects. Injection techniques (IV, IM, IP, etc.) are described in a separate section.

## 6.3 SAMPLING OF BODY FLUIDS

The skin or other sites of sampling should be properly cleaned with alcohol to assure maximum visibility and cleanliness.

### 6.3.1 Bleeding

The maximum amount of blood that should be withdrawn to insure survival is 1-2 ml/100 gm body weight once a week. Prolonged bleeding may result in anaemia and abnormalities in serum proteins.

#### 6.3.1.1 Bleeding from Peripheral Vessels (Code: GBPV)

Peripheral veins: Small amounts of blood can be obtained by venipuncture of the small veins on the dorsum of the rear feet or from the penile vein of males. An assistant should hold the guinea pig and extend the hind limb or penis. Swab the area with alcohol and use a 25 gauge needle to withdraw the blood. Alternately, use a needle to pierce the vessel; the drop of blood that forms is collected by capillary tube.

#### 6.3.1.2 Bleeding by Cardiac Puncture (Code: GBTP)

Heart (cardiac puncture): Since guinea pigs lack easily accessible peripheral veins, they are often bled by cardiac puncture. Anaesthetise the guinea pig with an injection or inhalation anaesthetic and restrain in a supine position. Insert a syringe with a 20 gauge needle to the left of the sternum midway into the rib cage. Approximately 2 ml of blood per 100 gm can be withdrawn. Allow the guinea pig to recover from anaesthesia.

**Bleeding by Surgical Approaches (See surgical section)**

Blood samples can also be obtained by direct puncture or catheter placement in major vessels such as the carotid artery, jugular vein or femoral artery. These techniques are considered survival surgery and are addressed in the surgical section.

#### 6.3.1.3 Bleeding by Terminal Procedures (Code GBTB)

Terminal bleeding: Anaesthetise the guinea pig or euthanase it and open the abdomen rapidly to expose the abdominal vena cava for blood withdrawal. The anaesthetic agents or euthanasia agents used will be present in the blood subsequently recovered.

### 6.3.2 Collection of Peritoneal Cells

#### 6.3.2.1 Macrophage Collection (Code: GBMC)

To stimulate yields of activated macrophages, animals are injected with thioglycolate solution or sterile mineral oil (dose 1 ml) IP. The macrophages are collected at the time of euthanasia (3-4 days later).

#### 6.3.2.2 Polymorphonuclear Leukocyte Collection (Code: GPLC)

To stimulate exudation of polymorphonuclear leukocytes into the abdominal cavity, inject a 0.1% glycogen solution IP and collect cells within 4 hours at the time of euthanasia.

## 6.4 INJECTIONS

The skin is cleaned with alcohol to assure maximum visibility and cleanliness.

In addition tot he routes listed below, injections can be made directly into major vessels by needle or catheter placement. The carotid artery, jugular vein or the femoral vessels can be used. These techniques are considered surgical procedures and are addressed in the surgery section.

Phosphate buffered saline (PBS) or other isotonic and physiological solutions are suitable for intravenous administration. Oil-based adjuvants are not to be administered intravenously.

IV: Must not exceed 1 ml/100 g with not more than 0.5 ml given at other tissue sites
IP: Must not exceed 10 ml
Intratracheal: Must not exceed 0.2 ml
Oral: Must not exceed 5 ml

### 6.4.1 Intravenous (IV) (Code: GIIV)

Intravenous injection can be accomplished via the penile vein of males. Restrain the guinea pig, extend the penis and clean the skin. A 25 gauge or smaller needle is inserted for injection.

Dorsal metatarsal vein: This procedure requires two people. The assistant holds the guinea pig and extends its leg at the ankle. The dorsal aspect of its foot is wet-shaved and wiped with alcohol. The assistant dilates the vessel by pressure at the ankle and the investigator flicks the vein with fingers to further dilate. With the animal's toes curved over the operator's finger and its skin stretched tightly, the operator inserts a 25 gauge needle at a shallow angle into the vein just where it starts to travel up the animals foot after crossing from its toes.

### 6.4.2 Subcutaneous (SQ) (Code: GISO)

SQ injections are done under the skin of the back and sides. Prep the skin and pass a needle through the skin at a shallow angle to the surface. Once through the skin, aspirate to assure that the needle is not in a vessel and inject. Maximum volume is 3 ml per site. Any bleeding that occurs after withdrawal can be controlled with pressure.

### 6.4.3 Intradermal (ID) (Code: GIID)

Shave the fur and clean the skin with alcohol. ID injections are done by inserting the needle bevel up very superficially into the skin of the back and sides. Shave and clean the skin to determine the exact location of the needle. Inject only very small quantities (about 0.1 ml). A successful ID injection is recognised as a small bleb.

### 6.4.4 Intramuscular (IM) (Code: GIIM)

IM injections are usually given in the heavy thigh muscles. Clean the skin and insert the needle in the direction away from the femur and sciatic nerve. Take care to avoid the bone. Aspirate the needle before injection to be sure the material does not enter a vessel. Maximum volume is 0.5 ml.

### 6.4.5 Intraperitoneal (IP) (Code: GIIP)

Restrain the guinea pig in an extended position, shave the abdomen and swab it with an antiseptic solution. Use a large-gauge needle since it is less likely to penetrate the viscera. Insert the needle tip through the abdominal wall in the lower quadrants away from the midline (bladder) and begin the injection immediately to push away any viscera. After injecting, withdraw the needle immediately.

### 6.4.6 Intragastric (Code: GIIG)

Approximate the distance from the mouth to the stomach first. Use a mount gag to prevent the guinea pig from biting the catheter (plastic or rubber). Moisten the empty catheter with saline and introduce into the guinea pig's mouth over the back of the tongue. care must be taken to advance the catheter during swallowing to avoid its inadvertent entrance into the trachea and the lung. Determine the position of the catheter by injecting a small amount of air while listening to the stomach area with a stethoscope. Administer the material to be injected slowly through the tube. Clear the catheter with a small amount of air before withdrawal to avoid contamination of the air passage with residual material from the injection.

## 6.5 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of Anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. Procedures requiring prolonged Anaesthesia (more than 3 hours) will require special approval and should be explained in item 8.3 of the Application Form. Surgical procedures require clean but not sterile techniques, and should be carried out in approved surroundings (please check with the Animal Ethics Sub-committee). Autopsy with sampling of organs after euthanasia is not considered a surgical technique. All survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 8.3 of the Application Form.

Body heat is rapidly lost during surgical procedures; a heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 6.5.1 Anterior Neck (Code: GSAN)

Anaesthesia is induced appropriate to the duration of the planned resource. Injection anaesthesia may be preferable since inhalation anaesthesia is difficult to maintain while working on the neck or trachea. Tranquillising levels of anaesthesia and local anaesthesia at the site of incision may be adequate. Restrain the guinea pig with its throat exposed. Shave the throat and wash the skin with 70% ethyl alcohol and betadine solution. Administer local anaesthetic and make a midline incision. Use blunt dissection to extend the incision down to the site of interest.

Locate the carotid artery, jugular vein, and vagus nerve adjacent to the tracheal area. These vessels are located by palpitation of the carotid pulse. Isolate the vessels by blunt dissection and place sutures are placed around them for control of haemorrhage during injection or catheter placement. Insert needles (P-10 or P-50 tubing) under direct vision. The direction of placement is dependant on the purpose. Removal of needles or catheters is followed by securing haemostasis.

Locate the trachea by palpitation of the tracheal rings. Accomplish direct intratracheal injection by inserting a 25 gauge needle between two tracheal rings. A small catheter can be inserted if desired. Tracheal intubation is accomplished in a similar manner, except that a larger diameter tube is inserted. After removing the catheter or larger tube, reconstruct the tracheal structure by using one or two 5-0 silk or absorbable sutures to reappose the divided tracheal rings. Clean the incision site.

### 6.5.2 Cannulation of Femoral Vessels (Code: GSFV)

Restrain the guinea pig in a supine position usually with tranquillising anaesthesia. Shave the femoral area and wash the area of incision with antiseptic solution (70% ethyl alcohol and betadine). Infiltrate the area with 1-2% procaine, xylocaine. Make a small 1 cm incision and isolate the femoral vessels by blunt dissection. Surround the vessels with 2-0 to 5-0 silk or absorbable sutures to control haemostasis and secure catheters or needles. Straight or hooked needles are used for simple injections or blood samples. For serial sampling, injection, or pressure monitoring, use bevelled P-10 or P-50 catheters which are threaded into the femoral artery and/or vein in a retrograde fashion to the desired level in the aorta or vena cava, respectively. Remove catheters at the conclusion of the experiment, establish haemostasis, close the skin with 2-0 silk or absorbable sutures and clean the site.

### 6.5.3 Abdominal Surgery (Code: GSA)

The techniques for abdominal surgery can be applied to the biopsy and/or removal of various organs as well as for other manipulations in the abdominal cavity, including vascular injection and body fluid sampling. Anaesthesia appropriate for the surgical approach and duration of the procedure is administered. Consult Appendix I and the veterinarian.

After inducing anaesthesia, restrain the guinea pig on an operating surface in an optimal position for the planned incision. For prolonged procedures, heated operating surfaces are used to maintain body heat. Monitor body temperature as needed.

Midline incision or flank approaches are most common for abdominal surgery. Shave the area of the incision and render the skin surface antiseptic with 70% alcohol and betadine solution. If additional anaesthesia is desired, intracutaneous administration of a local anaesthetic such as 1-2% procaine or lidocaine at the site of the skin incision is administered via a 25 gauge needle. A long-acting anaesthetic may obviate the need for post-operative analgesia. The volume of local anaesthetic is to be kept below 2.0 ml. Isolate the area of incision in a clean, but not necessarily sterile manner, and proceed using sterile instruments and clean technique.

Make an incision of appropriate length in the midline or flank. Midline incisions have less bleeding and less need for subsequent haemostasis than incisions through muscle layers such as in the flank area. The abdominal incision is divided into two layers (1. skin; 2. muscle/peritoneum) to avoid unintentional laceration of the abdominal contents. When entering the abdominal cavity, tent the muscle wall and make a very small initial incision to allow the abdominal contents to fall away from the site before the incision is extended. Establish haemostasis with small clamps or sutures. Move the abdominal contents aside using saline-dampened gauze sponges and appropriate retractors to isolate and expose the organ, vessel or duct of interest.

If organ biopsy procedures are carried out, expose the organ of interest and obtain tissue using an appropriate biopsy needle or a wedge-shaped incision. Bleeding is controlled by closing the incision with 5-0 silk or absorbable sutures. Gelfoam may also be used to advantage if bleeding is difficult to control in a friable organ such as the liver.

If organ removal is desired, isolate the vasculature and other related ducts and ligate with suture material. Either absorbable or nonabsorbable suture may be used, as appropriate, for the procedure and subsequent course of experiment. Silk suture of 3-0 to 5-0 is usually satisfactory and may be left in the abdominal cavity without subsequent infection. After isolating the organ remove it by transection of the ligated vessels and ducts and any remaining connective tissue.

If vascular or duct perfusion procedures are being done, isolate the vessel or duct and insert a needle or catheter appropriate for the procedure. After infusion or collection, the needle or catheter is removed and haemostasis re-established. In the case of vessels, apply gentle pressure and/or the application of Gelfoam. The latter can be left in place and will be reabsorbed by the animal.

If a chronic in-dwelling catheter has been placed, tunnel through the abdominal wall and under the skin to an appropriate external site. This is usually over the scapula, where it is secured in a way that the animal cannot damage it. A variety of connections can be used, as appropriate, for the subsequent experimentation. Chronic in-dwelling bladder catheters are usually placed in such a way that they exit directly into the lower abdominal wall.

After completion of the procedure, care is taken to assure that haemostasis is satisfactory and all gauze sponges and other restraining devices are removed. Close the abdominal incisions(s) in two layers. Close the muscle and peritoneal layer with a non-locking running stitch of absorbable or non-absorbable suture, usually of 2-0 silk. Care is taken to avoid inclusion of abdominal contents in the suture line. Use running stitch to provide a continuous closure to avoid subsequent herniation of abdominal contents through the suture line. Close the skin layer with a line of running or interrupted sutures. Skin staples may also be used to close the incision and are removed 10-14 days later. Be sure to include the deep fascial layers of skin in the superficial suture line. Clean the skin surface, remove restraints, and the allow the animal to recover from anaesthesia under frequent observation.

Post-operative analgesia is not normally required, particularly if methoxyflurane and/or long-acting local anaesthetic has been injected into the site of the skin incision. The animal should be watched carefully to insure it is up and moving in the cage. Use of post-operative analgesia should be decided upon in consultation with the veterinarian.

### 6.5.4 Castration (Code: GSCR)

Anaesthetise male guinea pig and prepare the scrotum well with 70% alcohol and betadine solution. Make a small (ca. 1cm) incision through the skin at the tip of the scrotum between the two bulges of the testicles. Clear the subcutaneous connective tissue and gently squeeze a testicle out of the scrotum. Make a small 5mm incision into the tip of the testicle sac and pull out the cauda epididymis, the testis, the caput epididymis, the vas deferens and the spermatic blood vessels. Place a single ligature of 2-0 silk around the vas deferens and the spermatic blood vessels and severe these distal to the ligature. Remove the testis and the epididymis. Repeat the procedure on the remaining testis. Suture the scrotum with one or two silk sutures and clean the wound with betadine solution. Post-operative analgesia is as described for abdominal surgery above.

## 6.6 PHYSIOLOGICAL MEASUREMENTS

The guinea pig is placed in a metabolism chamber and various measurements are done using specialist analysers connected to the chamber.

### 6.6.1 Respirometry (Code: GPRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. For rodents, burrow tube type chambers seem best, as the animals settle down more quickly. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline). Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 6.6.2 Measurement of food consumption (GPF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some animals, particularly fossorial animals are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 6.7 and quote reference number of relevant euthanasia procedure).

### 6.6.3 Measurement of total body water by tritium dilution (Code: GPW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April 1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste"*- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 6.4 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 6.2 and 6.3 and quote reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 6.6.4 Urine analysis (Code: GPU)

Guinea pigs are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 6.6.5 Non- shivering thermogenesis (Code: GPS)

Non-shivering thermogenesis (NST) or "chemical thermoregulation" is induced by

norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the animal. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 6.6.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 6.6.1) and injected with saline (for controls) or NE solutions (1.5 mg/kg) subcutaneously (see Sections 6.4 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of animals should be avoided, since high $T_b$s above 40$^o$C may inhibit thermogenesis.

### 6.6.6 Activity measurements (Code: GPA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The guinea pig is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 6.6.7 X-Ray techniques (Code: GPX)

The digestive tract of guinea pigs can be studied by X-rays following introduction of a suitable contrast agent such as barium sulfate or gastrografin. The guinea pig is trained before the experiment to be used to the position required for the x-ray measurement. The animal is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the animal is let into the room and all metals are removed form the area. The animal is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The animal is allowed to ingest appropriate feed coated with barium sulfate. It is important that the animal does not move during the experiment. If it was not possible to train the animal to maintain the appropriate position, soft materials and sandbags are used to keep the animal in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 6.7 EUTHANASIA

In any type of euthanasia care must be taken to assure death has occurred.

### 6.7.1 Overdose of Inhalant Anaesthetic (Code: GEOD)

Under a hood, place cotton gauze soaked in anaesthetic in the bottom of a bell jar and cover with wire mesh or grate so the animal does not come into direct contact with the anaesthetic' place the guinea pig into the jar and apply the cover. After the animal's respiration stops, check it for complete loss of heartbeat.

### 6.7.2 Injectable Anaesthetic or Euthanasia Agent (Code: GEIN)

An overdose of an injectable anaesthetic such as pentobarbital or an euthanasia agent such as T-61 may be given intravenously or directly into the heart.

### 6.7.3 Euthanasia in Which Drugs Cannot be Used (Code: GECO)

Carbon dioxide suffocation may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket or plastic bag. Place the guinea pigs in the container and close. After guinea pigs no longer move, check for loss of heartbeat.

Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel of Euthanasia (see Appendix II). Explain your special need for these alternative methods under item 8.3 of the Application Form.

# 7. STANDARD PROTOCOLS FOR RABBITS

## 7.1 HANDLING (Code: LH)

Rabbits must be handled with care since their backs fracture easily with spinal cord damage if they are allowed to struggle. They are also capable of inflicting rather severe scratches on handlers with their hind claws. However, rabbits rarely bite or exhibit aggressive behavior.

In working with rabbits, it should be emphasised that the mere holding or restraining of the animal at times produced a screaming reaction. The screaming usually does not result from pain since it most often occurs before any manipulations have taken place. The sound is very distressing to those handling the rabbit and anyone in the immediate area, but often cannot be avoided. Light anaesthesia sometimes seems to increase the tendency to scream.

Grasp the rabbit with both hands while it is on the floor of its cage. With one hand, control the rear quarters by holding the skin over the rear haunches. With the other hand, control the head by grasping the loose skin over the shoulders. Lift the rabbit from the cage and transfer it to a rabbit restraining box, insert all four extremities quickly and close the lid. The boxes have some form of adjustable slide to insure a snug fit. The rabbit's head or just it's ears can extend from the box. Care must be taken to assure that the rabbit has adequate room for ventilation.

Rabbits may also be restrained by hand on a suitable work surface. Prevent the rabbit from jumping from any elevated surface. The rabbit can be held firmly, for example, on a table against the handler's side, by controlling the front feet and head with one hand and the back and rear quarters with the elbow. The rabbit can also be held in a fully extended position with the front legs held alongside the head and the rear legs stretched out. The former position is most suitable for injection, and the latter position is used for cardiac puncture. Since large quantities of blood are easily obtained from the central artery of the ear, cardiac puncture with its attendant mortality is usually not needed.

## 7.2 ANAESTHESIA

Anaesthesia for rabbits is somewhat more difficult to achieve than for other commonly used research animals. The appropriate anaesthesia, including tranquilisation and post-operative analgesia, can best be selected with the advice of the veterinarian (see Appendix I). Analgesia requiring controlled substances will be the responsibility of the veterinarian. In completing the Application Form, simply indicate the method (and code) to be used. List the specific agents chosen in your response to item 8.2.

### 7.2.1 Inhalation Anaesthesia (Code: LAIH)

Surgical procedures on rabbits require sterile techniques and need to be done in an operating room. An anaesthesia machine is provided and yields the anaesthesia required for operative procedures. Non-flammable anaesthesia (methoxyflurane or halothane) are administered by face mask or endotracheal tube to the rabbit which has first been tranquilized with ketamine. The veterinarian will assist you in learning this technique.

### 7.2.2 Injection Anaesthesia (Code: LAIN)

Rabbits can be subjected to light anaesthesia by injection of hypnotic substances (see Appendix I). IV routes of administration are usually optimum and offer the best control over the level of anaesthesia achieved. Administration of these agents must be repeated as required to maintain the desired level of anaesthesia. The level used should minimize emotional and physical distress of the animal without jeopardizing its chances of survival.

## 7.3 SAMPLING OF BODY FLUIDS

The skin, or other sites of sampling, should be properly cleaned to assure maximum visibility and cleanliness.

### 7.3.1 Bleeding

#### 7.3.1.1 Bleeding from Peripheral Vessels (Code: LBPV)

Ear vessels: Ear bleeding is accomplished with the rabbit in a restraining box with its ears extended. Lightly shave the area over the central ear artery or the lateral ear vein to increase visibility. The ear vessels will become engorged by gently stroking, thumping, or exposure to dry or moist heat that is bearable to the handler's own hands. Xylene or skin irritants should not be used since they cause prolonged irritation to the delicate skin of the ears. Careful application of heat and manual stroking are all that is needed. For arterial bleeding, insert a needle of the size appropriate for the volume of blood desired into the most distal portion of the artery available. A 22 or 23 gauge needle is adequate for 2-10 ml of blood. An 18 or 19 gauge needle needs to be used to collect 50 ml. The needle may be connected to a catheter used to deliver blood directly into a tube. Once the blood has been obtained, remove the needle and apply pressure to the needle hole until bleeding has stopped. Removal of the rabbit's head from the box and withdrawing the compression slide will hasten the control of bleeding from the puncture site. Control the bleeding with a cotton swab and a bent paper clip which is removed before the rabbit is returned to its cage. A small volume of blood can be obtained from the lateral ear vein using a 22 or 23 gauge needle.

#### 7.3.1.2 Bleeding by Cardiac Puncture (Code: LBCP)

Heart: Blood may also be obtained by cardiac puncture. Anaesthesia is required. Restrain the rabbit in an extended position. Insert a needle, usually of 18 or 20 gauge, into the heart between the ribs lateral to the sternum on the left. Blood can be withdrawn directly from the heart. The mortality from intrathoracic bleeding and abdominal vessel bleeding is 5-10% depending on the skill of the investigator.

**Bleeding by Surgical Approaches (See surgical section)**

Blood samples can also be obtained by direct puncture or catheter placement in major vessels such as the carotid artery, jugular vein or femoral artery. These techniques are considered survival surgery and are addressed in the surgical section.

#### 7.3.1.3 Bleeding by Terminal Procedures (Code: LBTB)

The amount of blood that can be obtained from a rabbit at terminal bleeding can be increased by a combination of the ear bleeding and abdominal vessel bleeding techniques. In this case blood is first obtained from the ear; then the rabbit is euthanised with rapid exposure of the abdominal vena cava for additional blood withdrawal. The anaesthetic or euthanasia agents used will subsequently be present in the blood recovered from the abdominal vessels. Pentobarbital (IV) is the most satisfactory agent for this approach.

### 7.3.2 Bladder Catheterisation (Code: LBBC)

An anaesthetic is administered appropriate to the duration of the planned urine collection. The urethral orifice and surrounding area are washed with an antiseptic solution to reduce the chances of introducing infection into the bladder. A soft 8 or 19 French bladder catheter, moistened with saline, is gently inserted into the bladder through the urethral orifice. A Couday tipped catheter may be of help in males. The catheter can be removed after initial urine collection, or, if in-dwelling placement is desired, an in-dwelling catheter may be used

and the balloon filled with air or saline to retain the catheter in place. Long-term or repeated catheterisation may promote bladder infection which is screened by routine urinalysis and may require antibiotic therapy.

### 7.3.3 Collection of peritoneal cells

#### 7.3.3.1 Macrophage Collection (Code: LBMC)

To stimulate yields of activated macrophages, animals are injected with thioglycolate solution or sterile mineral oil (dose 1 ml) IP. The macrophages are collected at the time of euthanasia (3-4 days later).

#### 7.3.3.2 Polymorphonuclear Leukocyte Collection (Code: LPLC)

To stimulate exudation of polymorphonuclear leukocytes into the abdominal cavity, inject a 0.1% glycogen solution IP and collect cells within 4 hours at time of euthanasia.

## 7.4 INJECTIONS

The skin is cleaned with alcohol to assure maximum visibility and cleanliness.

Injections can be made directly into the major vessels by needle or catheter placement. The carotid artery, jugular vein or the femoral vessels can be used. These techniques are considered surgical procedures and are addressed in the surgical section.

Phosphate buffered saline (PBS) or other isotonic and physiologic solutions are suitable for intravenous administration. Oil based adjuvants are not to be administered intravenously.

IV: No more than 20 ml/kg, and no more than 1 ml at other tissue sites
IM: No more than 0.5 ml
Intragastric: No more than 20 ml

### 7.4.1 Intravenous (IV) (Code: LIIV)

Ear veins provide easy access for IV injection. Restrain the rabbit in a box and expose the area over the lateral ear vein by shaving. After cleaning, insert a 23 to 30 gauge needle, depending on the volume to be injected, into the vein at the most distal point possible. The rate of injection should be slow and can be judged by the visible extent of distention of the vein. For continuos injection or infusion, a needle with catheter attached can be inserted and held in place with tape. Restrain the rabbit in the box during the infusion. Periods of restraint greater than 1 hour require special approval; see item 8.3 or the Application Form. Infusion can also be given via catheters placed in the jugular or femoral veins by techniques described in the surgical section.

### 7.4.2 Subcutaneous (SQ) (Code: LISQ)

SQ injections are done under the skin of the back and sides. Prep the skin and pass a needle through the skin at a shallow angle to the skin surface. Once through the skin, aspirate to assure that the needle is not in a vessel and inject. Maximum volume is 1.0 ml. Any bleeding that occurs after withdrawal can be controlled with pressure.

### 7.4.3 Intradermal (ID) (Code: LIID)

ID injections are done by inserting the needle bevel up very superficially into the skin of the back and sides. Shave and prep the skin to determine the exact location of the needle. Only very small quantities can be injected (about 0.1 ml). Successful ID injection is recognized as a small bleb.

### 7.4.4 Intramuscular (IM) (Code: LIIM)

Clean the skin and insert the needle. IM injections are usually given in the heavy thigh muscles. Care must be taken to avoid the bone and to aspirate before injecting to be sure the material does not enter a vessel. Maximum volume is 0.5 ml.

### 7.4.5 Intraperitoneal (IP) (Code: LIIP)

Restrain the rabbit in an extended, head down position, then shave and swab the abdomen with an antiseptic solution. A large-gauge needle is used since it is less likely to penetrate the viscera. Insert the needle gently through the abdominal wall usually in the lower quadrant away from the midline (bladder), and begin the injection immediately to push away any viscera. Withdraw the needle immediately.

### 7.4.6 Intragastric (Code: LIIG)

Gastric intubation includes the use of a mouth gag to prevent the animal from biting the catheter. Plastic or rubber catheters can be used. The catheter should be moistened with saline and introduced into the animal's mouth over the back of the tongue. Care is taken to advance the catheter during swallowing to avoid its inadvertent entrance into the trachea and lungs. Determine the position of the catheter by injecting a small amount of air while listening to the stomach area with a stethoscope. Clear the catheter with air before withdrawal to avoid contamination of air passages. The maximum volume to prevent regurgitation is 20 ml.

## 7.5 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. procedures requiring prolonged anaesthesia (more than 3 hours) will require special approval and should be explained in item 8.3. Surgical procedures in rabbits require sterile techniques and must be carried out in operating rooms. Autopsy with sampling of organs after euthanasia is not considered a surgical technique. All survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose, and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 8.2.

Body heat is rapidly lost during surgical procedures. A heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 7.5.1 Minor Surgical Procedures

Minor surgical procedures (those that do not penetrate a body cavity or have the potential of providing a permanent handicap in an animal that is expected to recover) can be performed outside the operating room if they are performed in accordance with standard veterinary practice. Examples of minor surgical procedures are as follows:

tracheotomy
peripheral vessel cannulation
subcutaneous implants; portals
endoscopy
male castrations
thyroidectomy

skin grafting
peripheral lymph node injection

Standard veterinary practice includes the following:

1. Approved investigator work room
2. Aseptic preparation. The hair should be clipped and the skin cleaned with Betadine scrub followed by an alcohol wipe and a final prep with Betadine solution (or a comparable alternative)
3. Cap, mask, sterile gloves, and drape
4. All instrumentation should be sterilized with either gas (ethylene oxide) or steam

### 7.5.2 Popliteal Lymph Node Injection (Code: LSPL)

Inject the rabbit with a 1% solution of Evans blue dye in the dorsum of the hind feet. About 1 hour later, anesthetize the rabbit on its stomach with its hind legs extended. Shave the area behind the knee joint and treat the skin with an aseptic solution (70% ethyl alcohol and betadine). Anesthetize the skin with a local anaesthetic (1-2% procaine or lidocaine) in the area with a 1-2 cm incision in the popliteal fossa. Blunt dissection will reveal the small round lymph node which has been discolored with blue dye. Approximately 0.1 ml of antigen in adjuvant can be injected directly into the lymph node using a 23 to 25 gauge needle. Close the skin incision with two or three interrupted 2-0 silk or absorbable sutures and clean the site. The procedure is usually done bilaterally.

### 7.5.3 Anterior Neck (Code: LSAN)

An anaesthetic is administered appropriate to the duration of the planned procedure. Tranquilising levels of anaesthesia and local anaesthesia at the site of the incision are adequate. Restrain the rabbit with its throat exposed. Shave the large sublingual tuft of hair with 70% ethyl alcohol and betadine solution. After the anaesthesia takes effect, make a midline incision. Blunt dissection is used to move the incision down to the site of interest.

The carotid artery, jugular vein and vagus nerve can be located adjacent to the tracheal area. These vessels are located by palpitation of the carotid pulse and feeling the tracheal rings. Isolate the vessels by blunt dissection and place sutures around them for control of hemorrhage during injection or catheter placement. Insert P-50 or P-90 tubing under direct vision. The direction of placement is dependent upon the purpose. Removal of needles or catheters is followed by securing haemostasis.

Locate the trachea by palpitation of the tracheal rings. Direct intratracheal injection is accomplished by inserting a 25 gauge needle between two tracheal rings. Insert a small catheter if desired. Tracheal incubation is accomplished in a similar manner except that a larger diameter tube is inserted. Follow catheter or larger tube removal by reconstruction of the tracheal structure, using one or two 5-0 silk or absorbable sutures to reappose the divided tracheal rings.

The thyroid is approached in the same manner as the trachea. The two lobes of the thyroid are adjacent to the larynx. For thyroidectomy, the lobes are dissected bluntly and removed with haemostatic control. After completion of the procedure(s), close the incision with interrupted 2-0 silk or absorbable sutures. The skin is cleaned and the rabbit returned to its cage to recover from anaesthesia. Post-operative analgesia should be decided upon in consultation with the veterinarian.

### 7.5.4 Cannulation of Femoral Vessels (Code: LSFV)

Restrain the tranquilised rabbit in a supine position. Shave the femoral area and apply an

antiseptic solution (70% ethyl alcohol and betadine) to the area of incision. Infiltrate the area with 1-2% procaine or xylocaine. Make a small 1-2 cm incision and isolate the femoral vessels by blunt dissection. Surround the vessels with 2-0 silk or absorbable sutures to control haemostasis and to secure catheters or needles. Individual blood samples or simple injections are accomplished with straight or hooked needles. For serial sampling, injection, or pressure monitoring, beveled P-50 or P-90 catheters are threaded into the femoral artery and/or vein in a retrograde fashion to the desired level in the aorta or vena cava, respectively. The catheters are removed at the conclusion of the experiment, after which haemostasis is established and the skin is closed with 2-0 silk or absorbable sutures.

### 7.5.6 Major Surgical Procedure

#### 7.5.6.1 Abdominal Surgery (Code: LSAS)

The techniques for abdominal surgery can be applied to the removal or biopsy of abdominal organs as well as for other manipulation in the abdominal cavity including vascular injection and body fluid sampling.

Sterile surroundings in a surgical suite are needed to reduce any chance of subsequent infection. Use sterile instruments, drapes, masks, gloves, and aseptic technique.

Rabbit handling and anaesthesia are conducted as outlines in sections on handling and anaesthesia. If inhalation anaesthesia is used, it is maintained with methoxyflurane or halothane administered through a face mask or endotracheal tube using an anaesthesia machine to supply oxygen as well as anaesthetic. Use sufficient amounts to render the animal unresponsive to painful stimuli yet insufficient to induce respiratory depression. Ketamine can be used for immobilisation and the inhalation anaesthesia given by face mask. Injection anaesthesia using barbiturates or other agents including ketamine, along with local anaesthesia at the site of the skin incision, can also be used (see appendix I). The route of administration and frequency of additional doses will be determined by the length of the abdominal procedure. in procedures requiring assessment of vascular physiologic responses, the choice of anaesthetic must be carefully considered to avoid unwanted vascular effects. To reduce the depth of anaesthesia needed for the abdominal procedures, inhaled or injected anaesthetics are usually supplemented by the use of local anaesthesia at the site of skin incision.

Restrain the anesthetised rabbit on an operating surface in an optimal position for the planned incision. For procedures extending more than 5-10 min, heated operating surfaces are used to maintain body heat. Body temperature is monitored.

Midline incisions or flank approaches are most common for abdominal surgery. Shave the area of the incision and render the skin surface aseptic with 70% ethyl alcohol and betadine solution. If additional anaesthesia is desired, administer a local anaesthetic intracutaneously at this point. Isolate the area of the incision in a sterile field and follow sterile technique. For terminal surgery, a clean, but not sterile, procedure is used.

Use a scalpel to make an incision of the length and position desired for the procedure. Midline incisions have less bleeding and need for subsequent haemostasis than incisions through muscle layers, such as the flank position. The abdominal incision is carried out in two layers (1. skin; 2. muscle/peritoneum) to avoid unintentional laceration of the abdominal contents. When entering the abdominal cavity, tent the second layer and make a very small initial incision to allow the abdominal flank incisions, blunt dissection of the muscle layers is usually best. Haemostasis is established with small clamps or sutures as needed. Move the abdominal contents aside with saline dampened gauze sponges and appropriate retractors to isolate and expose the organ, vessel, or duct of interest.

For organ biopsy, the organ of interest is exposed and tissue obtained by using an appropriate biopsy needle or a wedge-shaped scalpel incision. Control bleeding by suturing the biopsy site with 5-0 silk or absorbable sutures. Gelfoam may also be used to advantage if bleeding is difficult to control in a friable organ such as the liver. If organ removal is desired, isolate the vasculature and any other related ducts and ligate with suture material. Either absorbable or non-absorbable suture may be used, as appropriate for the procedure and subsequent course of the experiment. Silk suture of 3-0 to 5-0 is usually satisfactory and may be left in the abdominal cavity without subsequent infection. The organ, once isolated, can then be removed by transecting the ligated vessels and ducts and any remaining connective tissue.

For vascular or duct perfusion/collection procedures, isolate the vessel or duct, and insert a needle or catheter appropriate for the procedure. After infusion or collection, remove the needle or catheter and reestablish haemostasis. For vessels, this is accomplished with gentle pressure and/or the application of Gelfoam. The latter can be left in place and will be reabsorbed by the animal. In some cases, the duct and organ, if not necessary for life, are simply removed at the end of the procedure.

If a chronic in-dwelling catheter has been placed, it is tunneled through the abdominal wall and under the skin to an appropriate external site. This is usually over the scapula, where it is secured in a way that the animal cannot damage it. A variety of connections can be used, as appropriate for the subsequent experimentation. Chronic in-dwelling bladder catheters are usually placed in such a way that they exit directly to the lower abdominal wall.

After completion of the procedure, be sure that haemostasis is satisfactory and that all gauze sponges and other restraining devices are removed. Close the abdominal incision(s) in two layers. The muscle and peritoneal layer is closed with a non-locking running stitch to provide a continuos closure and to avoid subsequent herniation of abdominal contents through the suture line. Carefully avoid inclusion of abdominal contents in the suture line. Close the skin layer with a line of running or interrupted sutures of absorbable or nonabsorbable suture usually of 2-0 silk. Clean the skin surface, remove restraints and allow the animal to recover from anaesthesia under frequent observation.

Post-operative analgesia should be decided upon in consultation with the veterinarian.

## 7.6 PHYSIOLOGICAL MEASUREMENTS

The rabbit is placed in a metabolism chamber and various measurements are done using specialist analysers connected to the chamber.

### 7.6.1 Respirometry (Code: LPRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. For rodents, burrow tube type chambers seem best, as the animals settle down

more quickly. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline). Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 7.6.2 Measurement of food consumption (LPF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some animals, particularly fossorial animals are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 7.7 and quote reference number of relevant euthanasia procedure).

### 7.6.3 Measurement of total body water by tritium dilution (Code: LPW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April 1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste"*- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 7.4 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 7.2 and 7.3 and quote reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 7.6.4 Urine analysis (Code: LPU)

Rabbits are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 7.6.5 Non- shivering thermogenesis (Code: LPS)

Non-shivering thermogenesis (NST) or "chemical thermoregulation" is induced by norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the animal. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 7.6.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 7.6.1) and injected with saline (for controls) or NE solutions (1.5 mg/kg) subcutaneously (see Sections 2.4 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of animals should be avoided, since high $T_b$s above 40$^o$C may inhibit thermogenesis.

### 7.6.6 Activity measurements (Code: LPA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The rabbit is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 7.6.7 X-Ray techniques (Code: LPX)

The digestive tract of rabbits can be studied by X-rays following introduction of a suitable contrast agent such as barium sulfate or gastrografin. The rabbit is trained before the experiment to be used to the position required for the x-ray measurement. The animal is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the animal is let into the room and all metals are removed form the area. The animal is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The animal is allowed to ingest appropriate feed coated with barium sulfate. It is important that the animal does not move during the experiment. If it was not possible to train the animal to maintain the appropriate position, soft materials and sandbags are used to keep the animal in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 7.7 EUTHANASIA

In any type of euthanasia, care must be taken to assure death has occurred.

### 7.7.1 Overdose of Inhalant Anaesthetic (Code: LEOD)

The rabbit must first be tranquilised with ketamine. Inhalation anaesthesia for rabbits must be done using a face mask with an anaesthesia machine or via intubation.

### 7.7.2 Injectable Anaesthetic or Euthanasia Agent (Code: LEIN)

An overdose of an injectable anaesthetic such as pentobarbital or an euthanasia agent such as T-61 may be given intravenously or directly into the heart.

### 7.7.3 Euthanasia in Which Drugs Cannot be Used (Code: LECO)

Euthanasia with carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket, or plastic bag. Place the rabbit in the container and close. After the rabbit no longer moves, check for loss of heartbeat.

Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel on Euthanasia (see Appendix II). Explain your special need for these alternative methods under item 8.3 of the Application Form.

# 8. STANDARD PROTOCOLS FOR WILD BIRDS

## 8.1 COLLECTING or CAPTURE (Code: BWC)

Parrots and lovebirds are purchased from recognised bird breeders, once the required permits are obtained from the KwaZulu-Natal Nature Conservation Services (KZNCS).

Small birds (bishops and sunbirds) are caught using mist nets. The mist nets are placed in the path of the birds flight. The birds fly into the nets and get trapped in the fine mesh of the mist net. Only trained personal are allowed to mist net birds.

Plovers are caught at midday near their nests using a spring loaded trap with bird netting**.**

Birds for ringing are caught using capture nets. Standard capture nets (16 - 60 mm mesh) are erected in series along a transect and supported by 3 m aluminium poles. Nets are checked at least once every 20 minutes and closed and furled if too hot.

Raptors: Balchatri traps are baited with a live rodent and used to capture raptors. When specific species are targeted a playback tape is used to call up birds into the net.

Captured birds are removed carefully by holding the feet first and carefully removing the net from the body of the bird. A crochet needle is used to assist in removing entangled birds when necessary. In stressful cases the net is cut. Birds are held in linen bags with drawstring closure. Birds bags are hung in the shade until the bird is processed soon as possible after capture.

## 8.2 HANDLING (Code: BWH)

Most birds tend to be easily alarmed or frightened. Sudden movement or loud noises may upset an entire flock.

Small birds are held in the hand with the neck of the bird between the index and fore fingers and the bird resting in the hand.

## 8.3 RINGING (Code: BWR)

Bird ringing is done according to international standards set by EURING. Ringing is carried out at various long term study sites, usually in the early morning or late afternoon, in KwaZulu-Natal under permit from KwaZulu-Natal Nature Conservation Services (KZNNCS), Department of Water Affairs and Forestry (DWAF) and SAFRING (Cape Town).

Birds are captured for ringing as outlined under Section 1. Birds are positively identified before being ringed. Birds are ringed with the appropriate open ring (recommended Aluminium, Incalloy or Stainless Steel ring sizes - SAFRING, University of Cape Town) using standard size ringing pliers. These rings are placed on the “ankle” of the bird so that they do not interfere with the general activities of the bird.

The following data are recorded: i) species (identified using Maclean, G.L. 1993. Roberts Birds of southern Africa, & SASOL Southern African birds, ii) sex (visually in dimorphic and dichromatic species), iii) mass (bird weighed in bag on Pesola balance), iv) wing length (Engineering rule), v) tail length (Engineering rule), vi) culmen length (Engineering rule), vii) tarsus length (Vernier Calipers), viii) moult score (visual), ix) breeding condition (visual

observation). The relevant data are inputted into a data base and are submitted on an annual basis to SAFRING (Cape Town).

Birds are released at their site of capture.

## 8.4 ANESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly use agents and dosages is provided in Appendix I.

### 8.4.1 Inhalation Anaesthesia (Code: BWAIH)

In birds, a series of air sacs communicates with relatively small lungs via bronchi. At inspiration, the caudal most groups of air sacs fill with air. Expiration and the following inspiration will move that air through the lungs and into the cranial air sacs. This is important to consider when using volatile anaesthetics in birds, because all inspired air is not expired prior to the next inhalation. The bird is easy to intubate with an endotracheal tube under direct visualisation. It can also easily be masked. Halothane or other precision vaporised gas anaesthesia are preferred because of the ability to control the amount of gas delivered.

### 8.4.2 Injection Anaesthesia (Code: BWAIN)

Injection anaesthesia can also be used. The route of administration and frequency of additional uses will be determined by the procedure. Injection techniques are described in a separate section.

## 8.5 SAMPLING OF BODY FLUIDS

The skin or other sites of sampling should be plucked and cleaned with alcohol to assure visibility and cleanliness.

### 8.5.1 Bleeding

The maximum amount of blood to be withdrawn to ensure survival is 10-15 ml/kg. If bleeding extends over 2-3 weeks, anaemia and abnormalities in serum proteins may result.

### 8.5.2 Bleeding From Peripheral Vessels (Code: BWBPV)

The two most common sources for blood collection in birds are the jugular vein and the brachial (wing) vein. The feathers at the site of the jugular vein must be plucked or wetted down with alcohol or water to allow visualisation and palpation of the vessel. With an assistant restraining the bird, occlude the vein in the area of the thoracic inlet. Insert a 23 gauge needle and slowly withdraw the blood.

The brachial vein is best accessed as it crosses the elbow joint. Then procedure for preparation and withdrawal is similar to the jugular vein site.

The needle should be withdrawn quickly and pressure applied to the site of venapuncture and hemostasis will result.

### 8.5.3 Bleeding by Cardiac Puncture (Code: BWBCP)

Anaesthetise the bird and place it on its back, head to right and feet to the left. With left hand, grasp sternum and palpate manubrium. With right index finger, palpate the heartbeat dorsocaudal to the manubrium. Remove finger and insert a 21 gauge needle directly into the heart. Do not move the needle around as this may lacerate the heart. If no blood flows,

withdraw the needle, carefully maintaining gentle negative pressure and try again. Approximately 30-40 ml of blood may be obtained from an adult chicken. Because of this inherent risk, this should be limited to a terminal procedure and euthanasia should follow when the bleeding is completed.

### 8.5.4 Bleeding by Terminal Procedures (Code: BWBTP)

Anaesthetise the bird and place on its back. Make a V-shaped incision through the skin and abdominal wall and proceed diagonally across each side. Locate the preferred vessel, post vena cava or aorta, and insert a 21-23 gauge needle. Gently apply negative pressure so as not to collapse the vein around the needle. If it does collapse, wait for vessel to refill and proceed.

## 8.6 INJECTION

The skin is prepared by plucking feathers and cleaning the site with alcohol. Injections can be made directly into the major vessels by needle or by catheter placement.

Phosphate buffered saline or other isotonic solutions are better than distilled water as a solvent/vehicle for injection. Oils are suitable for administration of lipid soluble substances or adjuvants, but absorption is delayed and this vehicle cannot be injected IV.

In general, the maximum quantity for an IV injection is 10 mg/kg body weight, but the dose depends on the route of administration.

IM: 0.25 ml/site
SQ: 5.0 ml/site
IV: 10 ml/kg

### 8.6.1 Intravenous (Code: BWIIV)

Insert a 23 gauge needle into the brachial or jugular vein. After removing the needle, blood flow stops with pressure.

### 8.6.2 Intramuscular (Code: BWIIM)

The preferred site for intramuscular injection is the pectoralis muscle. Palpate the large muscle mass on either side of the keel. Insert the needle, apply gentle negative pressure to assure that you are not in a vessel and inject. Three or four injections of 0.25 ml per site is acceptable.

### 8.6.3 Subcutaneous (Code: BWISQ)

The best sites for subcutaneous administration are the webbing, the axillary and inguinal areas. Large amounts of fluid (1 ml) can be deposited in these sites for absorption. Smaller amounts can be injected under the skin of the back or sides.

### 8.6.4 Intraperitoneal (Code: BWIIP)

An assistant can restrain the animal by holding the wings together with both hands. Place the bird in a slightly head down position to allow gravity to aid in moving the intestines cranially. Insert a 21-23 gauge needle in the lower right side. (In the female, a well developed ovary and oviduct is present on the left side.)

## 8.7 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. Only survival techniques are carried out on birds. Autopsy with sampling of organs after euthanasia is not considered a surgical technique. Surgical procedures require aseptic techniques, but not a formal operating room. They should be carried out in approved surroundings as specified by the presiding veterinarian. All survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 7 of the Application Form.

Body heat is rapidly lost during surgical procedures and a heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 8.7.1 Abdominal Surgery (Code: BWSA)

The techniques for abdominal surgery are used for surgical implants by a qualified veterinarian. The birds are anaesthetised using an inhalant anaesthetic, “Isoflor” mixed with oxygen.

After anaesthesia, the bird is restrained on an operating surface in an optimal position for the planned incision. For prolonged procedures, heated operating surfaces are used to maintain body heat.

Midline incision or flank approaches are most common for abdominal surgery. Feathers are removed from the area of the incision and the skin surface rendered aseptic with 70% alcohol and betadine solution. The area of the incision is then isolated in a sterile manner and the surgery proceeds using a sterile technique.

A scalpel is used to make an incision of the length and position desired for implantation of the minimitter in the abdominal cavity.

After completion of the procedure, care is taken to assure that hemostasis is satisfactory and all gauze sponges and other restraining devices are removed. The abdominal incision(s) is closed in two layers. The muscle and peritoneal layer is closed with a non-locking running stitch of absorbable or nonabsorbable suture, usually of 2-0 silk.

Care is taken to avoid inclusion of abdominal contents in the suture line. A running stitch is used to provide a continuous closure to avoid subsequent herniation of abdominal contents through the suture line. The skin layer is closed with a line of running or interrupted sutures. Alternatively, the skin may be closed with skin staples which are removed 10-14 days later. Care is taken to include the deep fascial layers of the skin in the superficial suture line. The skin surface is then cleaned, restraints are removed and the bird is allowed to recover from anaesthesia under frequent observation.

## 8.8 PHYSIOLOGICAL MEASUREMENTS

The bird is placed in a metabolic chamber and various measurements are done using specialist analysers connected to the chamber.

### 8.8.1 Respirometry (Code: BWPR)

Measurement of energetics and the response of a bird to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolic chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the bird and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

### 8.6.2 Thermoregulation (BWPT)

Selected adult birds are implanted with minimitters to measure adult body temperatures while the bird is incubating to determine how ground nesting birds are able to cope with nesting under severe thermal stress (see Sections 8.4 and 8.7 and quote the relevant anaestesia and surgery procedures). A single egg at nests where adults are implanted will have a thermocouple inserted 1-2mm below the shell surface in order to measure egg temperatures. This will involve drilling a small hole through the shell using a hand held drill, inserting a 0.5 mm diameter wire thermocouple and sealing the hole with superglue. Readings from the minimitter will be picked up by a circular aerial placed around the nest and linked to a receiver. Minimitters will be in the birds for ± 10 days before being retrapped and removed. Thermal experiments on chicks take place in a constant environment room where the chicks are exposed to different ambient temperatures (ranging from 15° to 30°C) to assess their ability to control their body temperature at different ages. A rectal thermocouple is carefully into the rectum of the bird to ascertain body temperatures of chicks under the different conditions. Depth of insertion is dependant on the size of the bird but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection. Birds are exposed to the various temperatures for no longer than 2 hours. Each bird is only be exposed to one temperature each day.

### 8.6.3 Measurement of food consumption (BWPF)

Birds are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some birds, particularly fossorial birds are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect droppings. The grid mesh size should be small enough to allow droppings to fall through but not damage the bird's feet. Daily weighing of birds, food eaten, water consumed and droppings produced are done.

Body composition of small birds, particularly water and fat content is determined following euthanasia of birds (see Section 8.9 and quote reference number of relevant euthanasia procedure).

### 8.8.4 Non- shivering thermogenesis (Code: BWPS)

Non-shivering thermogenesis (NST) or “chemical thermoregulation” is induced by norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of

NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the bird. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 8.8.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 8.8.1) and injected with saline (for controls) or NE solutions (1.5 mg/kg) subcutaneously (see Sections 8.6 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of birds should be avoided, since high $T_b$s above 40$^o$C may inhibit thermogenesis.

### 8.8.5 Activity measurements (Code: BWPA)

The responses of acclimated birds' activity to temperature, photoperiod and food availablity is established. Birds are acclimated to a photoperiod and ambient temperature for at least 1 week. The bird is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The bird is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the bird is removed from the metabolic chamber and returned to the vivarium.

### 8.8.6 X-Ray techniques (Code: BWPX)

The digestive tract of birds can be studied by X-rays following introduction of a suitable contrast agent such as gastrografin. The bird is trained before the experiment to be used to the position required for the x-ray measurement. The bird is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the bird is let into the room and all metals are removed form the area. The bird is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The bird is allowed to ingest appropriate feed coated with gastrografin. It is important that the bird does not move during the experiment. If it was not possible to train the bird to maintain the appropriate position, soft materials and sandbags are used to keep the bird in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 8.9 EUTHANASIA

In any type of euthanasia, care must be taken to assure death has occurred.

### 8.9.1 Overdose of Inhalant Anaesthesia (Code: BWEOD)

Place cotton or gauze soaked in anaesthesia in the bottom of a container and over it with a wire mesh or grate so the animal does not come into direct contact with the anaesthesia. After the animal's respiration stops, check it for complete loss of heartbeat.

### 8.9.2 Injectable Anaesthesia or Euthanasia Solution (Code: BWEIN)

An overdose of anaesthesia agent such as pentobarbital or a euthanasia solution such as T-61 may be given intravenously or directly into the heart.

### 8.9.3 Euthanasia in an Anaesthetised Animal by Physical Means (Code: BWEPM)

Cervical dislocation may be accomplished by holding the bird's head firmly in one hand and rapidly pulling firmly on the legs to dislocate the spine. This should only be accomplished by a trained, experienced technician.

### 8.9.4 Euthanasia in Which Drugs Cannot be Used (Code: BWECO)

Euthanasia by carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder via a plastic tube into a covered container. Place the bird in the container and close. After the bird no longer moves, check for loss of heart rate.

Other non-chemical means of euthanasia are described in the 1986 AVMA Panel Report on Euthanasia (see Appendix II).

# 9. STANDARD PROTOCOLS FOR DOMESTIC BIRDS

**(Galliformes- domestic fowl, pheasant, turkeys and quail)**

## 9.1 HANDLING (Code: BDH)

Most birds tend to be easily alarmed or frightened. Sudden movement or loud noises may upset an entire flock. At the time of capture, the wings must be restrained. With both hands, reach over the back of the bird and just prior to contact, drop the hands and pin the wings into the body while lifting the bird.

To carry- tuck the bird under one arm and maintain gentle pressure on the outer wing. This will calm the bird. Some birds may also require leg restraints in addition. Slip the hand between the legs and hold firmly in one hand.

The bird can be placed on a table on its back or side with the wings extended. Occasionally, a bird will lie quietly if a cloth is draped over its head or the head id tucked underneath the wing.

## 9.2 ANESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly use agents and dosages is provided in Appendix I.

### 9.2.1 Inhalation Anaesthesia (Code: BDAIH)

In birds, a series of air sacs communicates with relatively small lungs via bronchi. At inspiration, the caudal most groups of air sacs fill with air. Expiration and the following inspiration will move that air through the lungs and into the cranial air sacs. This is important to consider when using volatile anaesthetics in birds, because all inspired air is not expired prior to the next inhalation. The bird is easy to intubate with an endotracheal tube under direct visualisation. It can also easily be masked. Halothane or other precision vaporised gas anaesthesia are preferred because of the ability to control the amount of gas delivered.

### 9.2.2 Injection Anaesthesia (Code: BDAIN)

Injection anaesthesia can also be used. The route of administration and frequency of additional uses will be determined by the procedure. Injection techniques are described in a separate section.

## 9.3 SAMPLING OF BODY FLUIDS

The skin or other sites of sampling should be plucked and cleaned with alcohol to assure visibility and cleanliness.

### 9.3.1 Bleeding

The maximum amount of blood to be withdrawn to ensure survival is 10-15 ml/kg. If bleeding extends over 2-3 weeks, anaemia and abnormalities in serum proteins may result.

### 9.3.2 Bleeding From Peripheral Vessels (Code: BDBPV)

The two most common sources for blood collection in birds are the jugular vein and the brachial (wing) vein. The feathers at the site of the jugular vein must be plucked or wetted down with alcohol or water to allow visualisation and palpation of the vessel. With an assistant restraining the bird, occlude the vein in the area of the thoracic inlet. Insert a 23 gauge needle and slowly withdraw the blood.

The brachial vein is best accessed as it crosses the elbow joint. Then procedure for preparation and withdrawal is similar to the jugular vein site.

The needle should be withdrawn quickly and pressure applied to the site of venapuncture and hemostasis will result.

### 9.3.3 Bleeding by Cardiac Puncture (Code: BDBCP)

Anaesthetise the bird and place it on its back, head to right and feet to the left. With left hand, grasp sternum and palpate manubrium. With right index finger, palpate the heartbeat dorsocaudal to the manubrium. Remove finger and insert a 21 gauge needle directly into the heart. Do not move the needle around as this may lacerate the heart. If no blood flows, withdraw the needle, carefully maintaining gentle negative pressure and try again. Approximately 30-40 ml of blood may be obtained from an adult chicken. Because of this inherent risk, this should be limited to a terminal procedure and euthanasia should follow when the bleeding is completed.

### 9.3.4 Bleeding by Terminal Procedures (Code: BDBTP)

Anaesthetise the bird and place on its back. Make a V-shaped incision through the skin and abdominal wall and proceed diagonally across each side. Locate the preferred vessel, post vena cava or aorta, and insert a 21-23 gauge needle. Gently apply negative pressure so as not to collapse the vein around the needle. If it does collapse, wait for vessel to refill and proceed.

## 9.4 INJECTION

The skin is prepared by plucking feathers and cleaning the site with alcohol. Injections can be made directly into the major vessels by needle or by catheter placement.

Phosphate buffered saline or other isotonic solutions are better than distilled water as a solvent/vehicle for injection. Oils are suitable for administration of lipid soluble substances or adjuvants, but absorption is delayed and this vehicle cannot be injected IV.

In general, the maximum quantity for an IV injection is 10 mg/kg body weight, but the dose depends on the route of administration.

IM: 0.25 ml/site
SQ: 5.0 ml/site
IV: 10 ml/kg

### 9.4.1 Intravenous (Code: BDIIV)

Insert a 23 gauge needle into the brachial or jugular vein. After removing the needle, blood flow stops with pressure.

### 9.4.2 Intramuscular (Code: BDIIM)

The preferred site for intramuscular injection is the pectoralis muscle. Palpate the large muscle mass on either side of the keel. Insert the needle, apply gentle negative pressure to assure that you are not in a vessel and inject. Three or four injections of 0.25 ml per site is acceptable.

### 9.4.3 Subcutaneous (Code: BDISQ)

The best sites for subcutaneous administration are the webbing, the axillary and inguinal areas. Large amounts of fluid (1 ml) can be deposited in these sites for absorption. Smaller amounts can be injected under the skin of the back or sides.

### 9.4.4 Intraperitoneal (Code: BDIIP)

An assistant can restrain the animal by holding the wings together with both hands. Place the bird in a slightly head down position to allow gravity to aid in moving the intestines cranially. Insert a 21-23 gauge needle in the lower right side. (In the female, a well developed ovary and oviduct is present on the left side.)

## 9.5 SURGICAL PROCEDURES

Surgical procedures are classed in two groups, namely survival and terminal. Both require the same degree of anaesthesia and surgical care. In terminal procedures, the animal is not allowed to regain consciousness and is submitted to euthanasia at the conclusion of the procedure. Only survival techniques are carried out on birds. Autopsy with sampling of organs after euthanasia is not considered a surgical technique. Surgical procedures require aseptic techniques, but not a formal operating room. They should be carried out in approved surroundings as specified by the presiding veterinarian. All survival surgical techniques require consideration of post-operative analgesia. The veterinarian will be responsible for deciding the need and appropriate drug, dose and duration of post-operative analgesia. The procedure that you and the veterinarian decide to follow should be listed in your response to item 7 of the Application Form.

Body heat is rapidly lost during surgical procedures and a heated surface and monitoring of body temperature during surgery and recovery are required for procedures extending more than 5-10 minutes.

### 9.5.1 Abdominal Surgery (Code: BDSA)

The techniques for abdominal surgery are used for surgical implants by a qualified veterinarian. The birds are anaesthetised using an inhalant anaesthetic, “Isoflor” mixed with oxygen.

After anaesthesia, the bird is restrained on an operating surface in an optimal position for the planned incision. For prolonged procedures, heated operating surfaces are used to maintain body heat.

Midline incision or flank approaches are most common for abdominal surgery. Feathers are removed from the area of the incision and the skin surface rendered aseptic with 70% alcohol and betadine solution. The area of the incision is then isolated in a sterile manner and the surgery proceeds using a sterile technique.

A scalpel is used to make an incision of the length and position desired for implantation of the minimitter in the abdominal cavity.

After completion of the procedure, care is taken to assure that hemostasis is satisfactory and all gauze sponges and other restraining devices are removed. The abdominal incision(s) is closed in two layers. The muscle and peritoneal layer is closed with a non-locking running stitch of absorbable or nonabsorbable suture, usually of 2-0 silk.

Care is taken to avoid inclusion of abdominal contents in the suture line. A running stitch is used to provide a continuous closure to avoid subsequent herniation of abdominal contents through the suture line. The skin layer is closed with a line of running or interrupted sutures. Alternatively, the skin may be closed with skin staples which are removed 10-14 days later. Care is taken to include the deep fascial layers of the skin in the superficial suture line. The skin surface is then cleaned, restraints are removed and the bird is allowed to recover from anaesthesia under frequent observation.

## 9.8 PHYSIOLOGICAL MEASUREMENTS

The bird is placed in a metabolic chamber and various measurements are done using specialist analysers connected to the chamber.

### 9.8.1 Respirometry (Code: BDPR)

Measurement of energetics and the response of a bird to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolic chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the bird and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to $T_e$, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

### 9.6.2 Thermoregulation (BDPT)

Selected adult birds are implanted with minimitters to measure adult body temperatures while the bird is incubating to determine how ground nesting birds are able to cope with nesting under severe thermal stress (see Sections 9.4 and 9.7 and quote the relevant anaestesia and surgery procedures). A single egg at nests where adults are implanted will have a thermocouple inserted 1-2mm below the shell surface in order to measure egg temperatures. This will involve drilling a small hole through the shell using a hand held drill, inserting a 0.5 mm diameter wire thermocouple and sealing the hole with superglue. Readings from the minimitter will be picked up by a circular aerial placed around the nest and linked to a receiver. Minimitters will be in the birds for ± 10 days before being retrapped and removed. Thermal experiments on chicks take place in a constant environment room where the chicks are exposed to different ambient temperatures (ranging from 15° to 30°C) to assess their ability to control their body temperature at different ages. A rectal thermocouple is carefully into the rectum of the bird to ascertain body temperatures of chicks under the different conditions. Depth of insertion is dependant on the size of the bird but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection. Birds are exposed to the various temperatures for no longer than 2 hours. Each bird is only be exposed to one temperature each day.

### 9.6.3 Measurement of food consumption (BDPF)

Birds are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some birds, particularly fossorial birds are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect droppings. The grid mesh size should be small enough to allow droppings to fall through but not damage the bird's feet. Daily weighing of birds, food eaten, water consumed and droppings produced are done.

Body composition of small birds, particularly water and fat content is determined following euthanasia of birds (see Section 9.9 and quote reference number of relevant euthanasia procedure).

### 9.8.4 Non- shivering thermogenesis (Code: BDPS)

Non-shivering thermogenesis (NST) or “chemical thermoregulation” is induced by

norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the bird. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 9.8.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 9.8.1) and injected with saline (for controls) or NE solutions (1.5 mg/kg) subcutaneously (see Sections 9.6 and quote reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of birds should be avoided, since high $T_b$s above 40$^o$C may inhibit thermogenesis.

### 9.8.5 Activity measurements (Code: BDPA)

The responses of acclimated birds' activity to temperature, photoperiod and food availablity is established. Birds are acclimated to a photoperiod and ambient temperature for at least 1 week. The bird is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The bird is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the bird is removed from the metabolic chamber and returned to the vivarium.

### 9.8.6 X-Ray techniques (Code: BDPX)

The digestive tract of birds can be studied by X-rays following introduction of a suitable contrast agent such as gastrografin. The bird is trained before the experiment to be used to the position required for the x-ray measurement. The bird is preferably trained in the room where the experiment will be conducted. Everything required for the experiment is put into place before the bird is let into the room and all metals are removed form the area. The bird is food deprived at night and in the morning before the experiment. Experiments are done first thing in the morning. The bird is allowed to ingest appropriate feed coated with gastrografin. It is important that the bird does not move during the experiment. If it was not possible to train the bird to maintain the appropriate position, soft materials and sandbags are used to keep the bird in the correct position. The required shielding is used in such a way that it does not obscure the area of interest. The investigator should wear a lead apron. Serial micrographs are taken of the gastrointestinal tract or stools or fluoroscopy is used for continuous viewing of the contrast medium as it moves through the digestive tract. The gastrointestinal tract is measured by using a mixture of contrast medium and food. Retention time is measured by x-raying of first and last stools.

## 9.6 EUTHANASIA

In any type of euthanasia, care must be taken to assure death has occurred.

### 9.6.1 Overdose of Inhalant Anaesthesia (Code: BDEOD)

Place cotton or gauze soaked in anaesthesia in the bottom of a container and over it with a wire mesh or grate so the animal does not come into direct contact with the anaesthesia. After the animal's respiration stops, check it for complete loss of heartbeat.

### 9.6.2 Injectable Anaesthesia or Euthanasia Solution (Code: BDEIN)

An overdose of anaesthesia agent such as pentobarbital or a euthanasia solution such as T-61 may be given intravenously or directly into the heart.

### 9.6.3 Euthanasia in an Anaesthetised Animal by Physical Means (Code: BDEPM)

Cervical dislocation may be accomplished by holding the bird's head firmly in one hand and rapidly pulling firmly on the legs to dislocate the spine. This should only be accomplished by a trained, experienced technician.

### 9.6.4 Euthanasia in Which Drugs Cannot be Used (Code: BDECO)

Euthanasia by carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder via a plastic tube into a covered container. Place the bird in the container and close. After the bird no longer moves, check for loss of heart rate.

Other non-chemical means of euthanasia are described in the 1986 AVMA Panel Report on Euthanasia (see Appendix II).

# 10. STANDARD PROTOCOLS FOR CATTLE, SHEEP, GOATS AND OTHER FARM ANIMALS

## 10.1 HANDLING (Code: FH)

Handling procedures may include procedures associated with good farming practice. See **Standard Husbandry Practices** below.

In all experiments, cattle, sheep, goats or other farm stock are cared for, with expertise, by trained animal technicians. The investigators are not usually directly involved with handling large farm animals. When they are, it is with the assistance of trained personnel. . All procedures and practices applied to livestock must be competently performed and supervised by competent stockpersons or technicians.

For handling, the animals are herded into a suitable restraining area (for example, a crush pen or catch pens). The stock are evaluated daily by the technician and any problems are reported to the investigator in charge of the experiment.

Animal technicians and researchers should be familiar with acceptable ways of moving, driving, restraining, haltering, loading (boxing), dipping and weighing animals, and of lifting a recumbent animal and casting a standing animal. Techniques are described in “Practical Animal Handling” (X636.089.AND).

The people managing and handling cattle and sheep must be sensitive to the basic needs of the animals. The skills for managing and handling livestock include the ability to :

- work so that stress to the animal is minimised;
- use the natural behaviour of the animal;
- recognise the early signs of distress or disease and to initiate prompt and appropriate preventive or remedial action

Handling must be done, quietly and calmly, without cruelty or ill treatment.

- "Cruelty" means the wilful infliction of pain or suffering that in its kind or degree, or in its object, or in the circumstances in which it is inflicted, is unreasonable or unnecessary.
- "Ill treat" means beat, whip, kick, wound, maim, abuse, worry, torment, torture, terrify, infuriate, override, overdrive, overload, drive when overloaded, or by any act or omission whatsoever cause pain, suffering or distress.

Good stockpersons are flexible in their approach to animal management and handling and adapt to the needs of differing animals and circumstances.

***Cattle***

Cattle should always be handled quietly and, to the extent possible, in the cool of the day, especially during transport. Water should be available within the handling area. The number of cattle on the Research Farm should not exceed the capability of handling facilities or staff. When handling cattle, excessive noise, whips, canes, electric goads, etc. should be avoided. Races, spray-races, entrances and exits should be designed to take advantage of the social behaviour and movement patterns of cattle. The handling facilities should provide for efficient, quiet handling of cattle with non-slippery surfaces, and no projections into the yards or races which may bruise or injure cattle. Restraint should be the minimum necessary to perform management procedures efficiently and with operator safety.

Procedures and practices that cause pain should not be carried out if painless and practical methods of husbandry can be adopted to achieve the same result. Any injury, illness or

distress observed should be promptly treated. Frequency and level of inspection should be related to the potential risks to the welfare of the cattle, and may vary from daily to much longer periods. Cattle kept under intensive management in sheds, lots or yards should be inspected at least daily, fed daily and have ready access to water. Grazing cattle require supervision, according to the class of cattle, density of stocking, availability of suitable feed, reliability of the water supply, age, pregnancy and lactational status, climatic conditions and management practices.

***Sheep***

Practices that cause pain should not be carried out on sheep if painless and practical methods of husbandry can be adopted to achieve the same result. Sheep should not be allowed to suffer painful conditions for want of attention. Sheep should not be caught, dragged or lifted by the wool. They should not be lifted or dragged by the horns. The techniques used to restrain sheep should not cause injury or unnecessary distress. Generally, sheep should be handled or restrained by means of an arm under the neck and an arm around the rump. Propping the sheep on its rump so that it leans back on the handler's leg is a convenient method of restraint for many quick procedures such as foot trimming and shearing. Sheep should not be kept on their side or back for more than a few minutes at a time especially if the rumen is full or if they are heavily pregnant.

The frequency and level of inspection should be related to the likelihood of risk to the welfare of sheep. Hygienic precautions should be undertaken for all operations. Restraint used on sheep should be the minimum necessary to efficiently carry out procedures on them.

Sheep should be shorn annually; crutching, wigging and ringing may be required for hygienic reasons and to minimise impairment of vision and risk of fly-strike. Sheep affected with footrot may need to have diseased tissue pared away by a sharp instrument; paring should not be so severe as to make sheep unable to walk. Ingrowing horns should be trimmed or removed before they cause injury.

Sheep must not be tethered unless they can be adequately monitored. If they are tethered, sufficient feed and water must be available at all times. A collar which fits comfortably should be used and if it causes chafing or other injury it should be removed. Any sheep which continues to resist tethering after reasonable training has been undertaken should be released.

As a general rule, sheep should not be kept isolated from other sheep for any longer than necessary because they become distressed if left alone. Heavily pregnant sheep should be inspected at least once daily until they have lambed, to avoid them becoming cast.

## 10.2 IDENTIFICATION OF ANIMALS

***Cattle:*** The Royal College of Veterinary Surgeons has deemed hot branding of animals an unacceptable practice. There are a number of acceptable methods for permanently identifying cattle, which is a very necessary management procedure.

### 10.2.1 Freeze Branding of Cattle (Cryobranding) (Code FCIDF)

At the present time, the Research Farm does not possess the necessary equipment, nor have trained personnel, for freeze branding. A freeze branding expert should be appointed for this task until a technician has been trained and equipped. The area of hide to be branded should be shaved and cleaned with alcohol.

### 10.2.2 Ear-tagging of Cattle (Code FCIDG)

Ear tags should be inserted with the correct instrument for the design of tag used. A competent and confident stockperson or technician should perform the procedure. Animals must be firmly held in a crush pen.

Where ear tags are fitted to young animals enough space should be left between the fold of the tag and the edge of the ear, to allow for growth of the ear. Large plastic tags should not be fitted to young animals as the holes will become too big as the animal grows.

### 10.2.3 Ear Notching of Cattle (Code FCIDN)

Ear-notching should be performed by a competent animal technician, using the proper device. Wherever possible, an alternative method should be employed but, in extensive situations, notching may be the method of choice. It is less expensive than freezebranding, and avoids the use of ear-tags in situations where they are likely to be ripped out of the ear on trees and bushes.

### 10.2.3 Ear Tattoos of Cattle (Code FCINT)

Calves can be tattooed, using the correct instrument, as soon as possible after birth. The inside of the ear must be thoroughly cleaned with methylated spirits. The tattooed letters/numbers must be positioned between the ribs of the ear, and as deep into the ear as possible. A toothbrush is used to rub ink into the letter/number indentations.

***Sheep***: Ear tagging is the recommended method for permanent identification of sheep.

### 10.2.4 Ear-tagging of Sheep (Code FSIDG)

Tagging involves inserting a plastic or metal tag into the ear by punching a hole then clipping the tag into the hole or by clipping the tag directly through ear tissue. The number of tags used should be kept to a minimum, preferably not more than two per sheep.

When ear cutting or ear tagging, care should be taken to avoid cartilage ridges and blood vessels. Lambs should not be tagged within 24 hours of birth because of the risk of mismothering. If it is important to identify newborn lambs, a temporary raddle mark should be applied and every effort made to minimise disturbance of ewe and lamb.

## 10.3 STANDARD HUSBANDRY PRACTICES

The Royal College of Veterinary Surgeons (U.K.) views the following procedures as **acceptable**, provided they are governed by Codes of Practice: castration, disbudding, ear-notching, ear-tagging, ear-clipping and freeze branding. Dehorning and castration of adult animals is acceptable if performed by a veterinary surgeon.

Unless for therapeutic reasons, the following practices are **unacceptable**: teat removal and clipping of teeth in sheep. Corrosive chemical branding, hot branding and devoicing are unacceptable practices .

### 10.3.1 Castration

In all cases, the reasons for castration should be carefully justified. Researchers should give serious consideration to whether or not castration is really necessary. It should not be carried out unless it has significant management or experimental advantages, e.g. it may not be necessary in ram lambs that are to be slaughtered before 6 months of age.

The techniques of castration are best taught by a qualified or experienced person, through direct instruction in the field situation, and will not be described in detail here. All castrations must be performed by a suitably trained and experienced technician or, in certain cases, by a veterinary surgeon.

***Cattle***: there are three acceptable methods.

#### 10.3.1.1 Bloodless Cattle Castration: Burdizzo method (Emasculatome) (Code: FHCCL)

This method uses a large pair of pliers (Burdizzos) with two blunt crushing surfaces, which cut the blood vessel and vas deferens inside the scrotal sac. The sac itself is not cut. Eventually, the two testes shrivel and die. Although there is little risk of infection, there may be considerable pain at the time of the operation and swelling of the scrotal sac, for a period of weeks afterwards. Work is currently being conducted on the use of local anaesthetics/cortisone injections to alleviate transient but acute pain. This protocol is therefore subject to revision. Note, animals should be checked 3 to 4 weeks after the operation, to ensure successful castration has occurred.

This method is acceptable until the bull is TWO MONTHS old. Beyond two months of age, a veterinary surgeon must perform the operation.

Make sure the instrument is functioning properly (it can be tested on string folded between two pieces of thick brown paper). The joints in the instrument wear easily, making it ineffective. The animal must be firmly held in a crush pen, preferably in the standing position. It is important to ensure that the 2 cuts do NOT meet in the middle: it is possible to stagger the two cuts to avoid this possibility. It may be necessary to make two crush marks on each side of the scrotal sac. Care should be taken not to catch the skin on the inside of the leg.

#### 10.3.1.2 Bloodless Castration of Cattle: Rubber Rings (Code: FHCCR)

A tight rubber ring, designed specifically for this task, is placed above the testicles, with the proper instrument and with the calf in a sitting position. A check should be made that both testicles are in the scrotal sac, both before and after application of the ring. The ring grips the neck of the scrotal sac. Failure of the blood supply to the testes results in eventually shriveling up and destruction of the gonads. The scrotal sac falls away and there may be a slight risk of infection at this small open wound. It may be possible to slit open the rubber ring after three to four days, to relieve discomfort and possibly prevent total loss of the sac.

This method is acceptable until the animal is SEVEN DAYS old but, of the three methods is the least acceptable from a welfare point of view. It is rarely used.

#### 10.3.1.3 Surgical Castration of Cattle (Code: FHCCS)

This method involves an incision into the scrotal sac, cutting the vas deferens, pulling away the blood vessel and removing each testicle in turn. It is a very “sure” method of castration and the pain associated with this operation may be less than the methods above. However, there is an increased risk of infection.

This method is acceptable until the bull is TWO MONTHS old and should only be done from MAY onwards, when temperatures are dropping and flies are less of a problem. Thereafter, the operation must be performed by a veterinary surgeon, under local anaesthetic. This is not an easy operation below two months of age, so the use of a veterinarian is recommended at all ages.

A good knife, made of surgical steel must be used. The blade must be sharpened to razor

keenness. Ideally, a surgical scalpel should be used. There are two ways in which the cut can be made: removal of the whole bottom of the scrotal sac, or by making two vertical incisions, one for each testes. The former method is preferred as it allows free drainage of the wound. The animal must be firmly restrained in a crush pen, and the operator should be protected by a cross bar. The animal must be kept under observation for at least 48 hours to prevent excessive bleeding. The technician should have appropriate knowledge and drugs to deal with profuse bleeding. The use of cotton wool. soaked with adrenaline, has been advocated as an effective means of reducing bleeding.

***Sheep***

If lambs are to be castrated, the procedure should be carried out after maternal bonding has occurred (i.e. after 24 hours of age) and preferably before 6 weeks of age.

#### 10.3.1.4 Bloodless Castration of Sheep: Rubber Rings (Code: FHSCR)

The best method of lamb castration is the application of a rubber ring to the neck of the scrotum, using an elastrator, i.e. an applicator made for the purpose. This operation can be performed when the lamb is less than ONE WEEK old, by a trained stockperson. Anaesthetic is not necessary, but research is currently being conducted into alleviating the associated pain through the use of anti-inflammatory drugs, or local anaesthetic. The area where castration is carried out, the equipment used, the lambs themselves and the operator's hands should be as clean as possible and lambs should be dry. The elastrator and rubber rings can be dipped in dilute antiseptic before application.

#### 10.3.1.5 Surgical castration of Sheep (Code: FHSCS)

Surgical castration by laypeople is not recommended. It is likely to cause greater and more prolonged distress than other methods, together with an increased risk of adverse consequences following the operation, such as excessive bleeding, hernias (prolapse of intestine into the scrotum) and infection of the wound. If surgical castration is to be carried out by a layperson, veterinary advice should be obtained to ensure that the correct method is used to keep pain and distress to a minimum, and that the operation is carried out as hygienically as possible to reduce the risk of wound infections. Over THREE MONTHS of age, a veterinary surgeon must perform the castration, using anaesthetic.

### 10.3.2 Tail Docking (Code: FHTD)

The docking of lambs' tails is carried out to help prevent faecal dag formation and flystrike and to facilitate shearing.

To minimise the risk of infection, the operating area, equipment used, the lambs themselves and the operator's hands should be clean. The procedure should not be carried out on wet lambs or in wet weather, because this increases the risk of infection.

Tail docking must be done within ONE WEEK of age and performed by a competent stockperson. A rubber ring is used to restrict the blood flow to the tail. Anaesthetic is not necessary, but the same comments apply as for castration, as regards relieving discomfort.

Tail docking, at any age, is prohibited unless sufficient tail is retained to cover the vulve of female sheep and the anus of male sheep. This may help ensure that when the tail is lifted for defaecation the caudal folds on either side are raised and the faeces directed away from the body, thereby helping prevent faecal contamination of wool and helping prevent fly-strike. If tails are very short the caudal folds are not raised and soft faeces are more likely to soil the area below and on either side of the tail.

### 10.3.3 Disbudding (horns) (Code: FHD)

Disbudding of calves, other than by chemical cauterisation, within one week of age, is deemed inhumane, unless carried out under anaesthetic, according to the Protection of Animals (Anaesthetics) Act (amended 1982) in the U.K. However, chemical cauterisation is now widely out of favour, often resulting in severe caustic burns, and considerable, lasting discomfort for the animal. It has been common practice in South Africa to use a hot iron to cauterise and sever the nerves the horn bud, without use of local anaesthetic. The same act, mentioned above, states that disbudding of calves without local anaesthetic is an operation performed *without due care and humanity.* The Protection of Animals Act (1982) (U.K.) states that calves must be given a local anaesthetic before being dehorned or disbudded.

The procedure is best carried out between three to six weeks of age, the earlier the better. The horn bud should be big enough to be clearly felt, but not too large to fit in the disbudding iron. The nerve to the horn runs out from behind the eye and underneath a small overhanging ledge of bone which forms part of the skull. See attached diagram. Use a short needle to inject 2.0 ml of anaesthetic under the plate, on both sides. Slightly withdraw the plunger of the syringe before injecting the anaesthetic to check that veins and arteries running under the plate have been missed. Intravenous injection of anaesthetic can cause collapse. Leave the calf for about 5 minutes to allow the anaesthetic to take effect.

The technique of disbudding needs to be performed by a trained technician. The procedure is detailed in “A veterinary book for dairy farmers” (X636.214.2.BLO), pages 354 to 357. The wound should be treated with gentian violet or a similar antibiotic after the operation.

### 10.3.4 Dehorning and Trimming Horns (Adult Cattle) (Code: FHT)

The Protection of Animals (Anaesthetics) Act (amended 1982 and the Veterinary Surgeons Act (amended 1982) in the U.K. deem that the dehorning of any animal, of any age, should be carried out by a trained stockperson, or a veterinarian, with the use of local anaesthetic. Wherever possible, a veterinarian should be requested to perform this task. Dehorning should be performed on cool, dry days in the late afternoon, when flies are less of a problem.

The trimming of horns for therapeutic purposes (for example, where the horn is turning back into the face) should be performed by a trained technician who has detailed knowledge of the internal anatomy of the bovine horn and surrounding skull. Only the dead tissue at the tip of the horn should be trimmed, without anaesthetic.

### 10.3.5 Crutching, Dagging and Face-Wool Removal (Sheep) (Code: FHC)

Wool under and to either side of the anus can become laden by clumps of wet or dried faeces (faecal dags). These dags should be trimmed off as they can cause discomfort and inflammation of the underlying skin and they may attract flies.

Wool growing around the eyes of sheep should be trimmed if it obscures their vision.

Before lambing wool around the udder should be trimmed if necessary to allow the lamb unimpeded access to the teats. Wool around the vulva should be trimmed to facilitate lambing.

### 10.3.6 Shearing (Sheep) (Code: FHW)

It is strongly recommended that sheep be shorn at least once a year. Shearing equipment should be clean. Sheep should not be shorn if the forecast is for cold wet weather. Shearing must be carried out skilfully and/or carefully to ensure that shear cuts are kept to a minimum. Sheep should not be handled roughly. Extensive or severe cuts must be treated as soon as possible. Care should be taken to avoid accidental shearing cuts to teats and prepuces.

As sheep with a full rumen may suffer distress when shorn, those which have been on lush pasture should be rested off pasture for a minimum of 6 hours before shearing.

Provision should be made for extra feed and for appropriate shade and shelter for sheep after shearing. Newly shorn sheep require up to 40% more feed for 3 weeks or more after shearing to sustain body temperature and maintain body condition. Maintenance requirements may be increased for 6 to 8 weeks after shearing. There should be ready access to covered yards or effective shelter for several weeks after shearing in case of cold wet weather. After shearing sheep may be keen to get back to pasture, but without their wool they can be injured by crowding through gateways. Care should be taken not to hurry newly shorn sheep out of the yards and through gateways.

Prelamb shearing: If prelamb shearing is carried out effective shelter must be available. The ewes must be well fed because if they are hungry they may graze rather than shelter with their lambs. Prelamb shearing should be carried out early enough in the day to give ewes time to feed and find shelter before dark.

### 10.3.7 Dipping and Drenching

#### 10.3.7.1 Dipping (Code: FHDI)

***Cattle:*** no facilities exist on the Research Farm for plunge dipping. Cattle are dipped in a spray-race. The spray-race should be constructed, maintained and operated in a manner that minimises injury to animals.

The dipping chemicals should be diluted according to the manufacturer's instructions.

Dipping is a regularly repeated procedure. Animals should be handled considerately. Excessive use of force or goads should be avoided.

Calves are not usually dipped before two months of age. Where calves form part of the group of cattle to be dipped, the through rate must be slowed so that animals in the draining-area following the spray-race do not become over-crowded, endangering the calves.

***Sheep:***. sheep are dipped in a plunge dip. Experienced stockpersons and technicians ensure that the animals are not injured as they are moved through the dip. Sheep must not be held by the horns. The animals are lowered into the dip one by one.

***Goats:*** goats can present a special problem in a plunge-dip as they may try to jump across the water. To prevent injury, goats can be held by the horns as they are lowered into the dip, to improve control. The operator must ensure that the animal's full weight is not borne by the horns.

#### 10.3.7.2 Drenching (Code: FHDR)

The animal's head should be lifted and the drench gun inserted from one side. The head should be held up until the medication is swallowed. Patient, gentle handling is needed, particularly where drenching is to be done on a regular basis. The animal must be in the standing position to allow it to swallow properly, without water getting into the lungs. The medication should be delivered slowly for the same reason.

Care should be taken not to knock the teeth with the drenching gun or to damage the throat with the delivery system.

***Cattle:*** most cattle should be caught in a crush pen (dairy cattle may allow drenching in the milking parlour). Packing animals into a race in order to drench along the line is not recommended. Animals can get squashed, fall down or trampled. The animal should be caught in the crush pen and its head restrained through a neck clamp.

***Sheep:*** Sheep can most easily be drenched when tightly packed in a race, facing in the same direction. The operator should start at the back and move forwards along the line.

## 10.4 ROUTINE VACCINATIONS, OTHER INJECTIONS AND IMPLANTS

Cattle should be securely restrained in a crush pen. Sheep and goats should be restrained in a crush pen or held securely by a second worker. Suitable methods of restraint and handling are described in "Practical Animal Handling" (X636.089.AND). What defines appropriate restraint may depend on the size of the animal, its disposition and the type of injection. Appropriate restraint means that the operator is able to safely approach the animal and administer the medication or implant, and that the animal is not injured by either the restraint or injection/implantation. The technician should try to anticipate how the animal will react to the injection/implantation and use protective/restraining bars accordingly.

### 10.4.1 Injections

The decision to administer injectable medications should be based on sound principles. The effectiveness of some injectable medicines and biologicals depends on the appropriate timing of administration to the life cycle of the animal. Injection records should be kept to avoid unnecessary repeat treatments and to help in troubleshooting if there is a problem with animal health. The label on the injectable material should indicate the appropriate route of administration. If the choice exists between giving the material subcutaneously or intramuscularly, the former is often preferable to reduce the risk of lesions in the carcass.

The label recommendations for dosage, route of administration and recommended dosage per site must be followed. Use clean, sharp needles and wipe the rubber stopper of the bottle clean before use. The risk of infected sites can be reduced by maintaining clean working conditions and equipment. After use, syringes can be disinfected by washing in hot soapy water, rinsing with alcohol and drying before reassembly. Syringes for use with live or modified-live vaccines should be sterilised using dry or moist heat, as soaps, alcohol and other disinfectants may inactivate these vaccines. If it is necessary to rinse out a syringe during use, sterile saline or sterile water should be used. No more than 10 animals should be injected with the same needle and the technician should assess the risk of injection site blemishes and cross-contamination in using a needle on more than one animal. Where needles are not replaced after every usage, a separate, clean needle should always be used on the medicine container.

As a general rule, use the thinnest (largest gauge size needle) that the medicine will pass through. Needles with aluminium hubs should be used wherever possible, especially in cattle. Broken needles should be removed and, if necessary a veterinarian should be called to effect this removal so that needles do not enter the food chain. Do not straighten or reuse bent needles.

Routine inspection of injection and implant sites should happen every time the animals are confined in a race.

#### 10.4.1.1 Subcutaneous Injections (Code: FISO)

Subcutaneous injections should be administered in front of the shoulder. Needles should be 2.5 cm or less in length for any age animal. Give subcutaneous injections at the base of a

"tented" fold of loose skin, lifted away from the animal with the technician's free hand. This minimises the risk of injecting into the muscle, but care should be taken that the needle is not pushed through both layers of skin, and medication wasted. Maximum volume is 5.0 ml/site. Any bleeding after injection can be controlled with pressure.

#### 10.4.1.2 Intramuscular injections (Code: FIIM)

Usually given in the shoulder or thigh muscles. Clean the skin. Rub the injection site vigorously, and pat the area hard with the site of the fist. A clean sharp needle should be then rapidly placed into the site, the dose administered and the needle quickly removed. A longer needle is used than for subcutaneous injections and it is possible to connect the syringe after inserting the needle. If the medication should not be placed directly into the bloodstream, it may be necessary to draw back on the syringe a little and to observe for blood, before beginning an intramuscular injection. If the material is of an irritating nature, maximum volume is 1.0 ml/site.

#### 10.4.1.3 Intavenous Injections (Code: FIIV)

Use a rope around the animals neck, low down above the brisket, to get the vessels in the cavities on both sides of the neck to bulge out. A longer, thicker needle should be used than for the two procedures above (say, 6cm in cattle). Take care that the needle does not go all the way through the vein. The syringe is normally connected after insertion of the needle. Release the tension on the rope. The medication supplied should be at room temperature and allowed to enter the vessel at not more than 350 cc/3 minutes. Intravenous injections are normally given into the exterior jugular vein. Suitable intravenous vehicles are phosphate buffered saline (PBS) or other isotonic and physiologic solutions. The amount administered should not exceed 500ml / 45 kg.

### 10.4.2 Implants (Code: FIMP)

The manufacturer's directions should be read before using implants and use the proper device for the implant concerned. Unless other wise stated the site of implantation is always the middle third of the back side of the ear. If part of the ear has been lost, the implant can be placed further back. A competent technician should perform the implantation. The ear should be clean and dry and the implanting needle thoroughly cleaned. The needle should be cleaned between animals with diluted disinfectant. Do not use undiluted disinfectant as this can cause tissue irritation and expulsion of the implant. Alcohol should not be used. Six grammes of chlorhexine (blue disinfectant) in 1 litre of water works well. The needle can be coated in a non-irritating antibiotic. Visually inspect and palpate the implant site after the operation to ensure proper placement of the implant. The entry hole can be closed by pressure.

The technician should watch for abscesses, expelled implants, cartilage embedment and bunched pellets.

## 10.5 SAMPLING OF BODY FLUIDS

### 10.5.1 Bleeding from the Jugular Vein (Code: FBJV)

The area should be appropriately prepared and cleaned and the hair/wool clipped if necessary. The operator finds the jugular vein by compression at the sternal notch of the neck, with thumb or finger. The vein quickly becomes engorged and can be palpated with ease. A needle of appropriate size should be used, depending on how much blood is to be withdrawn. The needle is introduced into the vein in a cephalic direction. Use of a sealed Vacutainer makes withdrawal of blood an easy procedure. For small test bleedings, a 20 gauge needle is adequate. For larger volume bleedings, use blood drawing sets designed

for human use. These use 18 gauge needles and sterile tubing, connected to soft plastic blood bags, or to standard vacuum glass blood drawing bottles. The tubes, bottles or bags may contain an anticoagulant such as heparin. Blood drawing is normally limited to 500 ml/ 45 kg body weight.

### 10.5.2 Catheterisation of a Vein (Code: FBCV)

Where blood is to be sampled on a regular basis, over an extended period of time, it is advisable to fit a catheter into the vein (normally the jugular vein). This procedure should be carried out by a veterinary surgeon, under anaesthetic. Maintenance of the catheter is the responsibility of the researcher in charge of the experiment. The researcher should work closely with the animal technician to ensure that the site of entry of the catheter does not become infected and that the action of the catheter is maintained by regular flushing with sterile saline solution. Animals bled on a regular basis should be given some form of iron supplementation.

## 10.6 ELECTROEJACULATION

### 10.6.1 Electroejaculation of Bulls (Code: FLJC)

For reasons of operator safety, electroejaculation of bulls should only be performed under the supervision of a trained professional. At present, Mr Philip Jack, who has worked for Taurus for many years is the operator of choice. Electroejaculation of bulls is not to be used as a regular procedure. Wherever possible, the bull shoull be trained to mount a teaser bull and semen collected into an artificial vagina.

### 10.6.2 Electroejaculation of Rams (Code: FLJS)

**Electroejaculation** of rams is a potentially stressful and painful procedure, particularly where the operator is unskilled or insensitive. Electroejaculation of rams should only be perfomed for isolated samples of semen, and only when collection of a sample is **absolutely necessary**. Collection of semen by electroejaculation is only warranted where a thorough physical examination has suggested fertility problems. **It is emphasised that electroejaculation of rams is not to be used as a routine, repeated procedure**.

The procedure must be carried out with the correct, well-maintained equipment and an experienced operator should be present at all times, and preferably in control of the equipment. The most appropriately experienced operator in Natal at the present time is Butch Bosch of Allerton Veterinary Laboratories.

Where electroejaculation of rams has to be performed, the researcher, technician and experienced operator must all have read the comprehensive guidelines supplied by the Society for the Study of Animal Breeding (a division of the British Veterinary Association) - guidelines attached.

### 10.6.3 Training a Ram for Semen Collection (Code: FLJT)

Where repeated collections of semen are called for, the preferred method of collection is to use a teaser ewe and an artificial vagina. The ewes can be brought into heat using progesterone pessaries and pregnant mare serum gonadotropin (PMSG) injections. Alternatively, a more lasting oestrus can be achieved by using oestradial cypionate (ECP - Upjohn Co.), given as weekly intramuscular injections of 2.5 mg, for three weeks. Training requires planning and forethought, but the quality of the ejaculate produced is better than that obtained by electroejaculation. Where more than one collection is to be made on each ram, the ram must be trained for semen collection using an artificial vagina.

## 10.7 RUMEN FISTULATION AND DIGESTIVE TRACT CANNULATION (Code: FRC)

These are veterinary procedures and must not be attempted by an animal technician or researcher. The most appropriately experienced veterinarian in this area is Dr M MacFarlane. The animal must be tranquilised and the operations must be performed under local anaesthetic. Rumen fistulation is performed through the left flank of the animal. The rumen wall and the external skin of the animal are stitched togeter, forming an opening. A rumen cannula, made of pliable rubber material and large enough to allow passage of samples into and out of the rumen, is fitted and secured into this opening.

Post operative care, by trained and dedicated animal technicians, is very important in operations of this nature. The animal must be given a long acting antibiotic and the wound must be cleaned and disinfected regularly until completely healed. Regular checks and cleaning should continue as long as the animal is being used for research. The animal should be slaughtered when this level of care is no longer justified.

## 10.8 EUTHANASIA

***General principles***

All animals in research establishments must be treated effectively or humanely killed, **without delay,** if they are found to be suffering unnecessarily or will suffer adverse effects as the result of regulated procedures. If an animal undergoing a regulated procedure shows signs of severe pain or distress which cannto be allleviated, the researcher or technician must ensure tha the animal is killed painlessly, without delay.

A person who is competent to kill animals, using authorised procedures, must be available at all times when scientific experiments involving animals are being conducted.

Animals shoudl be handled sympathetically but firmly and with care. As a general rule, animals to be killed should be removed from the presence of other animals. Conditions in which animals become frightened or agitated should be avoided. The requirement for different species should be taken into consideration.

When using methods which do not result in instant death, the animal shoudl be rendered unconcious (stunned) using a method which **ensures** that the animal remains unconscious until it dies.

No person shoudl be expected to kill an animal unless they are willing and feel confident to do so in the prescibed manner. it is important that the killing is carried out quickly and confidently, in the context of routine handling, with which the animal is familiar.

It is accepted that killing a pregnant animal in late gestation will kill the unborn foetus. There is no evidence to suggest that the death of the unborn foetus would be distressful or inhumane, so long as the foetus is not physically damaged in any way.

***Ensuring the animals are dead***

Whatever method of humane killing is used, death must be confirmed in all cases before animals are disposed of or left unattended. Lack of a pulse or heart beat implies permanent cessation of the circulation system, but care should be taken - pulses can be hard to find. Death should not be assumed until all signs of reflex activity have ceased. In small animals, death can be ensured by dislocation of the neck. Exsanguniation will abolish the blood supply to the brain and ensure that the animal is dead. The onset of rigor morits, at which time the muscles become stiff, is an indication of death. However, the time taken to go into rigor mortis depends on the animal’s size, its physiological state before death and the environmental temperature.

### 10.8.1 Injectable Anaesthetic or Euthanasia Agent in Cattle (Code: FECIN)

Overdose of an injectable anaesthetic or euthanasia agent, which induces unconsciousness as quickly as possible. Intravenous injection of the agent is preferred. Direct injection into the heart, in the conscious animal, can be painful and should not be used.

**Note:** neuromuscular blocking agents are not appropriate for humane killing of animals. Death by inhalation of anaesthetic agents is too slow in alrger animals to be considered humane.

Personnel must be protected from anaesthetic gases and vapours.

### 10.8.2 Brain destruction in Cattle (Code: FECB)

Destruction of the brain by a free bullet or captive bolt pistol, followed by exsanguination. This method requires considerable skill and should be carried out, wherever possible, unless for emergency euthanasia, in a slaughterhouse.

### 10.8.3 Injectable Anaesthetic or Euthanasia Agent in Sheep (Code: FESIN)

Overdose of an injectable anaesthetic or euthanasia agent, which induces unconsciousness as quickly as possible. Intravenous injection of the agent is preferred. Direct injection into the heart, in the conscious animal, can be painful and should not be used.

### 10.8.4 Electrical stunning (Code: FEST)

Electrical stunning, followed by exsanguination. This procedure should be carried out in the abattoir facility at the Research Farm, by experienced and confident personnel.

### 10.8.5 Head-to-back Electrical stunning (Code: FESTB)

Head-to –back electrical stunning, in which cardiac fibrillation results in permanent insensibility. The carcass is then exsanguinated normally. This procedure must only be carried out by personnel experienced in the placement of electrodes and use of appropriate voltage and current.

# 11. STANDARD PROTOCOLS FOR BATS

## 11.1 CONSIDERATIONS FOR ETHICAL HOLDING, HANDLING AND CARE OF BATS:

**Social interactions:** Attempt to minimise the impact that holding or removal of individuals will have if they are from groups with complex social interactions, or solitary, or not tolerant of other species (Animal care and use committee, 1998).

**Holding and transporting captive bats:** The type of holding device will depend on the above consideration, the time to be held, and the number of bats. They should not be overcrowded, or held longer than necessary (Kunz and Kurta, 1998). However, the container should be small enough to minimise movement of the animals, thus reducing the risk of injury (Wilson, 1988). They should not be left in potentially stressful environments, and should be protected from extreme temperatures and forced convection (Kunz and Kurta, 1988). A variety of holding devices are suggested by Kunz and Kurta (1988).

**Maintenance of bats in captivity before processing:**
They must be maintained under conditions meeting their needs for food, moisture, space and microclimate.

**Cage design** - This will depend to some extent on the duration in captivity. For short periods a cage that is big enough to allow the bat to fully stretch its wings would be sufficient. Over longer periods most species do better in cages sufficiently large enough to allow flight (Wilson, 1988). Attempting to mimic the natural roost requirements of different species will determine whether there are perches for bats to hang free from, cloths to hide behind, and so on.

**Food** – Insectivores can be maintained on a diet of *Tenebrio molitor* larvae (mealworms). Frugivores can be fed a variety of different foods (Wilson, 1998).

**Feeding schedule** – May be a single daily feeding, which is best during the bats night cycle.

**Water** – Provided *ad libitum*.

**Temperature** – Attempt to mimic the normal ambient temperature of the species.

**Light** – Attempt to mimic the natural situation of the species. During the light period keep disturbances to the individuals to a minimum to allow then to sleep.

**Sanitation** – Nothing different to the rodents.

## 11.2 HANDLING (CODE: CH)

At the time of capture, the wings must be restrained. With both hands, reach over the back of the bat and just prior to contact, drop the hands and pin the wings into the body while lifting the animal.

To carry, tuck the bat under one arm and maintain gentle pressure on the outer wing. This will calm the bat. Some bats may also require leg restraints in addition. Slip the hand between the legs and hold firmly in one hand.

The bat can be placed on a table on its back or side with the wings extended. Occasionally, a bat will lie quietly if a cloth is draped over its head or the head id tucked underneath the wing.

### 11.3 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. A list of commonly used agents and dosages is provided in Appendix I. The veterinarian(s) will assist in selections that are adequate and yet convenient for the investigator. Analgesia requiring controlled substances will be the responsibility of the veterinarian. In completing the Application Form, indicate the method and code (inhalation/injection, etc.) to be used and list the specific agents in your response to item 8.2. Remember that ether is both flammable and explosive. It may not be used unless an explosion-proof hood or room is available and must be disposed of in explosion-proof containers. Proper ventilation is needed with all inhaled anaesthetics for user safety.

#### 11.3.1 Inhalation Anaesthesia (CODE: CAIH)

A vaporising anaesthetic machine, calibrated for isoflurane, is used with pure medical oxygen to supply a gas mixture of 0-5% isoflurane in oxygen, as required. During initiation of anaesthesia, place the animal in a clear Perspex respirometer and vent an initiation gas mixture of 2-4% isoflurane, depending on the species, into the respirometer at flow rates of 1-2 litres/min, depending upon the size of the animal. Place a cloth over the respirometer to quieten the animal, but remove it periodically to monitor the progress of anaesthesia. Once anaesthetised (usually after ca. five minutes), remove the animal quickly from the respirometer and place it on the preparation table. Place a gas mask appropriate for the shape of the animal over the facial region covering all respiratory surfaces. Lower the maintenance gas mixture to 1-2% isoflurane in oxygen, depending upon the species. Once pre-operative preparation of the animal is complete, move it to the surgery table. Generally the gas mixture can be lowered on commencement of suturing in order to minimise the total time under anaesthesia, and hence hypothermia. At all times during anaesthesia, monitor the rectal or cloacal temperature with a digital thermometer and rectal or cloacal probe. If the body temperature decreases significantly during lengthy procedures, the animal must be heated with a heating pad or infra-red lamp until normothermia is restored.

#### 11.3.2 Injection Anaesthesia (Code: CAIN)

Injection anaesthesia, using barbiturates or other agents such as ketamine, can also be used (see Appendix I). The route of administration and frequency of additional doses will be determined by the length of the procedure. In procedures requiring assessment of vascular physiologic responses, the choice of anaesthetic must be carefully considered to avoid unwanted vascular effects. Injection techniques (IV, IM. IP, etc.) are described in a separate section.

### 11.4 SAMPLING OF BODY FLUIDS

The skin, or other sites of the sampling, should be properly prepped with alcohol to ensure visibility and cleanliness.

#### 11.4.1 Bleeding

The maximum amount of blood to be withdrawn to insure survival is around 200 microlitres per adult bat body weight once a week. Larger volumes (up to maybe 400 microlitres per week) may be appropriate for larger species of fruit bats. If bleeding

extends over 2-3 weeks, anaemia and abnormalities in serum protein may result.

#### 11.4.1.1 Bleeding from Peripheral Vessels (Code: CBPV)

Orbital sinus: Hold the anaesthetised animal's head firmly against a work surface with your thumb and press down just behind its eye; pull the skin back to open the internal angle of the lid. Gently slide in the microhaematocrit tube or Pasteur pipette through the conjuntiva of the medial canthus. When you reach the sphenoid region, slide the tube down to the orbital sinus, then rotate the tube until the blood is collected. Remove the tube, release pressure and clean blood from the area.

Tail clipping: Using a scalpel blade, transect the tail completely about 3 mm from the tip; blood flow can be increased by placing the tail in warm water for 1-2 minutes before transection. Finger pressure stops bleeding.

Toe clipping: The technique is similar to tail clipping. Blood can be drawn once from each toe.

#### 11.4.1.2 Bleeding by Cardiac Puncture (Code CBCP)

Anaesthetise the animal and place on its back with its length perpendicular to you. With your left thumb and forefinger placed on each side of the thorax, compress slightly and insert a 21 gauge needle and a 3 cc syringe under the xyphoid cartilage. Hold the needle at a 30 degree angle and push forward slowly while aspirating; your left fingers may hold the barrel as your right hand pulls the plunger slowly and steadily. To be sure of its correct placement, feel for the heartbeat against the needle. If no blood flows, withdraw the needle slowly with continued aspiration. Although blood may safely be withdrawn from each bat once per week, all the blood constituents may not have returned to normal values. This is a risky procedure and should be reserved for terminal procedures.

#### 11.41.3 Bleeding by Surgical Approaches (See surgical section)

#### 11.4.1.4 Bleeding by Terminal Procedures (Code: CBTP)

Posterior vena cava: Anaesthetise the bat and place it on its back. Make a V-shaped incision through the skin and abdominal wall at the base of the abdomen and proceed diagonally across each side ending dorsolaterally at the thorax. Lay the skin over the thorax and deflect the gut to the bat's left, then push the liver forward and enter the vena cava at the level of the kidneys. Straddle the vessel with he fingers of your free hand and insert an appropriately sized needle (27). Withdraw the plunger slowly until the vein collapses; wait for it to refill, then continue. Turning the needle's bevel away from the wall and tenting the vessel will increase flow. When completed, proceed with euthanasia.

Dorsal aorta: Perform similarly to the vena cava technique, but enter the aorta just anterior to the distal bifurcation. When completed, proceed with euthanasia.

Axillary vessels: Place the anaesthetised bat on its back with the tail towards you. Stretch one forelimb and hold it in place with a pin through the foot. Make a skin incision in the axillary region; the bottom skin edge can be held up and used as a bowl to collect blood. Cut the axillary vessels with scissors; as the blood wells up , collect it with a Pasteur pipette or syringe. Note: This blood will be contaminated with tissue fluids. When completed, proceed with euthanasia.

### 11.5 INJECTIONS

The skin is prepped to assure maximum visibility and cleanliness. Injections can be made directly into the major vessels by needle or catheter placement. The carotid artery, jugular vein or the femoral vessels can be used. These techniques are considered surgical procedures and are addressed in the surgery section. Preparing the skin may

include shaving, cleaning with alcohol or a full surgical prep.

Phosphate buffered saline (PBS) or other isotonic solutions are better than distilled water as a solvent/vehicle for injections. Distilled water causes some hemolysis when given IV and pain when given SQ. Oils are suitable for administration of lipid-soluble substances or in adjuvants, but absorption is delayed and this vehicle cannot be injected IV.

In general, the maximum quantity for an IV injection is 1 ml/100 gm body weight, but the dose really depends on the route of administration. The pH should be physiologic (~7.4).

IM: Maximum 0.1 ml at any site in adult
SQ: Maximum 0.25 ml at any site in adult
IV: More than 1ml/100 gm may cause pulmonary oedema

### 11.5.1 Intravenous (IV) (Code: CIIV)

Insertion of the needle (25-30 gauge) in the lateral tail veins should start distally and work proximally in relation to the heart (because of direction of blood flow) for subsequent injections. After removing the needle, blood flow stops with pressure.

### 11.5.2 Subcutaneous (SQ) (Code: CISQ)

Injections are under the skin of the back or sides. Clean the site and pass the needle through the bat's skin in an anterior direction. To assure the needle is positioned correctly, move up and down in position to assure that it is SQ; if tenting of skin is not discernible, withdraw slightly until SQ.

### 11.5.3 Intramuscular (IM) (Code: CIIM)

This site should be avoided if possible because of the small muscle mass in bats. A usual intramuscular site is the large muscle of the rear limb. Insert the needle in a posterior direction away from the femur and sciatic nerve. If the needle penetrates too deeply, you may encounter bone or miss the muscle mass and fall into the fascial plane. Aspirate before injecting to assure needle placement is not intravascular.

### 11.5.4 Intraperitoneal (IP) (Code: CIIP)

Immobilise the animal by holding the skin of its neck, and the wings. Tilt the animal's head down to allow gravity to push the viscera cranially. After cleaning the skin, insert the tip of the needle into the lower quadrant(s) of the abdomen, away from the midline. Inject immediately to push away the viscera and withdraw the needle. A large gauge needle is less likely to penetrate the viscera.

### 11.5.5 Footpad (FP) (Code: CIFP)

Materials can be injected into the rear footpads only, using a small gauge needle inserted under the skin from above the heel.

### 11.5.6 Intragastric (oral) (Code: CIIG)

Not recommended.

## 11.6 SURGICAL PROCEDURES

### 11.6.1 Anterior Neck (Code: CSAN)

### 11.6.2 Thymectomy in Adults (Code: CSTH)

### 11.6.3 Intrathymic Injection (Code: CSIT)

### 11.6.4 Abdominal Surgery (Code: CSA)

### 11.6.5 Skin Grafting (Code: CSSG)

### 11.6.6 Castration (Code: CSCR)

## 11.7 PHYSIOLOGICAL MEASUREMENTS

The bat is placed in a metabolism chamber and various measurements are done using specialist analysers connected to the chamber

### 11.7.1 Respirometry (Code: CPRT)

Measurement of energetics and the response of an animal to ambient temperature is done by quantification of oxygen consumption or carbon dioxide production using a respirometer and analyser. These measurements are carried out in a metabolism chamber, using either a flow through or closed system. Flow through systems require that there is a sufficient high flow rate for the size of chamber used. The flow rate of air through the system should be sufficiently high to prevent carbon dioxide build up. The flow rate should be high enough for the size of the animal and the chamber. Closed systems require a means of pumping sample air from a syringe into the analyser at a fixed rate. Respirometer (metabolism chamber) design is important as it must have a well defined temperature, preferably equal to Te, and stable wind conditions. Metal walls should be coated in a layer of matt-black paint. There should be smooth unidirectional flow at the sufficient velocity to ensure stable repeatable conditions. For rodents, burrow tube type chambers seem best, as the animals settle down more quickly. If possible, the respirometer should have a wire grid raising the bottom so that urine collect below in containers of liquid paraffin.

Body temperature is measured by inserting the probe of a temperature recorder carefully into the rectum of the animal. The probe should first be covered with petroleum jelly (Vaseline). Depth of insertion is dependant on the size of the animal but should be kept consistent. The probe should be cleaned with 70% alcohol to avoid infection.

### 11.7.2 Measurement of food consumption (CPF)

Animals are usually placed in metabolic cages and fed the experimental diet for several days before the commencement of the experiment. Although the use of metabolic cages improves measurement of food eaten, some animals, particularly fossorial animals are disturbed if they are required to spend extended periods in metabolic cages. Staggered periods in the cages is then necessary. A conventional metabolic cage consists of a box or cage with a metal grid base that has a mesh below to collect faeces and prevent contamination of urine. Below this is a funnel-shaped tray which collect urine into a bottle containing liquid paraffin to prevent evaporation. The grid mesh size should be small enough to allow faeces etc. to fall through but not damage the animal's feet. Daily weighing of animals, food eaten, water consumed and faeces produced, together with volume of urine produced are done.

Body composition of small animals, particularly water and fat content is determined following euthanasia of animals (see Section 3.7 and quote reference number of relevant euthanasia procedure).

### 11.7.3 Measurement of total body water by tritium dilution (Code: CPW)

The tritium-dilution technique, to measure total body water, has been developed to measure influx and/or efflux, or water turnover rates assuming water intake equals water loss. Laboratory investigations include studies on water budgets, measuring of water intake and loss, and determining the limits of avenues of water loss under maximal stress, particularly abilities of desert rodents to cope on dry seed diets or saline water sources.

Tritium is accepted for use in biological experimentation because it is a soft beta-emitter, having a maximum radiation distance of less than 1 mm and a half life of 12.3 years, and thus has a low radiological working hazard. **However, the required care should be taken when working with radionuclides and investigators have to acquaint themselves with the necessary safety precautions set out in the Department of Health's "*Requirements for the safe use of unsealed radioactive nuclides*"-UNSEAL April 1993, revised April 1994, Feb. 1997) and "*Code of Practise for the management and disposal of non-nuclear radioactive waste"*- WSCP91-1 Nov. 1991, revised Feb. 1997.**

**The Department of Health Authority number held by the School for the use of tritium must be provided in the application form.**

The animal is kept in a metabolic chamber. An initial dose of tritiated water, varying in activity dependent on the size and experimental period is injected into the animal (see Section 3.5 and quote reference number of relevant injection procedure).and left to equilibrate with the body water pool for 2 to 3 h depending an metabolic rate and size. The animal is deprived of food and water for this period. At the end of the equilibration period and at different time intervals blood samples are taken (see Sections 3.3 and 3.4 and quote reference numbers of relevant anaesthesia and bleeding procedures). Urine is collected in by a funnel-shaped tray below the metabolic cage into a bottle containing liquid paraffin to prevent evaporation. Disposal of all tritium containing samples is done as set out in the above Department of Health documents.

### 11.7.4 Urine analysis (Code: CPU)

Bats are kept in a metabolic chamber with collecting jars or trays containing liquid paraffin for urine collection over 24h periods. In the field urine is collected while handling.

### 11.7.5 Non- shivering thermogenesis (Code: CPS)

Non-shivering thermogenesis (NST) or "chemical thermoregulation" is induced by norepinephrine (NE) injection. To ensure a maximal NST response, a mass specific dose of NE (as derived by Heldmaier 1971, *Z. Vergl. Physiol* 73:222-248) must be injected into the animal. Animals are first acclimated to a temperature at the lower end of their thermoneutral zone (TZN) and their resting metabolic rate measured using a flow-through system (see Section 3.6.1). Animals are then removed from the respirometer chamber, body temperature ($T_b$ ) read (see Section 3.6.1) and injected with saline (for controls) or NE solutions (1.5mg/kg) subcutaneously (see Sections 2.3 and quote

reference number of relevant injection procedure). Animals are immediately returned to the metabolic chamber and oxygen consumption recorded for 30-40 min when oxygen, consumption peaks. Animals are then removed from the chamber and $T_b$ recorded. During NE tests excessive hyperthermia of animals should be avoided, since high $T_b$s above $40^o$ C may inhibit thermogenesis.

### 11.7.6 Activity measurements (Code: CPA)

The responses of acclimated animals' activity to temperature, photoperiod and food availablity is established. Animals are acclimated to a photoperiod and ambient temperature for at least 1 week. The bat is placed in an activity box or chamber with upper (surface) and lower (burrows) arenas and provided with food and water. The animal is acclimated in for a further 1 day before data is collected. Activity is recorded by detecting the disruption of infra-red light beams that traverse the arena. Experiments are run for three days before the animal is removed from the metabolic chamber and returned to the vivarium.

### 11.7.7 X-Ray techniques (Code: CPX)

See standard protocol for mice.

## 11.8 EUTHANASIA

In any type of euthanasia, care must be taken to assure death has occurred.

### 11.8.1 Overdose of Inhalant Anaesthetic (Code: CEOD)

Under a hood, place cotton or gauze soaked in anaesthetic in a bottom of a bell jar and cover with wire mesh or grate so the animal does not come into direct contact with anaesthetic; place the bat into the jar and apply the cover. After the animal's respiration stops, check it for complete loss of heart beat. Please clean the bell jar after the procedure is completed.

### 11.8.2 Injectable Anaesthetic or Euthanasia Agent (Code: CEIN)

An overdose of an anaesthetic such as pentobarbital or a euthanasia agent such as T-61 may be given intravenously or directly into the heart.

### 11.8.3 Euthanasia in an Anaesthetised Animal by Physical Means (Code: CEPM)

Cervical dislocation not applicable.

### 11.8.4 Brain Fixation by Intracardiac Perfusion (Code: CEBP)

After anaesthesia, the bat is placed supine on a support table with a down-draft hood. Animals whose tissues are to be examined by ultrastructure are maintained on artificial respirators through an acutely placed tracheostomy, or an intratracheal cannula. With a midline incision and bilateral transection of the rib cage in the mid-axillary line, expose the heart. The sternum should be reflected dorsally and fractured above the manubrium. Reflect the left lobe of the lung forward to expose the descending aorta and clamp with a haemostat. Puncture the left ventricle with a 12-16 gauge needle to which is connected an airtight pressurised chamber containing the fixative (recently depolymerised paraformaldehyde in phosphate buffer, 4 g/100 ml). Inject the perfusate under a pressure of 120-140 mm Hg and puncture the right atrium to provide outflow. Artificial respiration

is terminated. The perfusion will continue for 5-15 minutes, then excise the brain whole, dissect and process for cytological and cytochemical analysis.

### 11.8.5 Euthanasia in Which Drugs Cannot be Used (Code: CECO)

Euthanasia with carbon dioxide may be accomplished by precharging a container with carbon dioxide from a gas cylinder introduced via a plastic tube into a covered box, bucket or plastic bag. Place the bat in the container and close. If more than one animal is euthanised, make sure the animals are not overcrowded in the container. After the bat no longer moves, check for loss of heart rate. The container should be clear so that the animal can be observed to assure that the animal is not distressed.

Other non-chemical means of euthanasia are described in the 1986 Report of the AVMA Panel on Euthanasia (see Appendix II). Explain your special need for these alternative methods under item 8.3 of the Application Form.

### 11.8.6 Euthanasia by Decapitation (Code: CEDE)

See standard protocol for mice.

**Literature cited:**

- Animal care and use committee. 1998. Guidelines for the capture, handling, and care of mammals as approved by the American Society of Mammalogists. *Journal of Mammalogy*. 79(4): 1416-1431.
- Kunz, T. H. and Kurta, A. 1988. Capture methods and holding devices. Pp. 1-29, *in* Ecological and behavioral methods for the study of bats. (T.H. Kunz, ed.) Smithsonian Institution Press. Washington D.C., 533pp.
- Wilson, D.E. 1988. Maintaining bats for captive studies. Pp 247-264, *in* Ecological and behavioral methods for the study of bats. (T.H. Kunz, ed.) Smithsonian Institution Press. Washington D.C., 533pp.

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## APPENDICES

# 12. STANDARD PROTOCOLS FOR SQUAMATE REPTILES

## 12.1 CAPTURE (Code SC)

Reptile capture will vary based on the lifestyle of the species being studied. Generally, reptiles are more active during summer and are thus more readily found during the warmer months. Some reptiles may go into aestivation during winter, hiding under rocks or underground. Further research is needed on individual species as to the lifestyle habits of the species in question, however, see Branch, W. 1998. Field guide to the Snakes and Other Reptiles of Southern Africa for ideas on general capture methods.

Reptiles are most commonly transported in a breathable sack or pillowcase. In the case of chameleons, which have modified feet, add some crumpled, non-inked paper, or a few small twigs to provide a suitable foothold.

## 12.2 HANDLING (Code SH):

Reptiles are best held using both hands. Small reptiles can held similar to birds with one hand restraining the upper body and head area, with the other restraining the back legs and tail. Larger reptiles with long tails will need to have their tail stabilised against the holder's body. Arboreal reptiles should be supported from below to reduced stress from fear of falling. Smaller reptiles may also be held in a lightly closed hand with the head protruding and the body supported in the hand.

Chameleons may be grasped with one hand under the base of the tail and hindlegs and carried with the front portion of the body hanging. Be sure to take care not to pull on a chameleon's tail when it is attached to something as the tail bones are easily broken.

Small tortoises may be restrained by holding the shell with two hands, one on each side, or with one hand supporting the bottom of the shell and the other on top. Large tortoises can be restrained by holding the shell between both knees and pointing the head away from you.

Snakes should be handled gently and, where possible, allowed to move their bodies freely. They should not be pulled with any force. Venomous snakes should be handled with extreme care and a "grabstick" should be used. For larger, and venomous snakes, a snake hook or grabstick may be used, usually while holding the snake gently by the tail.

## 12.3 ANAESTHESIA

### 12.3.1 Local Anaesthesia (Code SALO)

Local anaesthesia should be administered by a trained veterinarian and should generally be performed on an individual basis (mg drug/g body weight). Reptiles are especially sensitive to overdose and precise calculations based on body weight are critical.

### 12.3.2 Injection Anaesthesia (Code SAIN)

Injection anaesthesia should be administered by a trained veterinarian. Route of administration and frequency of additional doses will be determined by the length of the procedure. Reptiles are especially sensitive to overdose and precise calculations based on body weight are critical.

### 12.3.3 Inhalation Anaesthesia (Code SAIH)

Inhalation anaesthesia should only be administered by a trained veterinarian and is instituted in an inhalation jar or chamber constructed so that the agent does not directly contact the animal. The amounts of inhalation anaesthesia used are sufficient to maintain the reptile unresponsive to painful stimuli, yet insufficient to induce respiratory depression. On completion of the procedure, it is sometimes beneficial to allow the reptile to inhale pure oxygen for a short period to help the reptile to regain consciousness. Reptiles are sensitive to overdose and should be monitored throughout the procedure.

## 12.4 SAMPLING OF BODY FLUIDS – BLEEDING

The skin should be properly cleaned with alcohol to assure maximum visibility and cleanliness. Methods such as cardiac puncture, venipuncture and toe-nail clipping are common methods of blood extraction. The amount of blood able to be withdrawn will depend on the size of the animal. One (1) ml is usually adequate for most biochemical blood analyses. All procedures must be supervised by a veterinarian.

### 12.4.1 Bleeding by toe-nail clipping (Code SBTNC)

For small reptiles, quantities in the order of 1 µl to 1 ml can be obtained. For example, no more than a drop (50 µl) should be extracted at any time for dwarf chameleon species. This is least invasively done by clipping the claw short enough with round nail clipping shears so that the main blood vessel feeding the nail is able to be milked into a heparinized tube.

## 12.5 INJECTIONS

### 12.5.1 Subcutaneous (Code SISQ)

Can be given in the neck by pinching up loose skin and restraining the head.

### 12.5.2 Intraperitoneal (Code SIIP)

Generally used for lizards. Insert needle into the peritoneal cavity near the centre of the lower half of the animals abdomen. Be careful not to push the needle in too far, but making sure that it passes through the abdominal wall into the body cavity with out puncturing viscera. To decrease chances of puncturing the viscera, tilt the animal head down such that the viscera fall forward.

## 12.6 SURGICAL PROCEDURES

All surgical procedures must be performed by a trained veterinarian. All survival procedures require consideration of post-surgical analgesia.

### 12.6.1 Implantation of radio transmitters and/or data loggers (Code SIRT)

Small radio transmitters and data loggers can be internally implanted into reptiles subcutaneously or intraperitoneally depending on the animal. Transmitter and/or data logger assemblages should not exceed 10 % of individual body mass, and should be made of stainless steel or should be coated in plastic or wax that will not react with the reptile's body. All assemblages should be sterilised prior to implantation. Anaesthetise the animal and place on a clean, dry cloth. Clean the area to be cut open with alcohol or betadine. Make an incision in the skin or body wall that is just sufficient to insert the transmitter assemblage. Carefully slide the transmitter aerial subcutaneously. The incision is closed with interrupted absorbable or non-absorbable suture followed by a layer of plastic skin, such as Tegaderm.

## 12.7 PHYSIOLOGICAL MEASUREMENTS

### 12.7.1 Respirometry (Code SPRT)

As in frogs.

Cutaneous respiration is evident in some reptiles. In (semi-) aquatic reptiles, this can be measured by evaluating oxygen content in water in which the reptile is gently restrained for a set period. In terrestrial reptiles, this can be measured using standard methods, but using separate compartments of a chamber for the head and body such that cutaneous respiration can be measured independently of pulmonary repsiration.

### 12.7.2 Measurement of food consumption (Code SPF)

As in frogs.

### 12.7.3 Measurement of total body water by tritium dilution (Code SPW)

As in frogs.

### 12.7.4 Urine analysis (Code SPU)

As in frogs.

### 12.7.5 Activity measurements (Code SPA)

As in frogs. Additional non-invasive field observations can also be used in reptiles.

### 12.7.6 Selected body temperature (Code SPST)

The preferred or selected body temperature of reptiles can be measured in a thermal mosaic or gradient, which provide a range of temperatures for the reptile to choose from. Body temperature is monitored at various intervals throughout the experiment to calculate the average body temperature maintained by the animal being studied. Body temperature can be measured at intervals by capturing the reptile by hand and inserting a small temperature probe into the reptile's cloaca, or by monitoring body temperature continuously by securing a temperature probe in the reptile's cloaca with hypoallergenic tape (Micropore) and allowing the free end of the probe to trail behind the animal and connecting it to the thermometer when taking readings only, thus reducing the period where the reptile is handled. Continuous monitoring of body temperature is also possible through inserting temperature data loggers into the body cavity of the reptile (see Surgical Procedures).

### 12.7.6 Rate of water loss (Code SPWL)

The rate of water loss can be measured as the mass specific rate of water loss and can be done by placing animals in a constant environment and measuring the mass of the animals at various intervals. A limit should be set on the proportion of body mass lost in order to reduce unnecessary dehydration stress on the animal.

## 12.8 EUTHANASIA

In any type of euthanasia, care must be taken to assure swift, humane death. Carbon dioxide inhalation is NOT recommended for reptiles since they have relatively low metabolic rates and can tolerate high levels of anoxia.

### 12.8.1 Injectable anaesthetic (Code SEIN)

An overdose of anaesthetic may be given directly into the heart (in reptiles, other than snakes, insert needle on the side of the body just posterior to the left hind limb) or intraperitoneally.

### 12.8.2 Euthanasia in which drugs cannot be used (*in situ* freezing) (Code SEF)

Place in the freezer in a secure container or cloth bag for approximately 24 hrs. Should only be used for small/medium sized reptiles since larger reptiles have some thermal inertia and may experience pain during the freezing process. Large reptile should be euthanased using injectable anaesthetic.

# APPENDIX I

## ANAESTHETICS AND ANALGESICS OF LABORATORY ANIMALS

### AMPHIBIANS

#### Inhalation anaesthesia

Methoxyflurane - 0.5 or 1 ml is sufficient to produce an induction concentration of 3% in a liter anaesthesia jar. Deep anaesthesia is produced in 2 minutes and maintained for about 40 minutes.

#### Immersion Anaesthesia

Tricaine methane sulfonate (MS-222) 1% solution. Anaesthesia develops in 5-10 minutes can be maintained by wrapping the animals in gauze moistened with the solution. After the procedure, the animals should be rinsed off and recovered. Recovery can take Ω to 1 hour.

Ethyl alcohol - 10% solution. Anaesthesia is induced in about 10 minutes but recovery can be prolonged.

#### Parenteral Anaesthesia

Tribromoethanol (Avertin) - 100-160 mg/kg of 0.25% solution IP or in the dorsal lymph sac.

Tricaine methane sulfonate (MS-222) - 0.1 ml of 1% solution IP or in the dorsal lymph sac. Induction is within 3-5 minutes and recovery completed in 20 minutes.

#### Local Anaesthesia

**0.5-1.0 ml Lidocaine, Procaine infiltrated IC and IM.**

## RODENTS AND RABBITS

Drug effects will be influenced by the size, strain, age, sex, etc., of the animals, as well as its health status. For example, Fischer 344 rats may be particularly susceptible to fluoride ion toxicity from methoxyflurane anaesthesia, halothane has been reported to cause hepatic necrosis in some guinea pigs with repeated use, and albino rats have a lower tolerance for pentobarbital than do pigmented rats. It is important to make sure that rabbits and rodents to be anaesthetised are not compromised by the respiratory diseases common in these species as these conditions may dramatically increase the risks associated with anaesthesia.

Because of their small size, these animals are very susceptible to hypothermia when tranquillised or anaesthetised. Care should be taken to prevent heat loss, as described in the general guidelines.

These small animals should be weighed carefully for proper drug dosage calculation as small miscalculations of absolute mass may represent relatively large percentages of their total body weights. Guinea pigs, in particular, should be fasted for 6 hours prior to anaesthesia to allow them to clear their mouths of food and to help in determination of an accurate body weight since gastrointestinal contents may contribute as much as 30% of total body weight in these animals.

It is often desirable to dilute drugs for injection in the smaller rodents 1:10 with physiological saline or distilled water to allow for more accurate dosing and reduce local tissue damage at the injection site.

It is very difficult, but nonetheless desirable, to be able to insure a patent airway in all but the smallest rodents. The small mouth and large tongue and teeth in these species make endotrachael intubation difficult. Visualisation of the larynx may be enhanced with a small laryngoscope blade or otoscope cone with a light source. If necessary, a tracheostomy may be performed.

The guinea pig is unique in that it has an extensive soft palate that extends down to the base of the tongue. The larynx is accessed through a small hole in the descending part of the palate (palatal ostium). The ostium should be visualised before placing the endotracheal tube, since blind probing in the back of the mouth can damage the palate and cause bleeding into the airway, asphyxiation, and death.

Commercially produced endotracheal tubes may not be available for the very small animals. For these, appropriately sized polyethylene tubing or intravenous catheters may be adapted for use. For the larger animals such as rabbits, the various types of paediatric endotracheal tubes (2.5-3.5) work well.

Carbon dioxide may be used in rodents for periods of less than 2 minutes to provide narcosis for simple procedures such as bleeding via cardiac, anterior vena cava, or orbital routes. This procedure requires Special Review.

## MICE

### Anticholinergics

Atropine - 0.04 mg/kg IM or SQ

### Tranquilisers

Chlorpromazine - 50 mg/kg IM or 5010 mg/kg SQ
Acepromazine - 0.75 mg/kg IM
Diazepam - 5 mg/kg IP

### Local Anaesthetics

Procaine, Lidocaine (2%) - 0.5-1.0 ml infiltrated IC or SQ

### Analgesics

Meperidine - 20, 40 mg/kg IP; premedication
Morphine - 5, 10 mg/kg IP; premedication
Innovar-Vet - 10% solution, 0.002-0.005 ml/gm IM

### Injectable Anaesthetics

Pentobarbital - 35-70 mg/kg IP
Thiamylal or Thiopental - 25-50 mg/kg IV
Ketamine - 44 mg/kg IM, minor procedure (orbital bleeding)
Ketamine - 22-44 mg/kg + Xylazine 2.5 mg/kg + Acepromazine 0.75/kg IM
Ketamine - 50 mg/kg + Xylazine 50 mg/kg IM
Alpha Chloralose - 114 mg/kg IP, not for survival procedures
Chloral Hydrate - 370-400 mg/kg IP, not for survival procedures
Tribromoethanol -125 mg/kg IP of a 0.25% solution; higher doses and concentrations may produce intra-abdominal and intestinal adhesions and subsequently ileus

### Inhalation Anaesthetics

Halothane - 0.5-1.5%
Methoxyflurane - 0.3-2.0%

## RATS

### Anticholinergics

Atropine - 0.04-0.1 mg/kg SQ; helpful as a premedication to reduce salivary and bronchial secretions.

### Tranquilisers

Chlorpromazine - 1-2 mg/kg IM
Diazepam - 2.5 mg/kg IM, SQ

### Local Anaesthetics

Procaine, Lidocaine (2%) - 0.5-1.0 ml infiltrated IC or SQ

### Analgesics

Meperidine - 5 mg/kg SQ
Morphine - 25-50 mg/kg IM, SQ
Innovar-Vet - 0.13 ml/kg IM; sedation for minor procedures
0.33 ml/kg IM; surgical plane for some procedures; withdrawal reflex may persist

### Injectable Anaesthetics

Pentobarbital - 30-40 mg/kg IV titrated to effect; 35-45 mg/kg IP, SQ
Thiamylal or Thiopental - 20 mg/kg IV
Ketamine - 80 mg/kg + Xylazine 12 mg/kg IP
Ketamine - 90 mg/kg + Xylazine 12 mg/kg IM
Ketamine - 60 mg/kg IM + Pentobarbital 21 mg/kg IP
Ketamine - 22-44 mg/kg + Xylazine 2.5 mg/kg IP + Acepromazine 0.075 mg/kg
Droperdiol - 9 mg/kg + Fentanyl 0.18 mg/kg + Pentobarbital 15 mg/kg + Atropine 0.018 mg/kg
Urethane - 1000-1250 mg/kg IP, IV; carcinogenic , not for survival
Alpha Chloralose - 55 mg/kg IP; not recommended for survival procedures
Chloral Hydrate - 1 ml/100 gm of 3.5% solution OR 300-400 mg/kg IP; not recommended for survival procedures due to a delayed development of adhesions and adynamic ileus

### Inhalation Anaesthetics

| | Induction | Maintenance |
|---|---|---|
| Methoxyflurane - | 2% | 0.5-1.0% |
| Halothane - | 3% | 1.5% |
| Enflurane - | 3% | 0.5-2.0% in NO (1:1) |

## HAMSTERS

### Anticholinergics

Atropine - 0.2-0.5 mg/kg SQ, prior to inhalants

### Tranquilisers

Diazepam - 5 mg/kg IP

### Local Anaesthetics

Procaine, Lidocaine (2%) - 0.5-1.0 ml infiltrated IC or SQ

### Analgesics*

Morphine sulfate - up to 150 mg/kg IM, Sq, Ip; analgesia

### Injectable Anaesthetics

Pentobarbital - 50-90 mg/kg (10 mg/kg) IP
Thiamylal or Thiopental - 30 mg/kg IV
Ketamine - 40 mg/kg IM, sedation
Ketamine - 50-200 mg/kg + Xylazine 10 mg/kg IP
Ketamine - 40-150 mg/kg IM or 100-200 mg/kg IP; analgesia and relaxation reported poor**
Methohexital - 7.5 mg/ml + Diazepam 1.25 mg/ml dosed at 4.0 ml/kg IP

### Inhalation Anaesthetics

Halothane - Similar to use in rats and mice
Methoxyflurane - Similar to use in rats and mice

*Innovar-Vet - is not recommended for use in hamsters as it is reported to cause CNS signs in this species.

**** High doses of Ketamine (100-200 mg/kg) and Xylazine (10 mg/kg) given IM in the hind leg are reported to be associated with local muscle necrosis and are, therefor, not recommended via this route.**

## GUINEA PIGS

### Anticholinergics

Atropine - 0.1-0.2 mg/kg SQ; especially useful prior to inhalants, ketamine and xylazine

### Tranquilisers

Diazepam - 5 mg/kg IP, sedation
Chlorpromazine - 2 mg/100 gm SQ
Acepromazine - 2 mg/kg IM, SQ

### Local Anaesthetics

Procaine, Lidocaine (2%) - 0.5-1.0 ml infiltrated IC or SQ

### Analgesics

Meperidine - 2 mg/kg IM; analgesic or pre-anaesthetic
Innovar-Vet - 0.08 ml/kg IM; sedation for cardiac bleeding
Innovar-Vet - 0.66 ml/kg IM; general anaesthesia
Innovar-Vet - 0.88 ml/kg IM; general anaesthesia, but not for survival procedures due to reported sciatic nerve damage and self-mutilation of the hind leg

### Injectable Anaesthetics

Ketamine - 44 mg/kg IM; sedation and loss of righting reflexes, minor surgery
Ketamine - 44 mg/kg + Acepromazine 2 mg/kg IM, SQ
Ketamine - 25 mg/kg + Xylazine 5 mg/kg IM, SQ; sedation
Ketamine - 60 mg/kg + Xylazine 8 mg/kg IM, sedation and relaxation
Pentobarbital - 25-40 mg/kg IP or titrated diluted solution IV; reported associated with 13% mortality
Chloral Hydrate - 400 mg/kg IP; not recommended for survival procedures due to possible delayed adhesions and ileus

### Inhalation Anaesthetics

| | Induction | Maintenance |
|---|---|---|
| Methoxyflurane - | 2% | 0.5-1.0% |
| Halothane - | 3-5% | 0.75-1.5% |
| Nitrous Oxide - | Add in a 1:1 ratio to supplement other anaesthetics | |

## RABBITS

### Anticholinergics

Atropine - 0.05-0.5 mg/kg SQ, 1-3 mg/kg SQ; some rabbits have atropinesterases that make atropine less effective in these animals

### Tranquilisers

Acepromazine - 0.5-1.0 mg/kg IM or SQ
Chlorpromazine - 1-2 mg/kg IM
Diazepam - 5-10 mg/kg IM

### Local Anaesthetics

Procaine, Lidocaine (2%) - 0.5-1.0 ml infiltrated IC or SQ

### Analgesics

Innovar-Vet - 0.125 ml/kg; sedation for blood collection
0.22 ml/kg; anaesthesia
Meperidine - 5-10 mg.kg IM, 25 mg/kg IV

### Injectable Anaesthetics

Thiopental - 20-50 mg/kg IV; use diluted solutions
Thiamylal - 31 mg/kg IN; use diluted solutions
Methohexital - 4010 mg/kg IV, use diluted solutions
Pentobarbital - 20-45 mg/kg IV; use diluted solutions; narrow margin for safety
Ketamine - 30-50 mg/kg IM + Xylazine 2-5 mg/kg IM + Acepromazine 0.5-1 mg/kg IM; commonly used combination; very depressing when all three drugs used at the higher doses
Alpha Chloralose - 120 mg/kg IV; not recommended for survival procedures

### Inhalation Anaesthetics

| | Induction | Maintenance |
|---|---|---|
| Methoxyflurane - | 2% | 0.5-1.0% |
| Halothane - | 3% | 1.5% |
| Enflurane - | 3% | 0.5-2.0% in NO (1:1) |

**RUMINANTS**

Most of the ruminants used in the research environment are small ruminants (sheep, goats) and calves. In general, ruminants present many problems with regard to general anaesthesia, including excessive salivation, tympanitis and regurgitation. It is generally preferred with the large animals such as adult cattle to use local or regional anaesthesia and to do the procedure with the animal standing whenever possible. However, for the smaller ruminants usually used for research, many techniques for general anaesthesia using injectable and inhalant agents have been deployed.

Fasting prior to general anaesthesia is recommended to reduce the volume of rumenal contents. This fast may vary from 24 to 48 hours except in very young animals where the time of fasting is shorter. This will hopefully reduce the amount of regurgitation and will also help alleviate ventilatory depression resulting from pressure from the rumen on the diaphragm when the animals is in lateral or dorsal recumbency. The likelihood of regurgitation necessitates a rapid smooth endotracheal tube. In larger animals, these tubes may be placed by hand. In the smaller ones, use a long-bladed laryngoscope and long 6-10 mm tube, while calves will usually require 10-20 mm tubes.

The passage of a stomach tube after endotracheal intubation is recommended to help prevent the development or rumen tympany (bloat) during anaesthesia. It is best to keep the ruminant in a sternal position whenever possible. When recumbent, it may help to position the animals so that the abdominal contents are not pressing on the diaphragm (i.e. hindquarters slightly lowered) and the neck is slightly lowered relative to the chest and neck to allow passive drainage of saliva and fluids from the stomach tube without precipitating passive regurgitation from the rumen. In many cases, it will be necessary to support ventilation with a mechanical ventilator.

## RUMINANTS (SHEEP, GOATS, CALVES)

### Anticholinergics

Atropine - 0.2-0.8 mg/kg IV, SQ, IM
- 0.7 mg/kg IM (goats)
Scopolamine - 0.03 mg/kg IM (sheep, goats)

### Sedation/Tranquilisers

Diazepam - 1-2 mg/kg IM, IV; premedication for Ketamine
Acepromazine - 0.05-0.1 mg/kg IM
Promazine - 0.3-1.0 mg/kg IV, IM

### Local Anaesthetics

Procaine, Lidocaine (2%) - 5-10 ml infiltrated IC or SQ

### Injectable Anaesthetics

Pentobarbital - 25-30 mg/kg IV; recovery may be prolonged; not for survival procedures for calves less than 8 weeks old
Thiamylal, Thiopental - 10-25 mg/kg of 2-4% solution IV; not recommended for calves less than 8 weeks old or lambs/kids less than 3 months old due to prolonged recovery
Methohexital - 1-6 mg/kg IV, preferred for young calves
Xylazine - 0.05-0.1 mg/kg IV or 0.05-0.2 mg/kg IM
Ketamine - 2-10 mg/kg + Diazepam 1 mg/kg or Xylazine 0.05-0.1 mg/kg IV; surgical anaesthesia
Pentobarbital - 3-6 mg/kg IM, sedation

### Inhalational Anaesthetics

| | Induction | Maintenance |
|---|---|---|
| Halothane - | 2-4% | 0.5-2.0% |
| Nitrous Oxide - | Can be added in 1:1 ratio to oxygen to supplement other anaesthetics. Discontinue use if rumenal tympany appears to be developing | |

## BIRDS

### Anticholinergics

Atropine - 0.04 mg/kg IM

### Sedation/Tranquilisers

Xylazine - 20-40 mg/kg IM
Chlorpromazine - 1-1.5 mg/kg IM
Promazine - 0.5 mg/kg IM

### Local Anaesthetics

Procaine, Lidocaine (2%) - 0.5-1.0 ml infiltrated IC or SQ

### Parental Anaesthetics

Ketamine - 50-200 mg/kg IV
Pentobarbital - 30-45 mg/kg IV
Tiletamine zolazepam (Telazol) - 10-35 mg/kg IM
Tribromoethanol (Avertin) - 100-150 mg/kg IM

### Inhalational Anaesthetics

Halothane - 2-4% (induction) in oxygen at 1-2 liters/min
- 1-1.5% (maintenance) in oxygen at 1-2 liters/min
Methoxyflurane - 3% solution via mask or chamber

# 2000 Report of the AVMA Panel on Euthanasia

# 2000 Report of the AVMA Panel on Euthanasia

## Members of the AVMA Panel

Bonnie V. Beaver, DVM, MS, DACVB, (Chair) Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, Texas A&M University, 4474 TAMU, College Station, TX 77843-4474, representing the AVMA Executive Board.

Willie Reed, DVM, PhD, DACVP, DACPV, Animal Health Diagnostic Laboratory, College of Veterinary Medicine, Michigan State University, B646 W. Fee Hall-AHDL, East Lansing, MI 48824-1316, representing the AVMA Council on Research.

Steven Leary, DVM, DACLAM, Division of Comparative Medicine, Washington University, Box 8061, St Louis, MO 63110, representing the AVMA Animal Welfare Committee.

Brendan McKiernan, DVM, DACVIM, Denver Veterinary Specialists, 3695 Kipling St, Wheat Ridge, CO 80033, representing the American Animal Hospital Association.

Fairfield Bain, DVM, DACVIM, DACVP, DACVECC, Hagyard-Davidson-McGee Associates PSC, 4250 Iron Works Pike, Lexington, KY 40511-8412, representing the American Association of Equine Practitioners.

Roy Schultz, DVM, MS, DABVP, 1114 N Frost Ave, Avoca, IA 51521, representing the American Board of Veterinary Practitioners.

B. Taylor Bennett, DVM, PhD, DACLAM, Biologic Resources Laboratory (MC533), University of Illinois at Chicago, 1840 W Taylor St, Chicago, IL 60612-7348, representing the American College of Laboratory Animal Medicine.

Peter Pascoe, BVSc, DVA, DACVA, DECVA, Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, CA 95616-8745, representing the American College of Veterinary Anesthesiologists.

Elizabeth Shull, DVM, DACVB, DACVIM (Neurology), Veterinary Specialty Consultation Services, 1505 Bob Kirby Rd, Knoxville, TN 37931, representing the American College of Veterinary Behaviorists.

Linda C. Cork, DVM, PhD, DACVP, Department of Comparative Medicine, School of Medicine, Stanford University, MSOB Building, Room X347, Stanford, CA 94305-5415, representing the American College of Veterinary Pathologists.

Ruth Francis-Floyd, DVM, MS, DACZM, Department of Large Animal Clinical Sciences, College f Veterinary Medicine, University of Florida, Box 100136, Gainesville, FL 32510-0136, representing the International Association of Aquatic Animal Medicine.

Keith D. Amass, DVM, Safe-Capture International Inc, PO Box 206, Mount Horeb, WI 53572, representing wildlife regulatory/conservation agencies.

Richard Johnson, PhD, Department of Physiological Sciences, College of Veterinary Medicine, University of Florida, Box 100144, Gainesville, FL 32610-0144, representing the Society for Neuroscience.

Robert H. Schmidt, MS, PhD, Department of Fisheries and Wildlife, Utah State University, Logan UT 84322-5210, representing the wildlife damage management profession.

Wendy Underwood, DVM, MS, DACVIM, Lilly Corporate Center, Eli Lilly and Co, Indianapolis, IN 46285, representing the National Institute for Animal Agriculture Euthanasia Task Force.

Gus W. Thornton, DVM, DACVIM, Massachusetts Society for the Prevention of Cruelty to Animals (MSPCA), American Humane Education Society (AHES), 350 S Huntington Ave, Boston, MA 02130, representing an animal protection agency.

Barbara Kohn, DVM, USDA/APHIS/Animal Care, 4700 River Road, Unit 84, Riverdale, MD 20737-1234, representing the USDA/APHIS.

## PREFACE

At the request of the AVMA Council on Research, the Executive Board of the AVMA convened a Panel on Euthanasia in 1999 to review and make necessary revisions to the fifth Panel Report, published in 1993.[1] In this newest version of the report, the panel has updated information on euthanasia of animals in research and animal care and control facilities; expanded information on ectothermic, aquatic, and fur-bearing animals; added information on horses and wildlife; and deleted methods or agents considered unacceptable. Because the panel's deliberations were based on currently available scientific information, some euthanasia methods and agents are not discussed.

Welfare issues are increasingly being identified in the management of free-ranging wildlife, and the need for humane euthanasia guidelines in this context is great. Collection of animals for scientific investigations, euthanasia of injured or diseased wildlife species, removal of animals causing damage to property or threatening human safety, and euthanasia of animals in excess population are drawing more public attention. These issues are acknowledged in this report and special considerations are described for handling animals under free-ranging conditions, where their needs are far different from those of their domestic counterparts.

This report is intended for use by members of the

veterinary profession who carry out or oversee the euthanasia of animals. Although the report may be interpreted and understood by a broad segment of the general population, a veterinarian should be consulted in the application of these recommendations. The practice of veterinary medicine is complex and involves diverse animal species. Whenever possible, a veterinarian experienced with the species in question should be consulted when selecting the method of euthanasia, particularly when little species-specific euthanasia research has been done. Although interpretation and use of this report cannot be limited, the panel's overriding commitment is to give veterinarians guidance in relieving pain and suffering of animals that are to be euthanatized. The recommendations in this report are intended to serve as guidelines for veterinarians who must then use professional judgment in applying them to the various settings where animals are to be euthanatized.

## INTRODUCTION

The term euthanasia is derived from the Greek terms *eu* meaning good and *thanatos* meaning death.[2] A "good death" would be one that occurs with minimal pain and distress. In the context of this report, euthanasia is the act of inducing humane death in an animal. It is our responsibility as veterinarians and human beings to ensure that if an animal's life is to be taken, it is done with the highest degree of respect, and with an emphasis on making the death as painless and distress free as possible. Euthanasia techniques should result in rapid loss of consciousness followed by cardiac or respiratory arrest and the ultimate loss of brain function. In addition, the technique should minimize distress and anxiety experienced by the animal prior to loss of consciousness. The panel recognized that the absence of pain and distress cannot always be achieved. This report attempts to balance the ideal of minimal pain and distress with the reality of the many environments in which euthanasia is performed. A veterinarian with appropriate training and expertise for the species involved should be consulted to ensure that proper procedures are used.

Criteria for painless death can be established only after the mechanisms of pain are understood. Pain is that sensation (perception) that results from nerve impulses reaching the cerebral cortex via ascending neural pathways. Under normal circumstances, these pathways are relatively specific, but the nervous system is sufficiently plastic that activation of nociceptive pathways does not always result in pain and stimulation of other (non-nociceptive) peripheral and central neurons can give rise to pain. The term nociceptive is derived from the word *noci* meaning to injure and *ceptive* meaning to receive, and is used to describe neuronal input caused by noxious stimuli, which threaten to, or actually do, destroy tissue. These noxious stimuli initiate nerve impulses by acting at primary nociceptors and other sensory nerve endings that respond to noxious and non-noxious stimuli from mechanical, thermal, or chemical activity. Endogenous chemical substances such as hydrogen ions, potassium ions, ATP, serotonin, histamine, bradykinin, and prostaglandins, as well as electrical currents, are capable of generating nerve impulses in nociceptor nerve fibers. Activity in nociceptive pathways can also be triggered in normally silent receptors that become sensitized by chronic pain conditions.[3,4]

Nerve impulse activity generated by nociceptors is conducted via nociceptor primary afferent fibers to the spinal cord or the brainstem where it is transmitted to two general sets of neural networks. One set is related to nociceptive reflexes (eg, withdrawal and flexion reflexes) that are mediated at the spinal level, and the second set consists of ascending pathways to the reticular formation, hypothalamus, thalamus, and cerebral cortex (somatosensory cortex and limbic system) for sensory processing. It is important to understand that ascending nociceptive pathways are numerous, often redundant, and are capable of considerable plasticity under chronic conditions (pathology or injury). Moreover, even the transmission of nociceptive neural activity in a given pathway is highly variable. Under certain conditions, both the nociceptive reflexes and the ascending pathways may be suppressed, as, for example, in epidural anesthesia. Under another set of conditions, nociceptive reflex actions may occur, but activity in the ascending pathways is suppressed; thus, noxious stimuli are not perceived as pain. It is incorrect to use the term pain for stimuli, receptors, reflexes, or pathways because the term implies perception, whereas all the above may be active without consequential pain perception.[5,6]

Pain is divided into two broad categories: (1) sensory-discriminative, which indicates the site of origin and the stimulus giving rise to the pain; and (2) motivational-affective in which the severity of the stimulus is perceived and the animal's response is determined. Sensory-discriminative processing of nociceptive impulses is most likely to be accomplished by subcortical and cortical mechanisms similar to those used for processing other sensory-discriminative input that provides the individual with information about the intensity, duration, location, and quality of the stimulus. Motivational-affective processing involves the ascending reticular formation for behavioral and cortical arousal. It also involves thalamic input to the forebrain and the limbic system for perceptions such as discomfort, fear, anxiety, and depression. The motivational-affective neural networks also have strong inputs to the limbic system, hypothalamus and the autonomic nervous system for reflex activation of the cardiovascular, pulmonary, and pituitary-adrenal systems. Responses activated by these systems feed back to the forebrain and enhance perceptions derived via motivational-affective inputs. On the basis of neurosurgical experience in humans, it is possible to separate the sensory-discriminative components from the motivational-affective components of pain.[7]

For pain to be experienced, the cerebral cortex and subcortical structures must be functional. If the cerebral cortex is nonfunctional because of hypoxia, depression by drugs, electric shock, or concussion, pain is not experienced. Therefore, the choice of the euthanasia agent or method is less critical if it is to be used on an animal that is anesthetized or unconscious, provided that the animal does not regain consciousness prior to death.

An understanding of the continuum that represents stress and distress is essential for evaluating techniques that minimize any distress experienced by an animal being euthanatized. Stress has been defined as the effect of physical, physiologic, or emotional factors (stressors) that induce an alteration in an animal's homeostasis or adaptive state.[8] The response of an animal to stress represents the adaptive process that is necessary to restore the baseline mental and physiologic state. These responses may involve changes in an animal's neuroendocrinologic system, autonomic nervous system, and mental status that may result in overt behavioral changes. An animal's response varies according to its experience, age, species, breed, and current physiologic and psychologic state.[9]

Stress and the resulting responses have been divided into three phases.[10] Eustress results when harmless stimuli initiate adaptive responses that are beneficial to the animal. Neutral stress results when the animal's response to stimuli causes neither harmful nor beneficial effects to the animal. Distress results when an animal's response to stimuli interferes with its well-being and comfort.[11]

As with many other procedures involving animals, some methods of euthanasia require physical handling of the animal. The amount of control and kind of restraint required will be determined by the animal's species, breed, size, state of domestication, degree of taming, presence of painful injury or disease, degree of excitement, and method of euthanasia. Proper handling is vital to minimize pain and distress in animals, to ensure safety of the person performing euthanasia, and, often, to protect other people and animals.

An in-depth discussion of euthanasia procedures is beyond the scope of this report; however, personnel who perform euthanasia must have appropriate certification and training, experience with the techniques to be used, and experience in the humane restraint of the species of animal to be euthanatized, to ensure that animal pain and distress are minimized during euthanasia. Training and experience should include familiarity with the normal behavior of the species being euthanatized, an appreciation of how handling and restraint affects that behavior, and an understanding of the mechanism by which the selected technique induces loss of consciousness and death. Prior to being assigned full responsibility for performing euthanasia, all personnel must have demonstrated proficiency in the use of the technique in a closely supervised environment. References provided at the end of this document may be useful for training personnel.[12-21]

Selection of the most appropriate method of euthanasia in any given situation depends on the species of animal involved, available means of animal restraint, skill of personnel, number of animals, and other considerations. Available information focuses primarily on domestic animals, but the same general considerations should be applied to all species.

This report includes four appendices that summarize information from the text. Appendix 1 lists acceptable and conditionally acceptable methods of euthanasia, categorized by species. Appendices 2 and 3 provide summaries of characteristics for acceptable and conditionally acceptable methods of euthanasia. Appendix 4 provides a summary of some unacceptable euthanasia agents and methods. Criteria used for acceptable, conditionally acceptable, and unacceptable methods are as follows: acceptable methods are those that consistently produce a humane death when used as the sole means of euthanasia; conditionally acceptable methods are those techniques that by the nature of the technique or because of greater potential for operator error or safety hazards might not consistently produce humane death or are methods not well documented in the scientific literature; and unacceptable techniques are those methods deemed inhumane under any conditions or that the panel found posed a substantial risk to the human applying the technique. The report also includes discussion of several adjunctive methods, which are those methods that cannot be used as the sole method of euthanasia, but that can be used in conjunction with other methods to produce a humane death.

## GENERAL CONSIDERATIONS

In evaluating methods of euthanasia, the panel used the following criteria: (1) ability to induce loss of consciousness and death without causing pain, distress, anxiety, or apprehension; (2) time required to induce loss of consciousness; (3) reliability; (4) safety of personnel; (5) irreversibility; (6) compatibility with requirement and purpose; (7) emotional effect on observers or operators; (8) compatibility with subsequent evaluation, examination, or use of tissue; (9) drug availability and human abuse potential; (10) compatibility with species, age, and health status; (11) ability to maintain equipment in proper working order; and (12) safety for predators/scavengers should the carcass be consumed.

The panel discussed the definition of euthanasia used in this report as it applies to circumstances when the degree of control over the animal makes it difficult to ensure death without pain and distress. Slaughter of animals for food, fur, or fiber may represent such situations. However, the same standards for euthanasia should be applied to the killing of animals for food, fur, or fiber, and wildlife or feral animals. Animals intended for food should be slaughtered humanely, taking into account any special requirements of the US Department of Agriculture.[22] Painless death can be achieved by properly stunning the animal, followed immediately by exsanguination. Handling of animals prior to slaughter should be as stress free as possible. Electric prods or other devices should not be used to encourage movement of animals and are not needed if chutes and ramps are properly designed to enable animals to be moved and restrained without undue stress.[23-27] Animals must not be restrained in a painful position before slaughter.

Ethical considerations that must be addressed when euthanatizing healthy and unwanted animals reflect professional and societal concerns.[28,29] These issues are complex and warrant thorough consideration by the profession and all those concerned with the welfare of animals. Whereas the panel recognizes the need for those responsible for the euthanasia of ani-

mals to be cognizant of these issues, it does not believe that this report is the appropriate forum for an in-depth discussion of this topic.

It is the intent of the panel that euthanasia be performed in accordance with applicable federal, state, and local laws governing drug acquisition and storage, occupational safety, and methods used for euthanasia and disposal of animals. However, space does not permit a review of current federal, state, and local regulations.

The panel is aware that circumstances may arise that are not clearly covered by this report. Whenever such situations arise, a veterinarian experienced with the species should use professional judgment and knowledge of clinically acceptable techniques in selecting an appropriate euthanasia technique. Professional judgment in these circumstances will take into consideration the animal's size and its species-specific physiologic and behavioral characteristics. In all circumstances, the euthanasia method should be selected and used with the highest ethical standards and social conscience.

It is imperative that death be verified after euthanasia and before disposal of the animal. An animal in deep narcosis following administration of an injectable or inhalant agent may appear dead, but might eventually recover. Death must be confirmed by examining the animal for cessation of vital signs, and consideration given to the animal species and method of euthanasia when determining the criteria for confirming death.

## ANIMAL BEHAVIORAL CONSIDERATIONS

The need to minimize animal distress, including fear, anxiety, and apprehension, must be considered in determining the method of euthanasia. Gentle restraint (preferably in a familiar and safe environment), careful handling, and talking during euthanasia often have a calming effect on animals that are used to being handled. Sedation and/or anesthesia may assist in achieving the best conditions for euthanasia. It must be recognized that any sedatives or anesthetics given at this stage that change circulation may delay the onset of the euthanasia agent. Preparation of observers should also be taken into consideration.

Animals that are wild, feral, injured, or already distressed from disease pose another challenge. Methods of pre-euthanasia handling suitable for domestic animals may not be effective for them. Because handling may stress animals unaccustomed to human contact (eg, wildlife, zoo, and feral species), the degree of restraint required to perform any euthanasia procedure should be considered when evaluating various methods. When handling these animals, calming may be accomplished by minimizing visual, auditory, and tactile stimulation. When struggling during capture or restraint may cause pain, injury, or anxiety to the animal or danger to the operator, the use of tranquilizers, analgesics, and/or anesthetics may be necessary. A route of injection should be chosen that causes the least distress in the animal for which euthanasia must be performed. Various techniques for oral delivery of sedatives to dogs and cats have been described that may be useful under these circumstances.[30,31]

Facial expressions and body postures that indicate various emotional states of animals have been described for some species.[32-37] Behavioral and physiologic responses to noxious stimuli include distress vocalization, struggling, attempts to escape, defensive or redirected aggression, salivation, urination, defecation, evacuation of anal sacs, pupillary dilatation, tachycardia, sweating, and reflex skeletal muscle contractions causing shivering, tremors, or other muscular spasms. Unconscious as well as conscious animals are capable of some of these responses. Fear can cause immobility or “playing dead” in certain species, particularly rabbits and chickens. This immobility response should not be interpreted as loss of consciousness when the animal is, in fact, conscious. Distress vocalizations, fearful behavior, and release of certain odors or pheromones by a frightened animal may cause anxiety and apprehension in other animals. Therefore, for sensitive species, it is desirable that other animals not be present when individual animal euthanasia is performed.

## HUMAN BEHAVIORAL CONSIDERATIONS

When animals must be euthanatized, either as individuals or in larger groups, moral and ethical concerns dictate that humane practices be observed. Human psychologic responses to euthanasia of animals need to be considered, with grief at the loss of a life as the most common reaction.[38] There are six circumstances under which we are most aware of the effects of animal euthanasia on people.

The first of these is the veterinary clinical setting where owners have to make decisions about whether and when to euthanatize. Although many owners rely heavily on their veterinarian's judgment, others may have misgivings about making their own decision. This is particularly likely if an owner feels responsible for allowing an animal's medical or behavioral problem to go unattended so that euthanasia becomes necessary. When owners choose to be present during euthanasia, they should be prepared for what will happen. What drugs are being used and how the animal could respond should be discussed. Behaviors such as vocalization, muscle twitches, failure of the eyelids to close, urination, or defecation can be distressing. Counseling services for grieving owners are now available in some communities[39] and telephone counseling is available through some veterinary schools.[40,41] Owners are not the only people affected by euthanasia of animals. Veterinarians and their staffs may also become attached to patients they have known and treated for many years and may continue to struggle with the ethical implications of ending an animal's life.

The second is animal care and control facilities where unwanted, homeless, diseased, and injured animals must be euthanatized in large numbers. Distress may develop among personnel directly involved in performing euthanasia repeatedly. Emotional uneasiness, discomfort, or distress experienced by people involved with euthanasia of animals may be minimized. The person performing euthanasia must be technically proficient, use humane handling methods, understand the reasons for euthanasia, and be familiar with the

method of euthanasia being employed (ie, what is going to happen to the animal). When the person is not knowledgeable about what to expect, he or she may mistakenly interpret any movement of animals as consciousness and a lack of movement as loss of consciousness. Methods that preclude movement of animals are more aesthetically acceptable to most technical staff even though lack of movement is not an adequate criterion for evaluating euthanasia techniques. Constant exposure to, or participation in, euthanasia procedures can cause a psychologic state characterized by a strong sense of work dissatisfaction or alienation, which may be expressed by absenteeism, belligerence, or careless and callous handling of animals.[42] This is one of the principal reasons for turnover of employees directly involved with repeated animal euthanasia. Management should be aware of potential personnel problems related to animal euthanasia and determine whether it is necessary to institute a program to prevent, decrease, or eliminate this problem. Specific coping strategies can make the task more tolerable. Some strategies include adequate training programs so that euthanasia is performed competently, peer support in the workplace, professional support as necessary, focusing on animals that are successfully adopted or returned to owners, devoting some work time to educational activities, and providing time off when workers feel stressed.

The third setting is the laboratory. Researchers, technicians, and students may become attached to animals that must be euthanatized.[43] The same considerations afforded pet owners or shelter employees should be provided to those working in laboratories.

The fourth situation is wildlife control. Wildlife biologists, wildlife managers, and wildlife health professionals are often responsible for euthanatizing animals that are injured, diseased, in excessive number, or that threaten property or human safety. Although relocation of some animals is appropriate and attempted, relocation is often only a temporary solution to a larger problem. People who must deal with these animals, especially under public pressure to save the animals rather than destroy them, can experience extreme distress and anxiety.

The fifth setting is livestock and poultry slaughter facilities. The large number of animals processed daily can take a heavy toll on employees physically and emotionally. Federal and state agricultural employees may also be involved in mass euthanasia of poultry and livestock in the face of disease outbreaks, bioterrorism, and natural disasters.

The last situation is public exposure. Because euthanasia of zoo animals, animals involved in roadside or racetrack accidents, stranded marine animals, nuisance or injured wildlife, and others can draw public attention, human attitudes and responses should be considered whenever animals are euthanatized. Natural disasters and foreign animal disease programs also present public challenges. These considerations, however, should not outweigh the primary responsibility of using the most rapid and painless euthanasia method possible under the circumstances.

## MODES OF ACTION OF EUTHANATIZING AGENTS

Euthanatizing agents cause death by three basic mechanisms: (1) hypoxia, direct or indirect; (2) direct depression of neurons necessary for life function; and (3) physical disruption of brain activity and destruction of neurons necessary for life.

Agents that induce death by direct or indirect hypoxia can act at various sites and can cause loss of consciousness at different rates. For death to be painless and distress-free, loss of consciousness should precede loss of motor activity (muscle movement). Loss of motor activity, however, cannot be equated with loss of consciousness and absence of distress. Thus, agents that induce muscle paralysis without loss of consciousness are not acceptable as sole agents for euthanasia (eg, depolarizing and nondepolarizing muscle relaxants, strychnine, nicotine, and magnesium salts). With other techniques that induce hypoxia, some animals may have motor activity following loss of consciousness, but this is reflex activity and is not perceived by the animal.

A second group of euthanatizing agents depress nerve cells of the brain, inducing loss of consciousness followed by death. Some of these agents release inhibition of motor activity during the first stage of anesthesia, resulting in a so-called excitement or delirium phase, during which there may be vocalization and some muscle contraction. These responses do not appear to be purposeful. Death follows loss of consciousness, and is attributable to cardiac arrest and/or hypoxemia following direct depression of respiratory centers.

Physical disruption of brain activity, caused by concussion, direct destruction of the brain, or electrical depolarization of neurons, induces rapid loss of consciousness. Death occurs because of destruction of midbrain centers controlling cardiac and respiratory activity or as a result of adjunctive methods (eg, exsanguination) used to kill the animal. Exaggerated muscular activity can follow loss of consciousness and, although this may disturb some observers, the animal is not experiencing pain or distress.

## INHALANT AGENTS

Any gas that is inhaled must reach a certain concentration in the alveoli before it can be effective; therefore, euthanasia with any of these agents takes some time. The suitability of a particular agent depends on whether an animal experiences distress between the time it begins to inhale the agent and the time it loses consciousness. Some agents may induce convulsions, but these generally follow loss of consciousness. Agents inducing convulsions prior to loss of consciousness are unacceptable for euthanasia.

Certain considerations are common to all inhalant agents. (1) In most cases, onset of loss of consciousness is more rapid, and euthanasia more humane, if the animal is rapidly exposed to a high concentration of the agent. (2) The equipment used to deliver and maintain this high concentration must be in good working order and in compliance with state and federal regulations. Leaky or faulty equipment may lead to

slow, distressful death and be hazardous to other animals and to personnel. (3) Most of these agents are hazardous to personnel because of the risk of explosions (eg, ether), narcosis (eg, halothane), hypoxemia (eg, nitrogen and carbon monoxide), addiction (eg, nitrous oxide), or health effects resulting from chronic exposure (eg, nitrous oxide and carbon monoxide). (4) Alveolar concentrations rise slowly in an animal with decreased ventilation, making agitation more likely during induction. Other noninhalant methods of euthanasia should be considered for such animals. (5) Neonatal animals appear to be resistant to hypoxia, and because all inhalant agents ultimately cause hypoxia, neonatal animals take longer to die than adults. Glass et al,[44] reported that newborn dogs, rabbits, and guinea pigs survived a nitrogen atmosphere much longer than did adults. Dogs, at 1 week old, survived for 14 minutes compared with a 3-minute survival time after a few weeks of age. Guinea pigs survived for 4.5 minutes at 1 day old, compared with 3 minutes at 8 days or older. Rabbits survived for 13 minutes at 6 days old, 4 minutes at 14 days, and 1.5 minutes at 19 days and older. The panel recommends that inhalant agents not be used alone in animals less than 16 weeks old except to induce loss of consciousness, followed by the use of some other method to kill the animal. (6) Rapid gas flows can produce a noise that frightens animals. If high flows are required, the equipment should be designed to minimize noise. (7) Animals placed together in chambers should be of the same species, and, if needed, should be restrained so that they will not hurt themselves or others. Chambers should not be overloaded and need to be kept clean to minimize odors that might distress animals subsequently euthanatized. (8) Reptiles, amphibians, and diving birds and mammals have a great capacity for holding their breath and anaerobic metabolism. Therefore, induction of anesthesia and time to loss of consciousness when using inhalants may be greatly prolonged. Other techniques may be more appropriate for these species.

## Inhalant anesthetics

Inhalant anesthetics (eg, ether, halothane, methoxyflurane, isoflurane, sevoflurane, desflurane, and enflurane) have been used to euthanatize many species.[45] Halothane induces anesthesia rapidly and is the most effective inhalant anesthetic for euthanasia. Enflurane is less soluble in blood than halothane, but, because of its lower vapor pressure and lower potency, induction rates may be similar to those for halothane. At deep anesthetic planes, animals may seizure. It is an effective agent for euthanasia, but the associated seizure activity may be disturbing to personnel. Isoflurane is less soluble than halothane, and it should induce anesthesia more rapidly. However, it has a slightly pungent odor and animals often hold their breath, delaying onset of loss of consciousness. Isoflurane also may require more drug to kill an animal, compared with halothane. Although isoflurane is acceptable as a euthanasia agent, halothane is preferred. Sevoflurane is less soluble than halothane and does not have an objectionable odor. It is less potent than isoflurane or halothane and has a lower vapor pressure. Anesthetic concentrations can be achieved and maintained rapidly. Desflurane is currently the least soluble potent inhalant anesthetic, but the vapor is quite pungent, which may slow induction. This drug is so volatile that it could displace oxygen ($O_2$) and induce hypoxemia during induction if supplemental $O_2$ is not provided. Methoxyflurane is highly soluble, and slow anesthetic induction with its use may be accompanied by agitation. It is a conditionally acceptable agent for euthanasia in rodents.[46] Ether has high solubility in blood and induces anesthesia slowly. It is irritating to the eyes and nose, poses serious risks associated with its flammability and explosiveness, and has been used to create a model for stress.[47-50]

With inhalant anesthetics, the animal can be placed in a closed receptacle containing cotton or gauze soaked with an appropriate amount of the anesthetic,[51] or the anesthetic can be introduced from a vaporizer. The latter method may be associated with a longer induction time. Vapors are inhaled until respiration ceases and death ensues. Because the liquid state of most inhalant anesthetics is irritating, animals should be exposed only to vapors. Also, sufficient air or $O_2$ must be provided during the induction period to prevent hypoxemia.[51] In the case of small rodents placed in a large container, there will be sufficient $O_2$ in the chamber to prevent hypoxemia. Larger species placed in small containers may need supplemental air or $O_2$.[51]

Nitrous oxide ($N_2O$) may be used with other inhalants to speed the onset of anesthesia, but alone it does not induce anesthesia in animals, even at 100% concentration. When used by itself, $N_2O$ produces hypoxemia before respiratory or cardiac arrest. As a result, animals may become distressed prior to loss of consciousness.

Occupational exposure to inhalant anesthetics constitutes a human health hazard. Spontaneous abortion and congenital abnormalities have been associated with exposure of women to trace amounts of inhalation anesthetic agents during early stages of pregnancy.[52] Regarding human exposure to inhalant anesthetics, the concentrations of halothane, enflurane, and isoflurane should be less than 2 ppm, and less than 25 ppm for nitrous oxide.[52] There are no controlled studies proving that such concentrations of anesthetics are safe, but these concentrations were established because they were found to be attainable under hospital conditions. Effective procedures must be used to protect personnel from anesthetic vapors.

*Advantages*—(1) Inhalant anesthetics are particularly valuable for euthanasia of smaller animals (< 7 kg) or for animals in which venipuncture may be difficult. (2) Halothane, enflurane, isoflurane, sevoflurane, desflurane, methoxyflurane, and $N_2O$ are nonflammable and nonexplosive under ordinary environmental conditions.

*Disadvantages*—(1) Animals may struggle and become anxious during induction of anesthesia because anesthetic vapors may be irritating and can induce excitement. (2) Ether is flammable and explo-

sive. Explosions have occurred when animals, euthanatized with ether, were placed in an ordinary (not explosion proof) refrigerator or freezer and when bagged animals were placed in an incinerator. (3) Induction with methoxyflurane is unacceptably slow in some species. (4) Nitrous oxide will support combustion. (5) Personnel and animals can be injured by exposure to these agents. (6) There is a potential for human abuse of some of these drugs, especially $N_2O$.

*Recommendations*—In order of preference, halothane, enflurane, isoflurane, sevoflurane, methoxyflurane, and desflurane, with or without nitrous oxide, are acceptable for euthanasia of small animals (< 7 kg). Ether should only be used in carefully controlled situations in compliance with state and federal occupational health and safety regulations. It is conditionally acceptable. Nitrous oxide should not be used alone, pending further scientific studies on its suitability for animal euthanasia. Although acceptable, these agents are generally not used in larger animals because of their cost and difficulty of administration.

## Carbon dioxide

Room air contains 0.04% carbon dioxide ($CO_2$), which is heavier than air and nearly odorless. Inhalation of $CO_2$ at a concentration of 7.5% increases the pain threshold, and higher concentrations of $CO_2$ have a rapid anesthetic effect.[53-58]

Leake and Waters[56] reported the experimental use of $CO_2$ as an anesthetic agent for dogs. At concentrations of 30% to 40% $CO_2$ in $O_2$, anesthesia was induced within 1 to 2 minutes, usually without struggling, retching, or vomiting. For cats, inhalation of 60% $CO_2$ results in loss of consciousness within 45 seconds, and respiratory arrest within 5 minutes.[59] Signs of effective $CO_2$ anesthesia are those associated with deep surgical anesthesia, such as loss of withdrawal and palpebral reflexes.[60] Time to loss of consciousness is decreased by use of higher concentrations of $CO_2$ with an 80 to 100% concentration providing anesthesia in 12 to 33 seconds in rats and 70% $CO_2$ in $O_2$ inducing anesthesia in 40 to 50 seconds.[61,62] Time to loss of consciousness will be longer if the concentration is increased slowly rather than immersing the animal in the full concentration immediately.

Several investigators have suggested that inhalation of high concentrations of $CO_2$ may be distressing to animals,[63-66] because the gas dissolves in moisture on the nasal mucosa. The resulting product, carbonic acid, may stimulate nociceptors in the nasal mucosa. Some humans exposed to concentrations of around 50% $CO_2$ report that inhaling the gas is unpleasant and that higher concentrations are noxious.[67,68] A brief study of swine examined the aversive nature of $CO_2$ exposure[69] and found that 90% $CO_2$ was aversive to pigs while 30% was not. For rats, exposure to increasing concentrations of $CO_2$ (33% achieved after 1 minute) in their home cage produced no evident stress as measured by behavior and ACTH, glucose, and corticosterone concentrations in serum.[70]

Carbon dioxide has been used to euthanatize groups of small laboratory animals, including mice, rats, guinea pigs, chickens, and rabbits,[5,71-76] and to render swine unconscious before humane slaughter.[22,63, 64] The combination of 40% $CO_2$ and approximately 3% CO has been used experimentally for euthanasia of dogs.[65] Carbon dioxide has been used in specially designed chambers to euthanatize individual cats[77,78] and other small laboratory animals.[51,72,79]

Studies of 1-day-old chickens have revealed that $CO_2$ is an effective euthanatizing agent. Inhalation of $CO_2$ caused little distress to the birds, suppressed nervous activity, and induced death within 5 minutes.[73] Because respiration begins during embryonic development, the unhatched chicken's environment may normally have a $CO_2$ concentration as high as 14%. Thus, $CO_2$ concentrations for euthanasia of newly hatched chickens and neonates of other species should be especially high. A $CO_2$ concentration of 60% to 70% with a 5-minute exposure time appears to be optimal.[73]

In studies of mink, high concentrations of $CO_2$ would kill them quickly, but a 70% $CO_2$ concentration induced loss of consciousness without killing them.[80] Some burrowing animals, such as rabbits of the species *Oryctolagus*, also have prolonged survival times when exposed to $CO_2$.[81] Some burrowing and diving animals have physiologic mechanisms for coping with hypercapnia. Therefore, it is necessary to have a sufficient concentration of $CO_2$ to kill the animal by hypoxemia following induction of anesthesia with $CO_2$.

*Advantages*—(1) The rapid depressant, analgesic, and anesthetic effects of $CO_2$ are well established. (2) Carbon dioxide is readily available and can be purchased in compressed gas cylinders. (3) Carbon dioxide is inexpensive, nonflammable, nonexplosive, and poses minimal hazard to personnel when used with properly designed equipment. (4) Carbon dioxide does not result in accumulation of tissue residues in food-producing animals. (5) Carbon dioxide euthanasia does not distort murine cholinergic markers[82] or corticosterone concentrations.[83]

*Disadvantages*—(1) Because $CO_2$ is heavier than air, incomplete filling of a chamber may permit animals to climb or raise their heads above the higher concentrations and avoid exposure. (2) Some species, such as fish and burrowing and diving mammals, may have extraordinary tolerance for $CO_2$. (3) Reptiles and amphibians may breathe too slowly for the use of $CO_2$. (4) Euthanasia by exposure to $CO_2$ may take longer than euthanasia by other means.[61] (5) Induction of loss of consciousness at lower concentrations (< 80%) may produce pulmonary and upper respiratory tract lesions.[67,84] (6) High concentrations of $CO_2$ may be distressful to some animals.

*Recommendations*—Carbon dioxide is acceptable for euthanasia in appropriate species (Tables 1 and 2). Compressed $CO_2$ gas in cylinders is the only recommended source of carbon dioxide because the inflow to the chamber can be regulated precisely. Carbon dioxide generated by other methods such as from dry ice, fire extinguishers, or chemical means (eg, antacids) is unacceptable. Species should be separated and cham-

bers should not be overcrowded. With an animal in the chamber, an optimal flow rate should displace at least 20% of the chamber volume per minute.[85] Loss of consciousness may be induced more rapidly by exposing animals to a $CO_2$ concentration of 70% or more by prefilling the chamber for species in which this has not been shown to cause distress. Gas flow should be maintained for at least 1 minute after apparent clinical death.[86] It is important to verify that an animal is dead before removing it from the chamber. If an animal is not dead, $CO_2$ narcosis must be followed with another method of euthanasia. Adding $O_2$ to the $CO_2$ may or may not preclude signs of distress.[67,87] Additional $O_2$ will, however, prolong time to death and may complicate determination of consciousness. There appears to be no advantage to combining $O_2$ with carbon dioxide for euthanasia.[87]

### Nitrogen, argon

Nitrogen ($N_2$) and argon (Ar) are colorless, odorless gases that are inert, nonflammable, and nonexplosive. Nitrogen comprises 78% of atmospheric air, whereas Ar comprises less than 1%.

Euthanasia is induced by placing the animal in a closed container that has been prefilled with $N_2$ or Ar or into which the gas is then rapidly introduced. Nitrogen/Ar displaces $O_2$, thus inducing death by hypoxemia.

In studies by Herin et al,[88] dogs became unconscious within 76 seconds when a $N_2$ concentration of 98.5% was achieved in 45 to 60 seconds. The electroencephalogram (EEG) became isoelectric (flat) in a mean time of 80 seconds, and arterial blood pressure was undetectable at 204 seconds. Although all dogs hyperventilated prior to loss of consciousness, the investigators concluded that this method induced death without pain. Following loss of consciousness, vocalization, gasping, convulsions, and muscular tremors developed in some dogs. At the end of a 5-minute exposure period, all dogs were dead.[88] These findings were similar to those for rabbits[89] and mink.[80,90]

With $N_2$ flowing at a rate of 39% of chamber volume per minute, rats collapsed in approximately 3 minutes and stopped breathing in 5 to 6 minutes. Regardless of flow rate, signs of panic and distress were evident before the rats collapsed and died.[85] Insensitivity to pain under such circumstances is questionable.[91]

Tranquilization with acepromazine, in conjunction with $N_2$ euthanasia of dogs, was investigated by Quine et al.[92] Using ECG and EEG recordings, they found these dogs had much longer survival times than dogs not given acepromazine before administration of $N_2$. In one dog, ECG activity continued for 51 minutes. Quine also addressed distress associated with exposure to $N_2$ by removing cats and dogs from the chamber following loss of consciousness and allowing them to recover. When these animals were put back into the chamber, they did not appear afraid or apprehensive.

Investigations into the aversiveness of Ar to swine and poultry have revealed that these animals will tolerate breathing 90% Ar with 2% $O_2$.[69,71] Swine voluntarily entered a chamber containing this mixture, for a food reward, and only withdrew from the chamber as they became ataxic. They reentered the chamber immediately to continue eating. Poultry also entered a chamber containing this mixture for a food reward and continued eating until they collapsed.[71] When Ar was used to euthanatize chickens, exposure to a chamber prefilled with Ar, with an $O_2$ concentration of < 2%, led to EEG changes and collapse in 9 to 12 seconds. Birds removed from the chamber at 15 to 17 seconds failed to respond to comb pinching. Continued exposure led to convulsions at 20 to 24 seconds. Somatosensory-evoked potentials were lost at 24 to 34 seconds, and the EEG became isoelectric at 57 to 66 seconds. Convulsion onset was after loss of consciousness (collapse and loss of response to comb pinch), so this would appear to be a humane method of euthanasia for chickens.[93] Despite the availability of some information, there is still much about the use of $N_2$/Ar that needs to be investigated.

*Advantages*—(1) Nitrogen and Ar are readily available as compressed gases. (2) Hazards to personnel are minimal.

*Disadvantages*—(1) Loss of consciousness is preceded by hypoxemia and ventilatory stimulation, which may be distressing to the animal. (2) Reestablishing a low concentration of $O_2$ (ie, 6% or greater) in the chamber before death will allow immediate recovery.[69]

*Recommendations*—Nitrogen and Ar can be distressful to some species (eg, rats).[85] Therefore, this technique is conditionally acceptable only if $O_2$ concentrations < 2% are achieved rapidly, and animals are heavily sedated or anesthetized. With heavy sedation or anesthesia, it should be recognized that death may be delayed. Although $N_2$ and Ar are effective, other methods of euthanasia are preferable.

### Carbon monoxide

Carbon monoxide (CO) is a colorless, odorless gas that is nonflammable and nonexplosive unless concentrations exceed 10%. It combines with hemoglobin to form carboxyhemoglobin and blocks uptake of $O_2$ by erythrocytes, leading to fatal hypoxemia.

In the past, mass euthanasia has been accomplished by use of 3 methods for generating CO: (1) chemical interaction of sodium formate and sulfuric acid, (2) exhaust fumes from idling gasoline internal combustion engines, and (3) commercially compressed CO in cylinders. The first 2 techniques are associated with problems such as production of other gases, achieving inadequate concentrations of carbon monoxide, inadequate cooling of the gas, and maintenance of equipment. Therefore, the only acceptable source is compressed CO in cylinders.

In a study by Ramsey and Eilmann,[94] 8% CO caused guinea pigs to collapse in 40 seconds to 2 minutes, and death occurred within 6 minutes. Carbon monoxide has been used to euthanatize mink[80,90] and chinchillas. These animals collapsed in 1 minute, breathing ceased in 2 minutes, and the heart stopped beating in 5 to 7 minutes.

In a study evaluating the physiologic and behavioral characteristics of dogs exposed to 6% CO in air, Chalifoux and Dallaire[95] could not determine the precise time of loss of consciousness. Electroencephalographic recordings revealed 20 to 25 seconds of abnormal cortical function prior to loss of consciousness. It was during this period that the dogs became agitated and vocalized. It is not known whether animals experience distress; however, humans in this phase reportedly are not distressed.[96] Subsequent studies have revealed that tranquilization with acepromazine significantly decreases behavioral and physiologic responses of dogs euthanatized with CO.[97]

In a comparative study, CO from gasoline engine exhaust and 70% $CO_2$ plus 30% $O_2$ were used to euthanatize cats. Euthanasia was divided into 3 phases. Phase I was the time from initial contact to onset of clinical signs (eg, yawning, staggering, or trembling). Phase II extended from the end of phase I until recumbency, and phase III from the end of phase II until death.[54] The study revealed that signs of agitation before loss of consciousness were greatest with $CO_2$ plus $O_2$. Convulsions occurred during phases II and III with both methods. However, when the euthanasia chamber was prefilled with CO (ie, exhaust fumes), convulsions did not occur in phase III. Time to complete immobilization was greater with $CO_2$ plus $O_2$ (approximately 90 seconds) than with CO alone (approximately 56 seconds).[54] In neonatal pigs, excitation was more likely to precede loss of consciousness if the pigs were exposed to a rapid rise in CO concentration. This agitation was reduced at lower flow rates, or when CO was combined with nitrogen.[98]

In people, the most common symptoms of early CO toxicosis are headache, dizziness, and weakness. As concentrations of carboxyhemoglobin increase, these signs may be followed by decreased visual acuity, tinnitus, nausea, progressive depression, confusion, and collapse.[99] Because CO stimulates motor centers in the brain, loss of consciousness may be accompanied by convulsions and muscular spasms.

Carbon monoxide is a cumulative poison.[96] Distinct signs of CO toxicosis are not evident until the CO concentration is 0.05% in air, and acute signs do not develop until the CO concentration is approximately 0.2% in air. In humans, exposure to 0.32% CO and 0.45% CO for one hour will induce loss of consciousness and death, respectively.[100] Carbon monoxide is extremely hazardous for personnel because it is highly toxic and difficult to detect. Chronic exposure to low concentrations of carbon monoxide may be a health hazard, especially with regard to cardiovascular disease and teratogenic effects.[101-103] An efficient exhaust or ventilatory system is essential to prevent accidental exposure of humans.

*Advantages*—(1) Carbon monoxide induces loss of consciousness without pain and with minimal discernible discomfort. (2) Hypoxemia induced by CO is insidious, so that the animal appears to be unaware. (3) Death occurs rapidly if concentrations of 4 to 6% are used.

*Disadvantages*—(1) Safeguards must be taken to prevent exposure of personnel. (2) Any electrical equipment exposed to CO (eg, lights and fans) must be explosion proof.

*Recommendations*—Carbon monoxide used for individual animal or mass euthanasia is acceptable for dogs, cats, and other small mammals, provided that commercially compressed CO is used and the following precautions are taken: (1) personnel using CO must be instructed thoroughly in its use and must understand its hazards and limitations; (2) the CO chamber must be of the highest quality construction and should allow for separation of individual animals; (3) the CO source and chamber must be located in a well-ventilated environment, preferably out of doors; (4) the chamber must be well lit and have view ports that allow personnel direct observation of animals; (5) the CO flow rate should be adequate to rapidly achieve a uniform CO concentration of at least 6% after animals are placed in the chamber, although some species (eg, neonatal pigs) are less likely to become agitated with a gradual rise in CO concentration;[98] and (6) if the chamber is inside a room, CO monitors must be placed in the room to warn personnel of hazardous concentrations. It is essential that CO use be in compliance with state and federal occupational health and safety regulations.

## NONINHALANT PHARMACEUTICAL AGENTS

The use of injectable euthanasia agents is the most rapid and reliable method of performing euthanasia. It is the most desirable method when it can be performed without causing fear or distress in the animal. When the restraint necessary for giving an animal an intravenous injection would impart added distress to the animal or pose undue risk to the operator, sedation, anesthesia, or an acceptable alternate route of administration should be employed. Aggressive, fearful, wild, or feral animals should be sedated or given a nonparalytic immobilizing agent prior to intravenous administration of the euthanasia agent.

When intravenous administration is considered impractical or impossible, intraperitoneal administration of a nonirritating euthanasia agent is acceptable, provided the drug does not contain neuromuscular blocking agents. Intracardiac injection is acceptable only when performed on heavily sedated, anesthetized, or comatose animals. It is not considered acceptable in awake animals, owing to the difficulty and unpredictability of performing the injection accurately. Intramuscular, subcutaneous, intrathoracic, intrapulmonary, intrahepatic, intrarenal, intrasplenic, intrathecal, and other nonvascular injections are not acceptable methods of administering injectable euthanasia agents.

When injectable euthanasia agents are administered into the peritoneal cavity, animals may be slow to pass through stages I and II of anesthesia. Accordingly, they should be placed in small cages in a quiet area to minimize excitement and trauma.

### Barbituric acid derivatives

Barbiturates depress the central nervous system in descending order, beginning with the cerebral cortex,

with loss of consciousness progressing to anesthesia. With an overdose, deep anesthesia progresses to apnea, owing to depression of the respiratory center, which is followed by cardiac arrest.

All barbituric acid derivatives used for anesthesia are acceptable for euthanasia when administered intravenously. There is a rapid onset of action, and loss of consciousness induced by barbiturates results in minimal or transient pain associated with venipuncture. Desirable barbiturates are those that are potent, long-acting, stable in solution, and inexpensive. Sodium pentobarbital best fits these criteria and is most widely used, although others such as secobarbital are also acceptable.

*Advantages*—(1) A primary advantage of barbiturates is speed of action. This effect depends on the dose, concentration, route, and rate of the injection. (2) Barbiturates induce euthanasia smoothly, with minimal discomfort to the animal. (3) Barbiturates are less expensive than many other euthanasia agents.

*Disadvantages*—(1) Intravenous injection is necessary for best results and requires trained personnel. (2) Each animal must be restrained. (3) Current federal drug regulations require strict accounting for barbiturates and these must be used under the supervision of personnel registered with the US Drug Enforcement Administration (DEA). (4) An aesthetically objectionable terminal gasp may occur in unconscious animals. (5) These drugs tend to persist in the carcass and may cause sedation or even death of animals that consume the body.

*Recommendations*—The advantages of using barbiturates for euthanasia in small animals far outweigh the disadvantages. Intravenous injection of a barbituric acid derivative is the preferred method for euthanasia of dogs, cats, other small animals, and horses. Intraperitoneal injection may be used in situations when an intravenous injection would be distressful or even dangerous. Intracardiac injection must only be used if the animal is heavily sedated, unconscious, or anesthetized.

### Pentobarbital combinations

Several euthanasia products are formulated to include a barbituric acid derivative (usually sodium pentobarbital), with added local anesthetic agents or agents that metabolize to pentobarbital. Although some of these additives are slowly cardiotoxic, this pharmacologic effect is inconsequential. These combination products are listed by the DEA as Schedule III drugs, making them somewhat simpler to obtain, store, and administer than Schedule II drugs such as sodium pentobarbital. The pharmacologic properties and recommended use of combination products that combine sodium pentobarbital with lidocaine or phenytoin are interchangeable with those of pure barbituric acid derivatives.

A combination of pentobarbital with a neuromuscular blocking agent is not an acceptable euthanasia agent.

### Chloral hydrate

Chloral hydrate depresses the cerebrum slowly; therefore, restraint may be a problem for some animals. Death is caused by hypoxemia resulting from progressive depression of the respiratory center, and may be preceded by gasping, muscle spasms, and vocalization.

*Recommendations*—Chloral hydrate is conditionally acceptable for euthanasia of large animals only when administered intravenously, and only after sedation to decrease the aforementioned undesirable side effects. Chloral hydrate is not acceptable for dogs, cats, and other small animals because the side effects may be severe, reactions can be aesthetically objectionable, and other products are better choices.

### T-61

T-61 is an injectable, nonbarbiturate, non-narcotic mixture of 3 drugs used for euthanasia. These drugs provide a combination of general anesthetic, curariform, and local anesthetic actions. T-61 has been withdrawn from the market and is no longer manufactured or commercially available in the United States. It is available in Canada and other countries. T-61 should be used only intravenously and at carefully monitored rates of injection, because there is some question as to the differential absorption and onset of action of the active ingredients when administered by other routes.[1]

### Tricaine methane sulfonate (MS 222, TMS)

MS 222 is commercially available as tricaine methane sulfonate (TMS), which can be used for the euthanasia of amphibians and fish. Tricaine is a benzoic acid derivative and, in water of low alkalinity (< 50 mg/L as $CaCo_3$); the solution should be buffered with sodium bicarbonate.[104] A 10 g/L stock solution can be made, and sodium bicarbonate added to saturation, resulting in a pH between 7.0 and 7.5 for the solution. The stock solution should be stored in a dark brown bottle, and refrigerated or frozen if possible. The solution should be replaced monthly and any time a brown color is observed.[105] For euthanasia, a concentration ≥ 250 mg/L is recommended and fish should be left in this solution for at least 10 minutes following cessation of opercular movement.[104] In the United States, there is a 21-day withdrawal time for MS 222; therefore, it is not appropriate for euthanasia of animals intended for food.

### Potassium chloride in conjunction with prior general anesthesia

Although unacceptable and condemned when used in unanaesthetized animals, the use of a supersaturated solution of potassium chloride injected intravenously or intracardially in an animal under general anesthesia is an acceptable method to produce cardiac arrest and death. The potassium ion is cardiotoxic, and rapid intravenous or intracardiac administration of 1 to 2 mmol/kg of body weight will cause cardiac arrest. This is a preferred injectable technique for euthanasia of livestock or wildlife species to reduce the risk of toxicosis for predators or scavengers in situations where carcasses of euthanatized animals may be consumed.[106,107]

*Advantages*—(1) Potassium chloride is not a controlled substance. It is easily acquired, transported, and mixed in the field. (2) Potassium chloride, when used with appropriate methods to render an animal unconscious, results in a carcass that is potentially less toxic for scavengers and predators in cases where carcass disposal is impossible or impractical.

*Disadvantage*—Rippling of muscle tissue and clonic spasms may occur on or shortly after injection.

*Recommendations*—It is of utmost importance that personnel performing this technique are trained and knowledgeable in anesthetic techniques, and are competent in assessing anesthetic depth appropriate for administration of potassium chloride intravenously. Administration of potassium chloride intravenously requires animals to be in a surgical plane of anesthesia characterized by loss of consciousness, loss of reflex muscle response, and loss of response to noxious stimuli. Saturated potassium chloride solutions are effective in causing cardiac arrest following rapid intracardiac or intravenous injection. Residual tissue concentrations of general anesthetics after anesthetic induction have not been documented. Whereas no scavenger toxicoses have been reported with potassium chloride in combination with a general anesthetic, proper carcass disposal should always be attempted to prevent possible toxicosis by consumption of a carcass contaminated with general anesthetics.

### Unacceptable injectable agents

When used alone, the injectable agents listed in Appendix 4 (strychnine, nicotine, caffeine, magnesium sulfate, potassium chloride, cleaning agents, solvents, disinfectants and other toxins or salts, and all neuromuscular blocking agents) are unacceptable and are absolutely condemned for use as euthanasia agents.

## PHYSICAL METHODS

Physical methods of euthanasia include captive bolt, gunshot, cervical dislocation, decapitation, electrocution, microwave irradiation, kill traps, thoracic compression, exsanguination, stunning, and pithing. When properly used by skilled personnel with well-maintained equipment, physical methods of euthanasia may result in less fear and anxiety and be more rapid, painless, humane, and practical than other forms of euthanasia. Exsanguination, stunning, and pithing are not recommended as a sole means of euthanasia, but should be considered adjuncts to other agents or methods.

Some consider physical methods of euthanasia aesthetically displeasing. There are occasions, however, when what is perceived as aesthetic and what is most humane are in conflict. Physical methods may be the most appropriate method for euthanasia and rapid relief of pain and suffering in certain situations. Personnel performing physical methods of euthanasia must be well trained and monitored for each type of physical technique performed. That person must also be sensitive to the aesthetic implications of the method and inform onlookers about what they should expect when possible.

Since most physical methods involve trauma, there is inherent risk for animals and humans. Extreme care and caution should be used. Skill and experience of personnel is essential. If the method is not performed correctly, animals and personnel may be injured. Inexperienced persons should be trained by experienced persons and should practice on carcasses or anesthetized animals to be euthanatized until they are proficient in performing the method properly and humanely. When done appropriately, the panel considers most physical methods conditionally acceptable for euthanasia.

### Penetrating captive bolt

A penetrating captive bolt is used for euthanasia of ruminants, horses, swine, laboratory rabbits, and dogs.[108] Its mode of action is concussion and trauma to the cerebral hemisphere and brainstem.[109,110] Captive bolt guns are powered by gunpowder or compressed air and must provide sufficient energy to penetrate the skull of the species on which they are being used.[109] Adequate restraint is important to ensure proper placement of the captive bolt. A cerebral hemisphere and the brainstem must be sufficiently disrupted by the projectile to induce sudden loss of consciousness and subsequent death. Accurate placement of captive bolts for various species has been described.[109-112] A multiple projectile has been suggested as a more effective technique, especially for large cattle.[109]

A nonpenetrating captive bolt only stuns animals and should not be used as a sole means of euthanasia (see "Stunning" under "Adjunctive Methods").

*Advantage*—The penetrating captive bolt is an effective method of euthanasia for use in slaughterhouses, in research facilities, and on the farm when use of drugs is inappropriate.

*Disadvantages*—(1) It is aesthetically displeasing. (2) Death may not occur if equipment is not maintained and used properly.

*Recommendations*—Use of the penetrating captive bolt is an acceptable and practical method of euthanasia for horses, ruminants, and swine. It is conditionally acceptable in other appropriate species. The nonpenetrating captive bolt must not be used as a sole method of euthanasia.

### Euthanasia by a blow to the head

Euthanasia by a blow to the head must be evaluated in terms of the anatomic features of the species on which it is to be performed. A blow to the head can be a humane method of euthanasia for neonatal animals with thin craniums, such as young pigs, if a single sharp blow delivered to the central skull bones with sufficient force can produce immediate depression of the central nervous system and destruction of brain tissue. When properly performed, loss of consciousness is rapid. The anatomic features of neonatal calves, however, make a blow to the head in this species unacceptable. Personnel performing euthanasia by use of a blow to the head must be properly trained and monitored for proficiency with this method of euthanasia, and they must be aware of its aesthetic implications.

## Gunshot

A properly placed gunshot can cause immediate insensibility and humane death. In some circumstances, a gunshot may be the only practical method of euthanasia. Shooting should only be performed by highly skilled personnel trained in the use of firearms and only in jurisdictions that allow for legal firearm use. Personnel, public, and nearby animal safety should be considered. The procedure should be performed outdoors and away from public access.

For use of a gunshot to the head as a method of euthanasia in captive animals, the firearm should be aimed so that the projectile enters the brain, causing instant loss of consciousness.[51,112-114] This must take into account differences in brain position and skull conformation between species, as well as the energy requirement for skull bone and sinus penetration.[109,115] Accurate targeting for a gunshot to the head in various species has been described.[114,116-119] For wildlife and other freely roaming animals, the preferred target area should be the head. The appropriate firearm should be selected for the situation, with the goal being penetration and destruction of brain tissue without emergence from the contralateral side of the head.[120] A gunshot to the heart or neck does not immediately render animals unconscious and thus is not considered to meet the panel's definition of euthanasia.[121]

*Advantages*—(1) Loss of consciousness is instantaneous if the projectile destroys most of the brain. (2) Given the need to minimize stress induced by handling and human contact, gunshot may at times be the most practical and logical method of euthanasia of wild or free-ranging species.

*Disadvantages*—(1) Gunshot may be dangerous to personnel. (2) It is aesthetically unpleasant. (3) Under field conditions, it may be difficult to hit the vital target area. (4) Brain tissue may not be able to be examined for evidence of rabies infection or chronic wasting disease when the head is targeted.

*Recommendations*—When other methods cannot be used, an accurately delivered gunshot is a conditionally acceptable method of euthanasia.[114,122-125] When an animal can be appropriately restrained, the penetrating captive bolt is preferred to a gunshot. Prior to shooting, animals accustomed to the presence of humans should be treated in a calm and reassuring manner to minimize anxiety. In the case of wild animals, gunshots should be delivered with the least amount of prior human contact necessary. Gunshot should not be used for routine euthanasia of animals in animal control situations, such as municipal pounds or shelters.

## Cervical dislocation

Cervical dislocation is a technique that has been used for many years and, when performed by well-trained individuals, appears to be humane. However, there are few scientific studies to confirm this observation. This technique is used to euthanatize poultry, other small birds, mice, and immature rats and rabbits. For mice and rats, the thumb and index finger are placed on either side of the neck at the base of the skull or, alternatively, a rod is pressed at the base of the skull. With the other hand, the base of the tail or the hind limbs are quickly pulled, causing separation of the cervical vertebrae from the skull. For immature rabbits, the head is held in one hand and the hind limbs in the other. The animal is stretched and the neck is hyperextended and dorsally twisted to separate the first cervical vertebra from the skull.[72,111] For poultry, cervical dislocation by stretching is a common method for mass euthanasia, but loss of consciousness may not be instantaneous.[134]

Data suggest that electrical activity in the brain persists for 13 seconds following cervical dislocation,[127] and unlike decapitation, rapid exsanguination does not contribute to loss of consciousness.[128,129]

*Advantages*—(1) Cervical dislocation is a technique that may induce rapid loss of consciousness.[84,127] (2) It does not chemically contaminate tissue. (3) It is rapidly accomplished.

*Disadvantages*—(1) Cervical dislocation may be aesthetically displeasing to personnel. (2) Cervical dislocation requires mastering technical skills to ensure loss of consciousness is rapidly induced. (3) Its use is limited to poultry, other small birds, mice, and immature rats and rabbits.

*Recommendations*—Manual cervical dislocation is a humane technique for euthanasia of poultry, other small birds, mice, rats weighing < 200 g, and rabbits weighing < 1 kg when performed by individuals with a demonstrated high degree of technical proficiency. In lieu of demonstrated technical competency, animals must be sedated or anesthetized prior to cervical dislocation. The need for technical competency is greater in heavy rats and rabbits, in which the large muscle mass in the cervical region makes manual cervical dislocation physically more difficult.[130] In research settings, this technique should be used only when scientifically justified by the user and approved by the Institutional Animal Care and Use Committee.

Those responsible for the use of this technique must ensure that personnel performing cervical dislocation techniques have been properly trained and consistently apply it humanely and effectively.

## Decapitation

Decapitation can be used to euthanatize rodents and small rabbits in research settings. It provides a means to recover tissues and body fluids that are chemically uncontaminated. It also provides a means of obtaining anatomically undamaged brain tissue for study.[131]

Although it has been demonstrated that electrical activity in the brain persists for 13 to 14 seconds following decapitation,[132] more recent studies and reports indicate that this activity does not infer the ability to perceive pain, and in fact conclude that loss of consciousness develops rapidly.[127-129]

Guillotines that are designed to accomplish decapitation in adult rodents and small rabbits in a uniformly instantaneous manner are commercially available.

Guillotines are not commercially available for neonatal rodents, but sharp blades can be used for this purpose.

*Advantages*—(1) Decapitation is a technique that appears to induce rapid loss of consciousness.[127-129] (2) It does not chemically contaminate tissues. (3) It is rapidly accomplished.

*Disadvantages*—(1) Handling and restraint required to perform this technique may be distressful to animals.[83] (2) The interpretation of the presence of electrical activity in the brain following decapitation has created controversy and its importance may still be open to debate.[127-129,132] (3) Personnel performing this technique should recognize the inherent danger of the guillotine and take adequate precautions to prevent personal injury. (4) Decapitation may be aesthetically displeasing to personnel performing or observing the technique.

*Recommendations*—This technique is conditionally acceptable if performed correctly, and it should be used in research settings when its use is required by the experimental design and approved by the Institutional Animal Care and Use Committee. The equipment used to perform decapitation should be maintained in good working order and serviced on a regular basis to ensure sharpness of blades. The use of plastic cones to restrain animals appears to reduce distress from handling, minimizes the chance of injury to personnel, and improves positioning of the animal in the guillotine. Decapitation of amphibians, fish, and reptiles is addressed elsewhere in this report.

Those responsible for the use of this technique must ensure that personnel who perform decapitation techniques have been properly trained to do so.

## Electrocution

Electrocution, using alternating current, has been used as a method of euthanasia for species such as dogs, cattle, sheep, swine, foxes, and mink.[113,133-138] Electrocution induces death by cardiac fibrillation, which causes cerebral hypoxia.[135,137,139] However, animals do not lose consciousness for 10 to 30 seconds or more after onset of cardiac fibrillation. It is imperative that animals be unconscious before being electrocuted. This can be accomplished by any acceptable means, including electrical stunning.[25] Although an effective, 1-step stunning and electrocution method has been described for use in sheep and hogs, euthanasia by electrocution in most species remains a 2-step procedure.[25,63,140]

*Advantages*—(1) Electrocution is humane if the animal is first rendered unconscious. (2) It does not chemically contaminate tissues. (3) It is economical.

*Disadvantages*—(1) Electrocution may be hazardous to personnel. (2) When conventional single-animal probes are used, it may not a useful method for mass euthanasia because so much time is required per animal. (3) It is not a useful method for dangerous, intractable animals. (4) It is aesthetically objectionable because of violent extension and stiffening of the limbs, head, and neck. (5) It may not result in death in small animals (< 5 kg) because ventricular fibrillation and circulatory collapse do not always persist after cessation of current flow.

*Recommendations*—Euthanasia by electrocution requires special skills and equipment that will ensure passage of sufficient current through the brain to induce loss of consciousness and cardiac fibrillation in the 1-step method for sheep and hogs, or cardiac fibrillation in the unconscious animal when the 2-step procedure is used. Although the method is conditionally acceptable if the aforementioned requirements are met, its disadvantages far outweigh its advantages in most applications. Techniques that apply electric current from head to tail, head to foot, or head to moistened metal plates on which the animal is standing are unacceptable.

## Microwave irradiation

Heating by microwave irradiation is used primarily by neurobiologists to fix brain metabolites *in vivo* while maintaining the anatomic integrity of the brain.[141] Microwave instruments have been specifically designed for use in euthanasia of laboratory mice and rats. The instruments differ in design from kitchen units and may vary in maximal power output from 1.3 to 10 kw. All units direct their microwave energy to the head of the animal. The power required to rapidly halt brain enzyme activity depends on the efficiency of the unit, the ability to tune the resonant cavity and the size of the rodent head.[142] There is considerable variation among instruments in the time required for loss of consciousness and euthanasia. A 10 kw, 2,450 MHz instrument operated at a power of 9 kw will increase the brain temperature of 18 to 28 g mice to 79 C in 330 ms, and the brain temperature of 250 to 420 g rats to 94 C in 800 ms.[143]

*Advantages*—(1) Loss of consciousness is achieved in less than 100 ms, and death in less than 1 second. (2) This is the most effective method to fix brain tissue *in vivo* for subsequent assay of enzymatically labile chemicals.

*Disadvantages*—(1) Instruments are expensive. (2) Only animals the size of mice and rats can be euthanatized with commercial instruments that are currently available.

*Recommendations*—Microwave irradiation is a humane method for euthanatizing small laboratory rodents if instruments that induce rapid loss of consciousness are used. Only instruments that are designed for this use and have appropriate power and microwave distribution can be used. Microwave ovens designed for domestic and institutional kitchens are absolutely unacceptable for euthanasia.

## Thoracic (cardiopulmonary, cardiac) compression

Thoracic (cardiopulmonary, cardiac) compression is used to euthanatize small- to medium-sized free-ranging birds when alternate techniques described in this report are not practical.[144]

*Advantages*—(1) This technique is rapid. (2) It is apparently painless. (3) It maximizes carcass use for analytical/contaminant studies.

*Disadvantages*—(1) It may be considered aesthetically unpleasant by onlookers. (2) The degree of distress is unknown.

*Recommendations*—Thoracic (cardiopulmonary, cardiac) compression is a physical technique for avian euthanasia that has applicability in the field when other methods cannot be used. It is accomplished by bringing the thumb and forefinger of one hand under the bird's wing from the posterior and placing them against the ribs.[144] The forefinger of the other hand is placed against the ventral edge of the sternum, just below the furculum. All fingers are brought together forcefully and held under pressure to stop the heart and lungs. Loss of consciousness and death develop quickly. Proper training is needed in the use of this technique to avoid trauma to the bird. Cardiopulmonary compression is not appropriate for laboratory settings, for large or diving birds,[144] or for other species.

### Kill traps

Mechanical kill traps are used for the collection and killing of small, free-ranging mammals for commercial purposes (fur, skin, or meat), scientific purposes, to stop property damage, and to protect human safety. Their use remains controversial, and the panel recognizes that kill traps do not always render a rapid or stress-free death consistent with criteria for euthanasia found elsewhere in this document. For this reason, use of live traps followed by other methods of euthanasia is preferred. There are a few situations when that is not possible or when it may actually be more stressful to the animals or dangerous to humans to use live traps. Although newer technologies are improving kill trap performance in achieving loss of consciousness quickly, individual testing is recommended to be sure the trap is working properly.[145] If kill traps must be used, the most humane available must be chosen,[146-148] as evaluated by use of International Organization for Standardization (ISO) testing procedures,[149] or by the methods of Gilbert,[150] Proulx et al,[151,152] or Hiltz and Roy.[153]

To reach the required level of efficiency, traps may need to be modified from manufacturers production standards. In addition, as specified in scientific studies, trap placement (ground versus tree sets), bait type, set location, selectivity apparatus, body placement modifying devices (eg, sidewings, cones), trigger sensitivity, and trigger type, size, and conformation are essential considerations that could affect a kill trap's ability to reach these standards.

Several kill traps, modifications, and set specifics have been scientifically evaluated and found to meet the aforereferenced standards for various species.[151,152,154-167]

*Advantage*—Free-ranging small mammals may be killed with minimal distress associated with handling and human contact.

*Disadvantages*—(1) Traps may not afford death within acceptable time periods. (2) Selectivity and efficiency is dependent on the skill and proficiency of the operator.

*Recommendations*—Kill traps do not always meet the panel's criteria for euthanasia. At the same time, it is recognized that they can be practical and effective for scientific animal collection when used in a manner that ensures selectivity, a swift kill, no damage to body parts needed for field research, and minimal potential for injury of nontarget species.[168,169] Traps need to be checked at least once daily. In those instances when an animal is wounded or captured but not dead, the animal must be killed quickly and humanely. Kill traps should be used only when other acceptable techniques are impossible or have failed. Traps for nocturnal species should not be activated during the day to avoid capture of diurnal species.[168] Trap manufacturers should strive to meet their responsibility of minimizing pain and suffering in target species.

### Adjunctive methods

Stunning and pithing, when properly done, induce loss of consciousness but do not ensure death. Therefore, these methods must be used only in conjunction with other procedures,[123] such as pharmacologic agents, exsanguination, or decapitation to euthanatize the animal.

#### Exsanguination

Exsanguination can be used to ensure death subsequent to stunning, or in otherwise unconscious animals. Because anxiety is associated with extreme hypovolemia, exsanguination must not be used as a sole means of euthanasia.[170] Animals may be exsanguinated to obtain blood products, but only when they are sedated, stunned, or anesthetized.[171]

#### Stunning

Animals may be stunned by a blow to the head, by use of a nonpenetrating captive bolt, or by use of electric current. Stunning must be followed immediately by a method that ensures death. With stunning, evaluating loss of consciousness is difficult, but it is usually associated with a loss of the menace or blink response, pupillary dilatation, and a loss of coordinated movements. Specific changes in the electroencephalogram and a loss of visually evoked responses are also thought to indicate loss of consciousness.[60,172]

Blow to the head—Stunning by a blow to the head is used primarily in small laboratory animals with thin craniums.[9,173-175] A single sharp blow must be delivered to the central skull bones with sufficient force to produce immediate depression of the central nervous system. When properly done, consciousness is lost rapidly.

Nonpenetrating captive bolt—A nonpenetrating captive bolt may be used to induce loss of consciousness in ruminants, horses, and swine. Signs of effective stunning by captive bolt are immediate collapse and a several second period of tetanic spasm, followed by slow hind limb movements of increasing frequency.[60,176]

Other aspects regarding use of the nonpenetrating captive bolt are similar to the use of a penetrating captive bolt, as previously described.

Electrical stunning—Alternating electrical current has been used for stunning species such as dogs, cattle, sheep, goats, hogs, fish and chickens.[133,134,140,177,178] Experiments with dogs have identified a need to direct the electrical current through the brain to induce rapid loss of consciousness. In dogs, when electricity passes only between fore- and hind limbs or neck and feet, it causes the heart to fibrillate but does not induce sudden loss of consciousness.[139] For electrical stunning of any animal, an apparatus that applies electrodes to opposite sides of the head, or in another way directs electrical current immediately through the brain, is necessary to induce rapid loss of consciousness. Attachment of electrodes and animal restraint can pose problems with this form of stunning. Signs of effective electrical stunning are extension of the limbs, opisthotonos, downward rotation of the eyeballs, and tonic spasm changing to clonic spasm, with eventual muscle flaccidity.

Electrical stunning should be followed promptly by electrically induced cardiac fibrillation, exsanguination, or other appropriate methods to ensure death. Refer to the section on electrocution for additional information.

### Pithing

In general, pithing is used as an adjunctive procedure to ensure death in an animal that has been rendered unconscious by other means. For some species, such as frogs, with anatomic features that facilitate easy access to the central nervous system, pithing may be used as a sole means of euthanasia, but an anesthetic overdose is a more suitable method.

## SPECIAL CONSIDERATIONS

### Equine euthanasia

Pentobarbital or a pentobarbital combination is the best choice for equine euthanasia. Because a large volume of solution must be injected, use of an intravenous catheter placed in the jugular vein will facilitate the procedure. To facilitate catheterization of an excitable or fractious animal, a tranquilizer such as acepromazine, or an alpha-2 adrenergic agonist can be administered, but these drugs may prolong time to loss of consciousness because of their effect on circulation and may result in varying degrees of muscular activity and agonal gasping. Opioid agonists or agonist/antagonists in conjunction with alpha-2 adrenergic agonists may further facilitate restraint.

In certain emergency circumstances, such as euthanasia of a horse with a serious injury at a racetrack, it may be difficult to restrain a dangerous horse or other large animal for intravenous injection. The animal might cause injury to itself or to bystanders before a sedative could take effect. In such cases, the animal can be given a neuromuscular blocking agent such as succinylcholine, but the animal must be euthanatized with an appropriate technique as soon as the animal can be controlled. Succinylcholine alone or without sufficient anesthetic must not be used for euthanasia.

Physical methods, including gunshot, are considered conditionally acceptable techniques for equine euthanasia. The penetrating captive bolt is acceptable with appropriate restraint.

### Animals intended for human or animal food

In euthanasia of animals intended for human or animal food, chemical agents that result in tissue residues cannot be used, unless they are approved by the US Food and Drug Administration.[179] Carbon dioxide is the only chemical currently used for euthanasia of food animals (primarily swine) that does not result in tissue residues. Physical techniques are commonly used for this reason. Carcasses of animals euthanatized by barbituric acid derivatives or other chemical agents may contain potentially harmful residues. These carcasses should be disposed of in a manner that will prevent them from being consumed by human beings or animals.

Selection of a proper euthanasia technique for free-ranging wildlife must take into account the possibility of consumption of the carcass of the euthanatized animal by nontarget predatory or scavenger species. Numerous cases of toxicosis and death attributable to ingestion of pharmaceutically contaminated carcasses in predators and scavengers have been reported.[107] Proper carcass disposal must be a part of any euthanasia procedure under free-range conditions where there is potential for consumption toxicity. When carcasses are to be left in the field, a gunshot to the head, penetrating captive bolt, or injectable agents that are nontoxic (potassium chloride in combination with a nontoxic general anesthetic) should be used so that the potential for scavenger or predator toxicity is lessened.

### Euthanasia of nonconventional species: zoo, wild, aquatic, and ectothermic animals

Compared with objective information on companion, farm, and laboratory animals, euthanasia of species such as zoo, wild, aquatic, and ectothermic animals has been studied less, and guidelines are more limited. Irrespective of the unique or unusual features of some species, whenever it becomes necessary to euthanatize an animal, death must be induced as painlessly and quickly as possible.

When selecting a means of euthanasia for these species, factors and criteria in addition to those previously discussed must be considered. The means selected will depend on the species, size, safety aspects, location of the animals to be euthanatized, and experience of personnel. Whether the animal to be euthanatized is in the wild, in captivity, or free-roaming are major considerations. Anatomic differences must be considered. For example, amphibians, fish, reptiles, and marine mammals differ anatomically from domestic species. Veins may be difficult to locate. Some species have a carapace or other defensive anatomic adaptations (eg, quills, scales, spines). For physical methods, access to the central nervous system may be difficult because the brain may be small and difficult to locate by inexperienced persons.

### Zoo Animals

For captive zoo mammals and birds with related domestic counterparts, many of the means described previously are appropriate. However, to minimize injury to persons or animals, additional precautions such as handling and physical or chemical restraint are important considerations.[16]

### Wildlife

For wild and feral animals, many recommended means of euthanasia for captive animals are not feasible. The panel recognizes there are situations involving free-ranging wildlife when euthanasia is not possible from the animal or human safety standpoint, and killing may be necessary. Conditions found in the field, although more challenging than those that are controlled, do not in any way reduce or minimize the ethical obligation of the responsible individual to reduce pain and distress to the greatest extent possible during the taking of an animal's life. Because euthanasia of wildlife is often performed by lay personnel in remote settings, guidelines are needed to assist veterinarians, wildlife biologists, and wildlife health professionals in developing humane protocols for euthanasia of wildlife.

In the case of free-ranging wildlife, personnel may not be trained in the proper use of remote anesthesia, proper delivery equipment may not be available, personnel may be working alone in remote areas where accidental exposure to potent anesthetic medications used in wildlife capture would present a risk to human safety, or approaching the animal within a practical darting distance may not be possible. In these cases, the only practical means of animal collection may be gunshot and kill trapping.[13,180-184] Under these conditions, specific methods chosen must be as age-, species-, or taxonomic/class-specific as possible. The firearm and ammunition should be appropriate for the species and purpose. Personnel should be sufficiently skilled to be accurate, and they should be experienced in the proper and safe use of firearms, complying with laws and regulations governing their possession and use.

Behavioral responses of wildlife or captive nontraditional species (zoo) in close human contact are very different from those of domestic animals. These animals are usually frightened and distressed. Thus, minimizing the amount, degree, and/or cognition of human contact during procedures that require handling is of utmost importance. Handling these animals often requires general anesthesia, which provides loss of consciousness and which relieves distress, anxiety, apprehension, and perception of pain. Even though the animal is under general anesthesia, minimizing auditory, visual, and tactile stimulation will help ensure the most stress-free euthanasia possible. With use of general anesthesia, there are more methods for euthanasia available.

A 2-stage euthanasia process involving general anesthesia, tranquilization, or use of analgesics, followed by intravenous injectable pharmaceuticals, although preferred, is often not practical. Injectable anesthetics are not always legally or readily available to those working in nuisance animal control, and the distress to the animal induced by live capture, transport to a veterinary facility, and confinement in a veterinary hospital prior to euthanasia must be considered in choosing the most humane technique for the situation at hand. Veterinarians providing support to those working with injured or live-trapped, free-ranging animals should take capture, transport, handling distress, and possible carcass consumption into consideration when asked to assist with euthanasia. Alternatives to 2-stage euthanasia using anesthesia include a squeeze cage with intraperitoneal injection of sodium pentobarbital, inhalant agents ($CO_2$ chamber, CO chamber), and gunshot. In cases where preeuthanasia anesthetics are not available, intraperitoneal injections of sodium pentobarbital, although slower in producing loss of consciousness, should be considered preferable over intravenous injection, if restraint will cause increased distress to the animal or danger to the operator.

Wildlife species may be encountered under a variety of situations. Euthanasia of the same species under different conditions may require different techniques. Even in a controlled setting, an extremely fractious large animal may threaten the safety of the practitioner, bystanders, and itself. When safety is in question and the fractious large animal, whether wild, feral, or domestic, is in close confinement, neuromuscular blocking agents may be used immediately prior to the use of an acceptable form of euthanasia. For this technique to be humane, the operator must ensure they will gain control over the animal and perform euthanasia before distress develops. Succinylcholine is not acceptable as a method of restraint for use in free-ranging wildlife because animals may not be retrieved rapidly enough to prevent neuromuscular blocking agent-induced respiratory distress or arrest.[185]

### Diseased, Injured, or Live-Captured Wildlife or Feral Species

Euthanasia of diseased, injured, or live-trapped wildlife should be performed by qualified professionals. Certain cases of wildlife injury (eg, acute, severe trauma from automobiles) may require immediate action, and pain and suffering in the animal may be best relieved most rapidly by physical methods including gunshot or penetrating captive bolt followed by exsanguination.

### Birds

Many techniques discussed previously in this report are suitable for euthanasia of captive birds accustomed to human contact. Free-ranging birds may be collected by a number of methods, including nets and live traps, with subsequent euthanasia. For collection by firearm, shotguns are recommended. The bird should be killed outright by use of ammunition loads appropriate for the species to be collected. Wounded birds should be killed quickly by appropriate techniques previously described. Large birds should be anesthetized prior to euthanasia, using general anesthetics.

## Amphibians, Fish, and Reptiles

Euthanasia of ectothermic animals must take into account differences in their metabolism, respiration, and tolerance to cerebral hypoxia. In addition, it is often more difficult to ascertain when an animal is dead. Some unique aspects of euthanasia of amphibians, fishes, and reptiles have been described.[13,51,186,187]

Injectable agents—Sodium pentobarbital (60 to 100 mg/kg of body weight) can be administered intravenously, intraabdominally, or intrapleuroperitoneally in most ectothermic animals, depending on anatomic features. Subcutaneous lymph spaces may also be used in frogs and toads. Time to effect may be variable, with death occurring in up to 30 minutes.[1,187,188] Barbiturates other than pentobarbital can cause pain on injection.[189]

Clove oil—Because adequate and appropriate clinical trials have not been performed on fish to evaluate its effects, use of clove oil is not acceptable.

External or topical agents—Tricaine methane sulfonate (TMS, MS-222) may be administered by various routes to euthanatize. For fish and amphibians, this chemical may be placed in water.[190-193] Large fish may be removed from the water, a gill cover lifted, and a concentrated solution from a syringe flushed over the gills. MS 222 is acidic and in concentrations ≥ 500 mg/L should be buffered with sodium bicarbonate to saturation resulting in a solution pH of 7.0 to 7.5.[105] MS 222 may also be injected into lymph spaces and pleuroperitoneal cavities.[194] These are effective but expensive means of euthanasia.

Benzocaine hydrochloride, a compound similar to TMS, may be used as a bath or in a recirculation system for euthanasia of fish[184] or amphibians.[13] Benzocaine is not water soluble and therefore is prepared as a stock solution (100 g/L), using acetone or ethanol, which may be irritating to fish tissues. In contrast, benzocaine hydrochloride is water soluble and can be used directly for anesthesia or euthanasia.[105] A concentration ≥ 250 mg/L can be used for euthanasia. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.[104]

The anesthetic agent 2-phenoxyethanol is used at concentrations of 0.5 to 0.6 ml/L or 0.3 to 0.4 mg/L for euthanasia of fish. Death is caused by respiratory collapse. As with other agents, fish should be left in solution for 10 minutes following cessation of opercular movement.[195,196]

Inhalant agents—Many reptiles and amphibians, including chelonians, are capable of holding their breath and converting to anaerobic metabolism, and can survive long periods of anoxia (up to 27 hours for some species).[197-202] Because of this ability to tolerate anoxia, induction of anesthesia and time to loss of consciousness may be greatly prolonged when inhalants are used. Death in these species may not occur even after prolonged inhalant exposure.[203] Lizards, snakes, and fish do not hold their breath to the same extent and can be euthanatized by use of inhalant agents.

Carbon dioxide—Amphibians,[1] reptiles,[1] and fish[203-205] may be euthanatized with $CO_2$. Loss of consciousness develops rapidly, but exposure times required for euthanasia are prolonged. This technique is more effective in active species and those with less tendency to hold their breath.

Physical methods—Line drawings of the head of various amphibians and reptiles, with recommended locations for captive bolt or firearm penetration, are available.[13] Crocodilians and other large reptiles can also be shot through the brain.[51]

Decapitation with heavy shears or a guillotine is effective for some species that have appropriate anatomic features. It has been assumed that stopping blood supply to the brain by decapitation causes rapid loss of consciousness. Because the central nervous system of reptiles, fish, and amphibians is tolerant to hypoxic and hypotensive conditions,[13] decapitation must be followed by pithing.[188]

Two-stage euthanasia procedures—Propofol and ultrashort-acting barbiturates may be used for these species to produce rapid general anesthesia prior to final administration of euthanasia.

In zoos and clinical settings, neuromuscular blocking agents are considered acceptable for restraint of reptiles if given immediately prior to administration of a euthanatizing agent.

Most amphibians, fishes, and reptiles can be euthanatized by cranial concussion (stunning) followed by decapitation, pithing, or some other physical method.

Severing the spinal cord behind the head by pithing is an effective method of killing some ectotherms. Death may not be immediate unless both the brain and spinal cord are pithed. For these animals, pithing of the spinal cord should be followed by decapitation and pithing of the brain or by another appropriate procedure. Pithing requires dexterity and skill and should only be done by trained personnel. The pithing site in frogs is the foramen magnum, and it is identified by a slight midline skin depression posterior to the eyes with the neck flexed.[187]

Cooling—It has been suggested that, when using physical methods of euthanasia in ectothermic species, cooling to 4 C will decrease metabolism and facilitate handling, but there is no evidence that whole body cooling reduces pain or is clinically efficacious.[206] Local cooling in frogs does reduce nociception, and this may be partly opioid mediated. [207] Immobilization of reptiles by cooling is considered inappropriate and inhumane even if combined with other physical or chemical methods of euthanasia. Snakes and turtles, immobilized by cooling, have been killed by subsequent freezing. This method is not recommended.[13] Formation of ice crystals on the skin and in tissues of an animal may cause pain or distress. Quick freezing of deeply anesthetized animals is acceptable.[208]

## Marine Mammals

Barbiturates or potent opioids (eg, etorphine hydrochloride [M 99] and carfentanil) are the agents of choice for euthanasia of marine mammals,[209] although it is recognized their use is not always possible and can

be potentially dangerous to personnel. An accurately placed gunshot may also be a conditionally acceptable method of euthanasia for some species and sizes of stranded marine mammals.[51,209,210]

For stranded whales or other large cetaceans or pinnipeds, succinylcholine chloride in conjunction with potassium chloride, administered intravenously or intraperitoneally, has been used.[211] This method, which is not an acceptable method of euthanasia as defined in this report, leads to complete paralysis of the respiratory musculature and eventual death attributable to hypoxemia.[209] This method may be more humane than allowing the stranded animal to suffocate over a period of hours or days if no other options are available.

### Euthanasia of animals raised for fur production

Animals raised for fur are usually euthanatized individually at the location where they are raised. Although any handling of these species constitutes a stress, it is possible to minimize this by euthanatizing animals in or near their cages. For the procedures described below, please refer to previous sections for more detailed discussion.

Carbon monoxide—For smaller species, CO appears to be an adequate method for euthanasia. Compressed CO is delivered from a tank into an enclosed cage that can be moved adjacent to holding cages. Using the apparatus outside reduces the risk to humans; however, people using this method should still be made aware of the dangers of CO. Animals introduced into a chamber containing 4% CO lost consciousness in 64 ± 14 seconds and were dead within 215 ± 45 seconds.[80] In a study involving electroencephalography of mink being euthanatized with 3.5% CO, the mink were comatose in 21 ± 7 seconds.[212] Only 1 animal should be introduced into the chamber at a time, and death should be confirmed in each case.

Carbon dioxide—Administration of $CO_2$ is also a good euthanasia method for smaller species and is less dangerous than CO for personnel operating the system. When exposed to 100% $CO_2$, mink lost consciousness in 19 ± 4 seconds and were dead within 153 ± 10 seconds. When 70% $CO_2$ was used with 30% $O_2$, mink were unconscious in 28 seconds, but they were not dead after a 15-minute exposure.[80] Therefore, if animals are first stunned by 70% $CO_2$, they should be killed by exposure to 100% $CO_2$ or by some other means. As with carbon monoxide, only one animal should be introduced into the chamber at a time.

Barbiturates—Barbiturate overdose is an acceptable procedure for euthanasia of many species of animals raised for fur. The drug is injected intraperitoneally and the animal slowly loses consciousness. It is important that the death of each animal be confirmed following barbiturate injection. Barbiturates will contaminate the carcass; therefore the skinned carcass cannot be used for animal food.

Electrocution—Electrocution has been used for killing foxes and mink.[135] The electric current must pass through the brain to induce loss of consciousness before electricity is passed through the rest of the body. Electrical stunning should be followed by euthanasia, using some other technique. Cervical dislocation has been used in mink and other small animals and should be done within 20 seconds of electrical stunning.[213] Use of a nose-to-tail or nose-to-foot method[135] alone may kill the animal by inducing cardiac fibrillation, but the animal may be conscious for a period of time before death. Therefore, these techniques are unacceptable.

### Prenatal and neonatal euthanasia

When ovarian hysterectomies are performed, euthanasia of feti should be accomplished as soon as possible after removal from the dam. Neonatal animals are relatively resistant to hypoxia.[44,214]

### Mass euthanasia

Under unusual conditions, such as disease eradication and natural disasters, euthanasia options may be limited. In these situations, the most appropriate technique that minimizes human and animal health concerns must be used. These options include, but are not limited to, $CO_2$ and physical methods such as gunshot, penetrating captive bolt, and cervical dislocation.

## POSTFACE

This report summarizes contemporary scientific knowledge on euthanasia in animals and calls attention to the lack of scientific reports assessing pain, discomfort, and distress in animals being euthanatized. Many reports on various methods of euthanasia are either anecdotal, testimonial narratives, or unsubstantiated opinions and are, therefore, not cited in this report. The panel strongly endorses the need for well-designed experiments to more fully determine the extent to which each procedure meets the criteria used for judging methods of euthanasia.

Each means of euthanasia has advantages and disadvantages. It is unlikely that, for each situation, any means will meet all desirable criteria. It is also impractical for this report to address every potential circumstance in which animals are to be euthanatized. Therefore, the use of professional judgment is imperative.

Failure to list or recommend a means of euthanasia in this report does not categorically condemn its use. There may occasionally be special circumstances or situations in which other means may be acceptable. For research animals, these exceptions should be carefully considered by the attending veterinarian and the Institutional Animal Care and Use Committee. In other settings, professional judgment should be used.

The panel discourages the use of unapproved products for euthanasia, unless the product has a clearly understood mechanism of action and pharmacokinetics, and studies published in the literature that scientifically verify and justify its use. Those responsible for euthanasia decisions have a critically important responsibility to carefully assess any new technique, method, or device, using the panel's criteria. In the absence of definitive proof or reasonable expectation, the best interest of the animal should guide the decision process.

References cited in this report do not represent a comprehensive bibliography on all methods of euthanasia. Persons interested in additional information on a particular aspect of animal euthanasia are encouraged to contact the Animal Welfare Information Center, National Agricultural Library, 10301 Baltimore Blvd, Beltsville, MD 20705.

The Panel on Euthanasia is fully committed to the concept that, whenever it becomes necessary to kill any animal for any reason whatsoever, death should be induced as painlessly and quickly as possible. It has been our charge to develop workable guidelines for veterinarians needing to address this problem, and it is our sincere desire that these guidelines be used conscientiously by all animal care providers. We consider this report to be a work in progress with new editions warranted as results of more scientific studies are published.

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## References

1. Andrews EJ, Bennet BT, Clark JD, et al. 1993 Report on the AVMA panel on euthanasia. *J Am Vet Med Assoc* 1993;202:230–247.

2. *Webster's ninth new collegiate dictionary*. Springfield: Merriam-Webster Inc, 1990.

3. Wall PD. Defining pain in animals. In: Short CE, Poznak AV, eds. *Animal pain*. New York: Churchill-Livingstone Inc, 1992;63–79.

4. Vierck CJ, Cooper BY, Ritz LA, et al. Inference of pain sensitivity from complex behaviors of laboratory animals. In: Chapman CR, Loeser JD, eds. *Issues in pain measurement*. New York: Raven Press, 1989;93–115.

5. Breazile JE, Kitchell RL. Euthanasia for laboratory animals. *Fed Proc* 1969;28:1577–1579.

6. Zimmerman M. Neurobiological concepts of pain, its assessment and therapy. In: Bromm B, ed. *Pain measurement in man: neurophysiological correlates of pain*. Amsterdam: Elsevier Publishing Co, 1984;15–35.

7. Kitchell RL, Erickson NH, Carstens E, et al, eds. *Animal pain: perception and alleviation*. Bethesda: American Physiological Society, 1983.

8. Kitchen N, Aronson AL, Bittle JL, et al. Panel report on the colloquium on recognition and alleviation of animal pain and distress. *J Am Vet Med Assoc* 1987;191:1186–1191.

9. National Research Council. *Recognition and alleviation of pain and distress in laboratory animals*. Washington, DC: National Academy Press, 1992.

10. Breazile JE. Physiologic basis and consequences of distress in animals. *J Am Vet Med Assoc* 1987;191:1212–1215.

11. McMillan FD. Comfort as the primary goal in veterinary medical practice. *J Am Vet Med Assoc* 1998;212:1370–1374.

12. Grier RL, Clovin TL. *Euthanasia guide (for animal shelters)*. Ames, Iowa: Moss Creek Publications, 1990.

13. Cooper JE, Ewbank R, Platt C, et al. *Euthanasia of amphibians and reptiles*. London: UFAW/WSPA, 1989.

14. Greyhavens T. *Handbook of pentobarbital euthanasia*. Salem, Ore: Humane Society of Willamette Valley, 1989;1–126.

15. *Operational guide for animal care and control agencies*. Denver: American Humane Association, 1988.

16. Fowler ME, Miller RE, eds. *Zoo and wild animal medicine: current therapy 4*. Philadelphia: WB Saunders Co, 1999;1–747.

17. Clark R, Jessup DA. *Wildlife restraint series*. Salinas, Calif: International Wildlife Veterinary Services Inc, 1992.

18. Kreeger T. *Handbook of wildlife chemical immobilization*. Laramie, Wyo: Wildlife Veterinary Services Inc, 1996.

19. Nielsen L. *Chemical immobilization of wild and exotic animals*. Ames, Iowa: Iowa State University Press, 1999.

20. McKenzie A, ed. *The capture and care manual*. South Africa: Wildlife Decision Support Services/The South African Veterinary Foundation, 1993.

21. Amass K, Neilsen L, Brunson D. *Chemical immobilization of animals*. Mount Horeb, Wis: Safe-Capture International Inc, 1999.

22. Humane slaughter regulations. *Fed Reg* 1979;44: 68809–68817.

23. Grandin T. Observations of cattle behavior applied to design of cattle-handling facilities. *Appl Anim Ethol* 1980;6:19–31.

24. Grandin T. Pig behavior studies applied to slaughter-plant design. *Appl Anim Ethol* 1982;9:141–151.

25. Grandin T. Farm animal welfare during handling, transport, and slaughter. *J Am Vet Med Assoc* 1994;204:372–377.

26. Grandin T. Objective scoring of animal handling and stunning practices at slaughter plants. *J Am Vet Med Assoc* 1998;212: 36–39.

27. Grandin T. Effect of animal welfare audits of slaughter plants by a major fast food company on cattle handling and slaughter practices. *J Am Vet Med Assoc* 2000;216:848–851.

28. Tannenbaum J. Issues in companion animal practice. In: *Veterinary ethics*. Baltimore: The Williams & Wilkins Co, 1989;208–225.

29. Rollin BE. Ethical question of the month. *Can Vet J* 1992;33:7–8.

30. Ramsey EC, Wetzel RW. Comparison of five regimens for oral administration of medication to induce sedation in dogs prior to euthanasia. *J Am Vet Med Assoc* 1998;213:240–242.

31. Wetzel RW, Ramsay EC. Comparison of four regimens for oral administration of medication to induce sedation in cats prior to euthanasia. *J Am Vet Med Assoc* 1998;213:243–245.

32. Beaver BV. *Feline behavior: a guide for veterinarians*. Philadelphia: WB Saunders Co, 1992;1–276.

33. Houpt KA. *Domestic animal behavior for veterinarians and animal scientists*. 3rd ed. Ames, Iowa: Iowa State University Press, 1998;1–495.

34. Hart BL. *The behavior of domestic animals*. New York: WH Freeman & Co, 1985;1–390.

35. Beaver BV. *Canine behavior: a guide for veterinarians*. Philadelphia: WB Saunders Co, 1999;1–355.

36. Beaver BV. *The veterinarian's encyclopedia of animal behavior*. Ames, Iowa: Iowa State University Press, 1994;1–307.

37. Schafer M. *The language of the horse: habits and forms of expression*. New York: Arco Publishing Co, 1975;1–187.

38. Hart LA, Hart BL, Mader B. Humane euthanasia and companion animal death: caring for the animal, the client, and the veterinarian. *J Am Vet Med Assoc* 1990;197:1292–1299.

39. Neiburg HA, Fischer A. *Pet loss, a thoughtful guide for adults and children*. New York: Harper & Row, 1982.

40. Hart LA, Mader B. Pet loss support hotline: the veterinary students' perspective. *Calif Vet* 1992;Jan-Feb:19–22.

41. Pet loss support hotlines (grief counseling). *J Am Vet Med Assoc* 1999;215:1804.

42. Arluke A. Coping with euthanasia: a case study of shelter culture. *J Am Vet Med Assoc* 1991;198:1176–1180.

43. Wolfle TL. Laboratory animal technicians: their role in stress reduction and human-companion animal bonding. *Vet Clin North Am Small Anim Pract* 1985;15:449–454.

44. Glass HG, Snyder FF, Webster E. The rate of decline in resistance to anoxia of rabbits, dogs, and guinea pigs from the onset of viability to adult life. *Am J Physiol* 1944;140:609–615.

45. Booth NH. Inhalant anesthetics. In: Booth NH, McDonald LE, eds. *Veterinary pharmacology and therapeutics*. 6th ed. Ames, Iowa: Iowa State University Press, 1988;181–211.

46. Wixon SK, Smiler KL. Anesthesia and analgesia in rodents. In: Kohn DF, Wixson SK, White WJ, et al, eds. *Anesthesia and analgesia in laboratory animals*. New York: Academic Press Inc, 1997;165–203.

47. Knigge U, Soe-Jensen P, Jorgensen H, et al. Stress-induced release of anterior pituitary hormones: effect of H3 receptor-mediat-

ed inhibition of histaminergic activity or posterior hypothalamic lesion. *Neuroendocrin* 1999;69:44–53.

48. Tinnikov AA. Responses of serum conticosterone and corticosteroid-binding globulin to acute and prolonged stress in the rat. *Endocrine* 1999;11:145–150.

49. Zelena D, Klem DT, Barna I, et al. Alpha 2-adrenoreceptor subtypes regulate ACTH and beta-endorphon secretions during stress in the rat. *Psychoneuroendocrin* 1999;24:333–343.

50. Van Herck H, Baumans V, DeBoer SF, et al. Endocrine stress response in rats subjected to singular orbital puncture while under diethyl-ether anaesthesia. *Lab Anim* 1991;25:325–329.

51. *Humane killing of animals*. Preprint of 4th ed. South Mimms, Potters Bar, Herts, England: Universities Federation for Animal Welfare, 1988;16–22.

52. *Occupational exposure to waste anesthetic gases and vapors*. No. 77-140. Washington, DC: Department of Health, Education, and Welfare (National Institute for Occupational Safety and Health), 1977.

53. Lecky JH, ed. *Waste anesthetic gases in operating room air: a suggested program to reduce personnel exposure*. Park Ridge, Ill: The American Society of Anesthesiologists, 1983.

54. Simonsen HB, Thordal-Christensen AA, Ockens N. Carbon monoxide and carbon dioxide euthanasia of cats: duration and animal behavior. *Br Vet J* 1981;137:274–278.

55. Klemm WR. Carbon dioxide anesthesia in cats. *Am J Vet Res* 1964;25:1201–1205.

56. Leake CD, Waters RM. The anesthetic properties of carbon dioxide. *Curr Res Anesthesiol Analg* 1929;8:17–19.

57. Mattsson JL, Stinson JM, Clark CS. Electroencephalographic power—spectral changes coincident with onset of carbon dioxide narcosis in rhesus monkey. *Am J Vet Res* 1972;33:2043–2049.

58. Woodbury DM, Rollins LT, Gardner MD, et al. Effects of carbon dioxide on brain excitability and electrolytes. *Am J Physiol* 1958;192:79–90.

59. Glen JB, Scott WN. Carbon dioxide euthanasia of cats. *Br Vet J* 1973;129:471–479.

60. Blackmore DK, Newhook JC. The assessment of insensibility in sheep, calves and pigs during slaughter. In: Eikelenboom G, ed. *Stunning of animals for slaughter*. Boston: Martinus Nijhoff Publishers, 1983;13–25.

61. Coenen AML, Drinkenburg WHIM, Hoenderken R, et al. Carbon dioxide euthanasia in rats: oxygen supplementation minimizes signs of agitation and asphyxia. *Lab Anim* 1995;29:262–268.

62. Kohler I, Meier R, Busato A, et al. Is carbon dioxide ($CO_2$) a useful short acting anaesthetic for small laboratory animals? *Lab Anim* 1998;33:155–161.

63. Hoenderken R. Electrical and carbondioxide stunning of pigs for slaughter. In: Eikelenboom G, ed. *Stunning of animals for slaughter*. Boston: Martinus Nijhoff Publishers, 1983;59–63.

64. Gregory NG, Moss BW, Leeson RH. An assessment of carbon dioxide stunning in pigs. *Vet Rec* 1987;121:517–518.

65. Carding AH. Mass euthanasia of dogs with carbon monoxide and/or carbon dioxide: preliminary trials. *J Small Anim Pract* 1968;9:245–259.

66. Britt DP. The humaneness of carbon dioxide as an agent of euthanasia for laboratory rodents. In: *Euthanasia of unwanted, injured or diseased animals for educational or scientific purposes*. Potters Bar, UK: UFAW, 1987;19–31.

67. Danneman PJ, Stein S, Walshaw SO. Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. *Lab Anim Sci* 1997;47:376–385.

68. Anton F, Euchner I, Handwerker HO. Psycophysical examination of pain induced by defined $CO_2$ pulses applied to nasal mucosa. *Pain* 1992;49:53–60.

69. Raj ABM, Gregory NG. Welfare implications of gas stunning pigs 1. Determination of aversion to the initial inhalation of carbon dioxide or argon. *Anim Welfare* 1995;4:273–280.

70. Hackbarth H, Kppers N, Bohnet W. Euthanasia of rats with carbon dioxide-animal welfare aspects. *Lab Anim* 2000;34:91–96.

71. Raj ABM, Gregory NG. Investigation into the batch stunning/killing of chickens using carbon dioxide or argon-induced hypoxia. *Res Vet Sci* 1990;49:364–366.

72. Hughes HC. Euthanasia of laboratory animals. In: Melby EC, Altman NH, eds. *Handbook of laboratory animal science*. Vol 3. Cleveland, Ohio: CRC Press, 1976;553–559.

73. Jaksch W. Euthanasia of day-old male chicks in the poultry industry. *Int J Stud Anim Prob* 1981;2:203–213.

74. Kline BE, Peckham V, Hesic HE. Some aids in handling large numbers of mice. *Lab Anim Care* 1963;13:84–90.

75. Kocula AW, Drewniak EE, Davis LL. Experimentation with in-line carbon dioxide immobilization of chickens prior to slaughter. *Poult Sci* 1961;40:213–216.

76. Stone WS, Amiraian K, DueIl C, et al. Carbon dioxide anesthetization of guinea pigs to increase yields of blood and serum. *Proc Care Panel* 1961;11:299–303.

77. Euthanasia (carbon dioxide). In: *Report and accounts 1976-1977*. South Mimms, Potters Bar, Herts, England: Universities Federation for Animal Welfare, 1977;13–14.

78. Hall LW. The anaesthesia and euthanasia of neonatal and juvenile dogs and cats. *Vet Rec* 1972;90:303–306.

79. Blackshaw JK, Fenwick DC, Beattie AW, et al. The behaviour of chickens, mice and rats during euthanasia with chloroform, carbon dioxide and ether. *Lab Anim* 1988;22:67–75.

80. Hansen NE, Creutzberg A. Simonsen HB. Euthanasia of mink (*Mustela vison*) by means of carbon dioxide ($CO_2$), carbon monoxide (CO) and nitrogen ($N_2$). *Br Vet J* 1991;147:140–146.

81. Hayward JS, Lisson PA. Carbon dioxide tolerance of rabbits and its relation to burrow fumigation. *Aust Wildl Res* 1978;5:253–261.

82. Bereger-Sweeney J, Berger UV, Sharma M, et al. Effects of carbon dioxide-induced anesthesia on cholinergic parameters in rat brain. *Lab Anim Sci* 1994;44:369–371.

83. Urbanski HF, Kelly SF. Sedation by exposure to gaseous carbon dioxide-oxygen mixture: application to studies involving small laboratory animal species. *Lab Anim Sci* 1991;41:80–82.

84. Iwarsson K, Rehbinder C. A study of different euthanasia techniques in guinea pigs, rats, and mice. Animal response and postmortem findings. *Scand J Lab Anim Sci* 1993;20:191–205.

85. Hornett TD, Haynes AP. Comparison of carbon dioxide/air mixture and nitrogen/air mixture for the euthanasia of rodents: design of a system for inhalation euthanasia. *Anim Technol* 1984;35: 93–99.

86. Smith W, Harrap SB. Behavioral and cardiovascular responses of rats to euthanasia using carbon dioxide gas. *Lab Anim* 1997; 31:337–346.

87. Hewett TA, Kovacs MS, Artwohl JE, et al. A comparison of euthanasia methods in rats, using carbon dioxide in prefilled and fixed flow rate filled chambers. *Lab Anim Sci* 1993;43:579–582.

88. Herin RA, Hall P, Fitch JW. Nitrogen inhalation as a method of euthanasia in dogs. *Am J Vet Res* 1978;39:989–991.

89. Noell WK, Chinn HI. Time course of failure of the visual pathway in rabbits during anoxia. *Fed Proc* 1949;8:119.

90. Vinte FJ. *The humane killing of mink*. London: Universities Federation for Animal Welfare, 1957.

91. Stonehouse RW, Loew FM, Quine JP, et al. The euthanasia of dogs and cats: a statement of the humane practices committee of the Canadian Veterinary Medical Association. *Can Vet J* 1978;19: 164–168.

92. Quine JP, Buckingham W, Strunin L. Euthanasia of small animals with nitrogen; comparison with intravenous pentobarbital. *Can Vet J* 1988;29:724–726.

93. Raj ARM, Gregory NG, Wotton SR. Changes in the somatosensory evoked potentials and spontaneous electroencephalogram of hens during stunning in Argon-induced anoxia. *Br Vet J* 1991;147:322–330.

94. Ramsey TL, Eilmann HJ. Carbon monoxide acute and chronic poisoning and experimental studies. *J Lab Clin Med* 1932; 17:415–427.

95. Chalifoux A, Dallaire A. Physiologic and behavioral evaluation of CO euthanasia of adult dogs. *Am J Vet Res* 1983;44: 2412–2417.

96. Haldane J. The action of carbonic oxide in man. *J Physiol* 1895;18:430–462.

97. Dallaire A, Chalifoux A. Premedication of dogs with acepromazine or pentazocine before euthanasia with carbon monoxide. *Can J Comp Med* 1985;49:171–178.

98. Lambooy E, Spanjaard W. Euthanasia of young pigs with carbon monoxide. *Vet Rec* 1980;107:59–61.

99. Lowe-Ponsford FL, Henry JA. Clinical aspects of carbon monoxide poisoning. *Adverse Drug React Acute Poisoning Rev* 1989;8:217–240.

100. Bloom JD. Some considerations in establishing divers' breathing gas purity standards for carbon monoxide. *Aerosp Med* 1972;43:633–636.

101. Norman CA, Halton DM. Is carbon monoxide a workplace teratogen? A review and evaluation of the literature. *Ann Occup Hyg* 1990;34:335–347.

102. Eechter LD. Neurotoxicity of prenatal carbon monoxide exposure. Research report. *Health Effects Inst* 1987;Vol:3–22.

103. Wojtczak-Jaroszowa J, Kubow S. Carbon monoxide, carbon disulfide, lead and cadmium—four examples of occupational toxic agents linked to cardiovascular disease. *Med Hypotheses* 1989;30: 141–150.

104. Noga E. *Fish disease: diagnosis and treatment*. St. Louis: CV Mosby, 1996;1–367.

105. Stoskopf MK. Anaesthesia. In: Brown LA, ed. *Aquaculture for veterinarians: fish husbandry and medicine*. Oxford, UK: Pergamon Press, 1993;161–167.

106. Lumb W. Euthanasia by noninhalant pharmacologic agents. *J Am Vet Med Assoc* 1974;165:851–852.

107. Barbiturates. In: Ciganovich E, ed. *Field manual of wildlife diseases*. US Department of the Interior/US Geological Survey, Biological Resources Division, Information and Technical Report 1999-2001.

108. Dennis MB, Dong WK, Weisbrod KA, et al. Use of captive bolt as a method of euthanasia in larger laboratory animal species. *Lab Anim Sci* 1988;38:459–462.

109. Blackmore DK. Energy requirements for the penetration of heads of domestic stock and the development of a multiple projectile. *Vet Rec* 1985;116:36–40.

110. Daly CC, Whittington PE. Investigation into the principal determinants of effective captive bolt stunning of sheep. *Res Vet Sci* 1989;46:406–408.

111. Clifford DH. Preanesthesia, anesthesia, analgesia, and euthanasia. In: Fox JG, Cohen BJ, Loew FM, eds. *Laboratory animal medicine*. New York: Academic Press Inc, 1984;528–563.

112. Australian Veterinary Association. Guidelines on humane slaughter and euthanasia. *Aust Vet J* 1987;64:4–7.

113. Carding T. Euthanasia of dogs and cats. *Anim Reg Stud* 1977;1:5–21.

114. Longair JA, Finley GG, Laniel M-A, et al. Guidelines for euthanasia of domestic animals by firearms. *Can Vet J* 1991;32: 724–726.

115. Finnie JW. Neuroradiological aspects of experimental traumatic missle injury in sheep. *N Z Vet J* 1994;42:54–57.

116. Blackmore DK, Madie P, Bowling MC, et al. The use of a shotgun for euthanasia of stranded cetaceans. *N Z Vet J* 1995; 43:158–159.

117. Blackmore DK, Bowling MC, Madie, P, et al. The use of a shotgun for emergency slaughter or euthanasia of large mature pigs. *N Z Vet J* 1995;43:134–137.

118. Denicola AJ. Non-traditional techniques for management of overabundant deer populations. *Wildl Soc Bull* 1997;25:496–499.

119. McAninch JB, ed. Urban deer: a manageable resource? in *Proceedings*. Symp 55th Midwest Fish Wildl Conf 1993;1–175.

120. Finnie JW. Traumatic head injury in ruminant livestock. *Aust Vet J* 1997;75:204–208.

121. Blackmore DK, Daly CC, Cook CJ. Electroencephalographic studies on the nape shooting of sheep. *N Z Vet J* 1995;43:160–163.

122. *On-farm euthanasia of swine—options for the producer*. Perry, Iowa: American Association of Swine Practitioners and Des Moines, Iowa: National Pork Producers, 1997.

123. *Practical euthanasia of cattle: considerations for the producer, livestock market operator, livestock transporter, and veterinarian*. Rome, Ga: American Association of Bovine Practitioners, 1999.

124. *The emergency euthanasia of horses*. Sacramento: California Department of Food and Agriculture and Davis, Calif: University of California's Veterinary Medical Extension, 1999.

125. *The emergency euthanasia of sheep and goats*. Sacramento: California Department of Food and Agriculture and Davis, Calif: University of California's Veterinary Medical Extension, 1999.

126. Gregory NG, Wotton SB. Comparison of neck dislocation and percussion of the head on visual evoked responses in the chicken's brain. *Vet Rec* 1990;126:570–572.

127. Vanderwolf CH, Buzak DP, Cain RK, et al. Neocortical and hippocampal electrical activity following decapitation in the rat. *Brain Res* 1988;451:340–344.

128. Derr RF. Pain perception in decapitated rat brain. *Life Sci* 1991;49:1399–1402.

129. Holson RR. Euthanasia by decapitation: evidence that this technique produces prompt, painless unconsciousness in laboratory rodents. *Neurotoxicol Teratol* 1992;14:253–257.

130. Keller GL. Physical euthanasia methods. *Lab Anim* 1982;11:20–26.

131. Feldman DB, Gupta BN. Histopathologic changes in laboratory animals resulting from various methods of euthanasia. *Lab Anim Sci* 1976;26:218–221.

132. Mikeska JA, Klemm WR. EEG evaluation of humaneness of asphyxia and decapitation euthanasia of the laboratory rat. *Lab Anim Sci* 1975;25:175–179.

133. Warrington R. Electrical stunning, a review of the literature. *Vet Bull* 1974;44:617–628.

134. Lambooy E, van Voorst N. Electrocution of pigs with notifiable diseases. *Vet Q* 1986;8:80–82.

135. Loftsgard G, Rraathen S, Helgebostad A. Electrical stunning of mink. *Vet Rec* 1972;91:132–134.

136. Hatch RC. Euthanatizing agents. In: Booth NH and McDonald LE, eds. *Veterinary pharmacology and therapeutics*.6th ed. Ames, Iowa: Iowa State University Press, 1988;1143–1148.

137. Croft PG, Hume CW. Electric stunning of sheep. *Vet Rec* 1956;68:318–321.

138. Roberts TDM. Electrocution cabinets. *Vet Rec* 1974;95:241–242.

139. Roberts TDM. Cortical activity in electrocuted dogs. *Vet Rec* 1954;66:561–567.

140. Anil MH, McKinstry JL. Reflexes and loss of sensibility following head-to-back electrical stunning in sheep. *Vet Rec* 1991;128:106–107.

141. Stavinoha WR. Study of brain neurochemistry utilizing rapid inactivation of brain enzyme activity by heating and mirowave irradiation. In: Black CL, Stavinoha WB, Marvyama Y, eds. *Microwave irradiation as a tool to study labile metabolites in tissue*. Elmsford, NY: Pergamon Press, 1983;1–12.

142. Stavinoha WB, Frazer J, Modak AT. Microwave fixation for the study of acetylcholine metabolism. In: Jenden DJ, ed. *Cholinergic mechanisms and psychopharmacology*. New York: Plenum Publishing Corp, 1978;169–179.

143. lkarashi Y, Marvyama Y, Stavinoha WB. Study of the use of the microwave magnetic field for the rapid inactivation of brain enzymes. *Jpn J Pharmacol* 1984;35:371–387.

144. Gaunt AS, Oring LW. *Guidelines to the use of wild birds in research*. Washington DC: The Ornithological Council, 1997;1–52.

145. Federal Provincial Committee for Humane Trapping. *Final report: committee of the federal provincial wildlife conference*. Ottawa: Canadian Wildl Service, 1981;1–172.

146. *Agreement on international humane trapping standards*. The European Community, the Government of Canada, and the Government of the Russian Federation. Department of Foreign Affairs and International Trade, 1997;1–32.

147. Canadian General Standards Board. *Animal (mammal) traps—mechanically powered, trigger-activated killing traps for use on land*. No. CAN/CGSB-144.1-96. Ottawa: Canadian General Standards Board, 1996;1–36.

148. Nolan JW, Barrett MW. *Description and operation of the humane trapping research facility at the Alberta Environmental Centre, AECV90-R3*. Vegreville, AB: Alberta Environmental Centre, 1990.

149. *Animal (mammal) traps-part 4: methods for testing killing trap systems used on land or underwater, TC 191, ISO/DIS 10990-4E*. International Standardization Organization, 2000;1–15.

150. Gilbert FF. Assessment of furbearer response to trapping devices. In: Chapman JA, Pursley D, eds. *Worldwide furbearer conference proceedings*. Frostburg, Md: 1981;1599–1611.

151. Proulx G, Barrett MW. Evaluation of the Bionic Trap to quickly kill mink (*Mustela vison*) in simulated natural environments. *J Wildl Dis* 1991;27:276–280.

152. Proulx G, Barrett MW. Field testing of the C120 magnum trap for mink. *Wildl Soc Bull* 1993;21:421–426.

153. Hiltz M, Roy LD. Rating killing traps against humane trapping standards using computer simulations, in *Proceedings*. 19th Vertebrate Pest Conf 2000.

154. Proulx G, Barret M. Evaluation of the Bionic Trap to quickly kill fisher (*Martes pennanti*) in simulated natural environments. *J Wildl Dis* 1993;29:310–316.

155. Proulx G, Pawlina IM, Wong RK. Re-evaluation of the C120 magnum and bionic traps to humanely kill mink. *J Wildl Dis* 1993;29:184.

156. Proulx G, Barrett MW, Cook SR. The C120 Magnum with pan trigger: a humane trap for mink (*Mustela vison*). *J Wildl Dis* 1990;26:511–517.

157. Proulx G, Kolenosky AJ, Cole PJ. Assessment of the Kania trap to humanely kill red squirrels (*Tamiasciurus hudsonicus*) in enclosures. *J Wildl Dis* 1993;29:324–329.

158. Proulx G, Kolenosky AJ, Badry MJ, et al. Assessment of the Savageau 2001-8 trap to effectively kill arctic fox. *Wildl Soc Bull* 1993;21:132–135.

159. Proulx G, Kolenosky AJ, Cole PJ, et al. A humane killing trap for lynx (*Felis lynx*): the Conibear 330 with clamping bars. *J Wildl Dis* 1995;1:57–61.

160. Proulx G, Barret MW, Cook SR. The C120 Magnum: an effective kill trap for marten. *Wildl Soc Bull* 1989;17:294–298.

161. Proulx G, Cook SR, Barrett MW. Assessment and preliminary development of the rotating jaw Conibear 120 trap to effectively kill marten (*Martes americana*). *Can J Zool* 1989;67:1074–1079.

162. Naylor BJ, Novak M. Catch efficiency and selectivity of various traps and sets used for capturing American martens. *Wildl Soc Bull* 1994;22:489–496.

163. Hill EP. *Evaluation of improved traps and trapping techniques*. Alabama Department of Conservation and Natural Resources P-R Project Report W-44-5 Job IV-B:1-19.

164. King CM. The effects of two types of steel traps upon captured stoats (*Mustela erminea*). *J Zool (Lond)* 1995;553–554.

165. Cooper JE, Ewbank R, Platt C, et al. *Euthanasia of amphibians and reptiles*. London: UFAQ/WSPA, 1989.

166. Twitchell C, Roy LD, Gilbert FF, et al. Effectiveness of rotating-jaw killing traps for beaver (*Castor canadensis*), in *Proceedings*. North Am Aquatic Furbearer Symp 1999.

167. Warburton B, Hall JV. Impact momentum and clamping force thresholds for developing standards for possum kill traps. *N Z J Zool* 1995;22:39–44.

168. Guidelines for the capture, handling, and care of mammals as approved by the American Society of Mammalogists. *J Mammal* 1998;79:1416–1431.

169. *Improving animal welfare in US trapping programs*. Washington, DC: International Association of Fish and Wildlife Agencies, 1997.

170. Blackmore DK. Differences in behaviour between sheep and cattle during slaughter. *Res Vet Sci* 1984;37:223–226.

171. Gregory NG, Wotton SB. Time to loss of brain responsiveness following exsanguination in calves. *Res Vet Sci* 1984;37:141–143.

172. Blackmore DK. Non-penetrative percussion stunning of sheep and calves. *Vet Rec* 1979;105:372–375.

173. Canadian Council on Animal Care. *Guide to the care and use of experimental animals*. Vol 1. Ottawa: Canadian Council on Animal Care, 1980.

174. Green CJ. Euthanasia. In: *Animal anaesthesia*. London: Laboratory Animals Ltd, 1979;237-241.

175. Clifford DH. Preanesthesia, anesthesia, analgesia, and euthanasia. In: Fox JG, Cohen BJ, Loew FM, eds. *Laboratory animal medicine*. Orlando: Academic Press Inc, 1984;527–562.

176. Finnie JW. Neuropathologic changes produced by non-penetrating percussive captive bolt stunning of cattle. *N Z Vet J* 1995;43: 183–185.

177. Gregory NG, Wotton SB. Effect of slaughter on spontaneous and evoked activity of the brain. *Br Poult Sci* 1986;27:195–205.

178. Eikelenboom G, ed. *Stunning of animals for slaughter*. Boston: Martinus Nijhoff Publishers, 1983;1–227.

179. Booth NH. Drug and chemical residues in the edible tissues of animals. In: Booth NH, McDonald LE, eds. *Veterinary pharmacology and therapeutics*. 6th ed. Ames, Iowa: Iowa State University Press, 1988;1149–1205.

180. Acceptable field methods in mammalogy: preliminary guidelines approved by the American Society of Mammalogists. *J Mammal* 1987;68(Suppl 4):1–18.

181. American Ornithologists' Union. Report of committee on use of wild birds in research. *Auk* 1988;105(Suppl):1A–41A.

182. American Society of Ichthyologists and Herpetologists, Herpetologist League, Society for the Study of Amphibians and Reptiles. Guidelines for the use of live amphibians and reptiles in field research. *J Herpetol* 1987;21(suppl 4):1–14.

183. American Society of Ichthyologists and Herpetologists, American Fisheries Society, American Institute of Fisheries Research Biologists. Guidelines for use of fishes in field research. *Copeia Suppl* 1987;1–12.

184. Cailliet GM. *Fishes: a field guide and laboratory manual on their structure, identification, and natural history*. Belmont, Calif: Wadsworth, 1986.

185. Schwartz JA, Warren R, Henderson D, et al. Captive and field tests of a method for immobilization and euthanasia of urban deer. *Wildl Soc Bull* 1997;25:532–541.

186. Zwart P, deVries HR, Cooper JE. The humane killing of fishes, amphibia, reptiles and birds. *Tijdsehr Diergeneeskd* 1989; 114:557–565.

187. Burns R. Considerations in the euthanasia of reptiles, fish and amphibians, in *Proceedings*. AAZV, WDA, AAWV Joint Conference 1995;243–249.

188. National Research Committee on Pain and Distress in Laboratory Animals. *Recognition of pain and distress in laboratory animals*. Washington DC: National Academy Press, 1992.

189. Heard DJ. Principles and techniques of anesthesia and analgesia for exotic practice. *Vet Clin North Am Small Anim Pract* 1993;23:1301–1327.

190. Canadian Council on Animal Care. *Guide to the use and care of experimental animals*. Vol 2. Ottawa: Association of Universities and Colleges of Canada, 1984;1–16.

191. Harrell L. Handling euthanasia in production facilities. In: Schaeffer DO, Kleinow KM, Krulisch L, eds. *The care and use of amphibians, reptiles and fish in research*. Bethesda, Md: Scientists Center for Animal Welfare, 1992;129.

192. Ferguson HW. *Systemic pathology of fish*. Ames, Iowa: Iowa State University Press, 1989.

193. Letcher J. Intracelomic use of tricaine methane sulfonate for anesthesia of bullfrogs (*Rana catesbeiana*) and leopard frogs *(Rana pipens*). *Zoo Biol* 1992;11:242–251.

194. Brown LA. Anesthesia in fish. *Vet Clin North Am Small Anim Pract* 1988;18:317–330.

195. Josa A, Espinosa E, Cruz JI, et al. Use of 2-phenoxyethanol as an anesthetic agent in goldfish (*Cyprinus carpio*). *Vet Rec* 1992;131:468.

196. Noga EJ. *Fish disease. Diagnosis and treatment*. St Louis: Mosby, 1996.

197. Brannian RE, Kirk E, Williams D. Anesthetic induction of kinosternid turtles with halothane. *J Zoo Anim Med* 1987;18:115–117.

198. Calderwood HW. Anesthesia for reptiles. *J Am Vet Med Assoc* 1971;159:1618–1625.

199. Jackson OF, Cooper JE. Anesthesia and surgery. In: Cooper JE, Jackson OF, eds. *Diseases of the reptilia*. Vol. 2. New York: Academic Press Inc, 1981;535–549.

200. Johlin JM, Moreland FB. Studies of the blood picture of the turtle after complete anoxia. *J Biol Chem* 1933;103:107–114.

201. Moberly WR. The metabolic responses of the common iguana, *Iguana iguana*, to walking and diving. *Comp Biochem Physiol* 1968;27:21–32.

202. Storey KB. Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. *Am J Physiol* 1990;258:R559–R568.

203. Burns R, McMahan B. Euthanasia methods for ectothermic vertebrates. In: Bonagura JD, ed. *Continuing veterinary therapy XII*. Philadelphia: WB Saunders Co, 1995;1379–1381.

204. Cooper JE, Ewbank R, Platt C, et al. *Euthanasia of amphib-*

*ians and reptiles*. London: Universities Federation for Animal Welfare and World Society for the Protection of Animals, 1989.

205. Zwart P, deVries HR, Cooper JE. Humane methods of killing fish, amphibians and birds. *Tijdschr Diergeneedkd* 1989;114:557–565.

206. Martin B. Evaluation of hypothermia for anesthesia in reptiles and amphibians. *ILAR News* 1995;37:186–190.

207. Suckow MA, Terril LA, Grigdesby CF, et al. Evaluation of hypothermia-induced analgesia and influence of opioid antagonists in Leopard frogs (*Rana pipiens*). *Pharmacol Biochem Behav* 1999;63: 39–43.

208. Schaffer DO. Anesthesia and analgesia in nontraditional laboratory animal species. In: Kohn DF, Wixson SK, White WJ, et al. eds. *Anesthesia and analgesia in laboratory animals*. San Diego: Academic Press Inc, 1997;337–378.

209. Greer LL, Rowles T. Euthanasia. In: Dierauf LA, ed. *CRC handbook of marine mammal medicine: health, disease, and rehabilitation*. 2nd ed. Boca Raton, Fla: CRC Press, in press.

210. Blackmore DK, Madie P, Bowling MC, et al. The use of a shotgun for euthanasia of stranded cetaceans. *N Z Vet J* 1995;43:158–159.

211. Hyman J. Euthanasia in marine animals. In: Dierauf LA, ed. *CRC handbook of marine mammal medicine: health, disease, and rehabilitation*. Boca Raton, Fla: CRC Press, 1990;265–266.

212. Lambooy E, Roelofs JA, Van Voorst N. Euthanasia of mink with carbon monoxide. *Vet Rec* 1985;116:416.

213. *Recommended code of practice for the care and handling of mink*. Ottawa: Agriculture Canada, 1988;1–17.

214. Singer D. Neonatal tolerance to hypoxia: a comparative-physiological approach. *Comp Biochem Physiol* 1999;123:221–234.

215. Ludders JW, Schmidt RH, Dein J, et al. Drowning is not euthanasia. *Wildlife Soc Bull* 1999;27(3):1.

## Appendix 1

Agents and methods of euthanasia by species (refer to Appendix 4 for unacceptable agents and methods.)

| Species | Acceptable* (refer to Appendix 2 and text for details) | Conditionally acceptable† (refer to Appendix 3 and text for details) |
|---|---|---|
| Amphibians | Barbiturates, inhalant anesthetics (in appropriate species), $CO_2$, CO, tricaine methane sulfonate (TMS, MS 222), benzocaine hydrochloride, double pithing | Penetrating captive bolt, gunshot, stunning and decapitation, decapitation and pithing |
| Birds | Barbiturates, inhalant anesthetics, $CO_2$, CO, gunshot (free-ranging only) | $N_2$, Ar, cervical dislocation, decapitation, thoracic compression (small, free-ranging only) |
| Cats | Barbiturates, inhalant anesthetics, $CO_2$, CO, potassium chloride in conjunction with general anesthesia | $N_2$, Ar |
| Dogs | Barbiturates, inhalant anesthetics, $CO_2$, CO, potassium chloride in conjunction with general anesthesia | $N_2$, Ar, penetrating captive bolt, electrocution |
| Fish | Barbiturates, inhalant anesthetics, $CO_2$, tricaine methane sulfonate (TMS, MS 222), benzocaine hydrochloride, 2-phenoxyethanol | Decapitation and pithing, stunning and decapitation/pithing |
| Horses | Barbiturates, potassium chloride in conjunction with general anesthesia, penetrating captive bolt | Chloral hydrate (IV, after sedation), gunshot, electrocution |
| Marine mammals | Barbiturates, etorphine hydrochloride | Gunshot (cetaceans < 4 meters long) |
| Mink, fox, and other mammals produced for fur | Barbiturates, inhalant anesthetics, $CO_2$ (mink require high concentrations for euthanasia without supplemental agents), CO, potassium chloride in conjunction with general anesthesia | $N_2$, Ar, electrocution followed by cervical dislocation |
| Nonhuman primates | Barbiturates | Inhalant anesthetics, $CO_2$, CO, $N_2$, Ar |
| Rabbits | Barbiturates, inhalant anesthetics, $CO_2$, CO, potassium chloride in conjunction with general anesthesia | $N_2$, Ar, cervical dislocation (< 1 kg), decapitation, penetrating captive bolt |
| Reptiles | Barbiturates, inhalant anesthetics (in appropriate species), $CO_2$ (in appropriate species) | Penetrating captive bolt, gunshot, decapitation and pithing, stunning and decapitation |
| Rodents and other small mammals | Barbiturates, inhalant anesthetics, $CO_2$, CO, potassium chloride in conjunction with general anesthesia, microwave irradiation | Methoxyflurane, ether, $N_2$, Ar, cervical dislocation (rats < 200 g), decapitation |
| Ruminants | Barbiturates, potassium chloride in conjunction with general anesthesia, penetrating captive bolt | Chloral hydrate (IV, after sedation), gunshot, electrocution |
| Swine | Barbiturates, $CO_2$, potassium chloride in conjunction with general anesthesia, penetrating captive bolt | Inhalant anesthetics, CO, chloral hydrate (IV, after sedation), gunshot, electrocution, blow to the head (< 3 weeks of age) |
| Zoo animals | Barbiturates, inhalant anesthetics, $CO_2$, CO, potassium chloride in conjunction with general anesthesia | $N_2$, Ar, penetrating captive bolt, gunshot |
| Free-ranging wildlife | Barbiturates IV or IP, inhalant anesthetics, potassium chloride in conjunction with general anesthesia | $CO_2$, CO, $N_2$, Ar, penetrating captive bolt, gunshot, kill traps (scientifically tested) |

*Acceptable methods are those that consistently produce a humane death when used as the sole means of euthanasia. †Conditionally acceptable methods are those that by the nature of the technique or because of greater potential for operator error or safety hazards might not consistently produce humane death or are methods not well documented in the scientific literature.

*Continued on next page.*

## Appendix 2

Acceptable agents and methods of euthanasia—characteristics and modes of action (refer to text for details)

| Agent | Classification | Mode of action | Rapidity | Ease of performance | Safety for personnel | Species suitability | Efficacy and comments |
|---|---|---|---|---|---|---|---|
| Barbiturates | Hypoxia attributable to depression of vital centers | Direct depression of cerebral cortex, subcortical structures, and vital centers; direct depression of heart muscle | Rapid onset of anesthesia | Animal must be restrained; personnel must be skilled to perform IV injection | Safe except human abuse potential; DEA-controlled substance | Most species | Highly effective when appropriately administered; acceptable IP in small animals and IV |
| Benzocaine hydrochloride | Hypoxia attributable to depression of vital centers | Depression of CNS | Very rapid, depending on dose | Easily used | Safe | Fish, amphibians | Effective but expensive |
| Carbon dioxide (bottled gas only) | Hypoxia attributable to depression of vital centers | Direct depression of cerebral cortex, subcortical structures, and vital centers; direct depression of heart muscle | Moderately rapid | Used in closed container | Minimal hazard | Small laboratory animals, birds, cats, small dogs, rabbits, mink (high concentrations required), zoo animals, amphibians, fish, some reptiles, swine | Effective, but time required may be prolonged in immature and neonatal animals |
| Carbon monoxide (bottled gas only) | Hypoxia | Combines with hemoglobin, preventing its combination with oxygen | Moderate onset time, but insidious so animal is unaware of onset | Requires appropriately maintained equipment | Extremely hazardous, toxic, and difficult to detect | Most small species including dogs, cats, rodents, mink, chinchillas, birds, reptiles, amphibians, zoo animals, rabbits | Effective; acceptable only when equipment is properly designed and operated |
| Inhalant anesthetics | Hypoxia attributable to depression of vital centers | Direct depression of cerebral cortex, subcortical structures, and vital centers | Moderately rapid onset of anesthesia, excitation may develop during induction | Easily performed with closed container; can be administered to large animals by means of a mask | Must be properly scavenged or vented to minimize exposure to personnel | Some amphibians, birds, cats, dogs, furbearing animals, rabbits, some reptiles, rodents and other small mammals, zoo animals, fish, free-ranging wildlife | Highly effective provided that subject is sufficiently exposed; either is conditionally acceptable |
| Microwave irradiation | Brain enzyme inactivation | Direct inactivation of brain enzymes by rapid heating of brain | Very rapid | Requires training and highly specialized equipment | Safe | Mice, rats | Highly effective for special needs |
| Penetrating captive bolt | Physical damage to brain | Direct concussion of brain tissue | Rapid | Requires skill, adequate restraint, and proper placement of captive bolt | Safe | Horses, ruminants, swine | Instant loss of consciousness, but motor activity may continue |
| 2-Phenoxyethanol | Hypoxia attributable to depression of vital centers | Depression of CNS | Very rapid, depending on dose | Easily used | Safe | Fish | Effective but expensive |
| Potassium chloride (intracardially or intravenously in conjunction with general anesthesia only) | Hypoxia | Direct depression of cerebral cortex, subcortical structures, and vital centers secondary to cardiac arrest. | Rapid | Requires training and specialized equipment for remote injection anesthesia, and ability to give IV injection of potassium chloride | Anesthetics may be hazardous with accidental human exposure | Most species | Highly effective, some clonic muscle spasms may be observed |
| Tricaine methane sulfonate (TMS, MS 222) | Hypoxia attributable to depression of vital centers | Depression of CNS | Very rapid, depending on dose | Easily used | Safe | Fish, amphibians | Effective but expensive |

## Appendix 3

Conditionally acceptable agents and methods of euthanasia—characteristics and modes of action (refer to text for details)

| Agent | Classification | Mode of action | Rapidity | Ease of performance | Safety | Species suitability | Efficacy and comments |
|---|---|---|---|---|---|---|---|
| Blow to the head | Physical damage to brain | Direct concussion of brain tissue | Rapid | Requires skill, adequate restraint, and appropriate force | Safe | Young pigs < 3 weeks old | Must be properly applied to be humane and effective |
| Carbon dioxide (bottled gas only) | Hypoxia due to depression of vital centers | Direct depression of cerebral cortex, subcortical structures and vital centers; direct depression of heart muscle | Moderately rapid | Used in closed container | Minimal hazard | Nonhuman primates, free-ranging wildlife | Effective, but time required may be prolonged in immature and neonatal animals |
| Carbon monoxide (bottled gas only) | Hypoxia | Combines with hemoglobin, preventing its combination with oxygen | Moderate onset time, but insidious so animal is unaware of onset | Requires appropriately maintained equipment | Extremely hazardous, toxic, and difficult to detect | Nonhuman primates, free-ranging wildlife | Effective; acceptable only when equipment is properly designed and operated |
| Cervical dislocation | Hypoxia due to disruption of vital centers | Direct depression of brain | Moderately rapid | Requires training and skill | Safe | Poultry, birds, laboratory mice, rats (< 200 g), rabbits (< 1 kg) | Irreversible; violent muscle contractions can occur after cervical dislocation |
| Chloral hydrate | Hypoxia from depression of respiratory center | Direct depression of brain | Rapid | Personnel must be skilled to perform IV injection | Safe | Horses, ruminants, swine | Animals should be sedated prior to administration |
| Decapitation | Hypoxia due to disruption of vital centers | Direct depression of brain | Rapid | Requires training and skill | Guillotine poses potential employee injury hazard | Laboratory rodents; small rabbits; birds; some fish, amphibians, and reptiles (latter 3 with pithing) | Irreversible; violent muscle contraction can occur after decapitation |
| Electrocution | Hypoxia | Direct depression of brain and cardiac fibrillation | Can be rapid | Not easily performed in all instances | Hazardous to personnel | Used primarily in sheep, swine, foxes, mink (with cervical dislocation), ruminants, animals > 5 kg | Violent muscle contractions occur at same time as loss of consciousness |
| Gunshot | Hypoxia due to disruption of vital centers | Direct concussion of brain tissue | Rapid | Requires skill and appropriate firearm | May be dangerous | Large domestic and zoo animals, reptiles, amphibians, wildlife, cetaceans (< 4 meters long) | Instant loss of consciousness, but motor activity may continue |
| Inhalant anesthetics | Hypoxia due to depression of vital centers | Direct depression of cerebral cortex, subcortical structures, and vital centers | Moderately rapid onset of anesthesia; excitation may develop during induction | Easily performed with closed container; can be administered to large animals by means of a mask | Must be properly scavenged or vented to minimize exposure to personnel; ether has explosive potential and exposure to ether may be stressful | Nonhuman primates, swine; ether is conditionally acceptable for rodents and small mamals; methoxyflurane is conditionally acceptable for rodents and small mammals. | Highly effective provided that subject is sufficiently exposed |
| Nitrogen, argon | Hypoxia | Reduces partial pressure of oxygen available to blood | Rapid | Used in closed chamber with rapid filling | Safe if used with ventilation | Cats, small dogs, birds, rodents, rabbits, other small species, mink, zoo animals, nonhuman primates, free-ranging wildlife | Effective except in young and neonates; an effective agent, but other methods are preferable |
| Penetrating captive bolt | Physical damage to brain | Direct concussion of brain tissue | Rapid | Requires skill, adequate restraint and proper placement of captive bolt | Safe | Dogs, rabbits, zoo animals, reptiles, amphibians, free-ranging wildlife | Instant loss of consciousness but motor activity may continue |
| Pithing | Hypoxia due to disrution of vital centers, physical damage to brain | Trauma of brain and spinal cord tissue | Rapid | Easily performed but requires skill | Safe | Some ectotherms | Effective, but death not immediate unless brain and spinal cord are pithed |
| Thoracic compresion | Hypoxia and cardiac arrest | Physical interference with cardiac and respiratory function | Moderately rapid | Requires training | Safe | Small- to medium-sized free-ranging birds | Apparently effective |

## Appendix 4

Some unacceptable agents and methods of euthanasia (refer to text for details)

| Agent or method | Comments |
|---|---|
| Air embolism | Air embolism may be accompanied by convulsions, opisthotonos, and vocalization. If used, it should be done only in anesthetized animals. |
| Blow to the head | Unacceptable for most species. |
| Burning | Chemical or thermal burning of an animal is not an acceptable method of euthanasia. |
| Chloral hydrate | Unacceptable in dogs, cats, and small mammals. |
| Chloroform | Chloroform is a known hepatotoxin and suspected carcinogen and, therefore, is extremely hazardous to personnel. |
| Cyanide | Cyanide poses an extreme danger to personnel and the manner of death is aesthetically objectionable. |
| Decompression | Decompression is unacceptable for euthanasia because of numerous disadvantages.<br>(1) Many chambers are designed to produce decompression at a rate 15 to 60 times faster than that recommended as optimum for animals, resulting in pain and distress attributable to expanding gases trapped in body cavities.<br>(2) Immature animals are tolerant of hypoxia, and longer periods of decompression are required before respiration ceases.<br>(3) Accidental recompression, with recovery of injured animals, can occur.<br>(4) Bleeding, vomiting, convulsions, urination, and defecation, which are aesthetically unpleasant, may develop in unconscious animals. |
| Drowning | Drowning is not a means of euthanasia and is inhumane. |
| Exsanguination | Because of the anxiety associated with extreme hypovolemia, exsanguination should be done only in sedated, stunned, or anesthetized animals. |
| Formalin | Direct immersion of an animal into formalin, as a means of euthanasia, is inhumane. |
| Household products and solvents | Acetone, quaternary compounds (including $CCl_4$), laxatives, clove oil, dimethylketone, quaternary ammonium products*, antacids, and other commercial and household products or solvents are not acceptable agents for euthanasia. |
| Hypothermia | Hypothermia is not an appropriate method of euthanasia. |
| Neuromuscular blocking agents (nicotine, magnesium sulafte, potassiumchloride, all curariform agents) | When used alone, these drugs all cause respiratory arrest before loss of consciousness, so the animal may perceive pain and distress after it is immobilized. |
| Rapid freezing | Rapid freezing as a sole means of euthanasia is not considered to be humane. If used, animals should be anesthetized prior to freezing. |
| Strychnine | Strychnine causes violent convulsions and painful muscle contractions. |
| Stunning | Stunning may render an animal unconscious, but it is not a method of euthanasia (except for neonatal animals with thin craniums). If used, it must be immediately followed by a method that ensures death. |
| Tricaine methane sulfonate (TMS, MS 222) | Should not be used for euthanasia of animals intended as food. |

*Roccal D Plus, Pharmacia & Upjohn, Kalamazoo, Mich.

## APPENDIX III

## FREUND'S COMPLETE ADJUVANT

Suggested guidelines:

1. Alternatives to FCA should be considered especially for T cell stimulation and antibody production. FCA may be given once followed by Freund's Incomplete or another adjuvant as a booster.

2. The area should be clean and free of contamination.

3. The adjuvant should be free of extraneous microbial contamination.

4. Multiple small injections over a wider area may produce better antibody response as well as being less stressful for the animal.

5. The animal should be monitored after the immunisation of FCA for evidence of pain or distress resulting from infection or abscess formation. The animals will be treated appropriately.

Following is a Table of approved sites and maximum volume to be injected. The routes of administration of Freund's Complete Adjuvant are standard. If a Protocol does not contain these routes or amounts, it will receive special review by the Animal Ethics Sub-committee.

### FREUND'S COMPLETE ADJUVANT MAXIMUM VOLUME/SITE

| Species | Total Volume | Sub-cutaneous | Intra-dermal | Footpad | Tail Base | Intra-muscular |
|---|---|---|---|---|---|---|
| Mice | 0.25 ml | 0.1 ml | 0.1 ml | 0.05 ml | 0.2 ml | |
| Rats | 1.5 ml | 0.25 ml | 0.1 ml | | 0.2 ml | |
| Guinea pigs | 1.5 ml | 0.25 ml | 0.1 ml | | | |
| Hamsters | 1.0 ml | 0.25 ml | 0.1 ml | | | |
| Rabbits | 3.0 ml | 0.25 ml | 0.1 ml | | | |
| Farm Stock | 5.0 ml | 1.0 ml | 0.25 ml | | | |
| Avian Species | 1.0 ml | 0.25 ml | 0.1 ml | | | 0.25 ml |

**Note:** Intravenous, Intra-peritoneal or Footpad injection (where not indicated) require Special Review.

**SOUTHERN AFRICAN POULTRY ASSOCIATION / SUIDER-AFRIKAANSE PLUIMVEEVERENIGING**

# CODE OF PRACTICE 2001
# BEDRYFSKODE 2001

# SAPA CODE OF PRACTICE 2000

# INDEX

# FOREWORD

This is the fourth Code of Practice compiled by the Southern African Poultry Association. Members will once again be requested to undertake in writing that they will comply with this Code, and in return will receive a certificate advertising their undertaking in this regard.

The work and the amendments to this Code were carried out mainly by the Technical Committee of SAPA, but inputs were also received from all the other SAPA Committees. The latest amendments include a closer focus on free range, barn, and alternative systems of production. These amendments were approved at the 2000 Congress and were negotiated with the SPCA thereafter. A few minor amendments were made subsequent to the formal approval. This document is deemed to be a dynamic document and will be adjusted from time to time as the need arises.

The involvement of the SPCA with the SAPA Code of Practice is imperative and their qualified support is appreciated.

Z B Coetzee
Executive Director, SAPA

The National Council of Societies for the Prevention of Cruelty to Animals supports in principle the Southern African Poultry Association's Code of Practice, and welcomes the instituting of minimum standards of animal husbandry designed to improve the health and welfare of poultry.

We do not agree with certain aspects of the Code, such as cage sizes. We do however recognise the Code to be an important starting point in the efforts to improve conditions in the industry.

M French
SPCA National Council

**SAPA CODE OF PRACTICE 2001**

## 1. INTRODUCTION

This Code was compiled by the Southern African Poultry Association as an objective guide for all poultry produced in South Africa and in an endeavour to lay down the accepted norms of the industry, incorporating various legal requirements where necessary.

The Code provides defined standards of wellbeing for poultry in commercial operations, research and educational facilities. The recommendations are to be used as a guide and do not consider all possible conditions.

The Code considers safe and wholesome food for human consumption to be of the highest priority and therefore fully supports the implementation of applicable measures to comply with the requirements for safe food of poultry origin, as approved by the relevant Health Authorities.

Adequate facilities and resources must be available to supply proper housing, the supply of quality feed, attendance to sick and injured chickens and all else to ensure the wellbeing of the animals. Financial costs should not be considered a reason for neglecting a chicken obviously in distress or for failing to secure prompt and appropriate medical treatment or other care when necessary.

Persons working with chickens must understand and accept their responsibility to prevent any form of avoidable suffering.

## 2. IMPORTATION OF BREEDING MATERIAL

The Committee which advises the authorities on the need for the importation of genetic material under the Animal Improvement Act 1998, (Act No 62) will abide by the following:

### 2.1 Pure Lines

2.1.1 Layer lines are great-grandparents/grandparents

2.1.2 Broiler lines are a combination of great-grandparents and grandparents but not only grandparents.

### 2.2 Parents

Intended for trial purposes only for producers who have previously imported pure lines or intend to import pure lines.

2.2.1 Layers – a maximum of 4000 females – every second year plus approximately 15% males.

2.2.2 Broilers – a maximum of 20000 females annually plus approximately 15% males.

### 2.3 Turkeys, Ducks & Geese

The same numbers to apply as for broilers.

**2.4 Valid period of permit**

A permit is to be valid for 12 months as from the declared expected date of importation. If this period is exceeded, the importer must reapply (a section to be included in the forms declaring the expected date of import).

**2.5 Sub-Committee**

The Chick Producers' Organisation shall act as sub-committee as allowed for under the act and regulations and it will delegate the task to comment on imports in line with this code to the executive officer of SAPA.

**2.6 Deadlock**

In the case of a deadlock under Section 16 of the Act, an applicant can appeal to the Minister who will appoint a Board of Appeal consisting of a Chief Magistrate and two knowledgeable people to consider the implications.

## 3. HEALTH STATUS AND FOOD SAFETY

### 3.1 Health

The following are deemed to be the minimum requirements for meaningful monitoring of the health status of a flock used for saleable chick production:

### 3.1.2 Records

3.1.2.1 Health, vaccination and laboratory records of flocks of origin shall be kept for inspection for the normal expected lifetime of the progeny.

3.1.2.2 Hatchery hygiene records shall be kept for inspection for the normal expected lifetime of a particular hatch.

3.1.2.3 All vaccines administered to day old chicks must be stated on the delivery note, giving batch numbers, supplier numbers and expiry dates as well as method of application.

### 3.1.3 Blood testing

3.1.3.1 Breeding flocks should be serologically tested at regular intervals to confirm the negative status of the flocks before onset of lay and during the following production cycle for:

- Mycoplasma gallisepticum (Mg)
- Mycoplasma synoviae (Ms)
- Salmonella pullorum/gallinarum (BWD/Fowl Typhoid)

For this purpose, a representative sample is deemed to be at least:

- 15 samples from a house with up to 5000 chickens, or
- 30 samples from a house with over 5000 chickens

3.1.3.2 Health status of chicks delivered should be notified to the customer at the hatchery, or as soon as a change in status has become apparent.

3.1.3.3 Acceptable control measures must prevail in all flocks producing hatching eggs to assist in the prevention of vertical (transovarial) transmission of Avian encephalomyelitis, Egg Drop Syndrome and Salmonella enteritidis.

3.1.3.4 Chicks emanating from flocks affected by S.gallinarum or S.pullorum will not be distributed within the industry.

## 3.2 Food safety

### 3.2.1 Prudent use of Antibiotics and drugs

The Code supports the necessary professional discipline needed when the strategic treatment of a disease is required on advice of the poultry veterinarian in charge of the health of the flock.

All antibiotics, growth promotants (Enhancers/Antimicrobials)and coccidiostats for use in poultry in the country have to be registered in terms of Act 36/1947 (Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act) or Act 101/1965 (Medicines and Related Substances Control Act)

Registration of stock remedies and medicines:
The Code supports the very stringent evaluation of claims made for relevant drugs, which are based on internationally accepted scientific principles. These evaluations take into consideration the quality, safety and efficacy for the treatment of chickens, as well as the safety of the chicken products for human consumption.

Special emphasis is placed on the directions as per label or package insert of the product to ensure compliance to withdrawal periods, as approved by the Department of Health according to internationally accepted guidelines as determined by CODEX, USFDA, EMA (European Agency for the Evaluation of Medicinal Products) and JECFA (International Joint Expert Committee on Food Additives).

The Code supports the implementation of all the above measures to ensure the health of the consumer of chicken products.

### 3.2.2 Food borne pathogens

#### 3.2.2.1 Salmonella enteritidis

Subscribing to this Code implies the application of the Salmonella guidelines of SAPA.

**3.2.3** Any pathogen reduction plan for zoonoses or any health or production improvement plan devised and legislated for will form part of the Code of Practice**.**

## 4. HATCHERIES

Every person working with chicks in a hatchery shall be able to understand and accept responsibility to prevent unnecessary suffering. Hatchery operators shall be satisfied that attendants responsible for handling live chicks have the skills necessary to perform any required procedure without causing suffering to the chicks.

## 4.1 Handling of young chicks

4.1.1 Hatching trays with chicks shall be moved in a level position only and not thrown or dropped. Chicks shall be prevented from falling off hatching trays onto the floor.

4.1.2 Vent sexing to be performed by skilled and trained operators.

**4.2 Elective surgery or morphological alterations**

Beak trimming, claw trimming and identification marking shall be avoided except when it is considered necessary. Only competent persons shall perform any such procedure.

**4.3 Identification devices attached to chicks**

Identification devices that are permanently or temporarily attached to the chick's body must be lightweight and not cause physical injury to the chick. A responsible person has to check wing bands for physical injury post application within 14 days.

**4.4 Euthanasia and disposal of non-saleable chicks**

**4.4.1 Standards**

In all circumstances, the termination of life must be quick, humane and done in a manner that produces total and irreversible loss of consciousness with a minimum level of distress to the chick.

**4.4.2 Methods**

4.4.2.1 Drowning, smothering and thermal exhaustion are not acceptable under any circumstances.

4.4.2.2 High-speed maceration of chicks using properly designed macerators is a practical and acceptable method of euthanasia.

4.4.2.3 Carbon dioxide has been found a suitable agent for euthanasia of chicks. The method applied should cause death within a reasonable time. Chicks must be placed in such a way to allow penetration of the gas and prevent suffocation before gassing. Containers must be designed to allow continual refilling with carbon dioxide to maintain correct levels of the gas. Chicks must be exposed to the gas for a long enough period to cause death. Pending on more research results the industry could look at alternatives to $CO_2$ as it is being questioned at present. In the meantime the above arrangement holds.

4.4.2.4 Decapitation or cervical dislocation is acceptable if performed by trained and competent personnel.

**4.5 Euthanasia and disposal of unhatched chicks**

4.5.1 Drowning, smothering and thermal exhaustion or any other inhumane methods are not acceptable under any circumstances.

4.5.2 All unhatched chicks must be dead before disposal.

4.5.3 High-speed maceration is a practical, humane method of euthanasing a large number of unhatched chicks.

4.5.4 Rapid cooling and freezing are acceptable ways of euthanasing unhatched chicks.

4.5.5 A device for the crushing of unhatched chicks is acceptable provided that all unhatched chicks are crushed instantly and totally.

**4.6 Transportation of chicks**

4.6.1 Chicks dispatched shall be healthy and vigorous.

4.6.2 Delivery containers shall have clean, dry floor pads or absorbent mats where necessary and allow sufficient ventilation.

4.6.3 Containers must be stacked and spaced properly to allow free airflow around the chicks.

4.6.4 Outside temperature and duration of journey shall be considered when determining the optimal density in delivery containers.

4.6.5 Floor space allowed per chick should not be less than 20 $cm^2$ per chick.

4.6.6 Containers with live chicks should not be tilted more than 20 degrees from horizontal during any stage of loading or unloading.

4.6.7 Containers should always be moved smoothly and never be thrown or dropped.

4.6.8 A tie-down device preventing containers from overturning is advisable in chick trucks.

4.6.9 Transportation from hatchery to growing premises should be initiated properly and suitably. Transportation of chicks should never exceed 48 hours.

## 5. PRODUCTION OF HATCHING EGGS (LAYER AND BROILER STOCK) (including commercial table eggs where applicable.)

### 5.1 Receiving chicks on the premises.

5.1.1 Housing facilities shall be prepared to receive the chicks at the time of their arrival. The heating equipment shall be in operation to maintain the required environmental temperature.

5.1.2 Chicks shall be removed carefully from their containers.

5.1.3 Chicks shall be prevented from crowding or piling up in corners.

### 5.2 Housing

5.2.1 Light intensity for the first 3 days shall be sufficient to encourage chicks to start eating normally. Thereafter light intensity shall provide adequate illumination for normal feed and water intake.

5.2.2 Heating and ventilation systems shall maintain the recommended temperature and ventilation with reasonable accuracy in order to prevent either overheating or chilling of the chicks.

### 5.3 Chickens housed on the floor.

5.3.1 Chickens raised on the floor shall have enough freedom of movement to be able to stand normally, turn around and stretch their wings without difficulty.

5.3.2 Feed and water space for broiler breeders shall be the same as for layer type breeds except where nutritional control is practised. In this case feeding space must be increased to allow all birds to feed simultaneously.

5.3.3 Space requirements increase as the birds approach their mature weight

**TABLE 1 SPACE GUIDELINES FOR LAYER BREEDERS**

| Age Weeks | Weight g | Hens per $m^2$ | Feed trough cm | Water trough cm |
|---|---|---|---|---|
| 0 – 6 | 500 | 20 | 2.5 | 1.25 |
| 6 – 20 | 1400 | 12 | 3.5 | 1.25 |
| Mature | 1500+ | 7 | 6.0 | 1.25 |

| TABLE 2 | SPACE GUIDELINES FOR BROILER BREEDERS | | | |
|---|---|---|---|---|
| Age Weeks | Weight g | Hens per $m^2$ | Feed trough cm | Water trough cm |
| 0 – 6 | 750 | 20 | 2.5 | 1.25 |
| 6 – 14 | 1600 | 10 | 8.0 | 1.25 |
| 14 – 20 | 2300 | 10 | 10.0 | 1.25 |
| Mature | 2500+ | 6 | 10.0 | 1.25 |

5.3.4 Round feeders (tube or pan) can replace open troughs with each unit or diameter equalling 1.5 units of double-sided open trough or chain feeder.

5.3.5 During the rearing stage up to 50 birds per water cup or 20 per drinking nipple is a suitable level for chickens.

5.3.6 During the laying stage, there shall be 25 birds per cup or 10 per nipple drinker. One bell drinker may be used for every 70 birds.

5.3.7 Nesting space shall be provided to accommodate hens without crowding. Twenty individual nests are required for every 100 hens. Nesting material and bedding shall not contain any substances that are harmful.

5.3.8 Ammonia concentrations shall not exceed 25ppm at bird level.

## 5.4 Chickens housed in cages

5.4.1 Cages shall be designed to provide the chickens with a safe environment. Cage height shall permit standing chickens free head movement. The cage doors shall be designed for easy insertion and removal of chickens. A cage floor that causes injuries or deformities to the chicken during any period of the cycle is unacceptable under any circumstances.

| TABLE 3 | SPACE REQUIREMENTS FOR CHICKENS HOUSED IN CAGES | | | |
|---|---|---|---|---|
| Age Weeks | Body weight g | Cage floor (area/bird) $cm^2$ | Feed trough (length/bird) cm | Water (birds/cup/or nipple) |
| 0 – 6 | 650 | 220 | 2.5 | 15 |
| 6 – p.o.l. | 1200 | 290 | 5.7 | 8 |
| Adult | 2200 | 450 | 10.0 | 5 |

5.4.2 Cage doors shall be wide enough and door openings free from protrusions permitting the removal of birds without causing injury. (Doors shall not be less than 20cm wide and 20cm high).

## 5.5 Feed and water

5.5.1 Chickens shall not be deprived of water except for specific necessary management vaccination and therapeutic purposes. Drinking water must be fresh and suitable for poultry consumption. When house temperature exceeds 30°C interruption of supply shall not exceed 2 hours.

5.5.2 All chickens shall receive feed on a daily basis. When controlled feeding is necessary, any interruption of feed only shall not exceed 48 hours. The diet must not contain harmful ingredients for either chickens or human consumers of the chicken products. The producer must be prepared to immediately replace a diet proved harmful to the chickens.

5.5.3 Methods of moult inducement and controlled feeding, which deprive fowl of water for more than 24 hours or feed for more than 48 hours shall not be

performed. Both practises shall only be carried out on healthy fowls under close management supervision and conditions that will not cause stress.

### 5.6 Supervision and protection of chickens

5.6.1 Chicken flocks shall be observed at least twice a day. The arrangement of a chicken pen or cages shall permit easy inspection.

5.6.2 Sick or injured chickens must promptly be treated or killed humanely by dislocation of the neck by trained personnel. Dead chickens must be removed daily and disposed of in an appropriate manner.

5.6.3 Attendants shall periodically check the chickens for the presence of external and internal parasites. Should parasites be detected, corrective treatment must be administered immediately.

5.6.4 Live chickens with clinical signs of disease or flocks with abnormally high mortality rates shall be handed to a veterinarian or diagnostic laboratory for diagnosis and subsequent recommendations for treatment must be followed.

5.6.5 Wild birds shall be prohibited from entering poultry houses and rodents in houses shall be controlled using appropriate humane methods.

## 6. BROILER PRODUCTION

### 6.1 Receiving of chickens onto the premises

6.1.1 Housing facilities shall be prepared to receive the chicks at the time of their arrival. The heating equipment shall be operating to maintain an environmental temperature suitable for neonatal chicks.

6.1.2 Chicks shall be removed carefully from their containers.

6.1.3 Chicks shall be prevented from crowding or piling up in corners.

### 6.2 Housing

6.2.1 Light intensity for the first 3 days shall be sufficient to encourage chicks to start eating normally. Thereafter light intensity shall provide a period of adequate illumination for normal daily feed and water intake.

6.2.2 Heating and ventilation systems shall maintain the recommended temperature and ventilation with reasonable accuracy in order to prevent either overheating or chilling of the chickens.

6.2.3 Chickens raised in floor pens shall have enough freedom of movement to be able to stand normally, turn around and stretch their wings without difficulty. Density not to exceed 40kg per $m^2$.

6.2.4 Chickens shall be provided with the following feed and water space:

**TABLE 4 FEED AND WATER SPACE**

| Containers | Bird Density |
|---|---|
| *Feeders* | |
| Pans with a diameter of 30 cm | 70 birds/pan |
| Troughs | 2.5cm/bird |
| *Waterers* | |
| Troughs | 1.0cm/bird |
| Bell Drinkers | 1/100 birds |
| Nipples | 1/20 birds |

6.2.5 Bedding material shall not contain any material harmful to chickens.

## 6.3 Feed and Water

6.3.1 Chickens shall not be deprived of water except for necessary management vaccination and therapeutic purposes. Drinking water shall be fresh and suitable for use by poultry. When house temperature exceeds 30˚C interruption of water supply shall not exceed 2 hours.

6.3.2 All chickens shall receive food on a daily basis. Any feed interruption shall not exceed 24 hours. The diet must not contain harmful ingredients for either chickens or human consumers of the chicken products. The producer must be prepared to immediately replace any diet proved harmful to the chickens.

## 6.4 Supervision and protection of chickens

6.4.1 Chicken flocks shall be observed at least twice a day. The arrangements of a chicken pen shall permit easy inspection.

6.4.2 Sick or injured chickens must be treated promptly or killed humanely by dislocating the neck by trained personnel or any other acceptable method. Dead chickens must be removed daily and be disposed of in an appropriate manner.

6.4.3 Attendants shall periodically check the chickens for the presence of external and internal parasites. If parasites are detected, corrective treatment must be administered immediately.

6.4.4 Live chickens with clinical signs of disease or flocks with abnormal high mortality rates shall be handed over to a veterinarian or diagnostic laboratory for diagnosis and recommendations for treatment should be followed immediately. In any event where the administration of a suitable drug for the strategic treatment of a disease in chickens is necessary, only drugs registered in terms of the relevant Acts will be used and the prescribed withdrawal periods will be adhered to in broilers cropped for slaughtering or the collection of eggs destined for human consumption.

6.4.5 All mechanical systems shall be inspected daily. Chicken premises shall have an emergency plan with which every attendant shall be familiar. Defective mechanisms shall be repaired/replaced immediately.

6.4.6 Wild birds shall be prohibited from entering poultry houses and an effective rodent control programme must be maintained.

# 7. MINIMUM STANDARDS FOR FREE RANGE PRODUCTION

## 7.1 Introduction

7.1.1 International research has identified five basic freedoms that, if catered for, provide a generally acceptable welfare status for the confinement of livestock.

These criteria require that livestock are:-

- Free from hunger and thirst via the availability of fresh water and the appropriate feed.
- Free from abnormal discomfort via the provision of adequate shelter.

- Free from abnormal pain, injury or disease via the provision of appropriate prevention or alternatively, rapid diagnosis and treatment, of normal pathological conditions.
- Allowing for the freedom to express natural behaviour by providing sufficient space in suitable facilities and the company of the animals' own kind.
- By providing conditions and care which avoid undue suffering and thus permit freedom from fear and distress.

7.1.2 Free Range egg or broiler stock may never be confined in cage production systems.

## 7.2 Management

7.2.1 Managers and stockkeepers must be properly trained and competent to handle poultry livestock.

7.2.2 For flocks to qualify for Free Range classification, layers must be introduced to the Free Range system at no later than 130 days of age and broilers no later than 21 days of age.

7.2.3 Free Range stock must have access to an external range for a minimum of 6 hours per day, during natural daylight hours. It is accepted that it is counter-productive for birds to be outside during periods of extreme weather – routine external access may therefore be restricted at such times.

7.2.4 Access to the external range should be provided by means of doors, gates or popholes. When popholes are used, these should be provided at the rate of at least one pophole per 700 birds and be of a minimum size of 100 cm wide and 45 cm high.

7.2.5 Moulting shall not be artificially induced in any Free Range flocks.

## 7.3 Food

7.3.1 Livestock must have access to an appropriate wholesome diet, which is available in sufficient quantity so as to satisfy their diverse and complex nutritional needs.

7.3.2 Such access must be freely available, unless specifically prescribed to the contrary by an attending Veterinary surgeon.

7.3.3 Either mechanical or manual feed distribution systems are acceptable.

7.3.4 If using chain, trough or box feeders, which can be accessed from both sides, then a maximum of one adult hen per 5 cm of feeder length may be housed.

7.3.5 If only one side is accessible, then 10 cm per hen must be provided.

7.3.6 If pan or tube feeders are used, a maximum of 40 adult hens per feeder may be housed. In the case of broilers, 1 pan feeder of 330 mm diameter may cater for a maximum of 70 birds.

## 7.4 Water

7.4.1 Water is an essential nutrient and must be available at all times, unless otherwise prescribed by an attending Veterinary surgeon. Such water must be clean and fresh and dispensed in a manner which minimises water spillage.

7.4.2 If bell-type drinkers are provided, 100 adult hens or 120 broilers may be housed per single bell drinker.

7.4.3 Where nipples are used, 10 adult hens or 12 broilers may be housed per single nipple.

7.4.4 In pens containing less than 100 birds, access to at least two drinkers must be provided.

7.4.5 Where conditions so dictate, adequate provision must be made for the continuous supply of water in sub-zero temperatures.

## 7.5 Internal Environment ("The Chicken House")

7.5.1 The chicken house must be so constructed that it provides for the welfare needs of the birds, whilst simultaneously providing protection from inclement weather conditions and both physical and thermal discomfort.

7.5.2 Whilst concrete floors are desirable, these are not mandatory, provided that whatever flooring is used allows for effective cleansing.

7.5.3 Where open-type housing structures in excess of 6 metres wide are used, provision should be made for ridge openings to facilitate ventilation. Mechanical assistance to natural ventilation (e.g. fans) is an acceptable practice.

7.5.4 Where housing is predominantly enclosed, ventilation by fans with a minimum airflow of 8 cubic metres per hour per adult hen is required.

7.5.5 Litter must be provided on at least 33% of the floor area. Such litter must be of sufficient quality and quantity to allow for the proper dilution of droppings and to allow birds to dust bathe.

7.5.6 Stocking densities must be adequate to accommodate the birds' normal behaviour.

A maximum stocking density of 10 adult hens per square metre of available floor space is permitted. Such floor space shall exclude the area occupied by the egg collection/service area and in addition, shall exclude the area occupied by the enclosed portion of nest boxes where effective access to the area directly below is prevented. For broiler flocks, a maximum stocking density of 15 birds per square metre of available floor space is permitted.

7.5.7 In houses with appropriate perching/roosting facilities, stocking densities may be increased to 12 birds per square metre.

Such perches must be provided at not less than 10 cm per hen and must incorporate a gap on either side of no less than 1.5 cm in order to allow hens to grip the perches without injury to their claws. For the purposes of interpretation, perches will include the alighting rail immediately in front of nest boxes (if applicable).

7.5.8 Adequate nesting facilities must be provided (egg production only) in order to discourage birds from laying eggs on the floor. Where individual nest boxes are provided, this should not be less than 1 nest per 8 hens.

Where communal nests are provided, this should not be less than 1 square metre nest floor per 125 adult hens.

7.5.9 A lighting system for the provision of a minimum period of 9 hours continuous light in each period of 24 hours must be provided. Such light will either be artificial or via access to daylight. A minimum light intensity of 10 lux throughout the house during this time must be maintained.

A minimum period of 8 hours continuous darkness per 24-hour cycle must also be provided in order to accommodate the birds' requirement for adequate rest.

## 7.6 External Environment ("The Range")

7.6.1 The stocking rate of the external range should not exceed 5 birds per square metre. It is recognised that the prevalence of livestock theft is a reality, which restricts the provision of more extensive ranges.

7.6.2 The range must be maintained in a manner that allows for a minimum of 50% living vegetation present at all times. It is acknowledged that certain climatic conditions and locations make it difficult for this vegetation to always be green, but that this should be the objective.

7.6.3 The practice of rotational grazing is a desirable management tool, which allows for the active management of damaged ground, as well as minimising the risk of a build-up of parasites.

7.6.4 External shade by way of either trees or artificial structures must be provided at the rate of 4 square metres shade per 1 000 birds.

7.6.5 In locations where overhead predators frequently occur, provision must be made for outside cover to reduce stress reactions from such sightings.

7.6.6 Fencing should be adequate to provide protection from indigenous terrestrial predators. Domestic animals such as dogs and cats must not be allowed into the enclosed range area.

## 7.7 Record Keeping

7.7.1 For verification purposes, a notice must be displayed at the entrance of each chicken house depicting the following:

a) Total floor area available to the birds.
b) Total number of drinkers.
c) Total feeder availability.
d) Total number of nests (egg production only).
e) Area of external range.
f) Maximum bird stocking capacity.

7.7.2 Suitable records of all sales of all Free Range egg sales are to be retained for a minimum period of 18 months to allow for audited correlation of production versus sales as required from time to time.

## 7.8 Health

7.8.1 It is an accepted fact that only healthy birds are able to produce to their optimum potential.

Birds used in Free Range production must be adequately vaccinated against prevailing pathological conditions.

It is important for management and stockkeepers to have ready access to competent Veterinarians.

7.8.2 The environment provided must be conducive to good flock health as well as providing the necessary protection from pain, injury and disease.

7.8.3 Ailing birds and birds suffering external wounds or fractures must be removed from the flock as soon as possible and either appropriately treated, or disposed of in a humane manner. Appropriate treatment will be done as set out in 6.4.4 above – especially with regard to withdrawal periods.

7.8.4 The practice of professionally performed judicious beak-trimming is internationally recognised as being a humane alternative to the appalling effects of cannibalism. The continuing need for beak-trimming is being constantly reassessed and it is accepted that as soon as the causes, and possible alternate means of preventing cannibalism, have been identified, then the phasing out of beak-trimming will be a welcome evolutionary development.

## 7.9 Regulations Regarding the Grading, Packing & Marking of Eggs Destined for sale in the Republic of South Africa

7.9.1 The regulations relating to the above (Agricultural Product Standards Act No. 119 of 1990) as contained in Government Gazette No. 19657 dated 8th January 1999 will apply to all Free Range eggs.

## 8. BARN-TYPE HOUSING

The condition for poultry in barn-type housing should be such that poultry should:

8.1 not be kept in a cage, but in suitable housing with protection from inclement weather;
8.2 have sufficient space to move around and flap their wings;
8.3 be able to scratch and dust-bathe on a floor covered in suitable bedding (material);
8.4 be able to lay eggs in the privacy of a nest;
8.5 be able to roost on protected perches that are not so high above ground-level that birds have difficulty in using them and risk injury;
8.6 be provided with fresh air and be free from excessive ammonia by the provision of ventilators and manure removal systems;
8.7 may not exceed a housing density in excess of 10 per square meter (under an isolated roof) and should have access to double this space outside.

## 9. CATCHING AND TRANSPORT

### 9.1 Catching

The control of the catching operation is the sole responsibility of the farmer. The catching process must be closely supervised by a responsible person using the following guidelines:

9.1.1 Sufficient number of suitably trained handlers must be used, whilst the work must be divided and allocated to staff according to ability, skill and experience (with close supervision of labour by experienced operators).
9.1.2 The need for following sound handling and transport practices must be stressed to the spent hen buyers.
9.1.3 The producer must be satisfied that loading will take place within a reasonable period of time.
9.1.4 Equipment must be moved to allow handlers free movement and lighting must be reduced to calm the broiler chickens without hampering catchers.
9.1.5 Battery caged chickens should be removed from the cage individually and be held by both legs to avoid injury and suffering.
9.1.6 The number of chickens carried at a time will depend on the size of the chickens and the ability of the carrier, but a maximum of five chickens per hand must not be exceeded. If any chicken escapes, it shall be caught as quickly and quietly as possible.
9.1.7 Chickens shall be handed to the loader as quickly and as quietly as possible, consistent with his loading speed. Care must be taken to avoid injury when

chickens are placed in the container. The number of chickens per container must be adjusted according to climatic conditions.

9.1.8 Visibly unfit or injured chickens must not be loaded, but shall be killed on the farm as quickly and humanely as possible.

## 9.2 Transport

### 9.2.1 Driver and vehicle

9.2.1.1 The driver of a vehicle transporting poultry shall be a responsible person.

9.2.1.2 The drivers of vehicles used for transporting livestock shall be trained in the transporting of livestock.

9.2.1.3 It is compulsory for a driver to be in possession of a valid and appropriate driver's licence as well as telephone numbers of the owners of the animals and emergency telephone numbers at all times during a journey.

9.2.1.4 Drivers shall at all times be able to perform their duties in an expert and responsible manner.

9.2.1.5 Drivers shall not handle a vehicle in a manner that might cause the transported animals to slip, fall or suffer injury. The safety and welfare of the animals shall never be ignored or disregarded.

9.2.1.6 Chickens shall be transported in roadworthy vehicles.

9.2.1.7 The driver must be trained to make the necessary adjustments to the truck when air-conditioning is used.

9.2.1.8 Stops en-route shall only be made when absolutely necessary. When stops are made in hot weather, the vehicle must be parked in the shade where possible or for very short periods when in the sun.

9.2.1.9 In the case of a truck breakdown without a standby facility causing a subsequent rise in temperature in the load space, the load shall be off-loaded if the system permits or at least spaced to accommodate the circumstances where possible.

9.2.1.10 Vehicles used for the transportation of live poultry must be constructed to protect the poultry against adverse weather conditions during transportation.

### 9.2.2 Day-old chicks

9.2.2.1 Chicks dispatched shall be healthy and vigorous.

9.2.2.2 Delivery containers shall have clean, dry floor pads or absorbent mats where necessary and allows sufficient ventilation.

9.2.2.3 Containers must be stacked and spaced properly to allow free airflow around the chicks.

9.2.2.4 Outside temperature and duration of journey shall be considered when determining the optimal density in delivery containers.

9.2.2.5 Floor space allowed per chick should not be less than 20 $cm^2$ per chick.

9.2.2.6 Containers with live chicks should not be tilted more than 20 degrees from horizontal during any stage of loading or unloading.

9.2.2.7 Containers should always be moved smoothly and never be thrown or dropped.

9.2.2.8 A tie-down device preventing containers from overturning is advisable in chick trucks.

9.2.2.9 Transportation from hatchery to growing premises should be initiated properly and suitably. Transportation of chicks should never exceed 48 hours.

### 9.2.3 Adult Birds

(Commercial layers/culls, broiler breeders/culls and broilers)

9.2.3.1 The chickens should be loaded into clean transporting crates or purpose-made wire mesh cages in trolleys.

9.2.3.2 The height of the containers should allow chickens to move their heads freely when sitting on the floor.

9.2.3.3 The construction should prevent protrusion of the head, wings and legs.

9.2.3.4 All the containers should have a lid that can be secured to prevent the chickens from escaping.

9.2.3.5 The number of chickens per crate should correspond to the floor space and body size of the transported chickens, with due regard to environmental conditions and duration of transport.

9.2.3.6 Maximum density should not exceed 55kg body mass per square metre. Maximum density per container should be low enough to allow all chickens to rest on the floor when evenly distributed.

9.2.3.7 The journey should not exceed 24 hours.

9.2.3.8 Portable transporting crates with live chickens should preferably be moved in a horizontal position. Crates should not be thrown or dropped.

9.2.3.9 A tie-down device preventing containers from overturning is advisable.

### 9.2.4 Humane handling of culled poultry

9.2.4.1 The keeping of poultry awaiting sale should meet all the requirements of this production code inclusive of food safety requirements for human consumption.

9.2.4.2 The directions for the physical handling of culls are the same as the directions for the catching and transport of adult birds as set out in clause 9 of this Code as applicable to adult birds (excluding directions for handling of day-old chicks – item 9.2.2)

9.2.4.3 The directions for the number of birds carried by hand should be applied strictly according to clause 9.1.6, provided that larger birds such as broiler breeding stock may not be picked up more than three per hand.

## 10. PROCESSING PLANTS (ABATTOIRS)

10.1 On arrival at the abattoir, consignments must be monitored for chickens that are dead on arrival, moribund, injured or unfit for slaughter.

10.2 Reasons for DOA's, sick or injured chickens should be determined and the information transmitted to farm managers or veterinarians through management. Records of all treatments with any type of drug must be available to ensure compliance with the relevant withdrawal times to ensure that the chicken products are safe for human consumption.

10.3 Chickens awaiting slaughter must be stored in a room or bay that offers adequate ventilation and protection from the elements. Expeditious handling of slaughter consignment is expected.

10.4 Moribund chickens should be euthanased and condemned, whilst injured chickens fit for slaughter should be slaughtered as soon as possible.

10.5 Chickens must be handled carefully and in a humane manner when hanging for stunning to avoid stress and injury.

10.6 Except in the case of religious ritual slaughter, all chickens must be stunned before being bled. The ideal voltage for electrical stunning is debatable, but as a guideline, voltages between 50v and 70v should be adequate for wet stunning if the head and neck of the chicken is immersed in the electrified bath.

10.7 Bleeding requires severance of the neck vessels and a minimum bleeding time of 90 seconds. A sharp knife or blade should be used for this purpose.

# THE SOUTH AFRICAN PIG WELFARE CODE

## PREAMBLE

This code of practice, as a statement of intent, has been drawn up by representatives of the South African Pork Producers Organisation, the Pig Veterinary Society, the National Council of SPCA's, the Livestock Animal Welfare Association, the previous Meat Board & ABAKOR under the auspices of the Livestock Welfare Coordinating Committee and is intended to be a supplementary set of rules that in no way supersedes or contradicts existing legislation concerning the care and handling of pigs or the provisions of meat hygiene regulations.
This code is based on the knowledge and technology available at the time of publication and may need to be changed in the light of future knowledge. It does not replace the need for experience and common sense in the husbandry of animals.

## INTRODUCTION

This code is based on the belief that pigs should be afforded the five freedoms of Webster, namely:

1. **Freedom from thirst, hunger and malnutrition**

2. **Freedom from discomfort**

3. **Freedom from pain, injury and disease**

4. **Freedom to express normal behaviour**

5. **Freedom from fear and distress**

## STOCKMANSHIP, HUSBANDRY AND HEALTH CARE

1. Stock workers and owners must be appropriately trained to handle pigs, and perform routine procedures in a manner, which is clean and causes minimum discomfort.

2. Routine procedures, which may be performed by the owner or trained stock workers are the following:

    - Injections (the neck muscles are the preferred site).

    - Tail docking, teeth clipping, ear notching and castration of piglets up to 7 days of age.

    - Uncomplicated wound and injury care, claw trimming, tattooing, use of antiseptics, pesticides; manual assistance with farrowing; sheath washing of boars; semen collection and insemination on the home property, pregnancy testing with externally applied apparatus.

3. The following practices are forbidden: - Kicking pigs, the use of electric prodders, whips, metal rods, heavy sticks or other objects liable to injure or terrify pigs, picking up by the ears, tail or foreleg. The breaking or cutting or sawing of boars' teeth at any time is prohibited, except when performed by a veterinarian with appropriate anaesthesia.

4. Animal waste and waste products must be handled in such a way as to minimise the risk of discomfort and spread of disease to other animals or to humans, and in a way that keeps pollution to a minimum.

5. Strict control of parasites, flies and rodents must be maintained at all times.

6. For the measurement of back fat in live pigs, only non-invasive and painless methods such as the use of ultrasonic equipment are permissible.

## 1. Tethers

**1.1** No new tether systems are to be installed from date of publication. No neck tethers are to be used at all.

**1.2** Girth tethers must be made of broad bands of webbing or similar material that will not fold or cause damage to the skin, and must be secured by means of a smooth chain to a countersunk fixture in the floor.

The chain must be long enough to allow movement within the sow's stall, but not so long as to allow entanglement with the adjacent sow.

**1.3** All tether systems are to phase out by 31st December 1999.

## 2. Crates and creep areas

**2.1** Dry sow and farrowing crates must be designed and built to allow the sow to stand or lie comfortably with her legs naturally extended. The udders of lactating sows must not be obstructed by any structures or parts of the farrowing crate

It is recommended that sows be given the opportunity to spend time out of crates or tethers, in alternative accommodation. This accommodation should allow the pig to perform its natural body functions in a manner which is relatively unrestricted and which will not permit opportunities for active aggression from other animals in that area.

**2.2** A safe creep area with bedding and provision for warmth for piglets must be provided in the farrowing pen, so that a temperature suitable for piglets will be maintained in the creep by means of insulation and/or artificial heating.

Heating devices must be placed in such a manner that piglets cannot be harmed or the heater interfered with.

**2.3** Rapid fluctuation in temperature should be avoided in the farrowing house.

**3.** Floors must be finished to be non-slip and allow for easy cleaning without being abrasive or causing pigs difficulty in standing or moving. Slats must be aligned evenly and spaced to avoid danger of injury to feet and legs.

**4.** Housing and crates must be well drained and must be kept in a hygienic condition.

**5.** Pens indoor or outdoor, and handling facilities must be constructed and maintained to avoid animal discomfort and injuries. Ventilation or special cooling methods should be adequate to avoid overheating of pigs.

**6.** Pigs must have suitable shelter from direct sunlight, winds and inclement weather.

**7.** Separate pens or sufficient space must be provided for housing different categories of pigs to avoid unnecessary distress, bullying or injury. Consideration must be given to size, numbers, sex, maturity and purpose when mixing pigs in confined areas.

The floor allocation in pens should allow a minimum of 0,85 m2 per 100 kg of live mass.

**8. Pigs kept outdoors**

8.1 Outdoor pigs must be supplied with: -

Appropriate enclosures which allow a minimum of 5 m2 per adult pig, and are designed to provide a warm and adequately ventilated draught free area for all classes of pig, and which are free from water-logging or persistent muddiness other than in a wallow.

8.2 Feeding and watering facilities which minimise fouling and wastage, and which ensure access to clean water at all times.

8.3 Effective fencing to confine and control the stock and adequate handling/isolation facilities for dealing with animals undergoing routine procedures or which are sick or injured or require attention.

8.4 Farrowing hutches supplied with plentiful bedding and shelter from inclement weather. Hutches should be designed to minimise overlying of piglets by the sow, and to confine piglets to the hutch for the first week of life.

## 9. Boars

9.1 Boars may be kept on their own or in small compatible groups until mature. A boar may be kept with a group of breeding gilts or dry sows, provided bullying does not occur.

9.2 Individual accommodation for adult boars should have a floor area of not less than 7,5 m2, with the shortest side not less than 2,5 m. The pen divisions should not be less than 1,5 m high, and gates must be secure. If used for living purposes and service purposes, the floor area should not be less than 10m2.

## 10. Sick and injured pigs

10.1 Pigs should be inspected at least daily for signs of vices, bullying or injury, illness or distress. Lighting, natural or artificial, should be readily available to allow inspections at any time to enable remedial action to be taken in cases of vice, bullying, injury, parasitic infestation or disease.

*Vices*: - Pigs may develop vices, typically tail biting and ear biting. Personnel must identify and minimise their causes. Too much light, overcrowding, inadequate ventilation, competition at the feeding or watering place and barren environmental conditions can contribute to the problem.

10.2 Sick or bullied pigs require isolation from aggressive pen-mates.

10.3 Sick pigs must be given first aid or appropriate veterinary attention promptly.

10.4 Seriously injured or terminally sick animals must be immediately and humanely destroyed.

## NUTRITION

1. All pigs must have access to clean drinking water at all times.

2. All pigs must have access to adequate quantities of suitable wholesome feed every day.

**TRANSPORT** - *This should be read in conjunction with the SA Code of Practice for Handling and Transporting of Livestock*

1. Whenever possible, pigs must be transported during the cooler part of the day. They must in any case be protected from direct sun, excessive cold and wind-chill and exhaust gases.

2. Sick and injured pigs should only be transported for purposes of getting veterinary treatment or for humane slaughter and must be separated from other pigs if on the same vehicle.

3. The vehicle must have: -

   3.1 A free flow of air over all pigs being transported. The sucking of exhaust gases into the load area and wind chill in cold conditions must be prevented.

   3.2 Provision for at least 80 % shade cloth or solid covering over the top if pigs are transported during the heat of the day for periods in excess of two hours.

   3.3 Provision for appropriate partitioning to separate different categories of pigs, or to divide large numbers into smaller groups to avoid crushing, fighting or bullying.

   3.4 A floor, which is constructed, and/or covered to prevent slipping.

   3.5 Sides high enough to prevent pigs jumping out of the vehicle.

   3.6 Internal surfaces and structures that will not cause injury to pigs.

   3.7 Loading gate(s) large enough to allow free movement of pigs during loading and off-loading

4. Pigs being transported over long distances must be provided with water within 18 hours.

5. Loading and driving

   5.1 Loading ramps and platforms must be built or adapted to match vehicle height and loading gate and also to ensure that pigs cannot escape or fall off. Inclines should not exceed 20°.

   5.2 Loading and unloading must be done quietly and with minimum force - the use of boards or rolled plastic bags where their use is necessary is recommended. No electric prodders are to be used.

   **The vehicle must be abutted against the platform so as to avoid any gaps, which may lead to injury of pigs.**

   5.3 Drivers of motor vehicles must ensure a smooth ride and only stop the vehicle when absolutely necessary, and then on a level surface only.

   5.4 Owners/managers must at all times have pre-arranged contingency plans for emergencies such as breakdowns.

   5.5 Drivers of motor vehicles transporting pigs should under no circumstances drive at an excessive speed or at any speed, which could be detrimental to the well being of the pigs concerned.

## ABATTOIRS

### 1. Lairage

1.1 Off-loading areas and passageways must be such as to allow free movement of pigs from vehicle to holding pens.

1.2 Water sprays or hoses must be used for cleaning and cooling hot, dirty or fractious pigs.

1.3 Pigs from different origins must be penned separately, in accordance with their origins.

1.4 Pens and passage ways must have non-slip floors, rounded corners, secure gates and sides with no projections and be kept in good repair. Protection from sun, rain and cold winds must be provided.

1.5 The whole lairage must be cleaned regularly but at least once a day.

1.6 Personnel handling pigs must be trained in welfare standards and in the use of boards and non-damaging "slaps" to ensure that stress and injury are minimised. Electric prodders are not permitted for use on pigs.

1.7 All pens must be supplied with readily accessible clean water for pigs of all sizes. Both troughs and nipple drinkers should be provided.

1.8 Pigs should be slaughtered within a few hours of being penned unless they are exhausted or distressed, in which case they should be given appropriate attention on veterinary advice.

### 2. Stunning and sticking

2.1 The slaughter staff must be trained in the principles of efficient and humane stunning. Management and supervisory staff must ensure that proper methods are consistently used.

2.2 No pig shall be stunned until the previously stunned pig has been hoisted for sticking and moved away from the stunning area.

2.3 Wherever possible, pigs should be stunned by means of electrical apparatus. Captive bolt pistols may be used on individual animals (such as mature boars) where electrical stunning is impractical or inadvisable.

2.4 Stunning apparatus must be kept in faultless working order and be used only in accordance with the manufacturer's recommendations to achieve optimum stunning

2.5 Pigs must be restrained in a stunning race or closely confined in the stunning pen to avoid unnecessary chasing.

It is forbidden to use stunning apparatus for any purpose other than stunning, for example to immobilise pigs by application to parts other than head or neck.

2.6 Good contact between electrodes and the pig's head or neck is best achieved by applying wet electrodes on a dry pig to avoid scattering of current to earth.

2.7 The criterion for efficient stunning is to pass a current of at least 1,3 amperes through the brain of the pig with sufficient voltage and for long enough to ensure unconsciousness. With presently available equipment, the recommended amperage voltages and times of application are: -

| | **Amps** | **Voltage** | **Time** |
|---|---|---|---|
| Porkers and baconers | 1,3 | 220 - 240 | 5 - 10 sec |
| Adult pigs | 1,3 | 240 or more | 10 - 20 sec |

**NB: -** Research, both local and in collaboration with overseas institutions, is urgently needed to devise optimum electrical stunning methods and apparatus and the necessary monitoring equipment, to ensure that the stunning of pigs is done humanely and in the spirit of this welfare code.

2.8 Effective stunning can be presumed when the pig has stopped breathing, its hind legs are fully extended and the corneal reflex is absent (i.e. touching the eye surface produces no reaction of the eyelids). This may take up to 20 Seconds or even longer with low voltages e.g. 100 V.

2.9 Sticking must follow stunning without delay and should be achieved within 20 seconds.

2.10 Pigs must not be put into the scalding tank before they are dead.

# CODE OF PRACTICE FOR THE HANDLING AND TRANSPORT OF LIVESTOCK

## 1. PENNING OF ANIMALS

1.1 Cattle, sheep, goats and pigs, shall be penned separately.

1.2 Animals shall not be penned in overcrowded conditions. More animals shall not be housed in a pen than the permissible number of the particular species for which the particular pen was designed. Penning space provided shall be enough to permit all animals to lie down at the same time and shall not be less than:

1.2.1 For adult cattle: 1,74 sq.m of floor area for each individual.
1.2.2 For bacon and small porker pigs, sheep and goats: 0,56 sq.m of floor area.
1.2.3 For large pigs and young calves: 0,74 sq.m of floor area.

1.3 Fractious animals shall not be penned with other animals.

1.4 Young, weaned juvenile animals, shall not be penned with adult animals, except in the case of mother and offspring. If harassed by other animals, mothers with their young should be penned separately.

1.5 Provisions shall be made in pens for:

1.5.1 facilities such as racks, mangers or other suitable feed containers, which are easy to clean, which will allow the feeding of an animal off the floor and which can be serviced without disturbing the animals;
1.5.2 water troughs with an adequate supply of suitable fresh water at all times;
1.5.3 sufficient facilities for the adequate cleaning of pens;
1.5.4 facilities for the safe and humane keeping and handling of animals; and
1.5.5 facilities for separate keeping of mothers with their offspring born in transit or in holding pens

1.6 The pen shall at all times be maintained in a good state of repair: Sharp points such as wire ends, broken boards, jagged ends or protruding hinges or bolts, which could cause injury to animals, shall be removed or otherwise suitably covered.

1.7 The floor of the entire pen, including the off-loading banks, races, and passages, shall be so constructed as to provide adequate non-slip surfaces that can be efficiently and suitably cleaned and kept dry and in a condition fit for the holding of livestock.

## 2. HANDLING

2.1 At all times livestock must be handled with patience and tolerance with due allowance for their natural behaviour, e.g.:

- Livestock respond easier to being driven when the drover stands behind the animal but within its field of vision.

- Herd animals respond easier to being driven when in a group rather than singly.
- Livestock don't like being driven in the dark.

2.2 Animals may not be dragged by their legs.

2.3 Pigs may not be lifted or carried by their head, ears or tail.

2.4 Young piglets should be carried if they will not move freely with ease.

2.5 Young calves should be carried if they cannot walk with ease. They may be carried by lifting them around the chest and hindquarters. Alternatively they must be guided with one hand on the hindquarters and the other near shoulder/neck, and walked in the required direction at a suitable pace.

2.6 Should unweaned calves be transported unaccompanied by their mothers, they shall be rested for a few hours and then fed with milk or appropriate milk substitute.

2.7 Only sticks with canvas or belting flaps may be used when driving livestock. It is preferable to strike the ground behind the animal than to hit the animal itself.

2.8 Electric prodders, sticks or goads should never be used on young calves or pigs.

2.9 Electric prodders shall not be applied to the face, anal or genital areas of livestock and not only should they not be used excessively or indiscriminately but only as a last resort.

2.10 Livestock should never be struck in the face.

2.11 The loading, off-loading and herding of sheep must preferably be facilitated by appropriate use of trained "Judas" goats.

2.12 If animals have to be restrained by hobbling, only the area above the "knee" is to be used.

2.13 Lactating animals shall be milked when necessary to prevent discomfort.

2.14 Injured, disabled and blind animals shall be priority slaughtered. If necessary and to prevent further pain or distress emergency slaughter may be required.

## 3. MOVEMENT OF THE HOOF

3.1 Animals driven on the hoof shall at all times be under proper and competent supervision.

3.2 Animals on the hoof shall be driven in a calm manner at a gait that is relaxed, natural to that animal, and not faster than the pace of the slowest animal.

3.3 Animals shall not be driven for periods in excess of 10 hours without being given rest of at least one hour and provided with sufficient suitable fresh water that will be available to all the animals.

3.4 No animal on the hoof shall be moved in excess of the following distances:

3.4.1 During a journey of not more than one day's duration in the case of:
a) Sheep and goats: 20 Kilometres (25 km is excessive) and;
b) Cattle: 30 Kilometres

3.4.2 During a journey of more than one day's duration:
a) Sheep and goats: 20 Kilometres during the first day and 15 Kilometres during subsequent day; and
b) Cattle: 25 Kilometres during the first day and 20 kilometres during each subsequent day.

3.5 Animals shall be watered and fed immediately on reaching their night camp or final destination, with sufficient food of a quality and of a type compatible with the species.

3.6 Ideally animals should not be moved in the dark.

3.7 No sick, injured, disabled or heavily gravid animals shall be moved on the hoof.

3.8 Contingency plans must be in place to move by vehicle any animal that becomes exhausted, lame or otherwise unable to keep up with the herd.

3.9 Contingency plans must be in place to emergency-slaughter any animal in such a condition that failing to humanely slaughter it would constitute cruelty: e.g. a broken leg or exhaustion.

## 4. VEHICLES USED IN THE TRANSPORT OF LIVESTOCK.

4.1 Vehicles and all trailers used in the transport of hoofed livestock shall be suitable for the transport of livestock and in a roadworthy condition.

4.2 All such vehicles and trailers shall have: -

4.2.1 a suitable non-slip floor which should not impede the cleaning of the floor of the vehicle. Hinged or removable battens or steel grids are permissible;

4.2.2 adequate ventilation and light whilst in motion as well as when stationary: no vehicle shall be totally enclosed;

4.2.3 adequate protection from exhaust gasses - exposure to exhaust fumes could interfere with animals' respiration or cause distress;

4.2.4 adequate protection from direct sun in the case of pigs transported longer than an hour;

4.2.5 no projections from the floor, sides or roof unless they are adequately protected so as to prevent injury to animals transported therein;

4.2.6 adequate provision for inspection at floor level of all the animals being transported;

4.2.7 sidewalls high enough to prevent animals from escaping or falling out of the vehicle: The sides and partitions, when used in a vehicle to separate animals carried therein, shall be of a height not lower than the shoulder joint of the largest animal being transported. In the case of cattle other than calves, the minimum height shall be 1800 mm. The minimum height shall be 750 mm in the case of any smaller animals.

4.2.8 in multi-tier vehicles, heights between decks shall be adequate, and in case of sheep and pigs not less than 1000mm, to enable the largest animals to stand naturally, freely and fully erect and to allow adequate space for the free flow of air above the animals;

4.2.9 floors that are solid and impervious;

4.2.10 floors that provide adequate and proper drainage;

4.2.11 loading/offloading openings at the rear of the vehicle that are the full width of the vehicle or, if at the sides, a width not less than 2400mm;

4.2.12 gates shall be of a design and construction strong enough and suitable for the conveyance of the intended consignment; and

4.2.13 gates must open and close freely and, as well as partitions, must be able to be well secured.

4.3 Materials used in the construction of partitions, side-rails, sidewalls, gates and ramps shall be sturdy and suitably robust and not be liable to breakage, splintering or present any surfaces liable to cause injury or bruising to the animals.

4.4 Suitable bedding material of sufficient density and thickness to prevent slipping and sliding is permissible. (Coarse sawdust and wood shavings absorbs urine and wet droppings and give a good "footing.")

4.5 The density of animals packed into any given space shall be such as to ensure the safety and comfort of the animals during transport. The recommended floor space per animal is as follows:

4.5.1 1,4 sq.m per each adult cattle; or
4.5.2 0,3 sq.m per small calf; or
4.5.3 0,4 sq.m per sheep and goat; or
4.5.4 0,3 sq.m per porker; or
4.5.5 0,4 sq.m per baconer; or
4.5.6 0,8 sq.m per adult other pig.

4.6 In the case of the transport of pigs, the vehicle shall have:

4.6.1 a free flow of air at a level that will ensure adequate ventilation without subjecting them to wind-chill;

4.6.2 a roof-covering providing effective shade shall be provided when pigs are exposed to direct sunlight; and

4.6.3 an adequate supply of water for use in an emergency: e.g. spraying to reduce heat exhaustion.

4.7 Where the loading area of a fully loaded vehicle exceeds 4 meters in length, suitable partitions shall be provided so that no single loading area exceeds 3 meters in length.

4.8 The owner shall maintain all vehicles used in the transport of animals in a clean and hygienic condition. The vehicle shall be thoroughly washed down or otherwise cleaned as soon as practicable after the animals have been off-loaded.

## 5. WATERING AND FEEDING OF LIVESTOCK PRIOR TO LOADING.

5.1 Cattle, sheep and goats shall be provided with sufficient and suitable food and fresh water up to commencement of the journey.

## 6. LOADING AND OFF-LOADING PROCEDURE

6.1 Pigs from separate pens should not be mixed if this can be avoided. The time between removal from their pens and loading onto the vehicle should be kept to a minimum and the vehicle must depart as soon as it is loaded.

6.2 Loading and off loading of livestock into or out of a vehicle shall be accomplished as quietly and calmly as possible, with patience and tolerance and without undue harassment, terrifying of the animals, bruising, injury, suffering or undue stress.

6.3 No animals shall be loaded or off-loaded by lifting by head, fleece, skin, ears, tails, horns or legs.

6.4 No animals shall be loaded or off-loaded otherwise than:

- by means of a ramp with a non-slip surface, sturdy enough to support the weight of the species of livestock being handled, with side panels or bars adequate to prevent animals escaping or falling off the ramp and of an incline not steeper than 30 degrees for sheep and goats, 25 degrees for cattle and 20 degrees for pigs; or
- at a loading bank equal to the height of the floor of the vehicle or, at off loading, not more than 310mm below the level of the off-loading vehicle and with an incline not exceeding 30 degrees.

6.5 Where a truck is equipped with an onboard removable loading ramp it should have a non-slip surface and be of a sufficient length when lowered that the inclination is no steeper than the inclines prescribed in 6.3. The distance from the ground to the heel of the ramp shall not exceed 120 mm.

6.6 The vehicle shall be lined up flush with the loading/ off-loading ramps or banks.

6.7 Ramps shall be correctly adjusted to the exact height of the vehicle's floor.

6.8 Journeys should commence as soon as possible after the livestock have been loaded and the animals promptly off loaded on arrival at destinations.

6.9 Mixing of species: Unless adequate provision has been made for effective separation, different or antagonistic species of animals such as pigs and cattle, sheep and pigs, cattle and sheep, calves and other species should not be loaded and transported in the same vehicle.

6.10 Mixing of animals of different ages or sizes: Animals of different ages, sizes and sexes shall not be loaded and transported in the same vehicle unless adequate provision has been made for the effective separation of such animals.

6.11 The mixing of adult horned cattle with polled cattle shall not be allowed and they must also be penned separately.

6.12 No animal which is diseased, emaciated, injured, disabled, exhausted or otherwise unfit or cows with udders distended with milk, or animals blind in one or both eyes should be loaded onto a vehicle and transported unless with the purpose to minimise its suffering, and then with the least discomfort. Where a journey has already commenced if, by reason of such unfitness, the animal is likely to be subjected to avoidable suffering or distress during transport, the consigner, carrier or other person in charge of the animal shall ensure that it is not carried further for a period longer than is necessary to transport it to the nearest available place at which it can receive attention, such as a veterinary hospital, clinic, or an animal welfare centre.

6.13 Where the owner of an animal or his agent, or the consignor, carrier or other person in charge thereof, has reason to believe that the animal is likely to give birth in the course of a proposed journey, the animal may not be loaded onto a vehicle or transported except with the written authority of a veterinary practitioner, and in accordance with the terms and conditions (if any) subject to which authority is given.

6.14 Animals that are blind shall be identifiable by being clearly marked by having a wide circle painted around the blind eye and, in addition, a circle not less than 120 mm painted onto both rumps of the animal. The colour of the paint shall be in strong contrast to that of the animal. Animals blind in one or both eyes shall not be herded together with other animals but shall be handled separately and guided to their intended destination with due care and consideration for their being unable to respond or react as would normally sighted animals. Where blind animals are off-loaded in an abattoir, such animals shall be the first of their species to be slaughtered.

6.15 The loading, off-loading and herding of sheep will be facilitated by appropriate use of trained "Judas" goats

6.16 A Bill of Loading shall accompany every consignment of animals transported. It shall conform to the schedule prescribed in Appendix 1.

6.17 The following procedures must be avoided:

6.17.1 Yelling, kicking, tail twisting, beating, whipping, dragging by head, fleece, ears, tails, horns or legs, hitting in the face or elsewhere with bars, rods or sticks without flaps or indiscriminate prodding;

6.17.2 the excessive or indiscriminate use of any instrument, prodder or object used for driving the animal;

6.17.3 use of excessive force or attempts to drive livestock into or out of vehicles in such a manner as to cause panic or terror;

6.17.4 excessive use of an electric prodder to an obstinate animal;

6.17.5 applying an electric prodder to the face, anal or genital areas of livestock;

6.17.6 carrying any objects or accessories in the load-area of the vehicle which could cause an animal to trip, fall or be bruised or injured;

6.17.7 using ramps or platforms that do not provide secure footing;

6.17.8 marking (identification-stamping) of livestock while still on the truck will be done calmly with no harmful materials;

6.17.9 loading too many animals into the apportioned space causing overcrowding;

6.17.10 loading too few animals into the apportioned space causing danger of falling;

6.17.11 loading livestock longitudinally in the vehicle instead of transversely;

6.17.12 transporting sheep in the chest recumbent position;

6.17.13 transporting unfit or heavily gravid animals except in an emergency and to the animal's benefit;

6.17.14 loading cows with udders distended with milk; or

6.17.15 transporting animals blind in one or both eyes not identified with circles painted around the blind eye and on both rumps in a strongly contrasting colour.

## 7. DISTANCE, DURATION, FEEDING AND WATERING DURING A JOURNEY

7.1 Any animal transported shall be moved with a minimum of stress.

7.2 It must be ensured that adequate time is provided for the loading and off-loading of the animals within the scheduled time so as to allow for calm and orderly handling;

7.3 Animals should be consigned so as to arrive at the destinations during the hours when the process of receiving, off-loading and lairaging could be done under supervision. (All abattoirs shall display the relevant hours applicable prominently.)

7.4 If unweaned animals are transported they shall be conveyed together with their mothers each in separate compartments.

7.5 If unweaned calves should be transported without their mothers they should be provided with milk at regular intervals.

7.6 Weaned calves should not be transported for periods in excess of twelve hours.

7.7 When transporting pigs over a distance of more than 50 km, a sufficient supply of water should be carried for emergency use, e.g. spraying the pigs to reduce heat exhaustion.

7.8 Animals shall be promptly off-loaded on arrival at the destination.

## 8. DRIVERS' RESPONSIBILITIES DURING TRANSPORT OF LIVESTOCK

**Drivers shall:**

8.1 drive in strict compliance with the requirements of the Road Traffic Ordinance;

8.2 be in a possession of a valid driver's license appropriate to the class of vehicle driven;

8.3 be in possession of the appropriate documentation (vide Annexure 2) as well as telephone numbers to be phoned in case of emergencies or assistance being required;

8.4 be in possession of a written and approved route plan of the most suitable and shortest route to the destination, a contingency alternate route as well as a contingency plan for emergencies and the telephone numbers of the consignor, the consignee, the transporters and 24-hour emergency contact numbers;

8.5 have knowledge of the natural behaviour of the animals he is transporting: e.g visual fields, flight patterns as well as of the appropriate use of flap-sticks, boards, electric prodders as well as having knowledge of disallowed handling methods; and

8.6 be responsible for ensuring that the load-space of the vehicle is free of any objects or equipment such as wire, webbing, spades, spare wheels, drums, tools, etc. which may cause injury to the animals being transported therein;

8.7 be responsible for ensuring that there are no rough edges, projecting plates or boards or sharp ends, bent bars etc., which may cause injury to the animals;

8.8 be responsible for the correct aligning of the vehicle to the loading/off- loading platforms so as to ensure that there is no space through which an animal can fall or be trapped;

8.9 at all times be alert and in a fit state to be in responsible charge of a vehicle conveying animals;

8.10 not handle a vehicle in such a manner as to cause the animals conveyed therein to slip, fall or be injured. The vehicle shall not be driven in disregard of the safety or well being of the animals;

8.11 not stop for more than 30 minutes while transporting livestock;

8.12 park loaded vehicles conveying livestock only on level ground, preferably in shade in a quiet area away from inquisitive onlookers;

8.13 ensure that, barring unforeseen eventualities, he is able to deliver the consignment of livestock to its destination within the scheduled time of acceptance;

8.14 be aware that the faster the vehicle travels, the greater the wind-chill factor:

| **Wind-chill factor at various speeds and ambient temperatures*** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Speed | | | | | **Ambient air temperature (°C)** | | | | |
| km/h | 25 | 20 | 15 | 10 | 5 | 0 | -5 | -10 | -15 |
| 8 | 25 | 19 | 14 | 9 | 4 | -2 | -7 | -12 | -17 |
| 16 | 23 | 17 | 11 | 3 | -2 | -7 | -13 | -18 | -24 |
| 24 | 21 | 15 | 8 | 2 | -5 | -11 | -17 | -24 | -30 |
| 32 | 20 | 13 | 7 | 0 | -7 | -13 | -20 | -26 | -33 |
| 40 | 19 | 12 | 6 | -1 | -8 | -15 | -22 | -29 | -35 |
| 48 | 18 | 11 | 4 | -3 | -10 | -17 | -24 | -31 | -38 |
| 56 | 17 | 10 | 3 | -4 | -12 | -19 | -26 | -33 | -40 |
| 64 | 16 | 9 | 2 | -5 | -13 | -20 | -28 | -35 | -42 |
| 72 | 16 | 8 | 1 | -6 | -14 | -21 | -29 | -36 | -44 |
| 80 | 15 | 8 | 0 | -8 | -15 | -23 | -30 | -38 | -45 |

* These parameters are applicable to dry animals only. The wind-chill factor is exacerbated when animals are wet. The danger of pneumonia and death is greatly increased where the animals are transported insufficiently protected in wet conditions.

8.15 be required to visually observe the animals he is transporting as frequently as circumstances may permit, but not less than every two hundred kilometres to ensure that no animal is in obvious distress. Where any distress is observed, immediate measures to relieve such distress must be taken by the driver;

8.16 be competent to assess such distress and be competent to take the necessary measures to alleviate or resolve the situation (being in possession of a functional cellphone is desirable);

8.17 in the case of an animal giving birth during transport, immediately take the necessary measures to ensure the protection of the mother and offspring from being trampled or otherwise injured or harassed by other animals;

8.18 in the case of an animal that becomes unfit or severely injured in the course of a journey, ensure that it is not carried for a period longer than is necessary to transport it to the nearest available place at which it can receive attention, such as a veterinary hospital or clinic or an abattoir, or auction pens, or to a Police Station for emergency humane destruction;

8.19 in the event of any breakdown of the transport vehicle, accident or injury to any animal in transit, the carrier shall contact assistance en route, i.e. the South African Police, the traffic authorities and breakdown service without delay and report the relevant details to the official in charge.

8.20 Notwithstanding the foregoing, it shall not be unlawful in the case of an emergency for a vehicle to be used as an ambulance and for an unfit animal to be carried therein with all practical speed direct to a place for veterinary treatment, or to the nearest available place at which it can be humanely killed.

## 9. RESTRAINING OF LIVESTOCK DURING TRANSPORT

9.1 No person shall transport any animal which is likely to become panic-stricken or which may try to escape or may be liable to injure any other animal, other than in an escape-proof container. Such containers shall be so constructed as to prevent contamination, to be free from hurtful projections and which shall have adequate ventilation and shade and protection from wind chill, wet or damp and exhaust gasses, so as not to cause such animal undue suffering or distress and which will allow the animal to stand freely and naturally erect with sufficient space above its head to permit free flow of air.

9.2 Where the transport of any animal may cause injury to itself or any other animal, it shall be restrained in such a manner as to prevent such injury.

9.3 Such restraining shall be affected without causing that animal physical injury, or deprivation of such essential needs as adequate ventilation and protection from adverse climatic conditions, noxious fumes and provided that the measures taken will not amount to cruelty to the animal.

9.4 Sheep shall not be transported in compartments requiring their being constrained in the chest recumbent position.

9.5 No animals shall be kept in restraint for more than 4 hours in any 24-hour period.

9.6 A sack containing an animal must be so secured to the vehicle that it will prevent sliding or movement during transport.

9.7 No wire or bailing twine shall be used for tying the animal's legs or feet.

9.8 To avoid strangulation or neck-break, a slipknot may not be used where animals are secured to the vehicle by horns or neck. The rope must be attached to the vehicle at the level of the animal's "knees", so that in the event of the animal falling, the possibility of serious injury or death is reduced. The rope should be long enough to allow the animal to lie comfortably in a natural position with head upright.