Canadian Council on Animal Care

guidelines on:

choosing an appropriate endpoint in experiments using animals for research, teaching and testing

The CCAC *guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* were developed by the *ad hoc* subcommittee on endpoints of the CCAC Guidelines Committee:

Dr Ernest Olfert, University of Saskatchewan (Co-chair)

Dr Jag Bhasin, National Research Council (Co-chair)

Dr Richard Latt, McGill University

Dr Eileen Macallum, Warner-Lambert Canada Inc.

Dr Kathie McCutcheon, University of Manitoba

Dr Don Rainnie, University of Prince Edward Island

Dr Michael Schunk, Pasteur Mérieux Connaught

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The Canadian Council on Animal Care (CCAC) is responsible for the oversight of animal use in research, teaching and testing. In addition to the *Guide to the Care and Use of Experimental Animals*, Vol. 1, 2nd Edn., 1993 and Vol. 2, 1984, which lay down general principles for the care and use of animals, the CCAC also publishes guidelines on issues of current and emerging concerns. The CCAC *guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* is the third of this series and has been produced by the CCAC *ad hoc* subcommittee on endpoints.

The purpose of this document is to present guidelines for selecting an endpoint that reduces animal pain and/or distress, while still satisfying the experimental design requirements for objective evaluation when animals are used in biomedical research, teaching and testing. These guidelines are provided to assist animal care committee (ACC) members and investigators in fulfilling their ethical responsibilities in minimizing animal pain and/or distress in experimental protocols.

The refinement of animal use in biomedical research, teaching and testing is a gradual process which is never complete. Nowhere is this more true than in the process of seeking more humane endpoints to animal experiments. Therefore, these guidelines should be used, not as the final word on humane endpoints, but as a guide to the ongoing process of refinement in animal experimentation.

1. INTRODUCTION

The CCAC policy statement *Ethics of Animal Investigation* states:

"Animals must not be subjected to unnecessary pain or distress. The experimental design must offer them every practicable safeguard, whether in research, in teaching, or in testing procedures; ..."

(*Ethics of Animal Investigation*, CCAC, 1989)

The *Ethics of Animal Investigation*, also requires investigators to follow the "Three Rs" of Russell & Burch (1959): Replacement (of animals with other, non-sentient material or with animals of lower sentience); Reduction (of numbers of animals used); and Refinement (of technique, "to reduce to an absolute minimum the amount of distress imposed on those animals that are still used.").

The investigator's ethical responsibilities are clearly stated in this policy statement; however, some important questions need to be answered. How can an endpoint be chosen that satisfies these principles? How can experiments be "refined" through establishing earlier, more humane endpoints to invasive animal experiments, particularly those that may have used death as an endpoint in the past? Where should the line be drawn?

For the purposes of these guidelines, the term "Endpoint" is defined as the point at which an experimental animal's pain and/or distress is terminated, minimized or reduced, by taking actions such as killing the animal humanely, terminating a painful procedure, or giving treatment to relieve pain and/or distress.

There are several types of studies where the death of the animal may in the past have been the endpoint as part of the experimental design, but where this requirement is now questioned. These areas include regulatory toxicology, diagnostic toxicology, acute toxicity studies in research, infectious disease studies, microorganism virulence challenge studies, vaccine efficacy trials, cancer research, and cancer treatment evaluation.

In some research investigations, pain and/or distress is an unwelcome part of the disease or condition being studied (e.g., some models of human diseases such as arthritis or cancer). Also, in some experimental animal use, any pain and/or distress is a side effect to the animal use (e.g., monoclonal antibody production, Freund's Adjuvant use in antibody production). In these latter cases, endpoints are relatively easy to define (e.g., limiting the volume and number of times ascites fluid is collected from a mouse), and a policy statement pertaining to some of these procedures already exists (CCAC, 1991).

2. GENERAL GUIDELINE

In experiments involving animals, any actual or potential pain, distress, or discomfort should be minimized or alleviated by choosing the earliest endpoint that is compatible with the scientific objectives of the research. Selection of this endpoint by the investigator should involve consultation with the laboratory animal veterinarian and the animal care committee.

3. RECOMMENDED PROCEDURES FOR SELECTING AN APPROPRIATE ENDPOINT

The animal in a moribund state may be past suffering (and actually comatose). A moribund animal is one that is close to death and may be comatose or unresponsive to stimuli, exhibit dyspnea or other severe breathing problems, hypothermia, prostration, etc. However, before the animal gets to the point of being moribund, detailed observations of the animal can help to set an earlier endpoint and thereby reduce the actual cost to the animal, in terms of pain and/or distress.

There are several considerations in defining an appropriate endpoint in a given experiment. These all depend on an objective determination of any deviations from an animal's "normal" state, followed by a correlation of these changes with degrees of discomfort, pain and/or distress. Some of these considerations are:

- making the appropriate observations of the animals (of their behavior, physiology, etc.);
- assigning objective values to the observations of animal behavior and physiology;
- determining which observations are the most significant indicators of pain and/or distress in the specific circumstances of the research;
- determining which observations are the most significant predictors of further deterioration in the animal's condition, and then identifying the earliest point at which those signs appear;
- meeting the scientific demands for an objectively measured and significant endpoint;

- clearly defining the information/data being sought in the experiments.

a. **Making the Appropriate Observations of the Animals**

Morton & Griffiths (1985) laid the groundwork for developing a set of observations for assessing pain, distress and discomfort in laboratory animals, based on evaluating five aspects of an animal's condition:

- changes in body weight (and related changes in food and water intake);
- external physical appearance;
- measurable clinical signs (e.g., changes in heart rate, in respiratory rate, and in their nature);
- changes in unprovoked behavior; and,
- behavioral responses to external stimuli.

In each of these categories, a rating system of 0 (normal or mild) to 3 (severe changes from normal) was proposed. The cumulative rating obtained by adding the score for each category indicates increasing deviation from normal in the animal, which can be interpreted as an indication of increasing pain and/or distress. A total score can be identified, at which point the animal's pain and/or distress will be terminated or alleviated. The observations listed by Morton & Griffiths (1985) are only some of the observations that may be required to determine an endpoint in specific research models.

In addition to behavioral and physiological measurements, a number of hormonal indicators have been used to measure stress/distress in animals (catecholamines, corticosteroids, prolactin, tumor necrosis factor, interleukins) (NRC, 1992). There is little consensus on which of the many possible hormonal changes truly measure distress or pain. Nevertheless, if blood sampling is part of the research protocol the investigator should consider analysis of the blood collected for some of the stress indicators. This information would be a valuable addition to the data, and could provide a useful correlation to any noted behavioral changes.

It is important to be aware of the characteristic behavior of the species under observation. Animals such as non-human primates, rodents, rabbits and some livestock may not show many behavioral changes even when in severe pain. In addition, strain variations must be considered. It is, therefore, imperative that the investigator/observer understand these characteristics prior to setting endpoints.

In practical terms, the animal should first be observed carefully without disturbance. Its appearance and posture should be observed, and a determination made about whether its behavior is normal or abnormal. The animal's reaction to an external stimulus could also be checked (e.g., noise, change in light level) before directly approaching the cage to handle the animal. Depending on the species, the animal should be handled for a clinical examination. During this time, clinical signs are noted and measurements made (including weighing the animal). Any lesion or abnormality is also evaluated at this time (e.g., size of tumor). During the clinical examination, "provoked behavior" can also be assessed.

The use of observational "checklists" for scoring the animal's condition in a study provides an objective basis on which decisions about endpoints can be made. The advantages of checklists are that specific observations are not overlooked or taken for granted. The other real advantage is that such checklists help improve observational skills, particularly with the smaller laboratory animals where some of the conventional clinical observations made on larger animals are not readily determined (e.g., temperature, heart rate, respiratory rate). However, checklists do not cover all abnormalities or observations and thus are only a useful way to record certain findings. They cannot replace a thorough examination of the animal.

Morton & Griffiths (1985), and Sanford, *et al*. (1986), focussed attention on the need for more objective assessments of the pain and/or distress that may occur in an animal in the course of biomedical research. Efforts at refining the scoring of clinical signs have continued since 1985 (Morton, 1990; Morton & Townsend, 1995; Workman, *et al*., 1998, for example). More detailed observational checklists have been proposed for some specific scientific procedures, including: endotoxin administration in mice (Townsend & Morton, 1994); monoclonal antibody production in mice (Morton, 1997); cancer research (Workman, *et al*., 1998).

In addition to general signs of pain and/or distress, there are specific signs and symptoms related to the condition being studied. For most animal models of disease, information on organ system(s) affected, specific symptoms, progression of symptoms, time course of the disease condition, and expected lesions, is available from the comparative medicine and general veterinary literature (e.g., Armed Forces Institute of Pathology [AFIP] Fascicles). Such specific signs and symptoms must also be used in the overall evaluation of the animal's condition.

The result of previously published information or pilot studies, as well as information about the pharmacology or chemistry of the compound being tested should be used to predict any potential adverse effects on the animals. Implicit in this is a requirement that a complete literature search has been conducted.

b. **Scoring of Significant Physiological Observations to Select and Refine Endpoints**

Information on the general signs of pain and/or distress for the various animal species commonly used in biomedical research are readily available (CCAC, 1993; Sanford, *et al*., 1986; Wallace, *et al*., 1990). Of these signs, significant weight loss may be one of the more important signs of deterioration in the animal's condition (reflecting a change in food and water consumption). Weight loss in these circumstances must always be compared to the appropriate control animal. Body condition scoring charts, which are available for domestic livestock, and dogs and cats, may be useful for evaluating chronic weight loss in experimental animals in specific studies.

Hypothermia can also be an important indicator of a deteriorating condition in the animal, when it occurs in specific disease or toxic states. Wong, *et al*. (1997) found that a decrease in body temperature (below 32^{o}C) of mice infected with an influenza virus was predictive of mortality. Soothill, *et al*. (1992) found that in mice infected with bacteria, hypothermia of 34^{o}C was predictive of mortality. Thus, in specific experimental cases, the point at which the body temperature of

an experimental animal drops to a specified temperature could be set as the endpoint at which euthanasia is recommended.

The report of a committee of the British Laboratory Animal Science Association (Wallace, *et al*., 1990) includes an assessment of the severity of some procedures commonly performed on animals in the course of biomedical research. A number of other publications are also available to help identify the signs and symptoms of experimental animal pain and/or distress (Barclay, *et al*., 1988; Baumans, *et al*., 1994; British Veterinary Association, 1985; Butler, *et al*., 1985; De Castro Costa, *et al*., 1981; Flecknell, 1994; Keefe, *et al*., 1991; Wolfensohn & Lloyd, 1994; Soma, 1987).

c. **Identifying Significant Behavioral Indicators of Pain and/or Distress**

For any given animal model, there are many possible observations and measurements that can be made. Determining which are the most important or significant indicators of the condition of the animal, or perhaps more importantly from the investigator's perspective, which are the most important indicators of an irreversible deterioration in the condition of the animal, is not an easy task. Studies by Butler, *et al*. (1985) and De Castro Costa, *et al*. (1981), dealing with the adjuvant-induced arthritis model in the rat, provide insight into the difficulties in finding/choosing the correct observations. Although changes in the frequency of several behavior patterns were found (decreased rearing, running, eating, drinking and climbing; increased resting, freezing, scratching), the conclusion was that of all these changes in behavior, increased scratching was the most significant behavioral change that tied in with developing arthritis, indicating chronic pain.

Such behavioral evaluations (Butler, *et al*., 1985; De Castro Costa, *et al*., 1981) are research projects in themselves, involving many hours of technical time with expensive monitoring and analytical equipment. It may be unrealistic to demand a similar degree of preliminary evaluation each time an animal-based research program is initiated where the potential for pain and/or distress is high (mice in a liver cancer research program, for example). Nevertheless, conducting a pilot study to establish the observational criteria to be used to set endpoints may be a very useful exercise, particularly at the onset of a research program.

4. **USING PRELIMINARY OR PILOT STUDIES TO DETERMINE THE APPROPRIATE ENDPOINT**

The use of preliminary or pilot experiments can be very useful in determining endpoints (Olfert, 1995; Browder, 1995, Everitt & Griffin, 1995), particularly when the effects of the treatment on the animals are unknown. A pilot study, using a small number of animals, may help determine the morbidity, time course of effects, and frequency of observations required to set an earlier endpoint. A pilot study can also provide an indication of the variance of responses between treatment groups, which can then be used to estimate group sizes more accurately for the main study. Conducting a pilot experiment also provides the opportunity for all persons to become experienced with the expected signs and symptoms.

5. **DETERMINING THE REQUIRED FREQUENCY OF ANIMAL OBSERVATIONS**

Guideline: Based on previous knowledge, during critical periods of the experiment and at the onset of adverse reactions, a minimum of two or three observations should be made daily. The frequency of the observations should increase depending on the potential for increasing pain and/or distress.

The CCAC *Guide to the Care and Use of Experimental Animals* states that normal, healthy experimental animals should be observed at least once a day (CCAC, 1993). However, once an animal is in a potentially critical period with respect to impairment, more frequent observations must be made.

The frequency with which affected animals should be observed must be determined for each study. The required frequency of, and interval between, observations will depend on the expected or known time course of the condition. For example, in some experimental infections/toxicity cases, much more frequent (e.g., hourly) observations may be necessary to identify the point at which the selected "endpoint" has been reached and the animal's pain and/or distress must be terminated (Morton & Townsend, 1995; Townsend & Morton, 1994). Scheduling the study so the critical period for the animals occurs during normal working hours (when the lights are on in the animal room) may aid in ensuring that appropriate observations are made.

The appropriate monitoring schedule should be established by the investigator in consultation with the veterinarian, and approved by the ACC in its consideration of the protocol.

6. **DEFINING RESPONSIBILITY FOR ANIMAL OBSERVATION**

Guideline: With respect to setting and determining endpoints, the responsibility(ies) of each individual should be clearly defined, and a clear chain of reporting established. The ultimate authority for euthanasia must rest with the veterinarian, supported by the animal care committee.

It is essential that an appropriately trained and experienced person with the authority to euthanize, or order the euthanasia of, animals be constantly available during the study. Any observations of unusual behavior or signs of pain and/or distress should be reported immediately to that authority. This is important also for dealing with unanticipated adverse effects on the animals in an invasive study. The authority to euthanize animals that have reached the endpoint, or are experiencing severe unanticipated adverse effects should be clearly defined before the study begins. The ultimate authority must remain with the institutional veterinarian (CALAM, 1990).

7. **TRAINING OF PERSONNEL IN CLINICAL ANIMAL OBSERVATIONS**

Guideline: All persons responsible for making observations of the animals, from which an endpoint will be determined, should be competent in evaluating the normal physiology, behavior and body condition of the animals under observation, and the anticipated specific changes from normal.

It is the responsibility of the principal investigator or study director (and ultimately

the ACC) to ensure that all persons involved have the training appropriate to their responsibilities for animal observation. The training should be documented.

8. THE ROLE OF THE INSTITUTION'S ANIMAL CARE COMMITTEE IN SETTING ENDPOINTS

The role of the ACC is vital in establishing the structure to ensure that the earliest endpoints consistent with producing reliable data are considered, identified, and used. This is a joint responsibility with the investigator and the veterinary staff. ACCs should obtain information on the following questions, to ensure that an appropriate endpoint will be in place:

- what are the scientific justifications for using the proposed endpoint?
- what is the expected time course for the animals, from initial treatment to first signs of pain/distress, to the death of the animal, based on previous information with the specific model under study?
- when are the effects to the animal expected to be the most severe?
- if the course of the disease and expected signs of the adverse effects are unknown, could an initial (pilot) study, under close observation by the investigator and/or laboratory animal veterinary staff, answer these questions?
- has a checklist of observations, on which the endpoint will be based, been established?
- who will monitor the animals (identify all responsible) and keep records?
- has a clear chain for reporting observations been established?
- what will be the frequency of animal observations: a) during the course of the study; and b) during critical times for the animals?
- do the investigators, animal care and technical staff have the training and expertise to monitor the animals adequately?
- what provisions have been made to deal with any animals that show unexpectedly severe signs and symptoms?
- for toxicological studies, have existing toxicological data been evaluated?

9. GUIDELINES FOR SELECTING APPROPRIATE ENDPOINTS IN SPECIFIC AREAS OF BIOMEDICAL RESEARCH AND TESTING

For some specific areas of biomedical research and testing, more detailed guidelines for selecting an appropriate endpoint are provided in this section. Endpoint guidelines for animals used in monoclonal antibody production, cancer research, toxicology, infectious disease studies, and pain research are included. However, these are not the only areas where specific guidelines can be developed using the expertise of the attending laboratory animal veterinarian and the oversight of the ACC.

a. Monoclonal Antibody Production in Rodents

Guideline: That as long as rodents continue to be used for monoclonal antibody production, the following endpoints be established:

- the increase in body weight due to the accumulation of ascites fluid in the abdomen and/or tumor growth should not produce pain and/or distress to the animal;
- depending on the condition of the mouse, a maximum of two taps of the ascites fluid are allowed, with the second tap being a terminal procedure. Ascites fluid taps should be done under general anesthesia.

It is widely recognized that the production of monoclonal antibodies (mAb) in rodents raises important concerns regarding the potential for severe pain and/or distress in the animals. In view of the rapidly developing and widely available *in vitro* methodology to produce monoclonal antibodies, the continued use of animals for mAb production is increasingly difficult to justify. As a result several countries have taken the position that alternative *in vitro* methods should be used for monoclonal antibody production, and that the mouse ascites method could be justified (to an ACC) only under special circumstances (e.g., when the *in vitro* method had failed to produce the required antibodies). Some countries recently acted to ban the mouse ascites method of mAb production (Shalev, 1998). The CCAC supports the use of *in vitro* methods for mAb production wherever possible.

At several stages of mAb production; from intraperitoneal injection of the priming agent, to the accumulation of ascites tumor fluid, and to collection of the ascites fluid, the animals may experience pain and/or distress. Therefore, when monoclonal antibodies are produced in vivo, clearly defined limits and endpoints placed on the application of the various manipulations, and close monitoring of the condition of the animals are required to minimize the potential for distress.

Following injection of hybridoma cells, routine care should include daily observations by appropriately trained staff for the first week (approximately), and before ascites fluid accumulation is evident (as indicated by the swelling of the abdomen). Any observations of unusual behavior or symptoms during this time should be addressed in a timely fashion. Pertinent signs of distress include: decrease in activity; hunched appearance; ruffled hair coat; respiratory distress; weight loss (which may be masked by the accumulating fluid in the abdomen). Once the ascites fluid accumulation has resulted in obvious abdominal swelling, the condition of the animal must be assessed at least twice every 24 hours at regularly spaced intervals.

The production of mAb in rodents provides an excellent example of how both **replacement** of mice with *in vitro* methods, and **refinement** of procedures with establishment of endpoints can reduce the distress experienced by the animals. For a review and bibliography of antibody production and alternative methods, see Smith, *et al*., 1997. Refinements and endpoints for mAb in rodents are discussed by Gillette, 1987; Marx, *et al*., 1997; McGuill & Rowan, 1989; Mueller, *et al*., 1986; and Workman, *et al*., 1998.

b. **Cancer Research**

Guideline: For all cancer research in animal models, endpoints should be established that minimize the potential for pain and/or distress in the animals.

Some recommended endpoints are:

- the tumor mass should not proceed to the point where it significantly interferes with normal bodily functions, or causes pain or distress due to its location (solid tumors);
- weight loss exceeding 20% of the body weight of a similar normal animal (taking into account the tumor mass);
- ulceration/infection of the tumor site;

- invasion of surrounding tissues by a localized tumor;
- persistent self-induced trauma.

Tumor burden should not exceed 5% of the animal's normal body weight for routine tumor passage or 10% for animals involved in therapeutic experiments (10% typically represents a subcutaneous flank tumor diameter of 17mm in a 25g mouse or 35mm in a 250g rat). Calibration curves should be established as part of the characterization of any new tumor system (Workman, *et al*., 1998)

One of the scientific concerns that has been expressed about arbitrary assignment of an endpoint in cancer therapy studies is that early euthanasia may alter longevity or survival data which are important indicators of "successful" treatment. For example, the "successful" treatment of cancer in rats which resulted in them living a month longer, might be masked by early euthanasia based only on clinical observations. Workman, *et al*. (1998) recommend that the required information on response to therapy be obtained by tumor regrowth delay, clonogenic assay following tumor excision or an appropriate surrogate endpoint. In such cases finding the signs of disease and distress that point to an irreversible deterioration in the animal, is important.

Redgate, *et al*. (1991) in their examination of a brain tumor model in a rat (9L gliosarcoma in Fischer 344 rats), concluded that a weight loss period of more than six days had a high correlation with irreversible progression to death. In this model then, an endpoint that satisfied the scientific concerns could be established at the end of a six day period of consecutive weight loss, which in this case was about ten days before death of the animals.

A thorough discussion of the many welfare factors that must be considered when cancer research is undertaken is contained in the publication of the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) (Workman, *et al*., 1998), providing valuable information for investigators, and ACC. Additional criteria for selecting the endpoint in cancer research and in toxicology have been proposed by Montgomery (1987, 1990), Redgate, *et al*. (1991), and Tomasovic, *et al*. (1988).

c. Toxicological Studies and Toxicity Testing

i. Acute toxicity testing

Guideline: Before a protocol that includes safety/efficacy/toxicity testing with death as an endpoint for regulatory purposes can be accepted by the institution's animal care committee, there must be clear, written documentation obtained by the investigator from the appropriate regulatory agency that the proposed test is a necessary part of the submission for licensing/approval. The investigator must also demonstrate to the animal care committee that an alternative *in vitro* test will not be acceptable to the regulatory agency, and that this testing has not been previously done elsewhere.

For most toxicological studies, the investigator is interested in the primary or secondary interaction(s) of the compound with the body's cells and tissues, and not the tertiary effects (such as dehydration, anorexia, etc.) which may

cause the death of the animal. Thus, from a scientific viewpoint, it is important to collect as much data as possible during the early stages of the clinical effects. Any data collected from a moribund animal, or any unobserved animal death, represents lost information on the compound studied (Toth, 1997).

Guidelines on Acceptable Testing Standards: Toxicity tests should be done according to the guidelines of the Organization for Economic Cooperation and Development (OECD), International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Health Canada (HC), or US Food and Drug Administration (FDA), using the minimum number of animals possible, and with all possible consideration for the relief of animal pain and/or distress.

When initiating toxicological studies, there is usually little information available on the test compound. However, there may be data on the class of compounds, or compounds with similar chemical structures that can be used to guide the experimental design. Establishing doses and effects after single or repeated administration, toxicity and/or exaggerated pharmacological responses are the purpose of these studies.

The use of pilot experiments in determining endpoints (Olfert, 1995; Browder, 1995; Everitt & Griffin, 1995) are of particular value when dealing with compounds of unknown effects. Using a small number of animals and beginning with low-end doses, the morbidity, the time course of effects and the frequency of observations required to set earlier endpoints could be determined. Data collected in pilot studies that support earlier endpoints in specific toxicity studies, if provided to the regulatory agencies, could also assist the regulatory agencies in validating and approving earlier endpoints. As noted previously, conducting a pilot experiment also provides the opportunity for all persons to become experienced with the expected particular signs and symptoms.

Dose range finding studies are widely used in pharmaceutical toxicity research programs. Doses are selected based on pharmacology, pharmacokinetic characteristics, and previous experience with the class of drug under investigation. These studies also give an indication of potential target organs, and unexpected adverse effects that may be addressed in subsequent definitive studies, and significantly reduce the numbers of animals required.

The pharmaceutical industry initiative in the form of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) was organized to make recommendations on ways to achieve greater harmonization in the interpretation and application of technical guidelines and requirements for product registration in order to reduce or obviate the need to duplicate the testing carried out during the research and development of new medicines. There was agreement after the first ICH meeting (1991), and subsequent issuance of guidelines on single dose and repeat dose toxicity tests, that the LD_{50} (Lethal Dose 50) be abandoned for use in pharmaceuticals. Although an assessment of acute toxicity is required, a calculated LD_{50} is not (Federal

Register, 61(166), Single Dose Acute Toxicity Testing for Pharmaceuticals; Revised Guidance).

Similarly the OECD discourages the use of the classical LD50 test, and recommends alternatives that reduce animal numbers and use morbidity rather than mortality as the endpoint (OECD, 1987). The Interagency Research Animal Committee of the US (IRAC, 1993) Recommendations on LD_{50} Testing state:

1. "The Classical LD_{50} test should only be conducted when specifically justified for reasons of scientific necessity and approved by the institutional animal care and use committee (IACUC).
2. Toxicity testing procedures based on the principles of reduction and refinement (such as the Limit test) should be used until alternative test methods become validated."

The choice of toxicity tests should attempt to meet the collective requirements of the many national regulatory agencies to minimize the need for repeat studies for a specific country. In each case, the client must check with the regulatory agency(ies) to determine what testing is required.

Recalculation of previously published toxicological data should also be considered, to ensure that ED_{50} (Effective Dose 50) or LD_{50} values are accurate (Irvine, *et al.*, 1992).

Acute toxicity studies in fish. Acute toxicity tests with fin fish have been primarily for environmental protection from industrial effluents and the risk assessment of new pest control agents for agriculture and forestry. The concern has been primarily one of safety and there has been little interest in the pathology associated with the toxicity.

If no information is available on the potential toxicity to fish of the material to be tested, a range finding study should be conducted with a minimal number of fish. On the appearance of peracute clinical signs of irritation or toxicity, the fish should be immediately removed to fresh water. If there is no substantive reduction in the clinical signs within five minutes of transfer to fresh water, the fish should be euthanized.

Only when a concentration of the test materials which does not cause peracute clinical signs has been identified should a more substantial number of fish be exposed to this and lower concentrations to determine the acute toxicity. The minimum number of dilutions, minimum replications and minimum number of animals per dilution consistent with the accuracy and confidence interval required should be used.

In situations involving the monitoring of an effluent far exceeding required safety limits, the tests should be limited to a pass-fail approach rather than quantitating the dilution which produces a specified endpoint (e.g., LC_{50} - Lethal Concentration 50).

During initiation of exposure, fish should be observed continuously for the first 45 to 60 minutes. Subsequent observations should be made on a

geometric progression basis (e.g., 1.5h, 3h, 6h and 12h) and then a minimum of twice daily for the duration of the study. Frequency of observation should increase with the appearance of any clinical signs. On the appearance of severe clinical signs which are inconsistent with survival (e.g., gaping, forceful collisions with the side of the tank or aquarium, hyperactivity) the fish should be removed to fresh water. If the clinical signs persist without interruption for more than five minutes or are followed by loss of normal posture or position in the water column in the fresh water, the fish should be euthanized.

Animal Care Committee oversight in acute toxicity studies with death as an endpoint. In addition to the points already raised, some additional principles that will assist the institutional ACC in fulfilling its obligations when death as an endpoint is part of a protocol have been delineated by Hamm (1995). As noted above, written documentation justifying the need for death as the endpoint must be provided to the ACC prior to initiation of the study. The written documentation should include assurances that alternate earlier endpoints were considered, and the reasons why analgesia cannot be provided. The frequency of monitoring by trained personnel, identification of signs of distress or illness, and treatment of animals found with abnormalities, should be provided to the ACC and be part of the conditions for experiment. Complete documentation of all observations and actions taken should be retained and be available to the ACC and attending veterinarian (Hamm, 1995).

ii. **Chronic toxicity studies and studies in aging**

Guideline: Before a protocol that requires holding animals to an age close to or beyond the median survival age specific to the species or strain (e.g., chronic toxicity studies, carcinogenicity testing, or aging studies) is approved by the institution's animal care committee, the investigator in collaboration with the veterinary staff must establish the endpoint criteria for euthanasia of the animals, the persons responsible for monitoring the animals' condition, and the authority of the persons who will make the decision to euthanize.

Some of the most controversial areas concerning endpoints are in chronic toxicity or aging studies, especially the carcinogenicity studies required for New Drug Applications (NDA). In these studies, animals may receive the test compound for up to two years by the route(s) of administration to be used by humans. Most often this is oral (gavage or mixed in the diet), but other routes may be used. The conflict occurs when investigators are concerned with survival rates. To be considered a negative study, the US Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) require that survival be at least 50%. The investigator in collaboration with the veterinary staff can establish a criteria for euthanasia of animals. This should be defined prior to the start of the study, and addressed on a case-by-case basis.

Investigators should familiarize themselves with the current ICH recommendations for specific testing requirements prior to drafting their research/testing protocols.

The housing and care of aging animals requires special considerations for their comfort and well-being. Rats over 18 months of age, for example, tend to be large and develop weight related problems such as foot pad lesions,

obesity, and tumors. The use of solid bottom cages, along with appropriate bedding material will improve the comfort level for the animals. Aged animals may also require special attention regarding the way food and water are presented to them.

d. **Pain Research**

Because it is an inherent aspect of studies of pain in humans and animals that some pain must be produced, such experiments raise special ethical concerns. This difficulty was recognized early, as the study of pain became a more distinct discipline. The following guiding principles have been extracted from the literature cited in the accompanying discussion in this section.

Guidelines:

- the animals should be exposed to the minimal pain necessary for the purposes of the experiment;
- the duration of the pain must be as short as possible and the number of animals involved kept to a minimum;
- threshold levels of pain stimuli rather that supra-threshold levels should be used whenever possible;
- if models of acute pain, or acute pain tests are being used, where the pain is not terminated by the animal's reaction, but may extend beyond the time necessary to obtain results, the pain should be terminated as quickly as possible;
- tests other than avoidance tests are strongly discouraged;
- animal models experiencing chronic pain should be provided with adequate analgesia at all times. Exceptions to this should be restricted to those times justified to the institutional animal care committee by the investigator with evidence that the analgesics will interfere with the aims of the investigation.

The International Association for the Study of Pain (IASP) *Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals* (Zimmermann, 1983) contain seven points relating to the justification of the experiment, and to the potential severity and duration of pain produced in the experimental animal. These seven points are:

1. "It is essential that the intended experiments on pain in conscious animals be reviewed beforehand by scientists and lay-persons. The potential benefit of such experiments to our understanding of pain mechanisms and pain therapy needs to be shown. The investigator should be aware of the ethical need for a continuing justification of his investigations.
2. If possible, the investigator should try the pain stimulus on himself; this principle applies for most non-invasive stimuli causing pain.
3. To make possible the evaluation of the levels of pain, the investigator should give a careful assessment of the animal's deviation from normal behavior. To this end, physiological and behavioral parameters should be measured. The outcome of this assessment should be included in the manuscript.
4. In studies of acute or chronic pain in animals, measures should be taken to provide a reasonable assurance that the animal is exposed to the minimal pain necessary for the purposes of the experiment.

5. An animal presumably experiencing chronic pain should be treated for relief of pain, or should be allowed to self-administer analgesic agents or procedures, as long as this will not interfere with the aims of the investigation.
6. Studies of pain in animals paralyzed with a neuromuscular blocking agent should not be performed without a general anesthetic or an appropriate surgical procedure that eliminates sensory awareness.
7. The duration of the experiment must be as short as possible and the number of animals involved kept to a minimum."

For animal models of acute pain, careful design and conduct of the experiment will mean that results are obtained using the fewest numbers of animals necessary. An appropriate endpoint needs to be defined. The type of pain test proposed will influence the selection of the endpoint.

As noted in the IASP statement, it is incumbent on the investigator to be thoroughly familiar with the behavior of the animal species being used, and to document behavioral and physiological changes being observed in animals subjected to stimuli causing pain. This is also important so that unexpected severe levels of pain are recognized if they occur. Some of the tests for acute pain have been extensively used on humans, and those experiences should guide investigators regarding the nature of the pain produced.

Many pain tests use a threshold pain level, one that triggers avoidance or withdrawal behavior in the animal. If the stimulus intensity is above the threshold needed to elicit a response, special care must be taken in its application. Threshold levels of pain stimuli rather than supra-threshold levels should be used whenever possible. Franklin & Abbott (1989) indicate that in many instances supra-threshold pain stimuli are unnecessary.

In some pain tests the animals can terminate the painful stimulus by their action. For example, in the tail flick test, and the hot plate test, the animal's first response is taken as the endpoint, and the animal is removed from the stimulus. In other tests (e.g., the acute tissue injury-inducing tests, which include the writhing test and the formalin test), the painful stimulus presumably goes on longer than the test period, and the animals cannot escape from it. Using avoidance tests would be preferable to using tests where the pain continues after the results are obtained. If models of acute pain, or acute pain tests are being used where the pain is not terminated by the animal's reaction, then the pain should be terminated as quickly as possible. This may require humanely killing the animals as soon as the test is completed (e.g., the writhing test), or administering analgesic drugs.

Animal models of chronic pain are of particular concern, since the cost to animals where chronic pain is induced (adjuvant-induced arthritis in rats, for example) can be very high. It is well-known that such disease states in humans are often accompanied with severe, unremitting pain and distress. Unless particular attention is paid to the animal models used in such a study, pain and/or distress could easily extend beyond that necessary for the purposes of the research. Franklin & Abbott (1989) suggest that pain produced in adjuvant-induced arthritis in rats approaches an intensity such that, based on the rating proposed by

Morton & Griffiths (1985), relief (analgesics) should be given. The principal investigator, the institutional veterinarian, and the ACC should ensure that such animals are afforded every consideration for limiting or easing their discomfort and pain.

All animals with chronic pain should receive special attention not only to relieve their pain, but also with respect to their husbandry and housing. A great deal can be done, beyond the routine care given to normal laboratory animals, to make these special animal models comfortable (e.g., gentle handling, improved access to food and water, housing in solid bottom cages with deep, soft bedding). The expertise of the laboratory animal veterinarian and the animal health technicians should be consulted, and should be integral to the discussions of these aspects by the ACC.

e. **Infectious Disease Studies, Vaccine Trials, etc.**

Guideline: For all infectious disease research, including virulence tests in animal models, endpoints should be established that minimize the potential for pain and/or distress in the animals.

Some studies in infectious disease (e.g., tests to establish the virulence of an infectious organism) are still being conducted with mortality as the proposed endpoint (also referred to as the Rodent Protection Test). The use of PD_{50} (Protective Dose 50) tests in mice may be required when anti-infective studies are done.

Soothill, *et al.* (1992) found that in mice infected with bacteria, hypothermia of 34^{o}C was predictive of mortality. Siems & Allen (1989) recommended that the endpoint in a disease model (chronic infection with systemic *Candida albicans*) be set at (among other measurements) the point when the animals lose more than 20% of body weight, or when the body temperature drops more than 4^{o}C; both of which are easily monitored. The magnitude of these changes from normal would also give a maximum score on the Morton & Griffiths (1985) proposal, indicating severe negative effects on the animal.

The UK Rodent Protection Test Working Party considered the following as some of the general signs exhibited by rodents with systemic infections; ruffled or "spikey" fur, weight loss, ocular discharge, lethargy, hunched back, ataxia, tremor, hypothermia, cyanosis (Acred, *et al.*, 1994). Recommended clinical scoring systems useful for certain bacterial Rodent Protection Tests (RPT), for the *Candida albicans* RPT, and for the *Herpes simplex* RPT were provided by the RPT Working Group (Acred, *et al.*, 1994).

The use of observational checklists with sufficiently frequent observation times (see part 5 Determining the Required Frequency of Animal Observations) will assist the investigator in detecting the point at which there are signs of progressive deterioration of the animal's condition leading to death if no action is taken (Acred, *et al.*, 1994).

f. **Specific Animal Models with the Potential for Significant Levels of Pain and/or Distress**

Guideline: Any pain and/or distress, or deficits in function that negatively affect

the animal's well-being, not scientifically "necessary" for the study, should be alleviated or minimized. Cost or convenience should not deter from this. Further, as soon as the study is complete, the pain and/or distress should be alleviated through treatment or euthanasia.

Some animal models have inherent or induced functional deficiencies with the potential for pain and/or distress. There is a responsibility to attend to the special needs of these animals, beyond the care provided to normal animals. Animal models with inherited deficits (distressed phenotypes), transgenic animals, ischemia or seizure models, stereotaxic manipulations, are some examples of the kinds of laboratory animals for whom this recommendation is made.

10. SUMMARY

All those involved in the use of animals for research, teaching and testing; ACC members, investigators and their research staff, the laboratory animal veterinarian and the animal health technicians, have responsibilities for the humane care and use of experimental animals. In working with investigators to establish appropriate endpoints, the ACC should ensure that the appropriate criteria are used by the principal investigator to determine the endpoint. Through the use of observational checklists and animal condition scoring systems, the most objective, and more humane, endpoints can be identified. Responsibilities for observing and monitoring the animal's condition must be clearly delineated. Persons involved in establishing and validating endpoints in invasive experiments are encouraged to present and publish these data, to support efforts at continually refining the animal use practices that occur in biomedical research.

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APPENDIX A: SPECIES SPECIFIC SIGNS OF PAIN AND/OR DISTRESS

1. UNDERSTANDING NORMAL ANIMAL BEHAVIOR

Becoming familiar with the appearance, behavior, and physiology of the normal animal is of primary importance in assessing its well-being. It is also important to be aware of the variation in appearance, behavior and physiology depending on the age, strain, sex and time of day. There may also be seasonal differences in some species.

In addition to the normal behavior patterns of the individual animal (posture, grooming, feeding, sleeping, urinating, defecating, etc.) its interactions with cage mates, and its awareness of the surrounding environment should be noted. Included in the animal's normal behavior is the normal response to being handled and examined.

Most normal, healthy laboratory animals have a smooth, clean, well-groomed hair coat. Lack of grooming is usually a reliable indicator of dysfunction. Body condition can be assessed from observing the shape and posture of the animals.

Normal animals eat and drink, and void waste products, on a daily basis. An understanding of the normal food and water consumption, and the nature and amount of urine and feces produced daily are an important aspect of the knowledge of the normal healthy animal. The behavior of animals when eating or drinking should also be noted.

In general, most healthy small laboratory mammals are active, alert and inquisitive when they are approached. Normally any disturbance of the animals should produce a response. When handled the animals should feel warm to the touch.

For a more detailed physical examination, small rodents and rabbits will have to be handled and restrained. The normal reaction of these animals to handling should be understood, so that unusual or abnormal reactions are noted.

2. RECOGNIZING AND ASSESSING SIGNS OF PAIN AND/OR DISTRESS IN LABORATORY ANIMALS

One of the major problems in recognizing the signs of pain and/or distress in the laboratory rodents is the small size of the animals. Some of the clinical observations made in larger animals, to assess their health and well-being (e.g., temperature, pulse and respiration) are not easily accomplished in laboratory rodents. Thus it becomes even more important to assess the behavior of these animals for any deviations from normal behavior that may be signs of pain or discomfort. To do this both the individual animal, and its behavior in the group environment must be considered.

There is tremendous variation in the behavioral and physiological responses to pain and/or distress in animals, from species to species, and within a species there are individual, sex, and age differences. The signs suggestive of acute pain, and those of chronic pain, should be understood by all persons responsible for monitoring the animals during the research. Signs of chronic pain or distress are often more insidious, and careful observation is required to detect the changes in an animal's

appearance and behavior which indicate a deterioration in the condition of the animal. Observation of the animals should be done frequently, since signs of pain and discomfort are not constantly present.

Depending on the organ system(s) affected, there are some specific behavioral and physiological signs that can be used to assess the condition of the animal. For example, if the respiratory system is affected, the rate and nature of the respiration will change. Change in the nature and amount of feces (e.g., diarrhea) may indicate an intestinal effect. For a detailed listing of the potential signs of disease, pain and/or distress in relation to body systems affected, the reader is referred to the references noted below. The presence or absence of these specific signs should be recorded.

3. INFORMATION SOURCES ON SIGNS OF PAIN AND/OR DISTRESS IN EXPERIMENTAL ANIMALS

The Canadian Council on Animal Care (CCAC) *Guide to the Care and Use of Experimental Animals*, Vol. 1, 2nd Edn., 1993 contains several sections that provide information on the normal behavior of animals used in biomedical research, teaching and testing, and on the signs of pain and/or distress in these species. The reader is referred to these sections; specifically to Chapter X - Control of Animal Pain in Research, Teaching and Testing, Part E, pages 117-121, and to portions of Chapter VI - Social and Behavioral Requirements of Experimental Animals.

Additional selected publications that present sections on the signs of pain and/or distress in a number of animal species used in biomedical research, teaching and testing include *:

BAUMANS, V., BRAIN, P.F., BRUGERE, H., *et al*. (1994). Pain and distress in laboratory rodents and lagomorphs. Report of the FELASA Working Group on Pain and Distress. *Laboratory Animals* **28**(1):97-112.

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* This list will be updated on a regular basis.

APPENDIX B: SIGNS OF PAIN AND/OR DISTRESS IN FISH IN TOXICOLOGY

1. OBSERVING FISH BEHAVIOR

The following points are important in ensuring that observation of the fish occurs without missing important observations, or inadvertently altering normal behavior or biasing results:

a. Lighting

Many fish species "prefer" low light intensity, dark backgrounds or both in order to "feel" secure (as indicated by a reduction in what could be called anxious or nervous behavior). It is important that the investigator achieve an acceptable compromise between adequate lighting and background color combination which will permit reliable observations by research personnel while causing minimal distress to the test animal. A combination which often works well is shielded lighting located directly over the tank or aquarium combined with no or very low intensity room lighting. This accomplishes two goals: observer eye accommodation to low light; and reduced visibility of the observer to the fish. If the walls or portions of the wall of the tank are transparent, one must keep in mind that if the differential between the light intensity inside the tank (high) and that outside the tank (low) is sufficiently great the transparent areas of the wall act as mirrors and fish with strong territorial behavior will respond as though defending their territory and may not feed normally. The investigator should also keep in mind that the ability to make detailed observations in low light intensity is affected by age of the observer and by certain medications.

b. Comfort of the Observer(s)

The observation environment should be sufficiently comfortable for the research personnel so that they conduct their responsibilities thoroughly and in an unbiased manner (i.e., it must not be easier to observe one experimental unit than another).

c. Planes of Viewing the Fish

The unit for housing the fish (e.g., tank or aquarium) must be suitable for the species and for the observations required. If a change in the posture of the fish in the water column is an anticipated clinical sign (particularly a head or tail down attitude), it is important to be able to view the fish from the side, as a much more pronounced change in posture is required before it can be detected by observation from the top of the tank. If clinical signs such as respiration rate and depth of respiratory movements are to be documented, these are often best observed from the vertical plane of the fish (i.e., directly above or below the fish) for observing opercular movements, or the horizontal-lateral or horizontal-frontal plane of the fish for observing mouth and opercular movements.

d. **Observer's Clothing**

Individuals making observations should avoid wearing white or light colored clothing (e.g., white lab coats) as many of the fish species used in research are sufficiently "wild" or undomesticated that white remains an alarm signal.

2. CLINICAL OBSERVATIONS SPECIFIC TO FISH

The five aspects of an animal's condition which should be evaluated in assessing pain, distress and discomfort (Morton & Griffiths, 1985) apply equally to aquatic species, with some qualifications regarding the importance of body weight changes as an indicator of pain and/or distress.

a. In the more traditional laboratory animals, the concern is usually for abnormal or unanticipated weight loss. In endothermic aquatic animal species, particularly when housed in water at or below their species optimum temperature range, weight loss is extremely slow. A slower than normal rate of gain may be a more sensitive indicator of sub-optimal environment or health.

b. Certain species of fish undergo normal physiologic anorexia associated with environmental changes or sexual maturation or both. Lack of attention to environmental criteria can precipitate normal physiological anorexia.

c. Experience suggests that approximately 6-7% of Atlantic salmon in sea water in a tank will not eat. However, when moved to another tank, particularly if they are larger fish in a new situation, they will begin to feed normally.

d. Change in feeding activity or in feed consumption of the experimental unit (the tank) is a more immediate and sensitive indicator of abnormal environment or health than weight changes.

3. SOME SUGGESTED OBSERVATIONS FOR QUANTIFYING PAIN AND/OR DISTRESS IN FIN FISH

- **Feeding Activity/Feed Consumption**
  (normal is dependent on water temperature, fish size and palatability of feed)

- **Physical Appearance**
  - normal
  - fin and skin condition
  - mucus production
  - color change (usually a darkening associated with disease or bilateral blindness)

- **Measurable Clinical Signs**
  (normal is dependent on the environmental temperature to which the fish have been acclimated and fish size)
  - feed consumption
  - respiratory rate
  - posture in water column

- **Unprovoked Behavior**
  - position in the water column
  - social interactions
  - hyperactive
  - hypoactive

- **Provoked Behavior**
  - feeding activity
  - avoidance reaction to mechanical prod
  - avoidance reaction to light beam

APPENDIX C: EXAMPLES OF OBSERVATIONAL CHECKLISTS USED TO DETERMINE ENDPOINTS

***Note**: The observational checklists presented here are examples only. For each experiment where endpoints are required, the principal investigator, in consultation with the attending veterinarian and the ACC, should develop their own checklist specific to their research program.*

1. **CHECKLIST SAMPLE #1: ACUTE RESPIRATORY INFECTIOUS DISEASE IN CATTLE - A VACCINE/CHALLENGE STUDY**

   a. **Clinical Evaluation**

   Calves will be clinically examined each morning (8:00 a.m. - 12:00 p.m.) from day 0 to day 10 by the research veterinarian. In the afternoon, they will be examined in the pen by an animal health technician. Calves with a sickness score of >3.0, as determined by the research veterinarian, will be euthanized and necropsied that day.

   During the vaccination and evaluation period of the study, no concurrent medication will be administered except in the unlikely event of an anaphylactic type reaction to vaccination. Administration of antimicrobials during the study would render the trial useless since the outcomes of vaccine efficacy are sickness, weight change, death and severity of post-mortem lesions.

   b. **Response Variables and Scoring Method**

   A clinical examination on each calf will be conducted daily (day 0 to 10) by the research veterinarian, using the following standardized parameters to gauge the degree of clinical illness. Scores are described in increments of 0 through 4.

   i. **Weight**. The weight is recorded daily for each animal. Fluctuations in weight are possible depending on when the animal has eaten. Weight loss over a few days is typical in animals which show other clinical signs.

   ii. **Temperature**. Rectal temperature is taken with the animal restrained in a chute. Temperatures will be taken and recorded daily in all animals. Individual animal temperatures as well as mean group temperatures will be evaluated. *Caution must be exercised* in the evaluation process since severely ill animals on the verge of death usually have *subnormal* temperatures which will artificially lower the mean group temperatures. In such cases, if an animal has an elevated temperature followed by a declining temperature on the day of or day preceding death, that temperature may be excluded from calculating the daily mean group temperature for those respective days.

   iii. **Rhinitis or nasal score**.

0	*Normal* (mucosa pale pink, no visible nasal discharge).
1	*Mild Rhinitis* (mild serous rhinitis with focal mucosal necrosis, nostrils moist, secretions transparent, mucosa mildly hyperemic, focal mucosal vesicles, small white circular necrotic plaques < 2mm diameter, mild serous nasal discharge).

2	*Moderate Rhinitis* (moderately severe serous rhinitis with confluent areas of mucosal necrosis, mixture of focal plaques and confluent areas of necrosis, nostrils not occluded by exudates, mucosa intensely hyperemic, large confluent mucosal plaques >2mm diameter, serous nasal discharge with occasional globules or small strands of mucopurulent exudate).
3	*Severe Rhinitis* (necrotizing rhinitis, nasal discharge partially occluding nostrils, necrotic plaques cover large portion of nasal mucosa, intense mucosal hyperemia, may have halitosis, nostrils partially occluded by exudates, nasal secretion a mixture of serous and mucopurulent exudates, large confluent mucosal plaques, necrotic tissues forming diphtheritic pseudomembranes that peel from septum).
4	*Very Severe Rhinitis* (severe mucopurulent rhinitis with advanced mucosal necrosis, mucosal erosions bleed easily, halitosis, profuse nasal discharge, exudate hanging from nostrils, mouth breathing, head extended, thick coat of purulent of catarrhal exudate on nasal mucosa, exudates almost completely occlude nostrils).

iv. **Depression score**.

0	*Normal* (bright, alert, eyes bright, ears erect, chews cud, curious, attentive, stays with the group, stretches back muscles, hind legs when stands, licks nostrils frequently, usually within ten seconds of release from headgate).
1	*Mildly Depressed* (ears droop slightly, rarely stands alone, licks nostrils occasionally, tries to stay with the group, difficult to corner or nearly impossible to catch in corral).
2	*Moderately Depressed* (walks slowly, lethargic, stands alone for prolonged periods, sometimes stands with head low, easy to corner, but would be difficult to catch).
3	*Severely Depressed* (uninterested in environment, very lethargic, apathetic, stands with head lowered most of the time, a "straggler", lies in sternal recumbency frequently, often recumbent, reluctant to stand, but can stand when encouraged, easy for one person to catch).
4	*Moribund* (near death, no attempt to clean nostrils, recumbent almost continuously, rarely stands up, oblivious to surroundings, unable, unwilling, or very reluctant to stand, sternal or lateral recumbency).

v. **Strength**.

0	*Normal* (healthy, strong, stands square, runs fast, well-coordinated, impossible to catch, lays with head and legs positioned normally, curled under, easily keeps up with group).
1	*Mild Weakness* (unsteady gait, knuckles occasionally when walking, hind end wobbles, but calf does not stumble, walks slowly, but can

	trot and gallop when chased).
2	*Moderate Weakness* (staggers noticeably, may fall down when struggling, but rises again without delay, can walk and trot, but cannot gallop, rests head on ground when laying down, fairly easy to push off balance, could be laid on side with moderate difficulty).
3	*Severe Weakness* (rises with difficulty, knuckles frequently, stumbles occasionally, runs slowly, easily caught, has difficulty standing up, very easy to push off balance, often falls down when struggling).
4	*Advanced Deterioration* (very weak, unable to stand, emaciated, dehydrated, sunken eyes, skin tenting).

vi. **Respiratory distress**.

0	*Normal* (normal nasal breathing, mouth closed, lips dry).
1	*Mild Respiratory Distress* (intermittent mouth breathing, lips and jaw moist from salivation, but not observed holding mouth open, mucous membranes pink unless stressed).
2	*Moderate Respiratory Distress* (stands with head extended, salivates profusely, muzzle dripping wet).
3	*Severe Respiratory Distress* (mouth breathes when stressed, opens mouth frequently, occasionally extends tongue, breathes through mouth when disturbed, labored breathing, becomes cyanotic when stressed minimally).
4	*Very Severe Respiratory Distress* (cyanotic, stands with head lowered, neck extended, mouth open and tongue extended, severe drooling, anxious expression).

vii. **Sickness score**. Subjective - meant to emulate decision to select animals for treatment on the farm or at feedlot. This score is made after other scores recorded and clinician is informed of the animal's temperature. Since animals are not treated with antibiotics, this scoring system is used to determine the endpoint where an animal is euthanized rather than being allowed to die.

0	*Healthy Animal* (normal healthy animal, no treatment required, no fever, no clinical symptoms).
0.5	*Suspicion of Disease* (animal would not be treated, slight clinical signs and no fever, fever 39.5^{o}C-39.9^{o}C and no clinical signs, single chronic unresolved clinical sign and no fever, animal not depressed and still eating well).
1	*Mildly Sick Animal* (animal would be treated, febrile (40.0^{o}C) and/or clinical signs, disease progressed to where it would be selected to initiate therapy on the farm or feedlot, recovery would be expected with appropriate therapy).
2	*Obviously Sick Animal* (animal would be treated, obvious clinical signs usually febrile, disease is now serious - animal should have been treated before disease progressed to this point, recovery even

	with appropriate therapy would not be certain). These animals are watched to see if they progress to the next sickness score.
3	*Very Sick Animal* (animal would be treated in a clinical setting, severe clinical signs, including depression - fever may be present or temperature dropping, disease is very serious, recovery unlikely even with appropriate therapy). These animals are euthanized.
4	*Moribund* (near death, treatment would be futile; animal would be euthanized in a clinical or experimental setting).

2. **CHECKLIST SAMPLE #2: CHRONIC INFECTIOUS DISEASE IN MICE - LEISHMANIASIS**

a. **General**

i. The laboratory animal veterinarians are to be notified at the beginning of each experiment involving foot inoculation - date of inoculation, number of animals involved, etc.

ii. Any negative effects with respect to animal condition are to be recorded on a checklist by the research staff or animal technicians, and reported to the laboratory animal veterinarian.

iii. Animals on experiment are to be weighed weekly.

iv. Records relating to animal observations and endpoint measurements will be kept by the principal investigator. Such records to be kept in the animal room, accessible to both technical animal care and veterinary staff.

v. All unexpected negative effects on an animal's health and well-being are to be reported to the laboratory animal veterinarian immediately.

b. **Response Variables and Scoring System**

i. **Body weight changes**.

0	Normal
1	< 10% Weight loss
2	10-15% Weight loss
3	> 20% Weight loss.

ii. **Physical appearance**.

0	Normal
1	Lack of grooming
2	Rough coat, nasal/ocular discharge
3	Very rough coat, abnormal posture.

iii. **Behavior**.

0	Normal
1	Minor changes; limping, favoring inoculated limb
2	Abnormal; reduced mobility, inactive
3	Unsolicited vocalizations, self-mutilation, either very restless or immobile.

c. **Endpoint scoring**: When a total score of three or more is reached, based on above checklist, the laboratory animal veterinarian must be notified.

d. **Specific**

i. **Foot inoculation**. Foot swelling will be measured with a caliper. Measurements of foot swelling will be taken weekly. When the foot swelling is 4mm the animals will be humanely killed. In the event that this endpoint is reached and the principal investigator is unavailable for action, the decision regarding the immediate fate of the animals will be made by the veterinarian.

ii. **Rump skin inoculation**. The diameter of the skin lesion will be measured weekly. When the skin lesion reaches 8mm diameter, the animals will be humanely killed. In the event that this endpoint is reached and the principal investigator is unavailable for action, the decision regarding the immediate fate of the animals will be made by the veterinarian.

3. **CHECKLIST SAMPLE #3: CHEMICALLY INDUCED (AZOXYMETHANE) COLON CANCER IN RATS**

In this model, using Sprague Dawley rats, colonic tumors develop six to eight months after initiation of the carcinogenesis protocol, and grow relatively slowly after that. Routine animal care and routine monitoring is done for the first four months. Four months after initiation, the animals are examined three times weekly for signs of tumor burden. One animal is killed at this time to assess the progression of the tumor in each particular experiment. The examinations include palpation of the abdomen for presence of tumors or ascitic fluid, examination of the eyes for signs of anemia, testing the feces for blood, and examination of the general appearance and behavior of the animal.

a. **Response Variables and Scoring System**

i. **Body weight changes**.

0	Normal
1	< 10% Weight loss
2	10-15% Weight loss
3	> 20% Weight loss.

ii. **Physical appearance**.

0	Normal
1	Anemia (pale eye color)
2	Anemia, and blood in feces, diarrhea and/or small fecal pellets
3	Above signs, plus abdominal swelling
4	Ruffled fur coat, lack of grooming, abnormal posture.

b. **Endpoint scoring**: If weight loss exceeds 15% compared to control animals and the animals are not euthanized immediately, they will receive analgesics until the end of the experiment. Any animal reaching a total score of four or higher using the above checklist, will be euthanized and a necropsy done for tumor load evaluation and tissue sampling.