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Canadian Council on Animal Care
Conseil canadien de protection des animaux

CCAC guidelines: Scientific procedures (Part A – Administration of substances and biological sampling)

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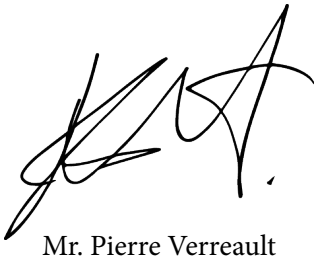

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PREFACE

The Canadian Council on Animal Care (CCAC) is the national peer-review organization responsible for setting, maintaining, and overseeing the implementation of standards for ethical animal care and use in science throughout Canada. CCAC standards are based on professional expertise and current interpretation of scientific evidence.

The *CCAC guidelines: Scientific procedures (Part A – Administration of substances and biological sampling)* is part of a series of general guidelines documents for the ethical use and care of all animals used in scientific activities, including wild animals in the field or brought into scientific facilities, and animals owned by third parties that are used in science. General guidelines streamline information for protocol authors, animal care committees, facility managers, veterinarians, technicians, and animal care personnel to help facilitate improvement in both the care given to animals and the manner in which scientific activities are carried out. More specific guidance on the application of this guidelines document for particular species or groups of animals can be found in the CCAC's types of animal guidelines documents.

This specific document describes the general guidelines for the administration of substances and biological sampling.

This guidelines document details the standards that are expected to be met by holders of the CCAC Certificate of GAP – Good Animal Practice®. For scientific activities conducted within Canada or outside of Canada, protocol authors based at CCAC-certified institutions are subject to these standards. Protocol authors are also subject to any relevant legislation and regulations in the jurisdiction where the scientific activity is conducted.

SUMMARY OF THE GUIDELINES LISTED IN THIS DOCUMENT

The following list of guideline statements serves as an executive summary covering the most important aspects of administering substances to, and biological sampling of, animals. These guideline statements are included throughout this document alongside details and references that provide support and context for their implementation. Throughout this document, the term 'should' is used to indicate an obligation, for which any exceptions must be justified to, and approved by, an animal care committee. The term 'must' is used for mandatory requirements.

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1 INTRODUCTION

Throughout this document, the term 'should' is used to indicate an obligation, for which any exceptions must be justified to, and approved by, an animal care committee. The term 'must' is used for mandatory requirements.

Administration of substances and biological sampling are procedures used for a wide variety of scientific purposes involving animals.

Protocol authors (investigators, instructors, and study directors) must collaborate with veterinarians and animal care committees to refine their studies to achieve the scientific objectives while minimizing the potential for negative welfare impacts on the animals. This guidelines document applies to administration and sampling procedures carried out for any scientific activity (i.e., research, teaching, training, or testing). The following guideline statements have been developed to provide information regarding specific administration and sampling procedures and volumes, and to set maximum limits.

1.1 GENERAL PRINCIPLES

Guideline 1

Administration of substances and biological sampling procedures should be carried out in a manner that minimizes negative animal welfare impacts on the animal.

When administering a substance or collecting a biological sample from an animal by any route, recognized veterinary practices should be used (CALAM, 2020).

The way substances are administered and biological samples are collected can significantly affect animals' welfare and the subsequent scientific value of the results. Once the particular species to be used has been selected, the protocol should be adapted to further refine the procedures and reduce negative welfare impacts on the animal (see Sections 1.3.2.2, "Handling the Animal", and 1.3.2.4, "Study Methodology"). Refinement of administration and sampling procedures provides opportunities for improving both welfare and science (Diehl et al., 2001; Turner et al., 2011a; Graham et al., 2015; Peterson et al., 2017). The welfare benefits are widely recognized; the scientific benefits result from better quality data obtained from more carefully prepared studies, and reduced variability associated with negative welfare states in animals (e.g., Stuart and Robinson, 2015; Barbee and Turner, 2019). Reduction in variability may ultimately reduce the number of animals required for a particular study. However, while reducing the number of animals used through sound study design and attention to the detailed steps of a procedure is important, minimizing any negative animal welfare impacts for the individual animal should be the primary objective (Morton et al., 2001).

Procedures should not be conducted in animal housing rooms; an exception to this can be made for procedures with minimal welfare impacts on the animal and other animals in the room. Steps should be taken to minimize the potential adverse welfare impacts on other animals in nearby locations (CCAC, 2024).

1.2 SPECIES-SPECIFIC CONSIDERATIONS

Guideline 2

The appropriateness of the species and of other pertinent characteristics of the animal for the administration of a substance or for the biological sampling required must be scientifically justified within the animal use protocol.

The appropriateness of the animal to be used (i.e., species, strain or line, age, sex, and other pertinent characteristics, including the vendor) should be determined for the intended route and dosing of the compound, and for the method of sampling, including the volume and frequency (CCAC, 2006). For example, some lines of common laboratory species are more docile and easily handled when dosing or sampling. Depending on the scientific goal of the protocol, it may be possible to select such a species or strain (line) to reduce undue negative welfare impacts of the procedure on the animal (Morton et al., 2001; Schapiro and Everitt, 2006).

1.3 PRE-PROCEDURE PLANNING

Preparation can reduce the likelihood of difficulties arising during procedures. Checklists may be developed for use in experimental planning to ensure that all factors have been considered; these factors should be evaluated during the review of any standard operating procedures (SOPs) or study protocols (Turner et al., 2011a; Morton et al., 2001). A checklist for planning procedures, adapted from Morton et al. (2001), is provided as Appendix 1.

1.3.1 Scientific Goals

It is important to fully consider the scientific goals of the study to determine the best approach to be used. The goal of the scientific activity should be met using the most appropriate administration and sampling routes and regimen; these routes should be verified (e.g., through the review of current literature and pilot studies, and consultation with the veterinarian and other experts; see Section 1.5, "Pilot Studies") prior to protocol commencement (CCAC, 2022). As with any animal-based study, the study design must be well described in the protocol and should include the necessary elements to assist animal care committees in making an informed decision on the ethical acceptability of the proposed work and meeting study reporting requirements (e.g., the PREPARE guidelines (Smith et al., 2017); the ARRIVE guidelines (Percie du Sert et al., 2020)). The study design should also include justification of the species, strain or line, age, and sex selection, recognizing that the procedures should be appropriate for the age of the animals and may have different impacts on male and female animals (CIHR, 2018). Unless there is scientific justification to require otherwise, both male and female animals should be used in biomedical research studies (CIHR, 2018).

Scientific endpoints must be clearly identified (see Section 1.4, "Scientific Endpoints, Humane Intervention Points, and Post-Procedure Monitoring"; Morton et al., 2001; CCAC, 2022).

1.3.2 The Technique

The techniques for administration of substances or biological sampling include the route to be used, the physical handling of the animals, and the skill of the operator. Potential technical problems should be identified (e.g., problems related to restraining the animal, or the inability to obtain the required volume of the sample from the proposed route) and whether the technique itself will have an adverse welfare impact on the animals. Relevant literature and other sources of expertise should be consulted to identify the most appropriate techniques. As part of this process, contingency plans and carefully chosen humane intervention points must be clearly identified (see Section 1.4, "Scientific Endpoints, Humane Intervention Points, and Post-Procedure Monitoring"; CCAC, 2022; Morton et al., 2001). A training protocol with clear competency benchmarks must be implemented to ensure proper technique to reduce technical problems.

1.3.2.1 The Route

The most suitable route for administration or sampling procedures must be determined; if various administration or sampling routes would achieve the same results, the route likely to cause the least negative welfare impact to the animal should be selected. In addition, if the procedure is to be performed more than once, the route should be appropriate for repetitive dosing or collection of biological samples. Methodologies that have been used in other studies and that have resulted in good welfare outcomes for the animals must be identified through a literature search or in discussion with veterinarians or other expert scientists. Many of the commonly used methods of administration or biological sampling require restraint, sedation, or general anesthesia; the potential impact of such manipulations should be evaluated when selecting any solution, administration or sampling route, and frequency (see Section 2, "Administration of Substances" and Section 3, "Biological Sampling").

1.3.2.2 Handling the Animal

Low-welfare-impact handling or restraint techniques should be planned (Marcotte et al., 2021; Russo et al., 2021). The first stage of any study should be to habituate the animals to being handled in their new surroundings. Even if the restraint period is of short duration, the most comfortable methods of restraint for the animals should be used, and the animals should be habituated or desensitized to being held in the position for dosing or sampling prior to starting the procedure, so that any negative welfare impacts are minimized when the procedure takes place (CCAC, 2017; McMillan et al., 2014; Meijer et al., 2006; Morton et al., 2001).

Many species become familiar with individual people, so it is helpful for the personnel who will be carrying out the procedure to develop this familiarity beforehand.

For field studies, handling is likely to have greater negative welfare impacts on wild animals compared to similar animals in a facility that are habituated to handling (CCAC, 2023).

When procedures for substance administration or biological sampling are to be carried out infrequently but on a long-term basis, the animals should be handled regularly to help reduce any negative welfare impacts when performing the procedure (Ujita et al., 2021). There are exceptions to this general rule (e.g., with neonates, with an animal unlikely to become habituated to handling, or with wildlife) that may compound the negative welfare impacts of procedures. In these cases, low-welfare-impact handling techniques should be used. For example, mice are unlikely to become habituated to conventional handling techniques (i.e., picking up by the tail), but low-welfare-impact handling techniques such as allowing a mouse to enter a tunnel

and then picking up the tunnel, make handling and subsequent restraint (e.g., scruffing) less aversive for the animal and easier for the handler (Gouveia and Hurst, 2019). Some species of animals (e.g., dogs, nonhuman primates) can be desensitized to handling (Clay et al., 2009) or trained to cooperate with the handler during handling and dosing, and this can further reduce negative welfare impacts. Animals may also learn to be less fearful of being handled by being housed with animals who have been desensitized to handling (de Lima Rocha et al., 2017). Methods of training-by-reward (positive reinforcement) that do not interfere with the study should be used to encourage animals to participate in administration and sampling procedures (see CCAC types of animal guidelines documents and other references; e.g., Morton et al., 2001). Positive reinforcement does not necessarily involve food rewards, as even brief pleasurable interactions with humans may make future handling less aversive (e.g., "tickling" in rats (LaFollette et al., 2017)).

1.3.2.3 Personnel

All personnel involved in performing the procedures must be competent in carrying out the techniques. A well-designed training program for personnel must be in place and should include specific competency assessment. The most appropriate personnel to perform the procedure should be identified, considering both the handling of the animals and the procedure itself. Sufficient personnel must be available to restrain, dose, or sample the animals and to monitor them post-procedure (some procedures can be carried out by one individual, provided the person is competent). Personnel directly in charge of monitoring animal welfare must be aware of the scientific endpoints, humane intervention points, and the plan of action to be followed when the endpoint or intervention criteria have been reached (Morton et al., 2001; CCAC, 2022).

1.3.2.4 Study Methodology

When planning an experiment and during protocol review, the dosing and sampling methods are important considerations, as they represent an essential opportunity for refinement (Turner et al., 2011a). For example, a Latin-square design may be used to determine appropriate doses (e.g., for a new drug or when used in conjunction with behavioural studies (NC3Rs, n.d.-c; Fry, 2013; Fry et al., 2022)), and the full experiment may be designed by using a small number of animals first (see Section 1.5, "Pilot Studies"). There are several other means of designing studies involving doses. The NC3Rs Experimental Design Assistant is a helpful resource for planning (NC3Rs, n.d.-c).

Complications may arise from the method of delivery, the factors associated with the substance administered or the biological sample withdrawn (e.g., the composition of the solution or volume administered, volume collected), and animal-related considerations such as species, strain or line, age, and sex. Study teams should account for these potential negative effects to avoid confounding effects with other aspects of study design and to permit accurate interpretation of research findings (Turner et al., 2011a).

If procedures are likely to result in pain for the animals, anesthesia and analgesia should be used (see the *CCAC guidelines: Scientific procedures (Part B – Analgesia, anesthesia, and surgery)* (2025)). The impact of the anesthetic and the anesthetic procedure on the animal and the possible interaction with the scientific procedures must be evaluated (Peterson et al., 2017). Reduction of negative welfare impacts while maintaining the validity of the results should be a priority, so the potential impact of withholding anesthesia and analgesia on the results should also be evaluated.

1.4 SCIENTIFIC ENDPOINTS, HUMANE INTERVENTION POINTS, AND POST-PROCEDURE MONITORING

Guideline 3

Scientific endpoints, humane intervention points, and monitoring procedures must be established for animal-based studies and approved by an animal care committee.

Animal care committees must ensure that the protocol authors and veterinarians have agreed to the scientific endpoints, humane intervention points, and post-procedure monitoring specific to the administration or sampling procedures prior to the commencement of the study. Similarly, animal care committees must ensure cumulative endpoints are established where multiple procedures will be carried out on the same animal (see *CCAC guidelines: Identification of scientific endpoints, humane intervention points, and cumulative endpoints* (2022) and *CCAC guidelines: Animal welfare assessment* (2021)).

Death as a study endpoint should not be accepted. Protocol authors must collaborate with veterinarians and animal care committees to determine early predictors of any severe scientific endpoints. Even for regulatory tests, surrogate endpoints should be used (OECD, 2000). If regulatory tests still require death as an endpoint, consultation with the relevant regulatory body should be sought to either replace the test with a humane alternative or make a case for an earlier or less severe scientific endpoint (RSPCA, 2019).

Personnel directly in charge of animal welfare must be aware of the protocol-specific scientific endpoints and humane intervention points, and the action plan if these points are reached (Morton et al., 2001; CCAC, 2022).

The action plan should include:

- a limit on the severity of the welfare impact for more invasive routes of administration or sampling (e.g., retro-orbital injection or bleeding, intracranial injections)
- an agreement to discontinue the administration of a test substance if unexpected side effects occur that negatively affect animal welfare
- a limit on the number of attempts to obtain a sample
- an agreement to stop a procedure if there is an unanticipated negative welfare impact on the animal

Animals must be closely monitored both during and after the procedure to ensure they receive any pre-established treatments, and to instigate follow up if animals are found to be experiencing negative welfare effects. Where monitoring identifies unanticipated adverse effects (including any post-procedure effects), these must be reported to the research team and the previously approved action taken, as agreed upon with the veterinarian.

The process for identifying and monitoring (e.g., clinical signs and checklists) may be developed and validated during a pilot study.

1.5 PILOT STUDIES

Pilot studies – small-scale studies involving small groups of animals – are both ethically and scientifically important in determining the most appropriate design and parameters to be used in complete studies (Johnson and Besselsen, 2002). Pilot studies are also important for establishing humane intervention points (CCAC, 2022).

Reducing negative animal welfare impacts may include refinements to the methodologies and increased monitoring; these refinements should be validated using pilot studies.

When there is insufficient evidence to establish scientific endpoints prospectively, pilot studies should be conducted to identify the earliest point at which the scientific activity can be terminated. Pilot studies must focus on determining welfare-appropriate endpoints rather than on generating useable scientific data.

Pilot studies must always be approved by the animal care committee, and the results must be reviewed by the animal care committee before the full protocol for the ensuing experiment is permitted to proceed (CCAC, 2022).

If the pooling of the pilot study and main study data is considered, this should be planned beforehand and described in the protocol, with a clear discussion of the statistical consequences and methods, to avoid potential bias (Thabane et al., 2010; Moore et al., 2011; NC3Rs, n.d.-b). Irrespective of the outcome of the pilot study, information relating to the pilot study should be included in publications of the main study, with a view to advancing knowledge about validated endpoints and to help inform future work (NC3Rs, n.d.-b).

2 ADMINISTRATION OF SUBSTANCES

Guideline 4

The properties of any substance and the dosing vehicle to be administered must be determined in relation to their likely impact on the animal.

When preparing a scientific protocol, protocol authors should collaborate with the veterinarian to fully consider the properties of the substance and the dosing vehicle, to ensure that the formulation is appropriate for the site, route, species, and purpose of the experiment (see Section 2.3, "Substance Formulation"). These properties and their potential impact on the animals should be considered by the animal care committee during the protocol review process.

In accordance with veterinary best practices, pharmaceutical-grade products should be used to ensure the safety and quality of administered formulations. Use of substances or dosing vehicles from non-pharmaceutical-grade suppliers must be scientifically justified to the animal care committee and approved before being used. Non-pharmaceutical-grade substances should be sterilized to minimize any negative welfare impacts on the animal. The concentration or dosing volume, nature of the formulation, or the potency of certain toxins (e.g., lipopolysaccharides (Luchi and Morrison, 2000)) may alter the expected effects and should be optimized to reduce any negative welfare impacts on the animal.

Pilot studies are important when new formulations are to be administered, for determining the balance between tolerated and effective dose levels when it is unknown for the specific strain, age, and sex of the animals to be used (see Section 1.5, "Pilot Studies"; Morton et al., 2001). A pilot study can also be helpful in determining appropriate volumes consistent with the study design.

2.1 PROPERTIES OF THE SUBSTANCE TO BE ADMINISTERED

It is important to understand as much as possible about the substance to be administered. This can be particularly important if pharmaceutical-grade products are not available, and the substance has been obtained from a non-pharmaceutical-grade supplier or prepared in-house. Information about the likely effects of the substance on the animals must be well researched, and if there are likely to be negative consequences for the animal, suitable steps to address these welfare impacts must be in place.

If the substance is known to be toxic or an irritant, the administration route chosen should be selected to minimize any unintended adverse welfare impacts; the dose should be minimized to the extent possible; the dose should be administered in a manner to minimize the impacts (e.g., splitting the dose over several sites); the frequency of dosing should be scheduled to minimize adverse impacts; and anesthetics, analgesics, and antibiotics should be used to minimize the severity of the welfare impacts.

When there is insufficient information about the substance to be administered, the literature pertaining to similar substances should be consulted, noting that even minor differences (e.g., isomeric forms) of the

substance to be administered can have a drastically different impact physiologically. If data is available from another species, it may be beneficial to use a dose equivalence equation to determine a starting point for the pilot studies (Sharma and McNeill, 2009; Nair and Jacob, 2016). This equation can be used directly from species to species using allometric scaling, which normalizes dose rate to body surface area.

For example, to determine the dosage for a dog based on that of a rat:

> dog dose (mg/kg) = rat dose (mg/kg) x (rat K_m/dog K_m).
> K_m values are calculated by dividing the animal reference body weight in kg by the body surface area in m^2 (Erhírhie et al., 2014).

2.2 VEHICLES FOR ADMINISTRATION

Guideline 5

The dosing vehicle selected should not be harmful to the animals.

A vehicle is a carrier or other medium used as a solvent or diluent in which the substance of interest can be administered. Vehicles can be food, liquids (solvents or solutions that act as emulsifiers), inert powders, and more. Vehicles should offer optimal dissolution and exposure to the substance of interest but should not influence the results obtained for the substance under investigation. Ideally, the vehicle chosen should be biologically inert, have no effect on the biophysical properties of the substance, and have no toxic effects on the animals at the quantities administered. If a component of the vehicle has biological effects, its total amount should be limited to reduce any negative welfare impacts. Many solvents are suitable for particular routes of administration only and must be evaluated to determine whether the solvent is appropriate for the intended route (Thackaberry et al., 2014; Gad et al., 2016).

In some cases, it may be possible to use commercially compounded food that includes the test substance in the animal's diet. When substances are to be dosed as solutions, sterile water or physiological saline are the commonly used solvents. For water-insoluble compounds, a suitable organic solvent should be used. Ideally, this solvent should lack pharmacological effects, be stable under conditions of use, and be non-toxic, non-irritating, and non-sensitizing.

Insoluble solids or immiscible chemicals may be dosed as suspensions or emulsions in suspending agents such as gum tragacanth, methyl cellulose, polysorbates, or polyethylene glycol conjugates (see Gad et al. (2016) for information about suitable vehicles for various species). Materials such as Tween™ and Cremophor™ can cause anaphylactic-like reactions when administered intravenously in some species (e.g., dogs (Qui et al., 2012)).

2.3 SUBSTANCE FORMULATION

A formulation includes the substance, vehicle, and additional carrier (if required). The formulation must remain fluid at the temperatures at which it will be used – except for oral administration or gel-based implants – and should have a boiling point that allows heat sterilization if required. For non-aqueous injectates, time should be allowed for absorption prior to re-dosing.

2.3.1 Pharmaceutical Formulation and Excipients

Pharmaceutical formulation is the process by which drugs or chemical substances are combined with materials called excipients to generate a usable dosage formulation. An excipient is a natural or synthetic substance that is combined with the active ingredient of a medication to generate the final active drug product. Nearly all drugs in human and veterinary medicine contain excipients (Narang and Boddu, 2015). The choice of the optimal excipient depends both on the final dosage form (e.g., oral tablets, parenteral solutions for injection) and the physicochemical properties of the chemical or active pharmaceutical ingredient. Excipients are classified according to their function and include materials such as bulking agents or fillers, diluents, preservatives, and vehicles or substances that enhance the solubility of the substance of interest. Excipients are often critical factors in drug function as they may act by transporting the substance of interest to a site where it is intended to exert its action, or by keeping the substance of interest from being released too early in the assimilation process, so that it does not damage tissues such as the gastric mucosa. Another critical function of excipients is to help solubilize the substance of interest into particles small enough to reach the circulation. Excipients may also protect a product's stability so it will be at maximum effectiveness at the time of use (IPEC, n.d.).

The formulation of a substance of interest that is both stable and acceptable to use in animal studies should involve the characterization of a substance's physical, chemical, and mechanical properties in order to choose the optimal excipient to be used in the preparation. Strategies and practical considerations in preclinical drug formulation, including commonly used excipients, are available (e.g., Shah et al., 2014).

2.3.2 Physicochemical Properties

There are constraints on the formulations that can be used for any particular route or species. The final formulation may have physicochemical properties that have the potential to adversely affect the animal recipient, such as osmolarity or osmolality, solubility, viscosity, pH, biocompatibility, purity, stability, and microbial contamination. The optimal ranges of these properties for formulations, according to the route of administration, are available (see Turner et al., 2011b). For ethical and scientific reasons, physicochemical compatibility studies (in vitro) and pilot studies (see Section 1.5, "Pilot Studies") should be carried out for new formulations.

Formulations should be used as soon after preparation as possible, or within the period defined as satisfactory for maintenance of the stability and quality of the formulation, to ensure accurate dosage (e.g., streptozotocin degrades rapidly in solution).

2.3.2.1 Bioavailability and Half-Life

The bioavailability and half-life of substances may be impacted by the formulation of the material (e.g., a progesterone derivative given to rats intramuscularly in an oil-based solution was 100% bioavailable, whereas an oral dose had very limited bioavailability, either due to lack of absorption from the gastrointestinal tract or loss to first-pass metabolism (Shaik et al., 2016)). If the bioavailability and half-life are unknown (e.g., for a novel drug), refinements such as an escalating dose or staggered dosing should be used to minimize adverse effects, and a pilot study may be required.

2.4 TEMPERATURE

Substances or formulations should be given at or near the body temperature of the animal, as this generally results in fewer adverse welfare impacts. Administration of substances or formulations below body temperature intraperitoneally or intravenously may induce hypothermia, and lead to negative welfare impacts for the animal. In addition, the local absorption rate can be influenced by the temperature of substances administered (Turner et al., 2011a). It is important to check for any exceptions for specific substances (e.g., the pain caused by an anti-emetic injection is reduced if the anti-emetic is refrigerated (Narishetty et al., 2009)), noting any species-specific considerations (e.g., the body temperature of ectotherms is reflective of their environment). For several compounds used in fish anesthesia (tricaine methanesulfonate (TMS), also known as MS-222, quinaldine sulfate, etc.), both induction and recovery times vary with the weight of the animal and water conditions such as temperature; however, these relationships may be species-specific (Schoettger and Julin, 1969; Houston and Corlett, 1976; Zahl et al., 2009; Zahl et al., 2011).

2.5 EFFECT OF FEEDING AND FASTING

The timing of dosing related to the species' circadian cycle may affect the absorption and the toxicity of the substance being administered (Orr and Benet, 1975). For example, laboratory mice often eat almost exclusively at night, consuming little during daylight hours if left undisturbed (Goulding et al., 2008). The presence of food may have an impact on the absorption and bioavailability of administered substances, particularly as it affects the gastric pH (Rouge et al., 1996; Lee et al., 2008). Tolerance to any fasting or water deprivation required for absorption of the substance must be evaluated for the species and the age of the animal to be used, as withholding food or water is not always appropriate (Kararli, 1995; Boillat et al., 2010; Padmanabhan et al., 2013). Evaluation should include consultation with veterinary personnel and a review of the published literature.

2.6 ADMINISTRATION QUANTITIES

Guideline 6

Dosing volumes should be minimized, consistent with the animal species, substance formulation, and route of administration.

The quantity of a formulation that can be safely administered varies with species (see Appendix 2, "Administration Volumes by Animal Species", and CCAC types of animal guidelines documents), strain, route, frequency of administration, speed of administration, and composition of the formulation. Dosing volumes should be the minimum that is compatible with the formulation and accuracy of administration (see Appendix 2, "Administration Volumes by Animal Species").

Particularly when high volumes are used, formulations, repeat dosing, half-lives, and physiological responses should be evaluated for potential welfare impacts (Diehl et al., 2001) by the inclusion of robust monitoring protocols and humane intervention points. Results may be confounded by the impacts of high volumes, which may include the potential for:

- increased side effects from the vehicle

- pain, tissue damage, necrosis, and changes in absorption, as well as leakage from the injection site if large volumes are administered via subcutaneous, intramuscular, and intradermal sites
- pain and respiratory distress from excessive pressure on the diaphragm from the administration of large dose volumes via the oral or intraperitoneal route; this may also influence the absorption of substances
- respiratory distress secondary to pulmonary edema from the administration of large volumes intravenously

These potential impacts of high dosing volumes emphasize the importance of starting with low dosing volumes and progressing to higher dosing volumes (i.e., to titrate) whenever possible, especially when the effects of the substance or formulation are unknown. A pilot study using a counterbalanced or Latin-square design can also be helpful in determining appropriate volumes when consistent with the study design. Further refinements should be sought where larger volumes are used.

2.7 ADMINISTRATION ROUTES

The route chosen should be suitable for the substance to be administered, including, where appropriate, consideration of the intended means of delivery to the animal (e.g., oral dose or injection). Any potential scientific problems should be identified (e.g., first-pass metabolism in the liver after oral or intraperitoneal dosing, degree or rate of absorption, local effects) so that the most appropriate route of administration can be selected.

Guideline 7

The route of administration should be determined by the purpose of the experiment, the species of the animal, the possible welfare impacts of the dosing technique on the animal, the properties of the substance, the expected dosing frequency, and the potential welfare impact of the formulation properties on the animal.

Factors to take into consideration when considering an administration route include:

- whether the substance is a solution, a suspension, an emulsion, or in semi-solid or solid form
- whether the substance is being administered for a local or systemic effect
- whether the administration route is enteral (through the digestive tract) or parenteral (outside the digestive tract)
- regulatory requirements (e.g., for pre-clinical safety testing, the route of delivery to animals should closely resemble the projected route of administration to humans)
- the level of negative welfare impacts for the animal – some routes and techniques are more impactful than others; the least impactful route consistent with the scientific goals should be selected
- the technical difficulty of certain routes for the individual carrying out the administration – personnel must be competent to perform the technique; otherwise, poor technique can lead to negative welfare impacts and complications, including loss of the animal (e.g., selecting subcutaneous delivery instead of intravenous)
- the possible effect of the substance on the selected route – e.g., some substances can be irritating if administered intraperitoneally or subcutaneously (Morton et al., 2001)
- the rate of metabolism of the substance to be administered via different routes

2.7.1 Sedation and Anesthesia

The administration of substances via some routes should be carried out under sedation or anesthesia. The pharmacokinetics and pharmacodynamics of the analgesic or anesthetic, including the possible interaction between the anesthesia and the substance being administered, should be evaluated (see the *CCAC guidelines: Scientific procedures (Part B – Analgesia, anesthesia, and surgery)* (2025)).

2.7.2 Enteral Route

Administering substances through the enteral route allows for the delivery of large amounts of non-sterile substances or solutions into the gastrointestinal tract, where the pH can be as low as 3 (Hirota and Shimizu, 2012). While substances do not need to be sterile, they must not be contaminated with pathogens, toxins, or other substances that may be harmful to the animals and that are not part of the study.

It is important to determine whether the substance of interest should be administered to an animal in a fed or fasted state. Absorption of the formulation and negative welfare impacts are important considerations when dosing via the enteral route. Some substances can have negative welfare impacts such as nausea or vomiting and gastric mucosal irritation that may be ameliorated by dosing orally after a meal. Some substances require food in the stomach for proper absorption. Conversely, substance absorption may be affected by stomach contents or stomach acid and should be dosed on an empty stomach. Knowledge of the biochemical properties and optimal absorption properties are, therefore, important considerations when designing studies.

If the presence of ingesta is likely to affect the absorption of the substance (Kararli, 1995) or fill the stomach, limiting accommodation of the dose, the animal's food intake may need to be restricted before dosing. The duration of fasting needed to achieve the required stomach emptying is highly species-dependent (see relevant CCAC types of animal guidelines documents). It includes consideration of the:

- feeding pattern of the species (Bachmanov et al., 2002; Longo and Panda, 2016)
- starting time for food restriction (Longo and Panda, 2016)
- digestive tract transit time (Padmanabhan et al., 2013)
- physiology of the species and its current physiological state (e.g., Vermeulen et al., 1997; Abbott et al., 2006)
- length of time of dosing (Turner et al., 2011a)
- diet (Turner et al., 2011a)
- light cycle (Turner et al., 2011a)

2.7.2.1 Oral

Oral dosing is a commonly used mode of administration of substances. Limitations of oral dosing include:

- slower onset of systemic action compared to parenteral delivery (sometimes this is desirable)
- potentially significant first-pass effect by the liver for substances metabolized through this route with reduced (or sometimes increased) efficacy
- lack of systemic absorption of a substance from the digestive tract due to chemical polarity or interference with absorption by ingesta

- poor compliance with voluntary consumption because of poor palatability, local irritation, taste aversion, or nausea
- degradation of substances by digestive enzymes and acid
- lack of suitability for animals that are unconscious or have clinically significant diarrhea or vomiting

2.7.2.1.1 Voluntary Consumption

Some animals can be trained to cooperate and voluntarily consume a substance of interest, depending on the formulation being administered. If possible, this can minimize negative welfare impacts for the animals. However, voluntary consumption may not be reliable in all animals or dose groups, or for long-term studies, because of individual preferences for flavours, palatability issues, and changes in behaviour over time. It is possible to introduce new substances to animals by mixing the substance with more palatable vehicles, such as chocolate syrup for swine, strawberry milkshake for rats, or canned dog food meatballs for dogs, then slowly diminishing the amount of the more palatable vehicle if necessary (Swindle and Smith, 2015).

In general, for voluntary consumption, animals should be separated to monitor food or water consumption. Separation may cause negative welfare impacts for socially housed species and thus may have an impact on the study results. Automated systems for regulating and monitoring individual intake of medicated foods or fluids may make separation unnecessary (e.g., mice: Santoso et al., 2006; rodents: Ali and Kravitz, 2018; cattle: Chapinal et al., 2007; pigs: Pomar et al., 2009).

2.7.2.1.2 Gavage

Gavage (esophageal or gastric) is often used to ensure precise and accurate dosing of animals. Substances can be administered through an orogastric or nasogastric route.

Administration of large volumes of substances or formulations by gavage is strongly discouraged as it may cause negative welfare impacts to the animals due to gastric distension, particularly for species that are unable to vomit, such as rodents and horses; it may also result in absorption changes associated with rapid shunting of the substance of interest or formulation to the duodenum. The administration of large volumes of a formulation can also result in reflux and aspiration into the lungs. Hence, the smallest volume possible should be used for the oral route of administration (see Appendix 2, Administration Volumes by Animal Species). When large volumes have to be administered by gavage, a slower delivery rate may be better tolerated by animals. The administration of large volumes of oil-based formulations by gavage is associated with greater toxicity than the administration of large volumes of aqueous-based formulations (Brown et al., 2000).

Selection of the appropriate size and type of needle (or tubing) for orogastric or nasogastric gavage is important to minimize discomfort while optimizing the delivery of substances (e.g., WSU IACUC, 2021). Damage to the esophagus is of particular concern for rodents if gavage is carried out by individuals who are not competent to perform the procedure, or when the size (length and gauge) of the gavage needle is not appropriate (Rao et al., 2001; Arantes-Rodrigues et al., 2012). Either flexible plastic or rigid stainless-steel tubes may be used for dosing rodents. There is a greater risk of esophageal tears with stainless-steel tubes (Jones et al., 2016). Although flexible plastic tubes are less likely to cause tissue damage or puncture the esophagus, if they are not well-placed in the mouth they may be chewed by the animal (Morton et al., 2001; Turner et al., 2011b). Larger animals may require the use of a mouth gag (Turner et al., 2011b). The gavage tubing should be measured externally (from mouth to end of ribs for intragastric administration), and a mark made on the outside of the tube to minimize placement errors.

2.7.3 Parenteral Routes

Most parenteral administration methods, except for the intraperitoneal route, result in the highest bioavailability of substances because these methods avoid the first-pass effect of hepatic metabolism. Parenteral routes also circumvent some of the unpredictability associated with enteral absorptive processes.

Factors of the formulation to be administered that should be evaluated for each route include the volume, stability of the formulation before and after administration, pH, viscosity, osmolarity or osmolality, buffering capacity, sterility, and biocompatibility. This is particularly important for studies involving multiple doses.

Materials to be injected should be sterile. Sterilization may be accomplished by heat, chemical methods, or filtration through a 0.2 μm filter, as applicable. Injection of non-sterile substances may result in infection and abscess formation, or even death.

Refinements for parenteral administration include the use of the least painful route, rotation of injection sites, use of small-diameter needles, and use of topical analgesics.

The smallest needle size should be used (see Appendix 3, "Needle and Cannula Gauges by Animal Species"), considering the:

- species of the animal
- age of the animal
- volume of the injection solution
- viscosity of the injection solution
- speed of injection
- frequency of injection

The needle used to withdraw a solution through a bottle with a rubber stopper can become dull even after a single withdrawal (Whitfield and Robinson, 2017); hence, a new sterile needle should be used for each injection. The rubber stopper must be disinfected before inserting the needle so as not to infect the solution. Care should also be taken to minimize the dead space in the syringe (Wong, 1982).

Formulations can be delivered via injection, infusion through an implanted catheter, or mini-pumps. Mini-pumps may be a suitable refinement because they avoid negative welfare impacts due to daily handling and repeated punctures; however, they require surgical intervention, the formulation must be dissolved, and the volume must be compatible with the size of the pump (Hrapkiewicz et al., 2013). For intravenous or intra-arterial injections, the site of injection or infusion should be monitored for signs of perivascular irritation and treated accordingly.

2.7.3.1 Subcutaneous

The subcutaneous route is frequently used as a rapid and simple method of parenteral substance administration for non-irritating substances. In addition, the subcutaneous space is an excellent site for large-volume fluid delivery in small or dehydrated animals. The rate and extent of absorption depend on the formulation. In general, substances administered subcutaneously are absorbed at a slower rate compared with other parenteral routes, providing a sustained effect (Kim et al., 2017). Substances or formulations delivered subcutaneously can be aqueous, lipid depots for slow absorption, or solid pellets. The skin overlying the site selected

for injection must be loose to minimize discomfort, and the needle should be inserted at a shallow angle to minimize damage to underlying tissues.

Substances can also be delivered by osmotic mini-pumps or other suitably sized implantable pumps, which are then surgically inserted into a subcutaneous pocket for precise and continuous sustained release.

If the substances to be injected have irritating qualities, the dose should be minimized to the extent possible, and administered in a manner to minimize the impacts (e.g., choosing another route, or splitting the dose over several sites); anesthetics, analgesics, and antibiotics should be used to minimize the severity of the welfare impacts.

2.7.3.2 Intraperitoneal

The intraperitoneal route is commonly used in laboratory rodents and other small species for which intravenous access is challenging. In general, intraperitoneal administration is not a preferred route for large animals, given that safer alternatives such as intravenous and subcutaneous routes of administration are available. Intraperitoneal injections may be performed in cattle and pigs, provided that an aseptic technique is used (Constable et al., 2017). It should be avoided in other species where safer routes exist. The intraperitoneal route can be used to administer relatively large volumes of fluid safely, or as a repository site for surgical implantation of a preloaded osmotic mini-pump. Despite some studies indicating daily injections via the intraperitoneal route can be carried out safely (Davis et al., 2014; Al Shoyaib et al., 2019), the intraperitoneal route should be avoided for studies involving multiple doses because of potential complications (e.g., injection into the intestinal tract (Zatroch et al., 2016); puncture of the urinary bladder (Ballard, 2009); formation of adhesions with multiple injections in late-term gestating animals (Ballard, 2009); neuroinflammation from injection of some vehicles (Freyssin et al., 2021); or the transfer of a small amount of injectate directly across the diaphragm (Morton et al., 2001)). In cases where multiple doses need to be administered, the animal care committee must approve a suitable limit to the number of permitted injections within a defined time frame. If osmotic mini-pumps are used, they should be appropriately sized for the species and should be capable of delivering substances at the appropriate rate.

Absorption of substances delivered intraperitoneally is much slower than for intravenous injection. In addition, the local absorption rate can be influenced by the temperature of substances administered intraperitoneally (Turner et al., 2011a). The pharmacokinetics of substances administered intraperitoneally are similar to that of oral administration because the primary route of absorption is into the mesenteric vessels, which drain into the portal vein and pass through the liver. Therefore, substances administered intraperitoneally may undergo hepatic metabolism before reaching the systemic circulation (Al Shoyaib et al., 2019).

Substances or formulations injected intraperitoneally must be sterile, isotonic, and non-irritating. Irritating substances injected intraperitoneally may induce painful ileus as well as peritonitis with subsequent adhesions. Substances may be irritating if they fall outside of the optimal physicochemical properties (see Section 2.3.2, "Physicochemical Properties", and Turner et al., 2011b).

Intraperitoneal administration may be conducted in conscious animals using low-welfare-impact handling techniques appropriate for the species. It is important to be aware of the internal anatomy of the species used, to avoid penetration of internal organs (e.g., Coria-Avila et al., 2007). Drawing back on the plunger may be useful in some instances (e.g., to ensure that substances or formulations are not being injected into the cecum or bladder (Laferriere and Pang, 2020)). If fecal material, blood, or urine is pulled into the needle

hub, the needle should be removed, and the entire syringe replaced before completing the injection; the animals must be carefully monitored if any of these complications occur.

2.7.3.3 Intravenous

The intravenous route is the most efficient means of delivering substances to animals because substances are delivered directly into the bloodstream and hence are 100% bioavailable. However, this is a difficult technique to perform on small mammals such as mice and rats, and those performing the technique must have received training and be competent. Substances can be administered as a bolus, or an infusion. The administration may be acute (i.e., once only) or chronic (i.e., repeated administration of a bolus, or a continuous infusion). The technique chosen should minimize the possibility of extravascular injection of material.

The maximum number of attempts to insert the needle into the blood vessel must be approved by the animal care committee prior to conduct of the procedure or when training, according to institutional SOPs. If more than one injection is required, sites should be rotated. The animals must be carefully monitored for any signs of damaged tissue or necrosis.

The maximum rate and total volume of intravenous substance administration must be set to prevent fluid overload (hypervolemia). Iatrogenic fluid overload can result in serious complications such as pulmonary edema (Boysen and Gommeren, 2021). Administration volumes that should be respected and maximum limits are given in Appendix 2, Administration Volumes by Animal Species, and CCAC types of animal guidelines documents).

2.7.3.3.1 Infusion

Infusion involves the aseptic preparation of the skin for percutaneous venous injection (except in species where it may be harmful; e.g., for aquatic species, the mucous layer should be retained).

Minimal effective restraint of animals with the least negative welfare impact on the animals is a key factor in any intravenous infusion. The total duration of an infusion is also an important factor. Depending on the species, the length of the procedure, and the potential welfare impact of the substance administered, the animal may need to be anesthetized.

Technique refinements include using the smallest needle or catheter size possible to minimize injection trauma (see Appendix 3, "Needle and Cannula Gauges by Animal Species"). Butterfly needles may be used for single injections to minimize perivascular trauma. Atraumatic indwelling catheters and vascular access ports should be used to improve animal comfort and locomotor freedom. The coating on the catheters should not interact with the substance to be administered. Topical anesthetic creams and ointments can be used prior to needle placement to minimize the negative welfare impacts of injection, and external pump packs contained in "jackets" can be used to minimize the restriction of animal movement associated with tethering (Nolan and Klein, 2002). Animals should be acclimated to devices such as jackets, e-collars, or tethering devices before their use.

There can be large differences in tolerated volume by intravenous infusion, depending on the vehicle used (Diehl et al., 2001).

2.7.3.3.1.1 Bolus Infusion

In a bolus infusion, the substance or formulation is typically administered in less than a minute. Substances given by bolus infusion often have a rapid onset of action; hence, any substances with unknown effects should be administered at the lowest dose first, and at the slowest rate possible. The volume of infusion is an important consideration. Reducing the volume of an infusion by concentrating it should be carefully evaluated as more concentrated infusions may cause a rapid onset of any negative effects. In addition, precipitation of substances that are poorly soluble may occur. By contrast, fluid overload can occur if the volume of the bolus is too high. For large volumes, the injectate should be warmed to body temperature. If the rate or volume of intravenous fluid administration is too rapid, fluid overload (hypervolemia) may result and may lead to the acute development of pulmonary edema. For information on administration volumes and maximum limits see Appendix 2, "Administration Volumes by Animal Species", and CCAC types of animal guidelines documents.

2.7.3.3.1.2 Slow Intravenous Infusion

A slow intravenous administration rate is sometimes indicated because of the expected clinical application of the compound or because of limiting factors such as solubility or irritancy. For slow intravenous infusion (typically lasting longer than one minute), a butterfly needle may be used, or an intravenous catheter may be taped in place (short term) or surgically placed before use (longer term or for multiple injections). For information on administration volumes and maximum limits see Appendix 2, "Administration Volumes by Animal Species", and CCAC types of animal guidelines documents.

2.7.3.3.1.3 Continuous Infusion

The volume and rate of administration for continuous infusion depend on the substance or formulation being administered (Li and Zhao, 2007) and the total amount of fluid being delivered, particularly when infusions are prolonged. Based on published guidance, 5 mL/kg/hr should be the maximum starting point (AAHA/AAFP, 2013; Morton et al., 2001; Crabtree and Epstein, 2021).

Depending on the types of substances to be administered and the duration of administration, various types of infusion pumps can be used for accurate chronic intravenous delivery (Turner et al., 2011b). As the insertion of infusion pumps requires surgery, animal care committees should require the use of the system best suited to the animal and the protocol, to minimize the overall animal welfare impact. In addition, there are various types of tether systems that allow animals to move around while receiving an infusion (Nolan and Klein, 2002).

2.7.3.4 Intramuscular

Intramuscular administration is a common parenteral route in large animals but is often avoided in smaller species because of the reduced muscle mass. Smaller volumes than for subcutaneous delivery are administered intramuscularly due to the lack of intramuscular space (see Appendix 2, "Administration Volumes by Animal Species", and CCAC types of animal guidelines documents). The procedure may result in negative welfare impacts for the animals because muscle fibres are under tension from the injected material. Possible refinements include adding a local anesthetic to the injectate (Zeydi and Khezri, 2012; Salari et al., 2018). Generally, intramuscular injections result in uniform and rapid absorption of substances because of the rich

vascular supply. However, absorption from intramuscular sites can be slow, and is different for aqueous and oily formulations (oily formulations are likely to remain as a depot for up to 24 hours).

Intramuscular administration sites should be chosen carefully to minimize the possibility of nerve damage. For livestock destined to be slaughtered for food, administration sites that will not interfere with meat quality should be used. Sites should be rotated for multiple-dose studies. With multiple-dose studies, the potential for inflammation and its sequelae requires that the animals are monitored carefully.

2.7.3.5 Intradermal

Intradermal administration of substances may be used for the assessment of immune, inflammatory, or sensitization responses. It may also be used for the inoculation of infectious organisms to mimic natural delivery. For these purposes, the material may be formulated with an adjuvant. Only small volumes must be used, dependent upon the thickness of the skin (see Appendix 2, "Administration Volumes by Animal Species", and CCAC types of animal guidelines documents), as this route involves a greater negative welfare impact for the animal than subcutaneous delivery.

Delivery of non-experimental treatment (e.g., local anesthetics) is often more effective if delivered via the intradermal route.

Clipping fur or hair allows for visualization of proper needle insertion. The smallest needle size appropriate for the animal type must be used. Needles with an intradermal bevel facilitate accuracy of the injections.

The type of substances injected intradermally often cause negative welfare impacts for the animals; hence, the use of sedation or analgesia is an important refinement. Clear humane intervention points should also be in place (CCAC, 2022).

2.7.3.6 Intranasal

The intranasal route is an effective, minimally invasive technique often used to deliver substances to the upper and lower respiratory tract and to the brain (Erdő et al., 2018; Le Net et al., 2019). For rodents, intranasal administration generally requires the use of restraint, and sedation may not be required. The volume of instillation and the use of light anesthesia can influence the distribution of the substance to the respiratory tract (Southam et al., 2002). Volumes administered intranasally should be small (compared with those of other routes) to minimize the potential for suffocation and death. One alternative method for intranasal administration without sedation is to place drops of liquid on the nose of obligate nasal breathing species such as mice (George et al., 2015). However, exact dosing is unlikely using this method.

2.7.3.7 Inhalational

Inhalational delivery of substances typically uses vapours (e.g., volatile anesthetic gases) or aerosols of powder or nebulized particles in solution. These compounds may be delivered in inhalation chambers, in which case the animals can be conscious and unrestrained. Otherwise, animals should be restrained (conscious or sedated), and a specialized nose mask used to optimize delivery to the animals. Animals should be habituated to the inhalation chamber or restraint devices and nose masks before the start of the study.

2.7.3.8 Intratracheal

Intratracheal instillation involves injecting small volumes of solutions directly into the trachea of animals and results in rapid but localized and uneven distribution of material over a relatively small volume of the lung (Driscoll et al., 2000). In general, animals should be sedated or anesthetized for intratracheal routes of delivery. Only non-irritating substances should be administered via the tracheal route to minimize pharyngeal edema, bronchial spasm, anaphylaxis, and chronic pulmonary fibrosis.

2.7.3.9 Other Parenteral Routes

The use of any of the following routes must have sound scientific justification and appropriate training to ensure competency before performing the procedures. For the following routes, the injectate must be sterile.

2.7.3.9.1 Intraosseous

The intraosseous route is not frequently used but can be helpful for the instillation of crystalloid fluids in emergency avian and rabbit treatment and as an alternative for the intravenous route in hypovolemic animals with inaccessible or collapsed veins. Cancer cells and stem cells are sometimes injected intraosseously (Cutrera et al., 2013).

2.7.3.9.2 Intra-Articular

The intra-articular injection route is used when it is necessary to access the joints; for example, to establish models of osteoarthritis (Adães et al., 2014). The injection volume should not exceed the normal volume of synovial fluid in the joints.

2.7.3.9.3 Epidural and Intrathecal

Epidural or intrathecal administration can be used when the rapid effects of substances on cerebrospinal tissues or meninges are required. These routes avoid problems related to absorption across the blood-brain barrier. Some species require heavy sedation or general anesthesia with a local anesthetic block over the spinal needle insertion site. In livestock species, the epidural is frequently used for standing procedures; therefore, sedation or a general anesthetic is generally not required. Goats are an exception: sedation should be given due to their low pain threshold (Hendrickson, 2010).

The maximum volume that can be administered is species-dependent due to the space available at the injection site. Extreme caution should be taken when calculating whole volume and rate of infusion. Drawing back the plunger prior to administration at this site helps to ensure that the substance is in the correct location. The expression of cerebrospinal fluid after spinal needle insertion will confirm intrathecal needle placement; however, if cerebrospinal fluid is encountered during an epidural procedure, the needle should be removed and a new needle used to reposition, as the kinetics of substance absorption from the epidural versus intrathecal space is quite different (Turner et al., 2011a).

2.7.3.9.4 Intracranial

Intracranial administration of substances typically requires stereotaxic equipment to ensure accurate placement of a transcranial port and instillation of the substance of interest, or placement of a cannula for re-

peated administrations (Mathon et al., 2015; De Vloo and Nuttin, 2019). Stereotaxic equipment may not be required for intracranial administration in neonatal rodents (Kim et al., 2014; Shimizu, 2004). Extreme care should be taken when calculating the volume to be administered. Generally, the injection volume should not exceed 2% of the brain volume (Morton et al., 2001); for example, injections for most brain regions in rodents should be less than 3 μL per site (Dülsner et al., 2017a). A slow rate of infusion should be used (Pardridge, 2016), as pressure in the brain can build up quickly (Leech and Miller, 1974; Belov et al., 2021). Adverse reactions caused by increased cerebrospinal pressure can be avoided by careful monitoring to ensure that cerebrospinal pressure does not increase (Morton et al., 2001; Belov, 2021). Personnel performing intracranial injections should use the smallest gauge guide cannula and injection needles appropriate to their stereotaxic target and species. Although cannula implantation must be done under anesthesia, intracranial injections are frequently performed in conscious animals that have been previously surgically prepared. Animals must be fully habituated to intracranial injection procedures.

2.7.3.9.5 Retro-Orbital

Retro-orbital injections should only be carried out where there are no viable alternatives. Retro-orbital injections may be used when other routes are technically more challenging (e.g., intravenous injection in rodent neonates (Steel et al., 2008; Yardeni et al., 2011; Wang et al., 2015)), or for very specific purposes, where other routes are inappropriate (e.g., injection of cancer cells and injection of contrast media for cardiac imaging (Socher et al., 2014)).

2.7.4 Topical

Some substances can be administered directly to the skin surface (cutaneous administration) for a topical effect. If the animal has a hair coat or fur, this should be clipped or removed to facilitate access to the skin and to allow visualization of any side effects. The substance should be applied to an area of skin that cannot be groomed easily by the animal (or group mates). The normal behaviour of the animal should be considered (e.g., pigs may roll over and rub against the side walls of pens, leading to variability in the administration of fentanyl by transdermal patches). There is the potential for significant negative welfare impacts associated with skin inflammation as a result of the topical application of substances, including the possibility of lesions or ulceration. As a result, clear humane intervention points should be established for this route, particularly if the likely effects of the substance are unknown.

The extent of absorption into the systemic circulation depends on:

- the surface area over which the substance is applied
- the concentration of the substance administered
- the lipid solubility of the material or vehicle
- whether the skin surface is intact
- the skin thickness and characteristics at the site of application
- the length of time that the material is in contact with the skin surface

3 BIOLOGICAL SAMPLING

Guideline 8

The species and sampling location must be carefully selected to minimize negative animal welfare impacts.

When preparing an animal use protocol for biological sampling, the protocol author should collaborate with the veterinarian to minimize negative welfare impacts for the animals. For any biological sampling, it is important to understand the relevant species-specific biological attributes, to ensure the proposed sampling location is appropriate for the purpose of the experiment, and that those carrying out the procedure are trained and competent. The potential impact of these factors on the selected animals must be reviewed by the animal care committee during the protocol review process.

Refinements should be implemented for sampling techniques that are likely to result in negative welfare impacts for the animals. These refinements may include the use of anesthesia; provision of analgesia; or choice of techniques that will minimize stress, tissue damage, and inflammation (see the *CCAC guidelines: Scientific procedures (Part B – Analgesia, anesthesia, and surgery)* (2025)). For example, a local anesthetic or indwelling catheter should be used when carrying out repeated venipunctures.

Biological sampling via some routes should be carried out under sedation or anesthesia. The impact of any anesthetic or analgesic on the animal and the possible interaction between the anesthetic or analgesic and the samples should be determined. The negative welfare impacts for animals when they are not sedated or anesthetized and the subsequent impact on the quality of samples should also be determined (see the *CCAC guidelines: Scientific procedures (Part B – Analgesia, anesthesia, and surgery)* (2025)); Peterson et al., 2017).

Social housing is particularly important for animals undergoing invasive sampling procedures. For social species, positive interactions with social partners can help minimize associated negative animal welfare impacts and promote rapid healing of tissue damage (Gouin and Kiecolt-Glaser, 2011), including the healing of injuries resulting from sampling procedures such as punch biopsies (Detillion et al., 2004). Whenever it is possible to group-house animals following procedures, they should be properly introduced (or re-introduced) into the enclosure to avoid social stressors that may impair the healing process.

Protocol authors, veterinarians, and animal care committee members should be aware of any emerging sampling techniques that might reduce the welfare impact of the procedures on the animals.

Pilot studies should be used when performing new procedures, to ensure that the procedure can be performed effectively before the full study commences. Pilot studies are also useful in validating refinements to procedures that may result in better welfare outcomes for the animals (e.g., the use of analgesia for venipuncture).

Humane intervention points must be in place prior to the start of the study to ensure that the impact of sampling does not exceed the agreed-upon welfare impact on the animals and that those involved are clear about the steps to be taken when the scientific endpoints are reached or when there is an unanticipated event (e.g., severe blood loss).

3.1 BLOOD SAMPLING

Protocol authors should always consult up-to-date information when developing protocols, including blood sampling, to ensure that the least invasive routes are used and that they are compatible with the study objectives (Tsai et al., 2015). In some cases, microsampling may be a viable option due to the improvements in bioanalytical sensitivities (Caron, 2015; Harstad et al., 2016). For other purposes, it may only be possible to collect samples as a terminal procedure. There are a number of publications and online resources that provide guidelines for blood removal from animals (Morton et al., 1993; Parasuraman et al., 2010; Herling, 2016; NC3Rs, n.d.-a).

3.1.1 Blood Sampling Volumes (Survival)

Guideline 9

Blood volumes collected must stay within the range for the individual animal.

The quantity of blood permitted to be collected in a sample is influenced by the frequency of sampling, species, age, and other characteristics of the individual animal. Techniques should be refined to reduce the blood sample volume, including the use of microsampling where possible, as this minimizes negative welfare impacts for the animal (e.g., Trudeau et al., 2007). For more information, the NC3Rs blood sampling site hosts a curated bibliography of microsampling references (NC3Rs, n.d.-d).

In general, a single withdrawal of up to 10% of an animal's total blood volume will not impact the animal's welfare (Herling, 2016). This applies to all species that need to be able to resume normal behaviour immediately after the sampling (Diehl et al., 2001). Removing volumes greater than 10% in a single sample has the potential to cause hypovolemic shock (Diehl et al., 2001). Multiple small samples over a 24-hour period are less likely to result in acute shock if the total blood volume collected is less than or equal to 15% of the animal's total blood volume (see Tables 1-3 below for limits; NC3Rs, n.d.-a for a comprehensive table of total blood volumes and safe blood sample volumes for laboratory animals, domestic species, and nonhuman primates; and CCAC types of animal guidelines documents for individual species-specific information).

As a general rule, when blood collections will take place over prolonged periods of time, a maximum of 1% of the animal's body weight in grams should be utilized as the weekly limit (where 1 g = 1 mL).

For prolonged periods of blood collection, the amount that may be collected in each sample can be determined using the following calculation:

> Blood volume allowed for collection at established frequency (blood sample size) = body weight of animal x average blood volume (mL/kg body weight; see Appendix 4) x % blood volume collected (limiting volume based on frequency of blood collection; see Tables 1-3).

For example:

Acute (single sample)

- 25 g mouse x 0.0585 mL/g = 1.5 mL total blood volume x 10% = 0.15 mL
- 5.0 kg Cynomolgus monkey x 65 mL/kg = 325 mL total blood volume x 7.5% = 24.4 mL

Chronic (multiple samples taken once daily over a two-week period)

- 10 kg dog (beagle) x 80 mL/kg = 800 mL total blood volume x 15% = 120 mL (e.g., 8.5 mL/day for 14 days)

Table 1 Limiting Volumes and Recovery Periods (Single Sample)

SINGLE SAMPLE % CIRCULATORY BLOOD VOLUME REMOVED	APPROXIMATE RECOVERY PERIOD[1]
1%	1 day
7.5%	1 week
10%	2-4 weeks
15% (maximum)	4 weeks

Table 2 Limiting Volumes and Recovery Periods (Multiple Samples)

MULTIPLE SAMPLES % CIRCULATORY BLOOD VOLUME REMOVED IN A 24-HOUR PERIOD	APPROXIMATE RECOVERY PERIOD[1]
7.5%	1 week
10-15%	2-4 weeks
20% (maximum)	4 weeks

Table 3 Limiting Volumes and Frequency of Sampling

MULTIPLE SAMPLES % CIRCULATORY BLOOD VOLUME REMOVED OVER AN EXTENDED PERIOD OF TIME	FREQUENCY OF SAMPLING
1% (Total volume to be removed)	Once per day
15% (Total volume to be removed)	Weekly over 28 days

Whenever chronic blood sampling is carried out over extended periods of time, investigators should consult a veterinarian to determine if the blood parameters should be monitored (e.g., through a hematocrit and hemoglobin test) to assess whether blood collection can continue or whether animals are experiencing anemia.

Animal welfare must be the prime consideration when the blood samples approach maximal limits. Additionally, an animal's physiological response to the withdrawal of volumes close to maximal limits may affect data interpretation and validity (see Diehl et al., 2001). If the animal's welfare is likely to be compromised by the volume or frequency of the sampling, either more animals should be used to permit a reduc-

[1] Time to return to normal hematological parameters

tion in the number of samples per animal, or compensatory volume replacement such as administration of warmed saline or a blood transfusion, should be performed.

3.1.2 Blood Sampling Methodology (Survival)

The smallest gauge needle should be used, as practical, and the impact of the required collection frequency should be minimized (e.g., by using intravenous catheters).

Humane intervention points should be used to determine the maximum number of blood samples that may be obtained, including the maximum number of needle punctures that can be carried out in a defined period (e.g., the use of an indwelling catheter when the number of individual needle punctures is in excess of 10 in a 24-hour period).

The maximum number of attempts to collect a single sample must be approved by the animal care committee prior to the conduct of the procedure or when training, according to institutional SOPs (see CCAC, 2006, 2022). In general, when it is not possible to use catheters, a maximum number of three venipunctures should be attempted per time point (see CCAC, 2022 and LASA, n.d.). If the blood vessel is not penetrated, the needle should be removed before reattempting to access the vein (i.e., it should not be redirected, to avoid damaging the vessel and surrounding tissues). Ideally, a fresh needle should be used. The animals must be carefully monitored for any signs of damaged tissue or necrosis, particularly when it has been challenging to obtain a sample.

Blood parameters may be affected by the methodology used (Hoggatt et al., 2016).

While it is preferable to habituate animals to any procedures, the use of a topical anesthetic may help minimize negative animal welfare impacts associated with acute sampling procedures. However, refinements such as sedation or light anesthesia should be used if animals have not been habituated or cannot be habituated to handling (see Section 1.3.2.2, "Handling the Animal"). Analgesics should also be used to minimize the negative animal welfare impacts of repeated sampling.

3.1.2.1 Catheters

For multiple samples to be collected over a short time frame, a butterfly needle, percutaneous (over the needle) catheter, or other types of catheter fixed in position (e.g., adhesive taped or sutured) should be used rather than repeated needle punctures. These catheters can be implanted non-surgically (Carroll et al., 1999; Matte, 1999) or surgically if intended for longer-term use. Ideally, commercially available catheters should be used, as these will have been sterilized. If using homemade catheters, they must be sterilized (by autoclave or chemical methods).

The biocompatibility of catheters is an important consideration, particularly if an anti-coagulant is used in the surface coating.

Catheter lock solutions are commonly used to fill the catheter lumen when the catheter is not in use. Lock solutions often contain anti-coagulants to help prevent thrombosis, or anti-microbials for infection prophylaxis. Refinements including sedation, general anesthesia, or topical anesthesia, should be used whenever catheters are implanted. Catheters should be flushed regularly with a sterile isotonic solution to ensure they remain patent.

3.1.2.1.1 Short-Term Catheters

Short-term catheterization generally refers to periods of up to 72 hours. Sterile indwelling catheters placed following surgical preparation of the skin can be used to collect samples for up to 72 hours (Parasuraman et al., 2010; Guérios et al., 2015). Over-the-needle, short-term catheters, commonly used with laboratory animals, are typically placed in a peripheral vein. They are short in length and made of radiopaque materials such as polyurethane or Teflon™. Due to their relatively short length, these catheters are technically easier to place and typically cause less tissue trauma than the longer intravenous catheters used for intermediate- and long-term purposes. Short-term catheters may be used to collect venous blood samples over shorter durations of time and may be a refinement for studies that involve multiple venipunctures (Elliott et al., 2010). Due to their short length and material of construction, complications commonly encountered with short-term catheters include occlusion secondary to thrombosis, extravasation, and phlebitis. Phlebitis is a sign of blood vessel damage that may be caused by chemical factors (e.g., due to the osmolarity of the solution), mechanical factors (e.g., from trauma at insertion or movement), or infections (e.g., microorganisms contaminating the device). Signs of phlebitis include swelling, redness, heat, induration, purulence, a palpable venous cord (hard vein), and negative welfare impacts related to local inflammation of the vein at or near the insertion site. Due to the relatively high rate of these complications, short-term catheters must be routinely examined and replaced if necessary.

3.1.2.1.2 Intermediate-Term Catheters

Intermediate-term catheters are typically used for up to approximately 30 days. These catheters are normally longer than those used short-term and are made of materials such as silicone and coated polyurethane, which tend to be less thrombotic and resist biofilm and subsequent infective colonization. Larger blood vessels, such as the jugular or femoral veins, or central vessels such as the vena cava, are utilized for intermediate-dwelling catheters as these catheters are much less likely to become occluded than those located in the smaller peripheral vasculature. Intermediate-term catheters may be placed into a blood vessel using an over-the-needle technique; however, given their length, guide wires are often used to aid in catheter placement within the vessel. Intermediate-term catheters exit the skin and are temporarily secured externally using methods such as sutures, tissue glue, and bandaging.

3.1.2.1.3 Long-Term Catheters

Long-term catheters are similar to intermediate-term catheters but can be used for longer than 30 days. As an example, chronic non-occlusive maintenance-free catheterization of the inferior vena cava in the rat is described by Kaufman (1980). Long-term catheters may be secured to implanted vascular access ports or buttons in a closed system, and so do not exit the skin (Guérios et al., 2015). The advantages of internally placed vascular access ports include the lack of a chronic exit site wound, reduction in the risk of infection (Graham et al., 2010), and the decreased likelihood that the vascular access can be disturbed or dislodged by the animal (Valentini et al., 2013). With proper placement, care, and maintenance, long-term intravenous catheter systems have been reported to remain unobstructed for over a year (Kosanovich et al., 2007; Graham et al., 2010; Mutch et al., 2020). A proper locking solution must be used (Aubert et al., 2011; Farrow et al., 2013). Animals should be carefully monitored, with periodic blood cultures, to be sure that infections are not developing (Blot et al., 1999). Even a small activation of the inflammatory response could affect the results of the experiment.

3.1.3 Blood Sampling Sites (Survival)

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The appropriate sampling site should be selected depending on the intended frequency of sampling.

Selection of the blood sampling site requires considering whether the site is fit for the sampling purpose, and whether it will minimize negative welfare impacts for the animal (Oruganti and Gaidhani, 2011). The optimal method of blood collection must be used for the purpose of the study (Sørensen et al., 2019) and should aim to minimize inflammation. Peripheral vessels should be used where possible, based on the number and volume of samples required and the risk to the animal, recognizing the possibility for a greater degree of trauma when sampling from larger blood vessels.

More detailed information about appropriate sampling sites for different species can be found in the relevant CCAC types of animal guidelines documents and in other publications (e.g., the National Institutes of Health's published guidelines for survival bleeding of mice and rats (NIH, 2022); the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) blood sampling microsite (NC3Rs, n.d.-a) for rodents and other animals commonly used in the laboratory; the *CCAC guidelines on: the care and use of fish in research, teaching and testing* (2005); *CCAC guidelines on: the care and use of farm animals in research, teaching and testing* (2009); and *CCAC guidelines: Wildlife* (2023)).

Rotation of blood sampling sites reduces the risk of injury to a single site and, therefore, has benefits for refinement and reducing the possibility of inaccurate results due to inflammation caused by repeated use of a site. However, samples taken from different sites may show differences in clinical pathology parameters (e.g., Christensen et al. (2009) describe differences in blood glucose levels in tail vein samples versus retro-orbital samples; Aasland et al. (2010) describe differences in hemolysis and blood glucose levels in saphenous vein compared to tail vein samples; and Mella et al. (2014) describe differences in cytokine concentration in samples from the facial vein, retro-orbital sinus, and heart). The reduction in inflammation may minimize subtle differences resulting from sampling from different sites. Different sites should be used for the administration of test compounds and withdrawal of blood samples.

3.1.3.1 Venous Sampling

Although blood samples can be taken from either arterial or venous blood vessels, venous routes are most often used due to their relative ease of collection. SOPs should be established for blood sampling procedures that are used frequently. The SOP should define the maximum number of venipuncture attempts permitted (i.e., the number of times before expert help must be sought), the maximum number of samples per 24-hour period, and the permissible collection volumes.

3.1.3.1.1 Location of Vein

For percutaneous sampling, the vein must be accurately located and, when possible, dilated by gentle obstruction or warming before sampling. If warming is used, the animal must be constantly observed to prevent hyperthermia. When samples are to be taken from deep vessels rather than a superficial vein, the

individual carrying out the procedure must have accurate knowledge of the vessel's location and the relevant sampling technique.

3.1.3.1.2 Jugular Vein

Sampling from the jugular vein is typically used for larger animals that can be more easily restrained (dogs, cats, cattle, sheep, etc.). It is also used in smaller mammals (e.g., guinea pigs (Birck et al., 2014), rabbits, rats, and mice). Smaller animals may require anesthesia for this technique, but jugular vein sampling has been performed without anesthesia in mice (Shirasaki et al., 2012).

The jugular vein can also be used to collect blood samples from birds. In many avian species, the jugular vein may be the only vessel large enough to collect blood from. Generally, a bird's right jugular vein is larger than the left jugular vein (although either vessel can be used) and can be accessed in the featherless tract, on the right side of the neck. However, sampling from the jugular vein at this site carries a risk of hematoma formation. Inserting the needle with the bevel facing down minimizes the risk of puncturing the far side of the vein wall (Kramer, 2015).

3.1.3.1.3 Cephalic Vein

Blood sampling from the cephalic vein can be used in dogs, nonhuman primates, cats, rabbits, and mini pigs. The use of indwelling catheters may be a refinement when multiple samples are required from this site (Elliott et al., 2010). The radial vein (a branch of the cephalic vein) can be used for mini pigs restrained in a sling (Mozzachio, 2019).

3.1.3.1.4 Lateral Saphenous Vein

This route has been used in many laboratory animals, including rats, mice, guinea pigs, ferrets, dogs, mini pigs, nonhuman primates, and mink (Hem et al., 1998). In rodents, an anesthetic is typically not required, making this route particularly suitable for repeated short-term blood sampling, especially in rats. See information from Norecopa (2023) and NC3Rs (n.d.-a) for further details.

3.1.3.1.5 Femoral (Medial Saphenous) Vein

Blood sampling from the femoral (medial saphenous) vein is commonly carried out with larger animals such as nonhuman primates. Nonhuman primates may be trained to cooperate with femoral vein blood sampling, thus minimizing the negative animal welfare impacts associated with the blood collection procedure (Reinhardt, 2003). However, the femoral vein can be technically challenging to access, and sampling from this vein may require anesthesia, particularly when the animals have not been habituated. The femoral vein is also used in non-anesthetized cats.

The procedures required for inserting a cannula in the femoral vein are similar between species (including rodents); as such, knowledge of general surgical techniques (Jespersen et al., 2012) and selection of the appropriate suture and tubing size or material for each species is required. Desjardins (1986) provides details on how to conduct femoral vein blood sampling in rodents.

3.1.3.1.6 Tail Vein (Coccygeal Venipuncture)

Coccygeal venipuncture (sampling from the tail vein) of mice and rats appears to have the least negative welfare impact, based on measures of corticosteroids (Madetoja et al., 2009). However, the technique requires restraint, followed by warming the tail to dilate the vessel, which can have negative welfare impacts for the animal (NC3Rs, n.d.-a). Alternatively, small animals may be anesthetized and placed on a heating pad to maintain body temperature, thus avoiding the negative animal welfare impacts associated with restraint. Warming the tail may result in a difference in blood parameters (Hoggatt et al., 2016). Sampling can be via venipuncture or tail snip, or a catheter may also be inserted into the tail vein to facilitate the collection of larger volumes.

Tail vein sampling is also commonly used in cattle to collect blood samples (Sears et al., 1978; for a clear description of the method, see BRISTOL (2020)). To prevent hematoma formation, it is important to apply pressure for a few seconds on the puncture site immediately after withdrawing the needle (Holtgrew-Bohling, 2012).

3.1.3.1.6.1 Tail Snip

Blood samples may be obtained from small rodents (e.g., mice), by tail snipping (Dudley et al., 2016). In tail snipping, a small section of tissue is removed from the tip of the tail using a new scalpel blade (see CCAC types of animal guidelines documents). Before collecting the blood, local anesthesia should be applied to the tail, unless it would affect the blood parameters to be measured, in which case, other methods of refinement, such as general anesthesia, should be used. Blood flow is stopped by dabbing the tail tip (Parasuraman et al., 2010).

Only small volumes of blood can be collected (i.e., < 200 µL (NC3Rs n.d.-a)), and hemostasis must be assured. Repeat samples can be taken by removing the scab formed at the site of the snip (Kim et al., 2018); however, tails must only be snipped once. Tail snipping should not be used if the tail has already healed after being snipped for genotyping purposes.

3.1.3.1.6.2 Tail Puncture

Another method of blood collection in rodents is a tail puncture. When small samples are required, the point of a hypodermic needle (of a suitable gauge e.g., 25-30 G for mice; 23-25 G for rats) can be used to puncture the vessel. Care must be taken not to damage the tail. It is inadvisable to use the point of a blade to puncture the tail, so extreme caution should be used if using a scalpel blade (specifically with small animals) so as not to damage the tail. This technique is only suitable for a small number of punctures, to avoid tissue trauma. Topical analgesia may be useful for some species; however, definitive proof of efficacy has not been established (David et al., 2014).

3.1.3.1.7 Caudal Vein

In most fish species, the caudal vein, located just posterior to the anal fin, is the preferred site of blood collection. In laterally compressed species, the needle insertion site may differ (i.e., laterally below the lateral line) from most other species in which ventral insertion provides the easiest access (i.e., salmonids, acipenserids, chondrichthyes). Blood flow is easily stopped through direct pressure to the needle insertion site, and repeated blood collection can be achieved from this site over time by tracking the vein anteriorly (Iwama

and Ishimatsu, 1994; Whitman, 2004; DFO, 2004). Lawrence et al. (2020) provide a list of best practices for non-lethal blood sampling in fish.

3.1.3.1.8 Retro-Orbital

Retro-orbital bleeding is variously described as peri-orbital, posterior-orbital, or orbital venous sinus bleeding. It may be used in swine and some other species (e.g., hamsters), where other routes are difficult to access (Dove and Alworth, 2015; McClure, 1999). Swine undergoing retro-orbital bleeding must be restrained.

In general, retro-orbital bleeding should not be a survival procedure for rodents (CCAC, 2019a, 2020).

Adverse effects of retro-orbital bleeding may include:

- retro-orbital hemorrhage, which may result in a hematoma and excessive pressure on the eye
- ulceration of the cornea, with potential rupture of the globe and micro-ophthalmia
- damage to the optic nerve and other intra-orbital structures, which can lead to deficits in vision and blindness
- fracture of the fragile bones of the orbit and neural damage by the micropipette
- penetration of the eye globe itself with a loss of vitreous humour

For this reason, retro-orbital bleeding is also listed in Section 3.1.4, "Blood Sampling Sites to be Avoided", and Section 3.1.5, "Blood Sampling Sites (Terminal)".

Samples obtained from retro-orbital bleeding are not representative of venous blood as it is a mixture of venous blood and tissue fluid (see Section 3.1.4.2, "Retro-Orbital in Rodents").

3.1.3.1.9 Submandibular (Facial) Vein

Sampling from the facial vein (cheek bleed) can be carried out in rodents. The facial vein method of blood collection has been advocated for use in mice as an alternative to retro-orbital bleeding (CCAC, 2019a). However, there is currently inconclusive evidence to suggest that this method is preferable in all instances (Whittikar and Barker, 2020).

Sampling from the facial vein carries the following potential concerns:

- considerable negative welfare impacts for the animal (Teilmann et al., 2014; Moore et al., 2017; Frohlich et al., 2018; Gjendal et al., 2020; Meyer et al., 2020)
- difficulty in controlling the blood flow (Teilmann et al., 2014; Frohlich et al., 2018)
- difficulty in locating the vein

Blood collection using this method does not require the use of an anesthetic (although the use of an anesthetic reduces the negative welfare impacts for the animal), but proper restraint is key to successful sampling. This is a technically difficult procedure, so those performing the technique must be trained and demonstrate competency prior to carrying out the technique on a live animal, particularly if the animal is unanesthetized (e.g., mice (Francisco et al., 2015)).

In horses, the sinus formed by the dilation of the transverse facial vein can be used as an alternative venipuncture site when the jugular vein is no longer patent or is unavailable due to hematoma formation, septic

thrombophlebitis, or focal cellulitis. Most horses are reported to tolerate this technique well (Walesby et al., 2007).

3.1.3.1.10 Inferior Labial Vein

Sampling from the inferior labial vein (chin bleed) has been suggested as a safer alternative to bleeding from the facial vein in small animals (Regan et al., 2016; Constantinescu and Duffee, 2017); however, there is limited evidence of its efficacy in practice.

The area under the chin is sparsely furred, making the blood vessels easier to access, with fewer muscles and no major glands. It appears to be easier to control the blood flow, as bleeding stops once the animal is released and resumes its normal head position, and there is no risk of arterial hemorrhage (Regan et al., 2016). Constantinescu and Duffee (2017) indicate the correct blood vessel for sampling as the inferior labial vein; the submental vein (referenced by Regan et al., 2016) is too small to collect useful blood sample volumes. Blood can be collected from alternating sides, provided that the daily maximums and rest periods are respected. Anesthesia is not necessary, provided that the person carrying out the procedure is well-trained. However, the use of anesthetic renders the procedure less stressful.

3.1.3.1.11 Sublingual Vein

Sublingual vein sampling can be performed in anesthetized rodents (Heimann et al., 2009) and can be used for the removal of large volumes of blood (e.g., 0.2 mL (mice) or smaller samples at frequent intervals (Diehl et al., 2001; NC3Rs n.d.-a)). However, the technique is more challenging in mice due to their small size (Sørensen et al., 2019; Harikrishnan et al., 2018). Post-collection monitoring is important, to ensure that the animals return to feeding.

3.1.3.1.12 Brachial Vein

The brachial vein is readily accessible in birds. As with other techniques, proper restraint is important. The technique often requires two individuals, one of whom should restrain the bird. The wing should be extended, and pressure applied to the humeral area to occlude the vein. Water can be used to keep the feathers separated. Pressure should be applied to the vein once the sample has been removed, to minimize the development of hematomas. Sheldon et al. (2008) and Owen (2011) reviewed the impact of blood sampling techniques on wild birds.

3.1.3.1.13 Tarsal Vein

Blood sampling from the tarsal vein is suitable for use in larger animals and is commonly used in guinea pigs (Parasuraman et al., 2010; Birck et al., 2014). While anesthesia is not required, the technique does require two individuals, one of whom should restrain the animal. The hind limb is extended downwards, and pressure is applied above the knee joint to provide stasis. The NC3Rs blood sampling site provides more details (NC3Rs n.d.-a). The medial metatarsal vein can be used for blood sampling in birds.

3.1.3.1.14 Anterior Vena Cava

Rats can be sampled from the anterior vena cava under anesthesia (Jekl et al., 2005). Use of this route is also one of the best techniques for the quick and safe collection of relatively large samples of blood in the ferret (Brown, 2006) and guinea pig (Williams and Kendall, 2015), and can be performed by experienced personnel without an anesthetic. In pigs, the anterior vena cava is the most suitable route for collecting single blood samples (NC3Rs n.d.-a). It is not suitable for multiple blood samples because of the risk of hematoma formation.

3.1.3.1.15 Mammary Vein

This technique is suitable for larger animals, such as swine (Scollo et al., 2019). It appears to require minimal restraint; hence, for lactating animals, it may be less stressful than sampling from the jugular vein. While the method has been used in cattle (Alvarenga et al., 2019), it is not advisable, particularly for lactating cows, as there is an increased risk of excessive bleeding, hematoma formation, and potential rupture of the vein. In addition, depending on the parameters to be measured, there can be a significant difference in values between samples taken from the jugular vein or tail vein compared to samples taken from the mammary vein (Linzell, 1960; Alvarenga et al., 2019).

3.1.3.1.16 Other less commonly used venous sampling sites

Other venous sites may be used for blood sampling, including the thoracic and pectoral veins in horses, the medial saphenous vein in cats, the subclavian vein in hamsters and mice (Yang et al., 2019), and the ear vein in guinea pigs (Birck et al., 2014; Dülsner et al., 2017b).

Sections 3.1.3.1.1-3.1.3.1.16 above are not an exhaustive list of venous blood sampling sites; in general, venous sites are suitable for blood sampling if the blood vessel is visible and venipuncture will not cause excessive trauma, pain, or tissue damage to the area being sampled. The vein should be easily punctured, and the bleeding stopped effectively once the sample has been obtained.

3.1.3.2 Arterial Sampling

Survival arterial blood sampling may be used to determine arterial blood gases or if arterial samples are specifically required for scientific purposes. Arteries have thicker walls and contain more musculature than veins; hence, some specific complications can be seen with arterial blood sampling. These potential complications include arteriospasm, hematoma formation, thromboembolism, or hemorrhage. Whether arterial puncture is intentional or unintentional, direct and prolonged pressure (i.e., for at least 5 minutes) should be applied to induce hemostasis (WHO, 2010).

3.1.3.2.1 Tail Artery

Rodents have two lateral tail veins and a central tail artery located on the ventral side of the tail. A greater volume of blood may be collected from the artery, but care must be taken to ensure hemostasis and to keep the lumen patent. Therefore, arterial blood collection should only be used if large volumes of blood are required. For tail artery sampling, the animal must be anesthetized and placed in dorsal recumbency. Homeostasis should be maintained whenever an artery is punctured.

The tail artery can be used for blood sampling in rats; however, this is technically difficult, and the tail artery may spasm if multiple sample collections are attempted. Hagmüller et al. (1992) described a method of tail-artery cannulation for sampling in freely moving rats.

3.1.3.2.2 Ear Vein or Artery

Sampling from the ear vein or artery is commonly used in rabbits and pigs, where the vessels are clearly visible (NC3Rs n.d.-a). The artery is normally used for larger volume samples or specifically when arterial blood samples are required, but it carries a greater risk of hematoma and bruising. Topical anesthesia should be used.

3.1.3.2.3 Dorsal Aorta

Sampling from the dorsal aorta is used mainly in salmonids and provides an alternative collection site to the caudal vein for repeated sampling and indwelling catheterization. The needle is inserted along the dorsal midline of the roof of the mouth, between the first and fourth gill arch, depending on the size of the fish (Iwama and Ishimatsu, 1994; Whitman, 2004).

3.1.4 Blood Sampling Sites to be Avoided

Obtaining blood samples from the following sites is not advisable because of the risks to the welfare of the animals. Sampling from these sites must only be carried out if there is sound scientific justification approved by an animal care committee, and must only be carried out by highly competent staff.

3.1.4.1 Toe and Ear Clipping

Blood collection from toe and ear clips should be avoided and may only be carried out when the procedures are also to be used for identification purposes (CCAC 2019a, 2020). Wever et al. (2017) provide a review of the level of discomfort reported in association with these procedures, which may be useful for decision-making.

3.1.4.2 Retro-Orbital in Rodents

Retro-orbital bleeding is not advisable for use in most rodents except as a terminal procedure (see Section 3.1.5, "Blood Sampling Sites (Terminal)"), despite contradictory evidence in the literature, as it is associated with negative animal welfare consequences, even when carried out by competent personnel (Freid et al., 2015; Jo et al., 2021).

In rare circumstances, retro-orbital bleeding may be used in rodents, with recovery, but it requires exceptional scientific justification and should only be used when other methods are not suitable. Further, the procedure should only be performed under anesthesia, and only by trained and competent individuals (see CCAC types of animal guidelines documents and NC3Rs, n.d.-a). Confirmation of the sample requirements should be sought to ensure that the sample is appropriate since this site is a "plexus" and not a vein, resulting in a different sample composition than that obtained from a blood vessel (Van Herck et al., 2001).

CCAC types of animal guidelines documents and the NC3Rs blood sampling microsite (NC3Rs, n.d.-a) provide additional information related to performing the procedure. To prevent additional blood loss when the

allowable amount of blood has been collected, the collection tube should be withdrawn, and slight pressure applied with a piece of gauze on the eye globe to prevent further bleeding.

3.1.4.3 Cardiac Puncture

In general, cardiac puncture should only be used as a terminal procedure (see Section 3.1.5.1, "Cardiac Puncture"). However, in exceptional circumstances, cardiac puncture may be carried out as a recovery procedure (e.g., in reptiles (see the CCAC guidelines on reptiles; in wild mice (Williams et al., 2020); and hamsters (NC3Rs, n.d.-a and the CCAC guidelines on hamsters and guinea pigs). All other options should be explored first.

Cardiac puncture must be performed under general anesthesia with an anticholinergic drug as premedication to prevent cardiac arrhythmia. The animal must be separated from other animals until it is fully conscious. The animal must be carefully monitored for adverse effects and euthanized if found to be exhibiting conditions such as dyspnea, tachycardia, or inability to ambulate, which may be indicative of complications such as bleeding into the pericardium or into the thorax (Herling, 2016).

3.1.5 Blood Sampling Sites (Terminal)

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Terminal blood sampling must only be carried out once the animal has been rendered unconscious via another method.

Terminal blood collection may involve: 1) exsanguination as a single process of blood removal to collect as much blood as possible and 2) multiple blood sampling during a terminal experiment under general anesthesia. Exsanguination must only be performed after the animal has been rendered unconscious or dead by another method (e.g., by general anesthesia or physical stunning (CCAC, 2010)). Methods used for exsanguination in unconscious animals include, but are not limited to:

- blood withdrawal from the vena cava caudalis or the aorta after laparotomy, when as much blood as possible may be removed in a sterile manner
- exsanguination after decapitation, incision of the jugular vein or carotid artery, or techniques used in the slaughterhouse (for large animals)
- retro-orbital bleeding of smaller laboratory animals such as gerbils, hamsters, mice, and rats, which can also be a method of exsanguination (Herling, 2016)

3.1.5.1 Cardiac Puncture

The terminal collection of blood by cardiac puncture can be performed in most rodents (e.g., NC3Rs, n.d.-a). A general anesthetic from which the animal will not recover should be administered.

3.1.5.2 Retro-Orbital Bleeding

In general, retro-orbital bleeding should only be performed as a terminal procedure for rodent species.

3.1.5.3 Venous System

For terminal blood sampling, cannulae can be implanted in several locations in the venous system (see Parasuraman et al. (2010) for an overview; Dülsner et al. (2017b) for a list of sites for various small animal species). These locations include the jugular vein, inferior vena cava (Kaufman, 1980), the renal, portal, and hepatic veins (Davis and Campbell, 1975; Yokota et al., 1976), and the pulmonary artery (Stinger et al., 1981; NC3Rs, n.d.-a).

For terminal blood sampling from the femoral vein, an effective and efficient approach is occlusive intravascular cannulation. This approach has the benefit of facilitating periodic or serial blood sampling in anesthetized animals during the conduct of a study, and the collection of a final, terminal blood sample of a larger volume.

3.1.5.4 Abdominal Aorta

The abdominal artery can be used for terminal blood collection in the hamster (Donovan and Brown, 2006) and other rodent species (see NC3Rs, n.d.-a).

3.2 URINE AND FECAL SAMPLING

Many nutrition or physiology studies require the use of metabolic housing. Metabolic cages, crates, pens, or stalls are also used to protect catheters or cannulae. Metabolic housing should provide for the animal's basic needs (e.g., shelter, ability to hide, thermoregulation). Smaller animals such as rodents may be held in metabolic cages for a short period (i.e., less than 24 hours), with rest periods in standard cages between repeat sampling. See *CCAC guidelines: Husbandry of animals in science* (2017) and CCAC types of animal guidelines documents for more species-specific information concerning metabolic housing.

Alternatives to metabolic cages or crates must be used whenever possible, as the negative welfare impacts of close confinement and loss of social contact may be extreme, especially if combined with food restriction (Appleby, 1995). The animals should be habituated to the metabolic housing by gradually introducing them to it for increasing periods of time, paired with some type of reward (e.g., food, "tickling" for rats (LaFollette et al., 2017)), if this does not interfere with the scientific goals of the protocol. This is particularly important for animals that have previously been group-housed. In general, metabolic cages, crates, and pens have some form of mesh flooring (to allow the collection of urine and feces), which can be detrimental to the animals' feet over the long term. The use of wire flooring must be avoided, and perforated plastic should be used instead. Information on individual species-specific requirements can be found in the CCAC types of animal guidelines documents.

The length of the habituation period should be subject to approval by the animal care committee, depend on the species and age of the animal, and depend on the study, including the amount of manipulation involved. For social species (e.g., rats, sheep), metabolic crates, pens, or stalls should be positioned so that the animal is in visual, auditory, and olfactory contact with conspecific animals, to minimize the effects of social isolation. If this is not possible, alternative methods to reduce the impact of isolation should be evaluated.

3.2.1 Urine Sampling

Urine sampling in laboratory animals is carried out for two main reasons: to ascertain the health status of animals and to assess the results of manipulations from scientific activities. In both cases, urine collection is

challenging, particularly to prevent contamination by feces. Methods of urine sampling for a wide variety of laboratory animals have been reviewed by Kurien et al. (2004).

The easiest method of urine collection does not involve any direct intervention. With smaller species, this can be accomplished by placing the animal on plastic wrap or by using non-absorbent material in the home cage (Hoffman et al., 2017). These methods are more appropriate when the volume of urine collected, and contamination of urine are not important factors.

Separating urine and feces when collecting via voluntary voiding presents challenges, but many commercially available apparatuses and other more economical methods are available to facilitate such separation (e.g., see Demirkan and Melli (2007) for an apparatus suitable for use with rats).

In large animals such as cattle, small volumes of urine may be obtained by manual stimulation, whereas collecting large volumes or total urine output requires keeping the animals in stalls and fitting them with a device that allows the collection of urine over an extended period (see Lascano et al. (2010) for an example of such a device for female cattle).

Urine collection from animals such as rabbits, cats, and dogs can be managed via voluntary voiding or, when this is not possible, by catheter or cystocentesis. The animals do not have to be anesthetized for the cystocentesis procedure, but physical restraint is required; the animals may also require sedation. As cystocentesis is a relatively invasive method of urine sampling, animal care committees should define the maximum frequency that this method can be used and establish clear humane intervention points.

Quantitative excretion analysis for scientific purposes may require the collection of timed urine samples (e.g., for 24 hours) and thus may require the use of metabolic cages or crates. As indicated above, the length of time that the animals are confined should be minimized. Catheterization of the urethra can be used as a means of collecting urine, particularly for long-term collection. An aseptic technique must be used when inserting the catheters to prevent contamination by bacteria, as this procedure can easily lead to urinary tract infections, and the catheters must be monitored regularly. Urine collection bags can be used in some instances (e.g., in male neonatal and weaned pigs (Gasthuys et al., 2017)); the collection of urine from female animals generally requires catheterization.

The use of indwelling catheters for urine collection in fish has been described by Schreck and Moyle (1990) and Black (2000); see CCAC, 2005.

Urine collection in nonhuman primates can be challenging. For a quantitative urinalysis, where the determination of urine volume is necessary, small species such as marmosets can be placed in rat metabolic cages for 24 hours after preliminary habituation to the cage to limit negative welfare impacts (Bluemel et al., 2015). Marmosets can also be trained to urinate in a special box after a light is turned on for a food reward (Anzenberger and Gossweiler, 1993).

Various methods have been used with macaques, including the placement of a metal plate and vat under a (wire-bottomed) cage and an adapted diaper (Kurien et al., 2004). Bladder catheterization in these animals is possible but requires anesthesia. Urine collection in these species is most reliably achieved by cystocentesis under anesthesia.

3.2.2 Fecal Sampling

The method and time of sampling feces should be carefully selected to ensure that it is appropriate for the scientific objective. For example, the collection of fresh feces rather than a random sampling of previously

voided feces (e.g., fecal pellets from cage bedding) avoids variability in the viability of any microbes or other biological materials being assayed in the feces (CCAC, 2019a). The least invasive method of collection should be used (i.e., natural voiding; Borrelli et al., 2020), and if manual extraction is to be used, then the individual carrying out the procedure must be trained and competent.

For animals remaining in group housing, fecal samples may be collected from specific individuals if identified using indigestible fecal markers (food colourants, seeds or grains, or glitter) (Griffin, 2002; Fuller et al., 2011). Information on species-specific requirements can be found in the CCAC types of animal guidelines documents.

3.3 GASTROINTESTINAL SAMPLING

Gastric or rumen sampling is generally carried out to assess digestion and the functioning of the gastrointestinal tract. Gastric sampling can be accomplished either by passing a tube into the stomach or rumen (Geishauser, 1993; Shomer et al., 1999; Muizelaar et al., 2020), or by cannulation (e.g., Kissing et al., 1998).

For ruminants, a rumen pump can be used to collect rumen samples. This requires inserting a tube into the mouth, down the esophagus, and into the rumen. Once the tube is inside the rumen, fluid can be pumped out and collected in less than a few minutes. This method is quick, inexpensive, and does not cause significant stress to the animal (Klopp et al., 2018).

Harmon and Richards (1997) provide guidance in the use of gastrointestinal cannulation in ruminants. They compare the advantages and disadvantages of various approaches, cannula types, and cannula materials. Rumen fistulation is a surgical procedure and should only be carried out by experienced surgeons. Proper equipment should be used to restrain the animals during fistulation, and proper procedures should be employed during sampling to prevent injury to personnel and animals (Klopp et al., 2018; de Assis Lage et al., 2020). Indwelling gastric or intestinal cannulae can also be surgically implanted in rodents (Flanagan et al., 1989) and exteriorized for the purpose of sampling (Conover et al., 1987) in conscious animals. Animals that have an indwelling cannula require additional care and sometimes particular environments. Personnel responsible for caring for these animals require special training to care for the animals and ensure early identification of any problems. SOPs must be in place to detail the requirements for post-operative care of a fistulated animal, or an animal with an indwelling vascular cannula, including the care and maintenance of the cannula itself. Protocols that include the use of fistulation and cannulation must have defined humane intervention points, both for complications and for the potential removal of the cannula.

Gastric sampling can be achieved in seabirds by inducing the birds to regurgitate their stomach contents (Yorio et al., 2017). This technique has also been used in other species, such as sea lions (McIntosh et al., 2006). Regurgitated samples are not necessarily representative of actual stomach contents, depending on the species.

3.4 CEREBROSPINAL FLUID SAMPLING

The collection of cerebrospinal fluid may be required, for example, to assess the penetration of drugs to the central nervous system or to evaluate other cerebrospinal fluid analytes. Surgical preparation of the sampling site and sterile technique are imperative to protect the animal from infection. The fluid may be collected at the cisterna magna (cerebromedullary cistern) via lumbar puncture or cannulation, or via direct cannulation of the lateral ventricles of the brain using stereotaxic surgical techniques. Šakić (2019) demonstrates a modified cisternal puncture method in mice. In larger species, permanent ports can be added for ventricular sampling (MacAllister et al., 2016). Potential complications of cerebrospinal fluid collections include spinal cord

trauma; iatrogenic hemorrhage due to inadvertent trauma of the venous sinuses or dural or arachnoid vessels; cerebellar tonsil herniation due to a substantial acute drop in cerebrospinal fluid pressure; meningitis; and a phenomenon known as post-dural puncture headache, which has been reported to occur in human patients, and hence may also occur in animals (Niraj et al., 2014; Gaitero, 2017; Smith et al., 2019). Non-specific clinical signs of post-dural puncture headache may include lethargy, inappetence, and more specific clinical signs of pain such as rigid body position and reluctance to change head or body position. At the current time, the pathophysiology and specific causes of post-dural puncture headaches are not fully understood. It is thought that intracranial hypotension results in vasodilation or traction on sensitive intracranial structures, or that fragments of tissues are released into the cerebrospinal fluid, resulting in meningeal irritation (Smith et al., 2019). In humans, post-dural puncture headache has been suggested to be associated with collection needle type and size, cerebrospinal fluid collection volume, and low cerebrospinal fluid pressure (Gaitero, 2017; Bakshi and Gehdoo, 2018; Headache Classification Committee of the IHS, 2018).

Refinements should be utilized to minimize all potential complications of cerebrospinal fluid collection (e.g., Amen et al., 2017; Bergadano et al., 2019). These refinements include the use of the smallest gauge and least traumatic needle type (Xu et al., 2017; Turnbull and Shepherd, 2003); placement of an indwelling catheter if multiple samples are required, with the use of anesthetics and analgesics; and reduction of collection volume and frequency to the lowest possible extent that will achieve the scientific objective (Monserrate et al., 2015).

Vigilant humane intervention point monitoring should be in place to facilitate the early detection and treatment of any complications, and should include monitoring of neurological function (e.g., cranial nerve examination, monitoring of ambulation, and postural reactions in appropriate species) and any indications of post-dural puncture headache or meningitis. The *Association of Primate Veterinarians' Position Statement: Cerebrospinal Fluid Aspiration for Nonhuman Primates in Biomedical Research* lists questions that an animal care committee should ask when reviewing protocols involving cerebrospinal fluid collection (APV, 2019).

Reference ranges for maximum collection volumes are not yet available for any nonhuman animal species. Hence, extrapolation from human-suggested maximums may be utilized as a general guide until better information is available (e.g., removal of 13% of human cerebrospinal fluid volume caused post-dural puncture headache (Kunkle et al., 1943), so it is advisable to limit collection volumes to less than 13%). Total cerebrospinal fluid volume and production rates are known in some laboratory animal species: cats and dogs (Thomas, 2010); cynomolgus macaques (Pardridge, 2016; Khani et al., 2019); rabbits (Harnish and Samuel, 1988); mice (Oshio et al., 2005); and rats (Harnish and Samuel, 1988; Murtha et al., 2014).

The welfare impact of cerebrospinal fluid collection is additionally influenced by the requirement for general anesthesia during cannulation; however, some published techniques permit the collection of cerebrospinal fluid from conscious animals via surgically placed subdural catheters (West et al., 2014; MacAllister et al., 2016; Abe et al., 2018; Xavier et al., 2018).

The frequency of cerebrospinal fluid collection should be minimized, particularly when multiple general anesthesias and multiple dural punctures are required (CCAC, 2022). Reducing collection time points per animal by increasing animal numbers should be considered.

3.5 SYNOVIAL FLUID

Sampling of synovial fluid may be required in protocols where the composition of synovial fluid is important in the assessment of inflammation or disease progression (e.g., arthritis). Synovial fluid can be harvested via joint arthrocentesis. This must be done using aseptic technique, and those doing the sampling must have

training to ensure proficiency and to reduce trauma to the tissues and joint structures. A protocol has been described for synovial fluid collection from the stifle joints of rodents under anesthesia (Yilmaz et al., 2013). In most cases, sedation or anesthesia is required for collecting synovial fluid.

3.6 MILK

Milk samples may often be required from livestock (e.g., cattle, goats, horses, pigs, sheep) and companion animals (e.g., dogs (Dokoupilová et al., 2016)) for various reasons, including the determination of milk composition, quantification of hormones, analysis of somatic cells and bacteria, and to detect the presence of antibiotics or other drug residues. Rodents are widely used to study the genetics and environmental influences on mammary gland function, in particular, in studies involving genetically modified rodents.

While milk samples can be obtained easily by manual expression (hand stripping) in most species, parenteral administration of oxytocin may be required in other species (e.g., pigs) to encourage milk let-down (Atwood and Hartmann, 1992). The dam may experience negative welfare impacts from the procedure and from the removal of offspring. DePeters and Hovey (2009) describe two methods for obtaining milk samples from mice, and Paul et al. (2015) describe a method using capillary tubes to collect milk samples from rats. In general, pups are removed from the dam for a couple of hours prior to sampling. The pups must be closely monitored during this time, and an additional external source of heat must be provided for the pups if they are not yet old enough to thermoregulate (Lagerspetz, 1966; Rink, 1969).

3.7 SALIVA

Sampling of saliva is a minimally invasive technique used to measure physiologically active substances that otherwise require blood sampling (Nohara et al., 2016). Because it is relatively non-invasive, it can be used to assess levels of cortisol as an indicator of physiological stress, if the negative animal welfare impacts of handling are minimized (Cook et al., 2013). Saliva can be collected using commercial collection devices or passive drool (Rapp-Santos et al., 2017); the method of collection may have an impact on the cortisol levels, which may not correspond to serum cortisol levels (Lutz et al., 2000; Poll et al., 2007). Salivary secretions can also be used to assess disease states (e.g., kidney disease (Romero et al., 2016; Bagavant et al., 2018)).

3.8 SEMEN

Collection of semen may be required for assisted reproduction and where manipulation of the genome is to be carried out via insertion of genes or manipulation of sperm DNA. A minimally invasive procedure (e.g., hand massage *per rectum* or an artificial vagina for bulls, rams, stallions; vaginal collection vial for rams; hand collection for boars; body massage technique for chickens) should be used; however, animals must be comfortable around people and habituated to the method of collection. Handling methods and facilities should be designed to prevent injury and minimize negative animal welfare impacts throughout all aspects of semen collection (e.g., for cattle, see Napolitano et al., 2020; for mice, see Val and Robledano, 2013).

If it is not possible for semen to be collected from a bull or ram with an artificial vagina, electroejaculation may be used. Veterinary skills are required to examine the suitability of the animal prior to the procedure and to ensure optimal analgesia, restraint of the animal, selection and operation of the equipment, and monitoring of the animal's responses, to minimize any discomfort associated with electroejaculation (CVMA, 2019). The use of analgesia may be a refinement to this procedure; however, there is currently insufficient evidence of its effectiveness to be considered a mandatory requirement.

3.9 TISSUE BIOPSY

Tissue biopsy may be carried out for a variety of purposes, including identifying the genotype of an animal, examining cellular structures or function, or pathogen diagnosis. A biopsy involves the removal of a small amount of tissue from a live animal and depending on the site of biopsy (e.g., liver, kidney), involves a surgical procedure. Information concerning biopsies for identification purposes is included in the relevant CCAC types of animal guidelines documents (e.g., mice (CCAC, 2019a), nonhuman primates (CCAC, 2019b), and rats (CCAC, 2020)). The *CCAC guidelines: Scientific procedures (Part B – Analgesia, anesthesia, and surgery)* (2025) should be consulted for more invasive procedures.

Tissue biopsy must only be performed after appropriate training and experience, to ensure that the individual carrying out the procedure is competent. Procedures and protocols that avoid or minimize negative animal welfare impacts must be chosen. Any biopsy that is likely to cause negative welfare impacts to the animal must be carried out under sedation or general anesthesia, with the provision of analgesia.

Studies of regeneration may require tissue removal without wound closure. These protocols require humane intervention points to minimize the potential for infection and attendant negative welfare impacts for the animals.

3.10 TISSUE HARVESTING

Necropsies may be performed on research animals for a variety of reasons. Any tissue samples should be collected and stored (formalin-fixed, frozen, etc.) according to established guidelines to maximize sample preservation and usefulness (Fiette et al., 2017; Scudamore et al., 2014; Treuting and Snyder, 2015).

Tissue harvesting includes the collection of fetuses. Death of the fetus must first be assured (CCAC, 2010).

3.11 COLLECTION OF UTERINE FLUIDS AND EMBRYOS

In rodents and other small laboratory animals, embryos and uterine fluids are typically collected from excised reproductive tracts. In swine, embryos are collected under general anesthesia after localizing the uterus by laparotomy or endoscopy. In nonhuman primates (e.g., the baboon and various species of monkeys), non-surgical (transcervical) collection of embryos is possible in anesthetized females. In bovine and equine species, transcervical embryo collection is a well-established and routinely performed procedure. Epidural anesthesia is almost always used in cattle before embryo collection, whereas in mares, no anesthesia is required, although the use of a sedative (e.g., xylazine, detomidine) is advisable if the animal is anxious. Acepromazine should be avoided as it could impair uterine fluid recovery due to extreme relaxation of the uterus (Gibbs and Troedsson, 1995; Pinto, 2020). Non-surgical (transcervical) collection of embryos is also becoming more common in small ruminants (e.g., goats, sheep). In species where a transcervical embryo collection procedure is well-established, obtaining samples of uterine fluids, cells, or tissue (biopsy) may be possible using a modified procedure and appropriately designed tools. Whenever a non-surgical procedure is used, cattle and horses should be restrained in a handling chute and should remain standing during the entire procedure.

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More information about documents marked "in prep." can be found in the Guidelines section of the CCAC website.

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APPENDIX 1
CHECKLIST WHEN PLANNING SUBSTANCE ADMINISTRATION PROCEDURES

(adapted from Morton et al., 2001)

Experimental aims:

- ☐ What is the scientific goal of the study?
- ☐ Will the administration regime selected meet the aims of the experiment?
- ☐ Consider not just whether it can be done but whether it should be done and whether there is a better way of doing it.

The administration route:

- ☐ Is the route suitable for the substance?
- ☐ Is the proposed route highly invasive?
- ☐ Would a less severe route achieve the same aim?
- ☐ Is the route suitable for repetitive doses (if needed)?

The substance:

- ☐ Are you certain you know what you are administering?
- ☐ Will the substance have any adverse effects on the animal, and is there any background data on these adverse effects? If so, have the necessary preparations been made?
- ☐ Could the nature of the formulation alter the expected effect?
- ☐ Does the substance need to be freshly prepared?
- ☐ Will the concentration and dosing volume alter the expected effect?
- ☐ Are there any additional concerns regarding the physicochemical properties of the substance or associated solvents (e.g., osmolarity)?
- ☐ Can the volume be reduced?
- ☐ Can the frequency of administration be reduced?
- ☐ If the substance is toxic, can the dose be reduced?
- ☐ Is the substance likely to be an irritant?
- ☐ Are pilot studies needed (e.g., to ascertain a tolerated or effective dose)?

The animal:

- ☐ Are there any problems with the particular individuals, species, or strain associated with the area or route chosen? Is the animal easily stressed by handling? Is the animal the most appropriate for the study in terms of these factors?

- ☐ Can the animal be trained to cooperate with the procedures? Does the animal need time to acclimatize to the procedures?
- ☐ Is an anesthetic, sedative, or analgesic required? Would it reduce stress or confound the experiment?
- ☐ Have you done a pilot study for tolerated and effective dose levels in the strain used?

The technique:

- ☐ What are the scientific problems (e.g., first-pass metabolism in the liver after oral or intraperitoneal dosing, degree or rate of absorption, local effects)?
- ☐ What are the technical problems (e.g., what is the correct way to hold an animal to allow insertion of a gavage tube with minimum negative welfare impacts)?
- ☐ Will the technique itself have any effect on the animals?
- ☐ Are the humane intervention points clearly defined?
- ☐ What refinements can be introduced to overcome any adverse effects?
- ☐ Is a pilot study necessary?
- ☐ Have you checked on references and sources of expertise in other organizations?

Personnel:

- ☐ Are the personnel competent in the technique and trained to deal with any unexpected effects?
- ☐ Who are the best personnel to carry out the procedure, when considering both the handling of the animals and the procedure?
- ☐ Are sufficient numbers of personnel available to restrain and dose the animals and to monitor them post-administration?
- ☐ Are the personnel aware of the intended effects of any agents administered?
- ☐ Are the personnel aware of the humane intervention points and do they have the delegated authority and skill to euthanize the animals if the endpoints are exceeded?

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APPENDIX 2
ADMINISTRATION VOLUMES BY ANIMAL SPECIES

This table has been prepared by the CCAC Subcommittee on Administration of Substances and Biological Sampling, based on the available literature and the experiences of subcommittee members. The table provides information on administration volumes by animal species, including volumes that should be respected and maximum volumes that must not be exceeded. This information on administration volumes is aligned with the CCAC principles for the ethical care and use of animals in science.

The administration volumes included in this reference table have been previously published. Animal care committees are encouraged to determine values for administration volumes for animal species that are not included. Animal care committees should also refer to the CCAC types of animal guidelines documents and the Codes of Practice for farm animals for further references on administration volumes.

SPECIES	ADMINISTRATION VOLUMES								
	GAVAGE	SUBCUTANEOUS	INTRAPERITONEAL	INTRAVENOUS	INTRAMUSCULAR	INTRADERMAL	EPIDURAL	INTRANASAL	INTRACEREBRO-VENTRICULAR
Bird (zebra finch)	10 mL/kg	10 mL/kg	-	5 mL/kg	0.05 mL/site	-	-	-	-
Cat (also ferret)	10 mL/kg	2 mL/kg	5 mL/kg	≤5 mL/kg (bolus)	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	0.15 mL/kg	200 µL**	80 µL/animal**
Cat (maximum volume that must not be exceeded)	15 mL/kg	5 mL/kg	10 mL/kg	10 mL/kg (slow infusion)	0.05 mL/kg/site (maximum 1 mL limit) (2 sites/day)	0.1 mL/site*	0.2 mL/kg	500 µL**	-
Chicken	10 mL/kg	10 mL/kg	-	1 mL/kg	1 mL/site	0.05 mL/site*	-	-	-
Dog	5 mL/kg	1 mL/kg	1 mL/kg	≤ 5 mL/kg (bolus)	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	0.15 mL/kg	200 µL**	-
Dog (maximum volume that must not be exceeded)	15 mL/kg	2 mL/kg divided over 2-3 sites	20 mL/kg	10 mL/kg (slow infusion)	0.5 mL/kg/site (maximum 3 mL limit) (2 sites/day)	0.1 mL/site*	0.2 mL/kg (6 mL maximum)	500 µL**	-

SPECIES	ADMINISTRATION VOLUMES								
	GAVAGE	SUBCUTANEOUS	INTRAPERITONEAL	INTRAVENOUS	INTRAMUSCULAR	INTRADERMAL	EPIDURAL	INTRANASAL	INTRACEREBRO-VENTRICULAR
Fish	5 mL/kg	1 mL/kg	10 mL/kg	<5 mL/kg (bolus)	0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	-	-	-
Fish (maximum volume that must not be exceeded)	-	-	-	-	-	0.1 mL/site*	-	-	-
Guinea Pig	10 mL/kg	5 mL/kg	10 mL/kg	≤ 5 mL/kg	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	-	-	5 µL/animal**
Guinea Pig (maximum volume that must not be exceeded)	20 mL/kg	10 mL/kg divided over 2-3 sites	20 mL/kg	20 mL/kg (slow injection)	0.05 mL/kg/site (2 sites/day)	0.1 mL/site*	-	-	-
Hamster	10 mL/kg	5 mL/kg	10 mL/kg	≤ 5 mL/kg (bolus)	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	-	-	3 µL/animal**
Hamster (maximum volume that must not be exceeded)	20 mL/kg	10 mL/kg divided over 2-3 sites	20 mL/kg	20 mL/kg (slow injection)	0.05 mL/kg/site (2 sites/day)	0.1 mL/site*	-	-	-
Minipig	10 mL/kg	1 mL/kg; 2 mL total over taut skin; 5-10 mL total maximum dose under loose skin	1 mL/kg	2.5 mL/kg (bolus)	0.25 mL/kg 5 mL/site (maximum) (2 sites/day)	0.1 mL/site*	0.15-0.2 mL/kg (6 mL total volume)	-	-
Minipig (maximum volume that must not be exceeded)	15 mL/kg	3 mL/kg divided over 2-3 sites, maximum	20 mL/kg	10 mL/kg (slow injection)	(0.5 mL/kg) (maximum 5 mL limit) (2 sites/day)	0.2 mL	-	-	-

SPECIES	ADMINISTRATION VOLUMES								
	GAVAGE	SUBCUTANEOUS	INTRAPERITONEAL	INTRAVENOUS	INTRAMUSCULAR	INTRADERMAL	EPIDURAL	INTRANASAL	INTRACEREBRO-VENTRICULAR
Mouse	5 mL/kg	5 mL/kg	10 mL/kg	≤ 5 mL/kg (bolus)	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	0.15 mL/kg	35 µL**	3 µL**
Mouse (maximum volume that must not be exceeded)	20 mL/kg	20 mL/kg divided over 2-3 sites	20 mL/kg	25 mL/kg (slow infusion)	0.05 mL/kg/site (2 sites/day)	0.1 mL/site*	0.2 mL/kg	50 µL**	-
Nonhuman primate (macaque)	5 mL/kg	2 mL/kg	3 mL/kg	≤ 5 mL/kg (bolus)	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	0.15 mL/kg	200 µL**	-
Nonhuman primate (macaque) (maximum volume that must not be exceeded)	15 mL/kg	2 mL/kg divided over 2-3 sites	10 mL/kg	10 mL/kg (slow infusion)	0.05 mL/kg (maximum 2 mL limit and 2 sites/day)	0.1 mL/site*	0.2 mL/kg	500 µL**	-
Nonhuman primate (marmoset)	10 mL/kg	2 mL/kg	5 mL/kg	≤ 2.5 mL/kg (bolus)	0.25 mL/kg (2 sites/day)	0.05 mL/site*	0.15 mL/kg	50 µL**	-
Nonhuman primate (marmoset) (maximum volume that must not be exceeded)	15 mL/kg	5 mL/kg	20 mL/kg	10 mL/kg (slow injection)	0.5 mL/kg (2 sites/day)	0.1 mL/site*	0.2 mL/kg	100 µL**	-
Rabbit	10 mL/kg	2.5 mL/kg	3-5 mL/kg	≤5 mL/kg (bolus)	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	0.15 mL/kg	200 µL**	80 µL/animal**

SPECIES	ADMINISTRATION VOLUMES								
	GAVAGE	SUBCUTANEOUS	INTRAPERITONEAL	INTRAVENOUS	INTRAMUSCULAR	INTRADERMAL	EPIDURAL	INTRANASAL	INTRACEREBRO-VENTRICULAR
Rabbit (maximum volume that must not be exceeded)	20 mL/kg (empty stomach)	10 mL/kg divided over 2-3 sites, maximum	10 mL/kg	10 mL/kg (slow infusion)	0.05 mL/site (1 mL limit) (2 sites/day)	0.1 mL/site*	0.2 mL/kg	500 µL**	-
Rat	5 mL/kg	5 mL/kg/site	10 mL/kg	≤ 5 mL/kg (bolus)	< 0.05 mL/kg/site (2 sites/day)	0.05 mL/site*	0.15 mL/kg	35 µL**	5 µL/animal**
Rat (maximum volume that must not be exceeded)	20 mL/kg	10 mL/kg divided over 2-3 sites	20 mL/kg	20 mL/kg (slow infusion)	0.05 mL/kg/site (2 sites/day)	0.1 mL/site*	0.2 mL/kg	50 µL**	-
Sheep	10 mL/kg	1 mL/kg	-	2 mL/kg	4 mL/site	0.1 mL/site*	-	-	-

* Maximum volume is dependent on skin thickness (International Consortium for Innovation and Quality in Pharmaceutical Development).

** Volumes indicated are based on the average adult weight of this species; administration volumes should be adjusted where necessary to reflect the weight or size of the animal.

\- No information available.

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APPENDIX 3
NEEDLE AND CANNULA GAUGES BY ANIMAL SPECIES

Table 1 Needle Gauge Sizes by Animal Species

SPECIES	INTRAPERITONEAL	INTRAMUSCULAR	INTRAVENOUS	SUBCUTANEOUS
Cat	21-23	23	21-25	21-23
Dog	21-23	21-23	21-23	21-23
Ferret	21-23	23-25	21-25	21-23
Guinea Pig	21-25	25	25-27	23-25
Hamster	23-25	25	25-27	25
Mouse	27	29-30	27-28	25
Nonhuman primate (rhesus macaque)	21-23	23-25	21-25	21-25
Rabbit	21-23	23-25	23-25	21-25
Rat	23-25	25	25-27	25
Sheep	19-21	21	19-21	19-21

Needle gauge sizes based on https://veteriankey.com/handling-and-techniques/

Table 2 Cannula Gauge Sizes by Animal Species

SPECIES	SITE	GAUGE	LENGTH
Cat	Cephalic or jugular vein	22-23	25 mm
Cattle or horse	Jugular vein	19-21	40 mm
Dog	Cephalic or jugular vein	20-21 (puppies 23 or 25)	25-40 mm
Ferret	Cephalic vein	24	19 mm
Nonhuman primate (rhesus macaque)	Cephalic or saphenous vein	21-24	19-25 mm
Pig	Ear vein	21-23	25-40 mm
Rabbit	Ear vein	24	19 mm
Rat	Tail vein	24-25	12-19 mm
Sheep or goat	Jugular vein	19-21	40 mm

Cannula gauge sizes based on https://veteriankey.com/handling-and-techniques/

APPENDIX 4
BLOOD SAMPLING SITES AND VOLUMES BY ANIMAL SPECIES

SPECIES	TYPICAL WEIGHT	PREFERRED BLOOD SAMPLING ROUTES	BLOOD VOLUME [mL/kg bodyweight]	7.5% [mL/kg bodyweight]	10% [mL/kg bodyweight]	15% [mL/kg bodyweight]	20% [mL/kg bodyweight]
Atlantic sturgeon	8-20 kg	Caudal vein	37	2.7	3.7	5.5	7.4
Bison (700kg+)	700 kg	Coccygeal vein Jugular vein	60	4.5	6.0	9.0	12.0
Cat	3 kg	Jugular vein Cephalic vein	56	4.2	5.6	8.4	11.2
Cattle (500kg+)	500 kg	Coccygeal vein Jugular vein	60	4.5	6.0	9.0	12.0
Chicken	1 kg	Brachial wing vein	60	4.5	6.0	9.0	12.0
Deer	85 kg	Jugular vein	60	4.5	6.0	9.0	12.0
Dog	10 kg	Jugular vein Cephalic vein	80	6.4	8.5	12.8	17.0
Ferret	0.5 - 2 kg	Saphenous vein Jugular vein	60	4.5	6.0	9.0	12.0
Gerbil	100 g (0.1 kg)	Saphenous vein	67	5.0	6.7	10.0	13.4
Goat	50 kg	Jugular vein	93	7.0	9.3	14.0	18.7
Guinea pig	200 g (0.2 kg)	Tarsal vein Saphenous vein Jugular vein	73	5.5	7.3	11.7	15.6
Hamster	100 g (0.1 kg)	Jugular vein Cranial vena cava Saphenous vein	78	5.8	7.8	11.7	15.6
Lamprey	200 g (0.2 kg)	Caudal vein Cardiac puncture	85	6.4	8.5	12.7	17.0

SPECIES	TYPICAL WEIGHT	PREFERRED BLOOD SAMPLING ROUTES	BLOOD VOLUME [mL/kg bodyweight]	7.5% [mL/kg bodyweight]	10% [mL/kg bodyweight]	15% [mL/kg bodyweight]	20% [mL/kg bodyweight]
Macaque	4-8 kg	Cephalic vein Saphenous vein Femoral vein Ear capillary	60	4.5	6.0	9.0	12.0
Marmoset	240 g (0.24 kg)	Femoral vein	60	4.5	6.0	9.0	12.0
Minipig	15 kg	Cranial vena cava Marginal ear vein (requires great care due to small size of veins)	65	4.8	6.5	9.7	13.0
Mouse	25 g (0.025 kg)	Saphenous vein Submandibular (Facial) vein Sublingual vein Lateral tail vein Tail tip amputation Cardiac puncture	58.5	4.4	5.9	8.8	11.7
Pig	130 kg	Cranial vena cava Marginal ear vein	65	4.8	6.5	9.7	13.0
Rabbit	4 kg	Jugular vein Marginal ear vein Femoral artery Cardiac puncture	56	4.2	5.6	8.4	11.2
Rainbow trout	50-200 g (0.05-0.2 kg)	Caudal vein	23	1.7	2.3	3.5	4.6
Rat	250 g (0.25 kg)	Jugular vein Saphenous vein Submandibular vein Sublingual vein Lateral tail vein Tail tip amputation Cardiac puncture	64	4.8	6.4	9.6	12.8
Sheep	75 kg	Jugular vein	60	4.5	6.0	9.0	12.0

The values in the above table are intended to be a general guide. Further information can be found in the reference list below. The scientific literature and the institutional veterinarian should be consulted for more specific information. In addition, it is important to note that for wildlife species, it is not appropriate to extrapolate values from domesticated animals. Consult with experienced wildlife veterinarians for more specific information.

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APPENDIX 5
HEMATOLOGICAL VALUES BY ANIMAL SPECIES

SPECIES	PCV (%)	RBC (10^{12}/L)	HB (G/DL)	WBC (10^{9}/L)
Atlantic salmon	44-49	0.8-1.0	8.9-10.4	17.5-37.3
Atlantic sturgeon	21-28	0.9-1.2	5.0-6.2	21.0-33.7
Cat	30-50	6.0-10.0	8.0-14.0	5.5-19.5
Cattle	24-46	5.0-10.0	8.0-15.0	4.0-12.0
Chicken	23-55	1.2-4.5	7.0-18.6	9.0-31.0
Dog	38-53	4.5-8.0	11.0-18.0	6.0-17.0
Goat	29-38	13.0-18.0	8.0-14.0	5.0-14.0
Guinea pig	35-42	4.5-7.0	11.0-17.0	10.0
Hamster	39-59	4.0-10.0	2.0-30.0	7.6
Macaque	39-43	4.5-6.0	12.7	11.5-12.4
Marmoset	45-48	2.5-10.4	15.1-15.5	3.0-15.0
Mouse	35-45	7.7-12.5	10.0-20.0	8.0
Pig	30-50	5.0-9.0	10.0-16.0	7.0-20.0
Rabbit	30-50	4.5-7.0	8.0-15.0	9.0
Rainbow trout	29-32	1.5-1.6	2.9	19.9-20.1
Rat	35-45	7.2-9.6	12.0-18.0	14.0
Sheep	29-38	8.0-14.0	10.0-12.0	4.0-12.0

GLOSSARY

Analgesia – drugs and technologies that reduce the ability to feel pain.

Anesthesia – an induced state of temporary loss of consciousness.

Bioavailability – the fraction of a substance (e.g., a drug) that reaches the intended location and is available to have the desired effect.

Cannula (or catheter) – a tube inserted into a body to allow fluid to enter or be drained.

Competency – the ability to effectively perform a particular task in relation to the care, maintenance, or use of animals, while ensuring the animals' welfare is protected as much as possible within the constraints of any approved studies that they are involved in. Focusing on competency rather than training acknowledges that there may be various ways of acquiring the necessary knowledge and skills and emphasizes learning outcomes. See the *CCAC guidelines on: training of personnel working with animals in science* (CCAC, 2015) for more details.

Enteral – a form of substance administration that uses the gastrointestinal tract.

Excipient – an inactive substance that is combined with an active ingredient to generate the final active product (usually a drug).

Exsanguination – the process of draining blood.

First-pass metabolism – a phenomenon of drug metabolism at a specific location in the body which leads to less of the active drug being available at the site of action.

Gavage – a method of force-feeding that uses a tube to deliver a substance to the recipient.

Habituation – a decrease in response to a stimulus after repeated presentations.

Lumen – a cavity within a structure such as an organ or vessel.

Osmolality – the concentration of particles dissolved in a kilogram of liquid.

Osmolarity – the concentration of particles dissolved in a litre of liquid.

Pain – an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage.

Parenteral route – a form of substance administration that does not involve the gastrointestinal tract.

Procedure – the part of the scientific activity specifically related to data collection (research and testing), or hands-on demonstration or interaction with animals (teaching and training). For example, this would not include routine husbandry activities such as cage cleaning.

Protocol author – the person who is ultimately responsible for the work performed under the protocol. Frequently, this person is the primary investigator (researcher), but may also be the course instructor, study

director, or testing lead. The protocol author may delegate tasks to other members of the scientific team (e.g., graduate students, post-doctoral fellows), but must always be considered responsible for the protocol.

Scientific activity – includes all aspects of any research, teaching, training, or testing activities.

Standard operating procedure (SOP) – a written document that describes in detail how a procedure should be carried out.