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Editorial

Refining canine experimental protocols

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Italy, and in particular the Istituto Superiore di Sanità (ISS), which has long represented a national reference point for animal experimentation, addressed the issue of regulating animal testing by initially implementing the European Directive 86/609/EEC (1986) [1-3]. When this Directive was adopted [4], unfortunately only in 1992, a further restriction was introduced [5], or rather a special legislative pathway was defined, for animal species considered vulnerable in terms of extinction risk (e.g., vulnerable, endangered, rare, near-extinct species, etc.). This pathway incorporated the classification of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, https://cites.org/) into national legislation. In March 2014, four years after the new European Directive on protection of animals used for scientific purposes had been issued [2, 6], new national legislation [7] modified the criteria and evaluation grid for research projects. The ISS remains actively engaged in promoting procedural and methodological ameliorations in 3Rs principles [8-11].

Over time, behavioural sciences and veterinary ethology at the international level have progressively developed refined methods for assessing psychophysical well-being, particularly in dogs, a species that was widely used in experimental research in the past. For many years now, legislation requires that, in addition to traditional model species (rats, mice, hamsters, quickly joined by zebrafish, etc.), a "non-rodent species" be included at the regulatory level. This requirement originates from the well-known thalidomide accident, in which pregnant women treated with the antiemetic drug experienced severe teratogenic effects in their fetuses. In fact, insufficient toxicological assessment in the rodent species used at the time to detect adverse effects before administration to human beings failed to reveal such outcomes due to absence of proper prenatal treatment evaluations, and to interspecific differences in metabolic processes [12].

At the end of this Editorial, aimed at refining the use of dogs in experimental settings, we provide a selective list of reference articles illustrating the progressive improvement of methodologies related to specific procedures (e.g., urine collection). Finally, we conclude with some helpful suggestions for adhering to the established 3Rs Principle, even at the international level.

Training laboratory animals has been widely recognised as critically important for biological sample collection, as it allows a substantial reduction in the reliance on forced manual restraint, which can rapidly induce aversion and stress responses, as well as on chemical restraint. Indeed, the induction of anesthesia or repeated pharmacological sedation may significantly alter physiological, endocrine, and immunological parameters, thereby introducing confounding variables that can ultimately affect study outcomes. By promoting voluntary cooperation through reward-based training, sample collection procedures can be performed more efficiently, with improved animal welfare and enhanced reliability of the collected data [13, 14].

In this context, it has long been established that, both in cognitive assessment and in routine experimental or veterinary procedures, reward-based training paradigms are significantly more effective than aversion-based approaches in many species, including dogs, cats and non-human primates (who are subject to the most rigorous preventive control).

Reward-based methods are associated with improved learning performance, reduced stress responses, and more stable behavioural and physiological outcomes, whereas aversion-based techniques are linked to increased fear, pessimistic bias, impaired learning ability, and compromised welfare [15]. In support of these principles, the extensive literature on positive reinforcement training (PRT) should be also considered [16-21]. Indeed, European Directive 2010/63/EU [6] and the corresponding Italian Legislative Decree 26/2014 [7], explicitly state in Annex III (General Section): "Establishments shall set up habituation and training programmes suitable for the animals, the procedures, and the length of the project". This provision is retained in the revised Annex III introduced by Legislative Decree n. 183/2025 [22], which will apply from December 4, 2026.

In many veterinary procedures involving animals in experimental settings (as well as in zoos), PRT has proven extremely useful when the animal is subjected to procedures for which a "standardized state of calmness" is essential for the reliable collection of scientific data. These procedures include blood or urine sampling, swab collection [23] repeated injections oral dosing/volun-

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tary ingestion of compounds, monitoring and measurement procedures (e.g., blood pressure measurement, electrocardiogram electrode placement), imaging and diagnostic procedures (e.g., ultrasound examinations, non-restrained radiographic positioning, ophthalmic examinations, and otoscopy) [24], handling management, and welfare-related procedures (e.g., voluntary kennel entry and exit, crate or transport box training, muzzle training, nail and fur toileting, habituation to novel environments or equipment, and target training for positioning, body condition scoring, nail trimming, fur grooming).

An important point concerns the scoring of the degree of suffering (mild, moderate, severe) in dogs. Although not universally validated, dog-specific severity scoring system is currently available, existing regulatory guidance clearly indicates that severity classification must consider not only physical pain, but also suffering and distress, including their emotional and psychological components [6, 25]. In dogs, a highly social and cognitively complex species, distress often manifests primarily as alterations in emotional state rather than as overt signs of physical pain. Indicators of emotional distress in this species include both overt, traditionally recognised behaviours, such as freezing responses and avoidance or withdrawal, and more subtle signs, including lip licking, yawning, head turning, and paw lifting, as well as negative shifts in affective state measurable through cognitive bias paradigms (e.g., pessimistic bias) [18]. These behavioural and cognitive indicators have been consistently associated with a measurable deterioration of welfare, even in the absence of detectable pain. Together, these considerations provide a strong scientific basis for explicitly incorporating emotional distress and its ethological markers into severity assessment for dogs. Such assessments should encompass both overt behaviours and more subtle ethological indicators, in full accordance with Directive 2010/63/EU [6, 7] and with the FELASA/ECLAM/ESLAV guidance [25].

With regard to the use or potential reuse in experiments of the same canine subject, focusing on a particularly critical species, a solid and progressively improving reference protocol has been developed over time [26].

Similarly to cats, dogs do represent a "special case", in which an enduring dialogue between the scientific community and general public becomes mandatory. The increasing prevalence in single-person households, especially in some Western countries, together with the strong dyadic relationships between human caregivers and family dogs, often fulfilling roles once occupied by broader human social network, has significantly heightened public sensitivity toward canine welfare. This sensitivity exerts powerful influence on how scientific research involving dogs is perceived. Consequently, for such an iconic species, there is a growing necessity to balance public sensitivity for canine quality of life and welfare, with the continued, albeit limited, necessity of experimental activities, conducted within an increasingly morally relevant (3Rs) principle.

Conflict of interest statement

The Authors declare that there are no conflicts of interest.

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