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A previous facts sheet (*Variables in Animal Based Research: Part 1. Phenotypic variability in experimental animals*, I. Harris, 1997) discussed the experimental variability due to animal inherent factors, including genetic variability (breed, age, sex), environmental variability (husbandry and food) and environment / genotype interactions.

This Facts Sheet will address the issues of variability associated with experimental conditions and techniques and will include the effects of stress, sampling techniques, anaesthesia and euthanasia, and how these may affect research data.

*An animal is a living sentient complex variable and varying organism. An animal reflects every change in its environment and management by modification to its body chemistry, behavioural patterns, physiological reactions or ability to combat infection. Such modifications may well invalidate or at least reflect on the reproducibility of animal tests.*

(G.H. Townsend, 1978).

The Working Committee for the Biological Characterisation of Laboratory Animals/GV-Solas chaired by A.W. Ellery in 1985 acknowledged that research in the natural sciences is based on the gradual accumulation of knowledge obtained by collating and comparing experimental data from various places and laboratories. Earlier experiments can only contribute effectively to knowledge and planning future studies if the conditions under which they were performed and the characteristics of the models used are described.

Results obtained using a particular animal are only valid for the conditions under which they are obtained and only useful for comparison if all relevant information concerning the experimental conditions is made available when reporting results in publications. Guidelines established by Ellery's Working Party provide a catalogue of information which should be included in publications concerning how animals are used and their conditions of husbandry. These data are essential to allow others to fully understand how the investigation was performed and how results are interpreted.

## 1. Stress

Stress in animals is difficult to identify and measure (Friend, 1980). For the issues discussed in this paper, the following definition may be useful, *Stress is an adaptive response to changes or challenges in the animal's environment involving a variety of interlocking anatomical, physiological, biochemical, immunological and behavioural adaptation mechanisms in an attempt to maintain a state of equilibrium.* 'Stressors' are environmental stimuli which evoke the stress response.

This definition of stress has a number of similarities to Townsend's and indicates how stress may influence experimental data. Release of adrenocortical hormones has biological effects which may confound measurements and interpretation of experimental results.

It is important to realise that there is a degree of stress associated with all aspects of existence for a living organism. This can be referred to as physiological stress. Normally the animal puts minimal effort into the response and is unconscious of the effort and adaptation required to maintain its biological equilibrium. This type of stress is part of the adaptive emotional reaction of any animal to normal daily situations, and is necessary for day-to-day functioning (Selye, 1973; Rowan and Moore, 1991).

If stress becomes extreme or continuous, as in over-stress or distress, effort put into the animal's response becomes substantial. The animal may become aware of the effort and could then be said to be 'suffering'. Diversion of effort to adapt is generally at the expense of other biological processes, for example, growth and reproduction. If this results in measurable physiological change, there could be profound effects on validity of research data derived from physiological measurements. Absence of stress on the other hand does not necessarily define well-being (Novak and Suomi, 1988). Boredom in laboratory animals may have as much effect as any stressor on the overall well-being of an animal. As demonstrated by Wemelsfelder (1990), animals will brave aversive conditions or engage in stereotypic behaviour, including self mutilation, to attain some stimulation.

There is currently considerable interest in testing the effects that variations in housing and cage environments have on the well-being of animals (Whary *et al.*, 1993; Blom *et al.*, 1993; Arnold and Estep, 1994;

Kitchen and Martin, 1996; Clark *et al.*, 1997). For example, Clark *et al.*, (1997) indicated that the well-being of dogs may not be improved by the provision of out-of-cage exercise, and that individual caging may actually affect dogs adversely. In addition, a lot of attention is currently being paid to the development of objects which can be placed into cages of laboratory animals for the provision of environmental enrichment (Huls *et al.*, 1991; Line and Morgan, 1991; Lambeth and Bloomsmith, 1992; Chmiel Jr and Noonan, 1995; Holmes *et al.*, 1995; Sherwin, 1998; Van de Weerd *et al.*, 1998).

It is important to establish that the introduction of environmental enrichment does not interfere with experimental data being measured (Watson, 1993). Despite this, Poole (1997) states that there is a growing body of evidence to show that animals whose well-being is compromised are often physiologically and immunologically abnormal and that experiments using them may lead to unreliable conclusions. There are a number of well known 'stressors' which have been shown to produce physiological effects. These are as follows:

#### **a) Transport**

Transporting animals is a potent stressor, and can produce a number of effects ranging from weight loss (Wallace, 1976) to changes in blood characteristics linked with stress and shock reactions (Gartner *et al.*, 1980). It can also produce changes in immune function (Landi *et al.*, 1982) and increases in corticosterone levels (Landi *et al.*, 1982 and Tuli *et al.*, 1994). Tuli *et al.*, (1994) showed that the simple act of moving mice from one room to another could be stressful, and even though the corticosterone levels returned to baseline within 24 hours, behavioural changes remained for up to, or beyond, four days. Martin (1989) and Bohus and Koolhaas (1991) concluded that the brain, behaviour, hormones and even the immune system are all inter-dependent and that disturbance of one system commonly influences one or all of the others.

Despite this, a period of acclimatisation may be useful following transportation, prior to the commencement of experimental studies. Landi *et al.*, (1982) recommended that a minimum stabilisation period of 48 hours was necessary to allow immunologic function and corticosterone levels to return to normal, whilst Damon *et al.*, (1986) recommended that there should be a period of acclimatisation of at least three to five days when moving rats to a new cage, to permit sufficient time for food and water consumption to return to normal. While Damon's studies referred specifically to toxicological testing, this finding should be considered for all studies where normal food and water intake is a prerequisite.

#### **b) Restraint and handling**

Restraint and handling are other potential stressors. Besch and Chou (1971) stated that plasma glucose levels in rats appeared to be directly related to the length

of handling time. Van der Wal *et al.*, (1986) concluded that even moderate stress (restraint) could cause significant changes in the acid-base equilibrium of pigs. Restraint stress in rats resulted in decreased plasma prolactin levels in female rats (Gala and Haisenleder, 1986). Other workers have shown that there are significant differences in many blood constituents, blood chemistry and enzyme levels in naive compared to handle-habituated animals (Barrett and Stockman, 1963; Gartner *et al.*, 1980; Corda *et al.*, 1980; Barnett and Hemsworth, 1986; Line *et al.*, 1987).

'Gentling' preweaning animals (removing them from the nest for short periods for stroking gently or handling) makes animals more habituated to humans and less fearful or reactive to procedures (Hirsjarvi and Junnila, 1986). This can also be achieved in adult rats with appropriate husbandry practices (Fox, 1986). There are species and strain differences, however, and Kersten *et al.*, (1989) found that while handling worked well with rabbits to produce habituation, it does not work consistently with mice, and hamsters may be adversely affected (Lawlor *et al.*, 1975).

#### **c) Surgery or injury**

It has long been established that surgical trauma can be a potent stressor (Selye 1936). Major body injury (surgical or accidental) evokes reproducible metabolic, hormonal and haemodynamic responses (Michell, 1974; Buckingham, 1985; Bevan, 1985). These responses are characterised by altered protein homeostasis, hypermetabolism, altered carbohydrate metabolism, sodium and water retention and increased lipolysis. The abnormal carbohydrate metabolism results in hyperglycaemia and there is negative nitrogen balance, reflecting accelerated net protein breakdown or catabolism (Weissman, 1990). The loss in body weight and leukocytosis which occurred for up to two weeks following thoracotomy in the rabbit led Warren and Ledingham (1972) to conclude that it was unlikely that cardiovascular studies would bear a close resemblance to normality within two weeks of surgery. Flecknell and Liles (1991) also reported that surgical procedures could have a detectable effect on locomotor activity and food and water intake.

Many of these responses, however, can be modified by anaesthetics (Weissman and Hollinger, 1988). The use of analgesics before surgery may block the stress response (George *et al.*, 1974; Savege, 1978) and stabilise the hyperactive autonomic nervous system by decreasing stress and pain (Freya, 1974).

#### **d) Communication of stress**

Valenta and Rigby (1968) reported that rats are able to discriminate between the odours of stressed and unstressed rats. The ability of untreated, control animals to recognise 'stressed' animals could lead to changes in the behaviour and responses in the control groups and affect results (de Laat *et al.*, 1989; Beynen, 1992). Placing a rat into a new cage or isolating it in a

new group was shown to lead to significant increases in plasma corticosterones, which would be augmented if the new environment was one in which animals had previously been subjected to stress (Mackay-Sim and Laing, 1980; Amario and Balasch, 1981). It is also well known that killing rats in the presence of others can have a significant effect on the remaining animals and may lead to variability in data collated (Knott *et al.*, 1977). In addition, the sequence of killing may produce variation in response between animals (Dunn and Scheving, 1971; Knott *et al.*, 1977).

## 2. Sampling techniques

Sampling of blood is one of the most common procedures undertaken during animal experimentation and a number of variables associated with the collection of samples can have significant effects on results obtained. As indicated previously, the stress of handling, or the discomfort associated with the sampling technique, with all the associated physiological changes, may invalidate results (O'Neill and Kaufmann, 1990; Sarlis, 1991.) In an attempt to refine techniques, to make them more humane (minimise pain and suffering and reduce variability), the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement produced a useful document describing techniques for taking blood from laboratory mammals and birds (Anon., 1993). They hoped that this report would be circulated widely and the recommendations therein adopted as 'best laboratory practice'. This point is also made with respect to clinical biochemistry of laboratory animals by Loeb and Quimby (1989).

### a) Site of collection

The site of collection of blood may have an effect on the parameters being measured (Upton and Morgan, 1975; Archer and Riley, 1981; Suber and Kodell, 1985; Horton *et al.*, 1986; Dameron *et al.*, 1992). Upton and Morgan (1975) showed that haemoglobin and haematocrit values were higher for tail blood than for blood removed from the heart or abdominal aorta in rats. For acid:base measurements, the site of removal was not significant except when comparing tail blood with aortic blood for pH and base excess measurements. Archer and Riley (1981) investigated the effects of varying sample site and the method of anaesthesia, and found some significant variation in results obtained. While there was little difference with respect to erythrocytes between samples taken from the heart or jugular vein (under halothane anaesthesia), or the tail vein (without anaesthesia), when leucocytes were considered there were substantial differences between the three sites. Suber and Kodell (1985) compared collection from the orbital sinus with tail vein incision, and cardiac puncture in rats anaesthetised with carbon dioxide and reported a significant problem with haemolysis in samples taken by cardiac puncture and tail vein. They felt that the variation in clinical chemical data compared to the haematological

parameters was due to the effect that this haemolysis had on analysis of the samples. They concluded that orbital puncture was the technique of choice with the least variance and greatest precision and if haemolysis could be minimised the results were comparable with those determined by tail vein incision. Dameron *et al.*, (1992) found that there were no differences in haematological values in blood collected from the orbital venous plexus compared to the posterior vena cava but that coagulation times and serum Mg and P differed significantly. They believed that these differences may have been due to tissue trauma associated with blood removal from the orbital venous sinus.

Given such variations, Archer and Riley's recommendation that standardisation and adequate quality control of tests is mandatory to obtain reliable and reproducible results is timely.

### b) Timing of samples

In an extensive study in rats, Gartner *et al.*, (1980) demonstrated the difficulties encountered in obtaining undisturbed control values for many blood characteristics and recommended that any sampling be completed within 100 seconds of first touching an animal's cage. This is particularly important if endocrine characteristics and plasma values linked with circulatory changes, capillary permeability, energy and mineral metabolism and acid:base balance are being measured. While some of these characteristics were only altered by 5-10% and for short periods of about 5-10 minutes (glucose, plasma protein, PCV and sodium), others were altered more than 100% and for more than 30 minutes (prolactin, TSH, corticosterone, lactate and pyruvate).

Bickhardt *et al.*, (1983) showed that if stress-sensitive characteristics such as PCV, cell counts, protein, lactate and glucose concentrations were being measured, quick decapitation of rats, previously undisturbed and with a maximum bleeding time of 10 seconds, gave representative results of the physiological status of the animal.

### c) Diurnal variation

Any diurnal, circadian or ultradian (incidence of regular secretory bursts) variation in the parameter being measured must be taken into account in the planning of experiments. It is well known that there is diurnal variation in plasma corticosteroids in man, monkey, dog, rat, mouse, sheep and other species (Yates and Urquhart, 1962; Yates *et al.*, 1971; McNatty *et al.*, 1972; McNatty and Young, 1973) and ultradian and circadian rhythms in the plasma concentrations of cortisol in sheep (Fulkerson and Tang, 1979). Plasma levels of other hormones, including insulin and growth hormones, and other parameters such as metabolites, heart rate and body temperature may show diurnal, circadian and ultradian rhythms (Bassett, 1974; Tannenbaum and Martin, 1976; Plonait *et al.*, 1979). Even the behaviour of animals in their home cages may vary with the time of day and with the cleaning

routine used in the facility (Saibaba *et al.*, 1996) and this should be taken into account.

**d) Volume of blood collected and multiple sampling**

The possible effects of the volume of blood removed may also be significant. Small volumes removed at too frequent intervals will produce anaemia, but if too large a volume is removed, either too rapidly or too frequently without fluid replacement, the animal may go into hypovolaemic shock, with an associated stress response. Removal of up to 10% of the circulating blood volume will initiate homeostatic mechanisms, but the animal will probably show no major ill effects. If 15-20% is removed, cardiac output and blood pressure will be reduced and without some fluid replacement the animal will be adversely affected. Removal of 30-40% can initiate hypovolaemic/haemorrhagic shock and if over 40% is removed this may result in 50% mortality in rats (McGuill and Rowan, 1989). Lawson and Gala (1974) found that stress indicators appeared after as little as 6-7% blood loss as a single event. They reported significant responses of plasma prolactin concentrations after removal of only 1.2 ml of blood from 225-300g rats within 20 minutes, but no changes when the lost blood was replaced by saline. In a study of repeated blood withdrawal, six, four ml samples of blood were taken from rats at one or two week intervals (Wiersma and Kastelij, 1986). Blood parameters were significantly affected in rats bled weekly but not in those bled every two weeks. It was estimated that adverse effects were seen when over 20% of blood volume was removed per week.

McGuill and Rowan (1989) cautioned that great care should be taken when estimating how much blood should be removed from an animal, because in addition to the variation in published blood volume values, a number of other variables must be taken into account. These include the rate of blood loss, site and technique of removal, skill of the bleeder, use and type of anaesthesia, age and sex of the animal and the nutritional and health status of the animal. They recommended a safe sampling volume for single removal of 10% blood volume (which was estimated to be approximately 10% of body weight) and for repeated sampling a 7.5% weekly limit. However, other unpublished data and advice from colleagues caution that removal of 7.5% of blood volume weekly is too high. Care must be taken to discriminate between a blood sample as a percentage of blood volume and as a percentage of body weight. These are not the same and the relationship varies between species and even within breeds of dog (see McGuill and Rowan, 1989 for a comprehensive coverage of this topic).

There may be other changes associated with repetitive blood sampling which may impact on experimental results. Cardy and Warner (1979) found that monthly one to two ml blood samples taken from young rats, even though not producing haematological changes, did produce a decrease in the rate of body weight gain. Another study indicated that repeated blood samples may have an effect on locomotor activ-

ity, evasion and wheel running activity in mice, (Pfeil, 1988). However, with appropriate techniques, it is possible to take repetitive blood samples from rats without perturbing some physiological parameters (Walsh *et al.*, 1980; Nachtman, 1985).

**e) Chronic cannulation**

The stress due to handling and restraint, timing of samples, site of removal and anaesthetic agents used may have a significant effect on the results obtained from sampling. Use of chronic cannulation has been recommended for a variety of species to allow sampling from, or infusion into, unrestrained, conscious 'physiologically normal animals' (Brown and Burr, 1985 (rats); Takahashi, 1986 (piglets); Dons and Havlik, 1986 (rats); Ladewig and Stribrny, 1988 (cattle); Moritz *et al.*, 1989 (pigs); Rath and Hutchinson, 1989 (rats); Spurlock *et al.*, 1990 (horses); Hodge and Shalev, 1992 (mice); Suzuki *et al.*, 1997 (rats). This can also be used for monitoring cardiovascular parameters which may also be modified by stress and anaesthetic agents (Parker and Martin, 1989).

In man, sepsis, septic thrombophlebitis and thromboembolism are significant hazards associated with chronic cannulation (Bentley and Lepper, 1968; Ryan *et al.*, 1974) and there is no reason to believe that this would not occur in animals, particularly associated with long-term cannulation, despite the widespread belief that rats are resistant to post-operative wound infection (Deem, 1981; Spurlock and Spurlock, 1990; Bradfield *et al.*, 1992). Popp and Brennan (1981) demonstrated that catheter infection occurred in five out of six non-sterilely catheterised rats and believed that this could induce artefacts in an experiment. This was reiterated by Pedersen *et al.*, (1989) and Bradfield *et al.*, (1992). Popp and Brennan (1981) felt that the incidence of infection was often not documented because of inadequate monitoring or short periods of observation after catheterisation. Treatment of catheter infections once established can be difficult, as found by Dennis *et al.*, (1989), who described their attempts to devise an effective method of sterilising catheters *in situ*.

Popp and Brennan (1981) and Fagin *et al.*, (1983) emphasised the need to allow animals to recover fully from the anaesthetic and surgical stress associated with cannulation, so that this did not interfere with experimental findings. Fagin *et al.*, (1983) showed that plasma corticosterones did not return to basal levels in rats until three to four days after cannulation. In addition, chronic catheterisation may be associated with appetite suppression and growth impairment and this must also be taken into account. This is particularly important if the physiological responses under study could be affected by alterations in nutrient intake and/or animal growth (O'Neill and Kaufman 1990).

Desjardins (1986) indicated that the chemical composition of synthetic polymers used in the production of catheters must be compatible with investigative needs and that artefact chemicals may leach out of implanted tubing and interfere with analytical techniques being used.

### 3. Anaesthesia.

It is well known that anaesthesia *per se* has a significant effect on cardiovascular, endocrine and respiratory function (Robertson and Frazer, 1958; Pettinger *et al.*, 1975; Bevan, 1985; James *et al.*, 1986; Field *et al.*, 1993; Muir and Mason, 1996; McDonnell, 1996) and on immune status (Walton, 1979). This is the same for adults and neonates (Eisenhauer *et al.*, 1994) though in the latter the effects will depend on the maturation of the nervous system and drug metabolising systems. It is likely therefore that anaesthesia will have an effect on some experimental measurements. In relation to electrophysiological studies, Goss-Sampson (1991) stated that it was essential to choose an appropriate anaesthetic agent with minimal confounding effects and to define systematically changes ascribed to the anaesthetic on the parameters to be measured. This would apply equally well to all types of studies. In addition, Flecknell and Liles (1991) confirmed that surgical procedures reduce activity levels and food and water intake in rats, but this was modified by anaesthesia and administration of analgesics in both normal and operated animals.

#### a) Direct effects of anaesthetics

Some anaesthetic agents are known to affect physiological parameters directly. For example, it was shown by early workers that diethyl ether causes a leucocytosis in man (Chadbourne, 1899). Similar results were demonstrated later in rabbits (Stier and Levy, 1933). Ether has also been shown to elevate glucose levels (Besch and Chou, 1971) and to cause a pronounced stress response (Gartner *et al.*, 1980) which, according to Van Herck *et al.*, (1991), is so great that it may mask any effects induced by orbital puncture. Ether also has significant effects on body temperature, heart rate and locomotor activity (Van Herck *et al.*, 1997).

The effects of pentobarbitone are also well recorded. Indirect effects may be elicited by circulatory changes resulting in a modification to erythrocyte, plasma and true blood volumes of organs and tissues (Rieke and Everett, 1957). Pentobarbitone also produces significant effects on heart rate, ventricular afterload and contractile state of the heart, which brings into doubt its usefulness in studies of canine cardiovascular physiology (Hansen *et al.*, 1986). Pentobarbitone may also have direct effects. It has been shown to affect the Atrial Natriuretic Peptide (ANP) system in rats, resulting in decreased tissue storage of the hormone and elevated plasma ANP (Lee *et al.*, 1994).

#### b) Specific effects on parameters

Different anaesthetic agents may produce specific effects on a variety of parameters. For example, Rogers *et al.*, (1980) compared the transport of horseradish peroxidase in nervous tissue when animals were anaesthetised with pentobarbitone or urethane

and found urethane to provide the best demonstration of peroxidase transport. Collado *et al.*, (1987) demonstrated an increased secretion of bile sodium following the use of pentobarbital or urethane and Laber-Laird *et al.*, (1992) showed that the effect of isoflurane on insulin secretion was sufficient to render the use of this anaesthetic agent unsuitable for studies where glucose tolerance was to be assessed.

#### c) Variability in the same parameter due to different anaesthetic agents

Different anaesthetics may produce different results in the same parameter being measured. Ajika *et al.*, (1972) showed that the use of ether to bleed rats resulted in a rise of prolactin, LH and FSH levels in ovariectomised rats. However, the elevation of plasma prolactin and LH was blocked by pentobarbitone, but it had no effect on the levels of FSH. Bruce *et al.*, (1984) found that when they compared progesterone levels in rats anaesthetised with ether or pentobarbitone there was a significant difference in the values obtained. Similar responses have been demonstrated in a number of other studies (Kaczmarczyk and Reinhardt, 1975; Fowler *et al.*, 1980; Rogers *et al.*, 1980; Archer and Riley, 1981; Naess *et al.*, 1986; Stringer and Seligmann, 1996).

#### d) Nil effect of anaesthetics

Some parameters may not be affected by anaesthetics and a number of papers have been published confirming that anaesthesia may have no effect in a particular instance. This may be significant if anaesthesia or analgesia are being used to reduce possible pain or distress associated with a procedure. Wiersma and Kastelijn (1986) showed that isoflurane in oxygen had no effect on the lungs, liver, kidney and spleen in a hind-limb ischaemia-reperfusion model and the use of an analgesic also had no effect on the parameters measured. Similar findings were demonstrated by Brown and Layman (1990), who found that a combination of ketamine and acetyl promazine did not alter plasma chylomicron clearance in rats, while halothane and pentobarbital decreased the clearance by 62% when compared with non-anaesthetised rats. The studies by Barzago *et al.*, 1994 and Carlberg *et al.*, 1995 also demonstrated the lack of effect of anaesthetic agents on measured parameters.

#### e) Long term effects of anaesthetic agents.

Anaesthesia and surgery may well affect an animal's responses, locomotor activity (Flecknell and Liles, 1991), blood flow to organs, absorption from the gut (Welling, 1977) and bile flow and secretion (Roberts *et al.*, 1967; Cooper *et al.*, 1976) for hours or even days afterwards. Even the use of a short acting inhalation agent, such as halothane, produced changes in the mean arterial blood pressure, cardiac output, heart rate and plasma renin activity in rabbits. In a study by Sartick *et al.*, (1979) the mean arterial blood pressure

and cardiac output returned to preanaesthetic levels 15 minutes after the anaesthetic was turned off but the heart rate and plasma renin activity took over 200 minutes to return to preanaesthetic levels. Rath and Hutchinson (1989) stated that a seven day recovery period following surgery to insert a bile duct cannula was required to minimise the possible effects of anaesthesia and surgery.

#### 4. Euthanasia.

Sampling after euthanasia to obtain blood, tissues and organs is a common practice and it is important that, in addition to euthanasia being rapid and as free of stress and pain as possible, the euthanasia technique should not interfere with the subsequent studies of samples taken. Methods of euthanasia can impact on data either directly, by changing the qualitative or quantitative characteristics of a parameter being measured, or indirectly, due to the circumstances associated with the euthanasia technique, such as handling, site and timing of samples and exposure to other animals being killed.

##### a) Direct effects of euthanasia techniques

There are numerous studies which indicate that both chemical and physical euthanasia methods may have a direct effect on the parameter being measured. For example, Seitz *et al.*, (1973), demonstrated the effect of anaesthetic drugs on the glycolytic intermediates in rat liver and Howard *et al.*, (1990) showed that a number of anaesthetics had a direct effect on two *in vitro* immune function assays. Others have shown that data obtained from animals euthanased by physical or chemical means may be significantly different from values obtained from cannulated or catheterised animals. This could be due to a generalised metabolic response secondary to the sympathoadrenal release which accompanies handling, stress, decapitation and anaesthesia *per se*. Besch and Chou (1971) showed that blood glucose levels in decapitated animals were much increased compared with animals restrained manually or stunned, and concluded that this was associated with a massive sympathetic discharge with decapitation. Others demonstrated that decapitation as well as gaseous anaesthetics produced a significant rise in the levels of circulating noradrenaline and catecholamines when compared with chronically cannulated rats (Depocas and Behrens, 1977; Popper *et al.*, 1977). Similar results were seen by Behrens and Madere, 1979; Milakofsky *et al.*, 1984 and Conahan *et al.*, 1985.

##### b) Humaneness of a technique

There is still some argument about the humaneness of euthanasia methods used, for example, decapitation without prior sedation or anaesthesia and the use of carbon dioxide (Mikesa and Klemm, 1975; Freed, 1983; Paton, 1983; Ewbank, 1983; AVMA, 1993; Reilly, 1993; Danneman *et al.*, 1997).

The use of an anaesthetic agent prior to decapita-

tion as recommended by the American Veterinary Medical Association Panel on Euthanasia (1993) may introduce another variable, as demonstrated by Kasten *et al.*, (1990), who showed that a number of anaesthetics including chloral hydrate, ketamine, pentobarbitone and chloralose/halothane used prior to decapitation significantly altered levels of fructose-2,6-biphosphate compared with decapitation alone. Engel *et al.*, (1996) also found that GABA<sub>A</sub> receptor function was altered and suggested that anaesthetics prior to decapitation should be used with caution. This is not a consistent finding, as Urbanski and Kelley (1991) and Berger-Sweeney *et al.*, (1994) found that sedation with carbon dioxide prior to decapitation did not alter circulating levels of LH, FSH and prolactin activities or cholinergic parameters in rat brain. The use of anaesthetics may reduce the sympathoadrenal response associated with decapitation, as demonstrated by Roizen *et al.*, (1978), but the anaesthetics may or may not have effects of their own (Scott and Trick, 1982).

##### c) Effects on tissue studies - histology

There are many reports that euthanasia methods have some significant effects on subsequent tissue studies. Both physical and chemical methods of euthanasia can alter tissue histology. For example, Fawell *et al.*, (1972) showed that oedema of perivascular connective lung tissue of rats was due to the use of carbon dioxide for euthanasia, whereas previously it was thought to be an artefact. Feldman and Gupta (1976) described histopathological changes associated with various types of euthanasia techniques in laboratory animals. They showed that each of the methods studied affected the lung tissue to some degree, but determined that some techniques were more suitable than others for particular studies. They indicated that carbon dioxide or intraperitoneal sodium thiopentone was suitable for pulmonary studies, whereas decapitation in mice, rats and guinea pigs, or cervical dislocation in mice was preferable if abdominal viscera were to be examined. This has been confirmed in studies by Iwarsson and Rehinder (1993), but is somewhat in contrast to Britt (1989), who showed that carbon dioxide affected lung histology. Port *et al.*, (1978) and Prien *et al.*, (1988) showed that concentrated barbiturate used as a euthanasia solution in cats, dogs and monkeys produced histological artefacts in lung and kidney tissue.

##### d) Effects on tissue studies - metabolic response

Euthanasia, as well as handling and anaesthesia, can modify metabolic responses of isolated tissues. In an extensive study, Faupel *et al.*, (1972) examined the effects of different types of handling, euthanasia and anaesthesia on the level of carbohydrate metabolism intermediates in rat liver. They found that all metabolites measured were very sensitive to tissue anoxia and some to handling stress. Kant *et al.*, (1980), and Zinn *et al.*, (1989) showed that different euthanasia techniques had a significant effect on metabolites in the brain of rats. In addition, the activity and responses of

various isolated tissues or organs can be affected by the method of euthanasia (Segel and Rendig, 1982 and 1986; Sage *et al.*, 1985; Butler *et al.*, 1990).

**e) Nil effects of euthanasia**

As in the case with anaesthesia, it is possible that the method of euthanasia may not influence the biological system under investigation. This was demonstrated by Kassay-Farkas and Wyse (1982) who showed that there was no difference in the response of helical strips from the ventral tail artery of rats killed either by cervical dislocation or by anaesthetic drugs.

**Conclusion**

This facts sheet has highlighted the importance of investigators being aware of, and taking into account, the effects on experimental animals of their environment, how they are handled and the procedures undertaken.

These variables should be defined, standardised, and minimised in order to obtain results which are meaningful, repeatable, and comparable with others. As indicated by Rowan (1990), refinement of research techniques using animals will lead to less animal distress and at the same time will usually lead to higher quality and more robust data.

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