Food for Thought …
Refinement

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Summary

The ultimate goal of the Three Rs is the full replacement of animals used in biomedical research and testing. However, replacement is unlikely to occur in the near future; therefore the scientific community as a whole must continue to devote considerable effort to ensure optimal animal welfare for the benefit of the science and the animals, i.e., the R of refinement. Laws governing the care and use of laboratory animals have recently been revised in Europe and the US and these place greater emphasis on promoting the well-being of the animals in addition to minimizing pain and distress. Social housing for social species is now the default condition, which can present a challenge in certain experimental settings and for certain species. The practice of positive reinforcement training of laboratory animals, particularly non-human primates, is gathering momentum but is not yet universally employed. Enhanced consideration of refinement extends to rodents, particularly mice, whose use is still increasing as more genetically modified models are generated. The wastage of extraneous mice and the method of their euthanasia are refinement issues that still need to be addressed. An international, concerted effort into defining the needs of laboratory animals is still necessary to improve the quality of the animal models used as well as their welfare.

Keywords: refinement, social housing, positive reinforcement training, animal euthanasia

Introduction

Components of animal welfare are generally accepted to include maintenance of good health, minimization of negative states such as pain and enhancement of positive states such as comfort and psychological well-being, and freedom to exhibit behaviors that are natural to the species (Fraser, 2009). In the case of laboratory animals, the first of these is ensured through appropriate veterinary care in compliance with various laws and guidelines (AWA, 1990; PHS, 2002; EU, 2010). These regulations also stipulate that pain and distress should be minimized or eliminated unless scientifically justified. While it is extremely important and indeed a challenge to recognize pain in laboratory animals, it is also imperative and at least as much of a challenge to enhance positive states and meet the needs of the animals to perform species-specific behaviors. In the absence of the opportunity to express normal behaviors, the animals may experience distress, which may be less evident than other aversive states like pain, but nonetheless can contribute to poor animal welfare and compromised data. Thus it is imperative that we learn how to assess the mental states of laboratory animals and ultimately address them to the point where research integrity can be preserved. Assessment of the mental states of animals, however, requires a comprehensive understanding of the normal behavioral needs of the species we use in research. Studies done on the domestication of animals, including laboratory animals, consider changes in shelter, space, feeding and drinking, predation, and social environment as all impacting animal welfare in a captive environment (Price, 1999). In considering the environment of laboratory animals, all of these factors play a role in contributing to their mental states. As we continue to use animals in research, we must be mindful of their species-specific needs. While it is reasonable to be pragmatic in our approaches, we should also be knowledgeable and creative in addressing animal welfare in biomedical research. Few biomedical scientists who use animals in research have an in-depth knowledge of laboratory animal science, or if they do, it is limited to one or two species. Even fewer have an appreciation for the normal behavior of the species they are using. The research culture appreciates the role of veterinarians in maintaining the health of research animals both in North America and Europe. However, behaviorists who can begin to evaluate the mental states of laboratory species are only now becoming recognized as essential participants in the research enterprise. This paper will examine the state of animal welfare in the context of refinement, as first posited by Russell and Burch (1959).

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Consideration 1: Why is refinement important?

When Russell and Burch (1959) first described the Three Rs, the recommendation to refine animal experiments came after one had ruled out the use of replacements and had taken steps to reduce the number of animals to the minimum required to achieve statistical significance. Most who work with animals in research would like to see their use ultimately replaced, but this goal is not likely to be achieved in the near future. Therefore, we have the responsibility to consider refinement as the R that can and must be implemented immediately. Optimal care for the physical and behavioral needs of animals contributes to optimal animal welfare and to research integrity.

It was not until the 1950's in the US that formal efforts were made to monitor the quality of laboratory animals with the establishment of the Animal Care Panel (later to become the American Association for Laboratory Animal Science) and the Institute of Animal Resources (later to become the Institute for Laboratory Animal Research or ILAR) at the National Academy of Sciences. At that time, most effort was focused on the sources of the animals and their physical health and microbial status, with recognition from both veterinarians and non-veterinary scientists that less than optimal health and underlying infectious diseases could compromise the quality of research (Wolfle, 2003). It took longer, however, to address the potential negative effects of aversive mental states, such as pain and distress, on both animal welfare and research integrity. It was not until 1985 that the amendments to the US Animal Welfare Act were passed (USDA-APHIS, 1991) mandating the establishment of Institutional Animal Care and Use Committees to review protocols to ensure that pain and distress were minimized. Unfortunately, even at that time, the assessment and recognition of pain were challenges even to the veterinary community (and remain so for some species). Less was known about distress and even today we have a limited comprehension of the extent of distress or its impact on research. Generally, distress is defined as an aversive negative state in which an animal cannot cope or adapt and fails to return to homeostasis (NRC, 2008). States of prolonged hunger and thirst, restraint, fear, boredom, and chronic pain are some examples of potential causes of distress in animals. Even though the concept of distress is relatively new, even Russell and Burch (1959) recognized that certain procedures induced a "state of excitement" in animals that would likely affect research results. Recognizing that we need to meet the normal behavioral needs of laboratory animals is only the first step in reducing distress – the challenge is in better understanding our research animals as complex organisms and then adjusting their environments so that they are able to exert some control over them.

Animal care legislation has been slow to address issues of refinement other than pain and "poorly defined" distress. The earliest mandates in US legislation for refinement came with the 1985 amendment to the Animal Welfare Act and included providing for environment enhancement to support the psychological well-being of non-human primates and exercise for dogs (USDA-APHIS, 1991). The regulations for non-human primates take into consideration the need for social housing, environmental enrichment, special consideration for young or distressed animals, and limiting the use of restraint devices. Regulations for canine exercise require institutions to develop an exercise plan and consider providing positive social interactions with humans as part of the plan (Kulpa-Eddy et al., 2005). More recently, the 8th edition of the *Guide for the Care and Use of Laboratory Animals* (the Guide) (NRC, 2011) and the revised European Union Directive 2010/63/EU (EU, 2010) specified that single housing of social species (i.e., most laboratory animals including rodents and rabbits) should be the exception, and this should only occur for special experimental circumstances or when an animal is aggressive and/or incompatible with other animals and when this is done, it should be for the shortest duration possible. The recognition of the social needs of animals in legislation was an important step in promoting animal welfare for laboratory species. However, the challenges of implementing these guidelines can be formidable without basic knowledge of the animals' normal behavioral needs, ability to recognize abnormal behavior, and training to implement behavioral interventions. The melding of physical and behavioral well-being for laboratory animals is essential to accomplish the goal of optimal welfare.

With the recognition that normal behavioral needs of laboratory animals should be met in a captive environment to the extent that is practically possible, there have been more publications focused on these areas. The number of universities and veterinary schools in Europe and North America with programs in animal welfare is increasing; initially many of these were focused on farm animals and now more emphasis is being placed on laboratory animals. The goal of these programs is to correlate physiological and behavioral endpoints, i.e., to find physiological biomarkers that can help to ascertain the mental state of the animals. However, addressing this task will necessitate considerable crosstalk and information sharing among neuroscientists, veterinarians, and behaviorists.

Consideration 2: Social housing is the rule, so we should stop focusing on the exceptions

The changing attitudes and practices regarding the social housing of laboratory animals are an excellent demonstration both of the impact that focusing on refinement can have on animal welfare and how much room for progress remains. While the importance of social housing for laboratory animals may seem to be self-evident given what we now know, this has not always been the case. The crucial role of social contact in normal development of mammals was most definitively demonstrated with work done in rhesus monkeys by Harry Harlow in his seminal paper, "The Nature of Love" (1958). However, it was not until more than 30 years later that the importance of that social contact was written into the US regulations governing the use of laboratory animals. Even then, the USDA regulations of 1989 stopped short of addressing social needs of any animals other than non-human primates; as mentioned above, recommendations for socialization of dogs were included in the initially proposed, but not final, rules (Kulpa-Eddy et al., 2005; CFR, 2011). Now, 25 years later, the body of evidence in favor of socially housing most laboratory animals has grown formidable. Building upon the work of Harlow and Harlow (1962), Novak (2003), Lutz et al. (2007), and others have further demonstrated the deleterious behavioral effects of singly housing non-human primates. Additionally, the literature has demonstrated that non-human primates are far from the only laboratory species for which social housing is important. For nearly every species examined, whether it be dogs (Hubrecht, 1995; Hetts et al., 1992), pigs (Barnett et al., 1985), rabbits (Chu et al., 2004), or mice (Van Loo et al., 2004), social housing has been demonstrated to be preferred by animals and/or to have a profound impact on measures reflecting their welfare. Industry standards have followed suit; as mentioned above, both European and US guiding documents now define social housing as the default for any social species. Indeed, a consensus has emerged among laboratory animal researchers, regulators, and caregivers that social housing is likely the single most important refinement that can be offered to a laboratory animal, so increased focus on this issue has great potential to increase well-being.

Despite this growing consensus, more than half of nonhuman primates in indoor caging or enclosures remained singly housed as recently as 2007 (Baker et al., 2007), to say nothing of the other common laboratory species that do not have the benefit of 60 years of literature supporting their social nature. The reason for this lack of progress is undoubtedly due in part to the laboratory animal community's comfort with exemptions from social housing, either for research or veterinary reasons. While there are surely some unique circumstances in which an exemption from social housing is truly necessary, the new EU and US guidelines, and a growing body of research suggest that it is well past time to rethink some of the common justifications for single housing. In their review of the benefits and risks of pair housing macaques, for instance, DiVincenti and Wyatt (2011) recount their own institution's shift to social housing of rhesus monkeys having cranial and corneal implants. While such surgical implants would still be considered a valid justification for single housing in many facilities, their experience is an example of how thin the justification may be for some long accepted justifications. Exemption from social housing based on experimentally induced infectious disease or drug treatment is another example in which the risks of social housing, i.e., cross contamination and/or difficulties of treatment, must be weighed against both the well-being of the animals and the impacts that single housing may have on the research itself. Laboratory environments and procedures can present any number of stressors to the animals housed within them, both foreseen by human handlers and not. The stress response, in turn, has been demonstrated to alter animal physiology in numerous ways, including profound effects on the immune system (Hutchinson et al., 2012; Tung et al., 2012) and even on how drugs are metabolized (Matamoros and Levine, 1996). On the other hand, social housing has been shown to provide a buffer against the effects of outside stressors, blunting the physiological response to outside events and likely increasing the validity of research done on socially housed animals (Gust et al., 1994; Gilbert and Baker, 2011). From a practical standpoint, there are daunting challenges to socially housing animals on such studies, but it is worth considering creative solutions rather than simply jumping straight to requests for exemption.

The current pressure toward social housing has also led to the questioning of what constitutes a "social animal," with adult male macaques and rabbits being two of the more frequently cited as "difficult to introduce." With respect to male macaques, their large canines and frequent dominance displays have led to a commonly accepted notion that they are more challenging or dangerous to introduce than females. DiVincenti and Wyatt (2011) summarized the literature that has largely debunked the ongoing concerns about the potential dangers of introducing adult male macaques; when done in a controlled manner, socializations of these putatively more risky animals need not be any more dangerous than introductions of adult females (Reinhardt, 1987) or even more dangerous than single housing (Schapiro and Bushong, 1994).

The hesitation to socially house rabbits largely stems from the very real tendency for males to castrate one another in fights during or after puberty. This led the laboratory community to throw the proverbial baby out with the bathwater, with many facilities housing all rabbits singly by default until the EU and US regulatory changes. However, as with primates, the evidence supporting the benefit of socially housing rabbits cannot be ignored. Studies have demonstrated that socially housed rabbits are less likely to engage in a number of abnormal behaviors (Held et al., 2001) and will work as hard for social contact as for food (Seaman et al., 2008). Additionally, several papers have been published describing the mechanics of successfully introducing and maintaining groups of rabbits, including castrated males (Love, 1994; Raje and Stewart, 1997). It should also be noted that the intense male fighting that leads to serious wounds does not typically happen until puberty (five to seven months of age in a New Zealand White), so studies using animals before they reach that age could take advantage of this to socially house intact male juveniles with decreased risk. By clearly stating that social housing is the default condition for laboratory animals, both the EU regulations and the Guide have challenged laboratory animal users to reconsider some of the dogma regarding what is a justifiable exemption and to think creatively to solve some of the practical issues of introducing and maintaining social groups.

Consideration 3: Positive reinforcement training for routine procedures is on a trajectory to, and should become, the default condition for laboratory animals

The increasing use of positive reinforcement training (PRT) to refine husbandry, medical, and experimental procedures for laboratory animals in many ways parallels the evolution of thinking about social housing. PRT has long been in use for many laboratory animals, but in the context of neuroscience to the extent that a separate set of US guidelines for neuroscientists devotes several chapters to the proper use and limitations of PRT (NRC, 2003). The use of PRT to train non-domesticated animals to cooperate with otherwise stressful or dangerous procedures, on the other hand, first gained real popularity with aquariums housing large aquatic mammals, whose size and environments greatly restrained the ability of veterinarians and husbandry staff to coerce the animals. The success of these techniques ensured their spread to other situations, such as zoos, where similar difficulties and risks of handling made the effort required for training pale in comparison to the benefit of having cooperative animals. In the laboratory setting, one of the first and most vocal advocates for training nonhuman primates to cooperate with husbandry and medical procedures was Viktor Reinhardt, who began advocating in the 1980s for training macaques to cooperate with venipuncture in their home cages (Vertein and Reinhardt, 1989), noting that this is likely the most frequent reason for sedation or restraint in laboratory primates (Reinhardt, 2003). In addition to making procedures faster and easier, another advantage of the positive effects of PRT of non-human primates is the increased integrity of the scientific data. As pointed out by Reinhardt (2003), significant physiological changes occur in animals that are forcibly restrained. On the other hand, PRT results in decreased cortisol levels, fewer stress-related abortions, and fewer fear-related responses, such as fear-grinning, screaming, and stress-induced diarrhea (Laule et al., 2003). Finally, it has also been demonstrated that just the act of being engaged in PRT may be beneficial to well-being, regardless of the usefulness of the behavior. Bloomsmith et al. (2007) cite several studies in which self-injurious behavior could be reduced by PRT. Similarly, Coleman and Maier (2010) demonstrated that PRT for complex tasks helped to decrease stereotypical behaviors. While much of the research regarding the benefits of PRT in a laboratory setting has centered on nonhuman primates, there is no reason to doubt its benefits for other laboratory species, and the cost/benefit ratio is likely to remain favorable for any of the large, potentially aggressive species. There are also extensive discussions of PRT implementation available online for both dogs and pigs, because of their status as pets, though controlled studies of the physiological benefits are somewhat lacking.

The growing consensus in support of PRT as a meaningful refinement for laboratory animals would seem to parallel that regarding social housing in the lead-up to the recent publication of EU regulations and US guidelines. In fact, PRT itself is addressed in both of these documents, with the EU Directive (2010) stating that "Establishments shall set up habituation and training programmes suitable for the animals, the procedures and length of the project" and the Guide (NRC, 2011) stating "Habituating animals to routine husbandry or experimental procedures should be encouraged whenever possible." Though these statements lack the specifics or force of the same documents' discussions of social housing, it is not unreasonable to assume that these may be tentative first steps toward an eventual acceptance of PRT as a prevailing standard. As the literature discussing the techniques and benefits of PRT continues to grow, laboratory animal facilities would do well to ensure they keep pace, both for their own benefit and that of the animals in their care.

Consideration 4: Challenging our nonchalant attitude toward rodent welfare

Cold stress and nesting material – optional?

The number of mice used for research has undoubtedly increased with the advent of genetically modified strains. (In the US, one cannot be sure because there is no public record of the numbers of mice and rats used in research due to their exclusion from the Animal Welfare Act.) Mouse use has also increased in the UK (UK Home Office, 2013) and the number hovers around 7 million in the EU (European Commission, 2013). Increased use of mice has necessitated changes in husbandry to allow for expediency and convenience for their care. For example, the use of individually ventilated cages contributes to the convenience of care, but mice, when given a choice, prefer cages that are not ventilated (Baumans et al., 2002). Several studies have been published recently that highlight the fact that mice in laboratory facilities are likely cold-stressed (Gaskill et al., 2012), and that the scientific reliability of such mice is questionable (e.g., Karp, 2012). Cold-stressed mice exhibit altered immune systems (e.g., Nguyen et al., 2011) and increased tumor growth (Kokolus et al., 2013) compared to mice housed at their thermoneutral temperature. The study by Gaskill et al. (2012) showed that mice of different strains and sexes prefer temperatures of 26-29°C, and if provided with 6-10 grams of nesting material, will build suitable nests to maintain body temperature. This small provision for mice can have tremendous advantages for both welfare and science. It allows the mice to regulate their body temperature better than by changing the ambient temperature of the animal room, since mice experience temperature fluctuations throughout the day and can move in and out of their nests as needed. Feed conversion in appropriately thermoregulated mice is more efficient and reproduction rates are higher with higher pup survival (Gaskill et al., 2013). Allowing mice to build nests provides them with the ability to control their environment and permits them to perform the natural behavior of nesting (Latham and Mason, 2004). The availability of nests affords the mice opportunities to retreat from other mice in the cage as well as regulate their body temperature, thus contributing to decreased stress and increased well-being.

Pain control is important in mice

Recognition and alleviation of pain in mice is important from both scientific and welfare perspectives. Pain has been shown to cause the stress response in animals, and thus can compromise scientific data through disturbance of homeostasis (e.g., Blackburn-Munro and Blackburn-Munro, 2001). Yet recognizing pain presents a formidable challenge in laboratory animal facilities solely from the perspective of the sheer number of animals to evaluate. Another factor is our collective inability to detect the signs of pain in a prey species such as the mouse, since obvious expression of physical compromise presents a distinct survival disadvantage. However, a significant article published by Langford et al. (2010) contributed to a change in the way the laboratory animal community viewed pain in mice. This exquisite study reported a "grimace scale" to score pain severity upon the administration of noxious stimuli to mice at different intensities. Similar to the reaction of human infants, mice express subtle but definite reactions to painful events that can be recognized by changes in their eyes, nose, cheeks, ears, and whiskers. This system was compared to both manual and automatic behavioral scoring of post-vasectomy pain in mice and found to produce concomitant evaluations (Leach et al., 2012). A grimace scale has also been established in rats (Sotocinal et al., 2011). It has also been suggested that ultrasonic vocalizations can be indicative of welfare states in rodents, thus perhaps providing an alternative type of assessment (Portfors, 2007).

Recognition of aversive states in mice has become more imperative given the number of genetically modified animals that are created. The welfare of these animals can be compromised because of the need to perform invasive procedures to create the animals or to obtain samples for genotyping as well as the fact that these strains many times are created without prior knowledge of the effects of the genetic modification on the phenotype (Ormandy et al., 2011). There is also a welfare issue due to the elevation in the numbers of mice needed to generate a particular genotype as the efficiency in 2003 was estimated to be only 1-30% (Robinson et al., 2003). While the efficiency may have increased since then, the number of mice that are killed because they do not have the desired genotype causes the total number of mice used for research to increase significantly. While this number may be expected to decline once all possible genetically-modified strains are generated, currently thousands of mice are killed daily, usually by CO_2 asphyxiation.

Euthanasia of rodents is still in need of refinement

In addition to the concerns discussed earlier, euthanasia of rodents is an area of increased scrutiny and an evolution of thought, but one where significant room for refinement remains. As mentioned above, CO_2 asphyxiation is the most common method of euthanasia for rodents. The popularity of this method is due in large part to its convenience, as many animals can be euthanized simultaneously using small amounts of a cheap gas, but it is also due somewhat to the knowledge that in humans and many other animals, CO_2 is an undetectable anesthetic gas at sub-lethal concentrations. Thus, if the air around an animal is slowly replaced with CO_2, that animal will slip into unconsciousness when the concentration reaches approximately 30%, then painlessly suffocate while in this anesthetized state with continued exposure and/or increasing concentrations of CO_2. This preference for slow filling has been endorsed by the American Veterinary Medical Association, which in its 2013 Guidelines for Euthanasia specifically recommended slow-filling systems rather than immersion in high concentration of CO_2 (AVMA, 2013). While immersion in "pre-filled" chambers had the advantage of rapid onset of unconsciousness and time to death, it has been demonstrated that exposure to high concentrations of gas leads to the formation of carbonic acid on the mucous membranes, leading to discomfort in humans at concentrations of 30% and pain at higher concentrations. Unfortunately, even with slow filling it is apparent that rodents are able to detect, and actively avoid, concentrations of CO_2 well below the anesthetic threshold (Makowska and Weary 2012; Wong et al., 2012). This is not entirely surprising given their evolution as a burrow living species, and has spawned any number of attempts to find a cheap, safe, inhaled agent that would be more humane. Unfortunately, these attempts have been largely unsuccessful. The most recent evidence would appear to suggest that inhaled anesthetic agents would be preferable, as rats given the choice between anesthetic concentrations of isoflurane and a bright light very often chose to remain in the gas to the point of unconsciousness. However, even this work is not without caveats, as the same animals quickly fled the gas on a second exposure, suggesting that isoflurane euthanasia would be a more humane alternative to CO_2 only in cases where the animals had not previously been exposed to it during surgery or other procedures (Wong et al., 2012).

Consideration 5: The future of refinement

The laboratory animal community – comprising scientists, veterinarians, behaviorists and animal care staff – has as its goal optimal animal welfare, both for the benefit of the science and the animals. Our knowledge base about the needs of the animals in a laboratory environment is increasing, but is still far below what is needed to ensure the best conditions for their care. However, even if we cannot achieve ideal refinement, then we need to be creative in approximating it as closely as possible given our current knowledge, and continue to improve it as we uncover more information about the needs of our laboratory species. In order to intensify our collective efforts to understand these needs, it would be useful to have a concerted international approach in conjunction with dedicated, multisource funding. While the studies needed to generate the knowledge to optimize the conditions for laboratory animals are not in the realm of the "cutting-edge science" that fuels the competition for funding, they are nonetheless necessary in order to ensure that our animal models are the best they can be – accurate models for disease and drug development – for the benefit of the science and the animals.

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