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ECOSYSTEMS

Refinement as ethics principle in animal research: Is it necessary to standardize the Environmental enrichment in laboratory animals?

MARTA L. FISCHER, GABRIELA S. RODRIGUES, WINDY P. AGUERO, RAFAEL ZOTZ & DAIANE P. SIMÃO-SILVA

Abstract: The Environmental enrichment technique, although scientifically recognized for raising the level of animal welfare, has led to the questioning of its influence on the results of experimental research. Thus, the goal is to promote reflection about the need for standardization of these procedures. For that, documents and experimental analysis were done, in order to quantify and characterize the types of environmental enrichment used and to evaluate the effect of that in the social behavior of *Rattus norvegicus*. Data from the document review confirmed the hypothesis that the researchers have used a variety of methods, not demonstrating a concern for standardization and prior assessment of its effects on the search results. Demand was corroborated in the experimental study in which, although there was available a simple object acting as refuge promotes behavioral improvements, the presence of the co-specific, as well as characteristics of the micro and macro environment can compromise the homogeneity of the sample. The data from this study endorse the need for validation procedures of environmental enrichment for specific proposals, to investigative data comparison, are possible and contribute to the refinement of the search to reduce the number of animals targeted for this purpose.

Key words: animal ethics, animal model, animal experimentation, animal welfare principle of 3R's, *Rattus norvegicus*.

INTRODUCTION

The use of animals for experimentation involves paradoxes (Feijó 2005), for example, the divergence between the need for the animal model for testing human health products, at the same time as it is attested that many of the effects are not transposable between species. There is also the argument of the importance of animal manipulation in teaching for the development of professional skills, *versus* researches showing a process of student careless throughout the course. Moreover, the contrast between the intention to provide the animal an environment that reduces suffering from confinement and experimental procedures, *versus* the obstacles imposed by the demand for standardization, and the rigid standards of production control and zootechnical management (Francione 2013, Bayne 2018). It is agreed that the use of animals promoted the scientific and technological development of humanity, as well as cognitive, phenotypic and genotypic improvement in domesticated species (Feijó 2005). In the same way, it is known that this process has come at the expense of a significant decrease in the quality of life of these species (Morris 1990).

The technique of the environmental enrichment is a set of procedures applicable to captive animals that live under human tutelage, in which physical, sensory, cognitive, and exploratory stimuli are inserted in the environment, aiming to simulate conditions similar to those natural that activate behaviors that promote animal welfare (Broom & Fraser 2010). The technique emerged with the intent of making captivity more complex and interactive, both for livestock intended for breeding, aiming the reduction of disease and mortality, as well as for wild species kept in conservation breeding, and other animal categories (Broom & Fraser 2010). Nevertheless, the implementation of environmental enrichment initially requires understanding the necessity of the animals (Malafaia et al. 2011).

The environmental enrichment for laboratory animals has found resistance by changing the strict production and management zootechnical routines, such as sanitary and nutritional control (Galef & Bennett 1999, Wolfle 2005), but especially in the last decade, numerous studies have shown significant improvement in the welfare of the animals, through simple environmental changes in the microenvironment (Orok-Edem & Key 1994). In Brazil, the National Council for Animal Experimentation Control (CONCEA) advises that, in order to alleviate the unwanted effects of captivity to the animal welfare, not only to meet its basic needs, such as appropriate water supply, food, temperature, proper selection, and anesthesia methods in experimental procedures, but it is necessary too to reflect on other ways to improve the environment (CONCEA 2015).

However, it is questioned how the environmental enrichment technique has been used in laboratory animals and what is the contribution to the refinement of the research, resulting, consequently, in more reliable data. Even in the face of scientific evidence of improvement in the physical and mental conditions of animals undergoing environmental enrichment, it has been questioned whether it would be a new variable capable of influencing the response of experimental animals to stimuli tested by conventional science. In this sense, if the modifications introduced are not well planned and applied, they can generate problems, because the control of the experimental group is lost, consequently generating the subsequent need to use a larger number of animals to reach a statistically testable response, besides making unfeasible the comparison between researches (Fischer et al. 2016). In the face of this demand, it is urgent to evaluate the applicability of ethical and legal recommendations based on the 3R principle (Russell & Burch 1959) in order to refine these studies promoting the reduction of suffering, reducing the number of animals, and developing alternatives methods. Thus, the present study sought to answer whether it is necessary to standardize environmental enrichment in order to promote the animal welfare while ensuring fidelity, reproducibility, and comparability of results. It is hypothesized that the use of a variety of environmental enrichment methods makes the comparison unfeasible, requiring reflection on the need for its standardization through the validation of items used in the referred technique. Thus, the objective was: a) to evaluate the existence of the environmental enrichment standardization of animals used in experimentation in the same area; b) to evaluate if the behavior analysis of *Rattus norvegicus* (Berkenhout 1769) is effective in identifying the influence of different types of the environmental enrichment; c) to reflect on the aspects that have to be considered in the planning of an investigation when using the environmental enrichment as a technique for research refinement; d) to discuss about

the demands for the implementation of the environmental enrichment as standardization of the animal welfare.

MATERIALS AND METHODS

This study was approved by the Animal Use Ethics Committee of the Pontifical Catholic University of Paraná (PUCPR) (protocol no. 685). All procedures employed, as well as those adopted by the animal origin bioterium, were in accordance with Brazilian law (CONCEA 2015). The selection of the animal model was due to its prevalence in the institution's experimental protocols. The experimental design sought to use the minimum necessary animals to perform statistical tests. Environmental enrichment was not provided as refinement for all animals since the absence of this was one of the variables. The habituation was performed before the tests, so that the animals became used to the presence of the researcher, as well as to monitor their reactions during the research, with minimal manipulation and permanence in the laboratory. The habituation started two weeks before the beginning of the experiment with the visit of the researcher to the animals every day, for 10 minutes, at different times, remaining close to the boxes for the animals to feel the odors and get used to their presence.

Document analysis: characterization of environmental enrichment application in laboratory animals

The documentary analysis took place in scientific articles retrieved through the CAPES Periodical Portal (Coordination for the Improvement of Higher Education Personnel), a Brazilian indexing platform for articles from various bases. It was applied as a search the term "environmental enrichment" conditional on the presence of the term "rats". The first 100 articles published between 2013 and 2014 involving the use of the environmental enrichment in neuroscience studies were rescued. This cut is justified due to the direct influence that has been attributed to the quality of life in relation to the quality of research results, more effectively in studies that aim to evaluate behavioral response. For quantification of environmental enrichment classified according to Neves (2013), the exclusion factor was inaccessibility to the full article, as well as literature review studies, so 80 articles were analyzed.

National and international guidelines, available on the Internet, regarding guidelines for conducting laboratory animal research, were analyzed. To this, were considered the global scope directing the search for application documents in Europe, Oceania, North America, South America, and Brazil. An exploratory analysis of the content of how the environmental enrichment is approached in these guidelines and the orientation to the researcher was performed.

Experimental analysis: animals and research site

With the data of the first stage, an experiment was designed to certify the need to consider certain variables in the planning of experimental research. To this, the behavior of 40 *R. norvegicus* males was analyzed, starting the tests 30 days after birth and followed for 6 months. Males were chosen because they have more pronounced territorial behaviors than females, promoting the establishment of hierarchies (Van Loo et al. 2004). The animals, originating from the vivarium of the higher education institution, were housed in the Behavioral Analysis Laboratory, in standardized boxes, placed on shelves. The boxes were made of polypropylene measuring 0.41 m long, 0.34 m wide and 0.17 m high, covered

with 0.41 m deep galvanized wire grid with 0.34 m wide and 00.7 m mesh. The bed was made up of pine wood sawdust. The maintenance of the animals was characterized by the daily *ad libitum* offer of water, food and the sawdust were change twice a week. Water was available in 0.7 L polypropylene drinkers with stainless steel spout and rubber stopper. The animals were weighed monthly on a digital precision scale.

Experiments

The animals were separated into 4 groups, with 10 animals each one: Group 1: isolated rat with environmental enrichment; Group 2: isolated rat without environmental enrichment; Group 3: rat in pairs (social enrichment) with environmental enrichment and Group 4: rat in pairs without environmental enrichment. With the intention of simulating the reality of many laboratories and, consequently, emphasizing the importance of the macro environment, no strict control of sanitary barriers was provided, nor control of temperature, humidity, and noise.

Physical enrichment

The physical enrichment consisted of the provision of a 180º bend PVC pipe with an open diameter of 0.75 m at the three ends. The tube was arranged in lateral position and over the sawdust bed.

Social enrichment

The social enrichment consisted of maintaining the animals in pairs. For recognition of the individuals, a mark was made on the tail, with a marker pen, which was reinforced weekly. In order to this variable did not influence on behavior, all the animals were marked. The animals were kept alone or in pairs in the standard boxes.

Behavior analysis

Video recordings of the animals were conducted twice a week, during the day and night, spending 2 minutes per cage, totaling 160 hours per week for 6 months. The filming was recorded with infrared light intended to exclude the interference of luminosity. Through the analysis of the records were qualified and quantified, the qualification was made for the elaboration of the ethogram, and then the behavioral acts exhibited by the rodents were quantified. From the records, it was possible to identify the establishment of social hierarchy distinguishing dominant and subordinate individuals.

Statistical procedures

The test results were compared between groups according to environmental and social enrichment. Comparisons between means were performed by the nonparametric Kruskall-Wallis and Mann-Whitney tests. For the homogeneity test of the samples (bibliographic and behavioral data), the "Goodeness of fit" test (G_{test}), was used, for the adherence test was used chi-square test. In all tests it was considered as null hypothesis the homogeneity of the sample and the significance level of 95%.

RESULTS

Document analysis: characterization of environmental enrichment application in laboratory animals

The neuroscience articles analyzed gathered 44 modalities of environmental enrichment categorized into: cognitive (running wheel and maze), physical (tunnel, tubes, igloo hut, shelters, nesting material, nets, ropes, spinning rod, blocks, cubes, lego, chain, boxes, barrel, mat, floor, ramp, ladder, platform, ball, bite toys, mirror, each of which can be made of

wood, plastic or paper), sensory (hissing toys, bells, music, objects moving, colorful toys) and nutritional (funnel with food and water bottle) and social (presence of co-specific). Physical enrichment presented greater diversity of items used (70%) than sensory (18%), cognitive (4.5%), nutritional (4.5%) and social (2%) ($G_{test\ (4)}$ =60,4; P<0.001), and was also the one that presented the highest representation, present in 92.5% of the analyzed articles. Most studies used two types of environmental enrichment (one = 12.5%, two = 45%, three = 31.2%, four = 11.3%) ($G_{test\ (4)}$ =25,3; P<0.001), being social / physical the most frequent combinations (31.2%).

Behavior analysis

The ethogram of *R. norvegicus* totaled 31,009 records related to 30 motor patterns grouped into the categories: maintenance (33%), exploration (16.7%), interactions (20%) and interactions with the object (30%). Although the maintenance category was the most diverse in motor patterns, exploitation, although represented by only 16.7% of the categorized motor patterns, was the one that accounted for the largest amount of sampled records (80.5%) ($G_{test\ (3)}$ = 44979; P<0.0001).

The environmental enrichment provided a reduction in behavioral acts for animals kept alone (without environmental enrichment = 8405; with environmental enrichment = 4804; $G_{test(1)}$ = 1642; P<0.0001), in contrast to animals kept in pairs (without environmental enrichment = 8461; with environmental enrichment = 9339; $G_{test\ (1)}$ = 7.54; P < 0.01).

The environmental enrichment was effective in reducing the acts of “cleaning”, “ingesting”, “sleeping” and “stopping” in the maintenance phase in isolated animals, “biting”, and “ingesting” in pairs while “scratching” decreased in dominant with environmental enrichment (Figure 1). During exploration,

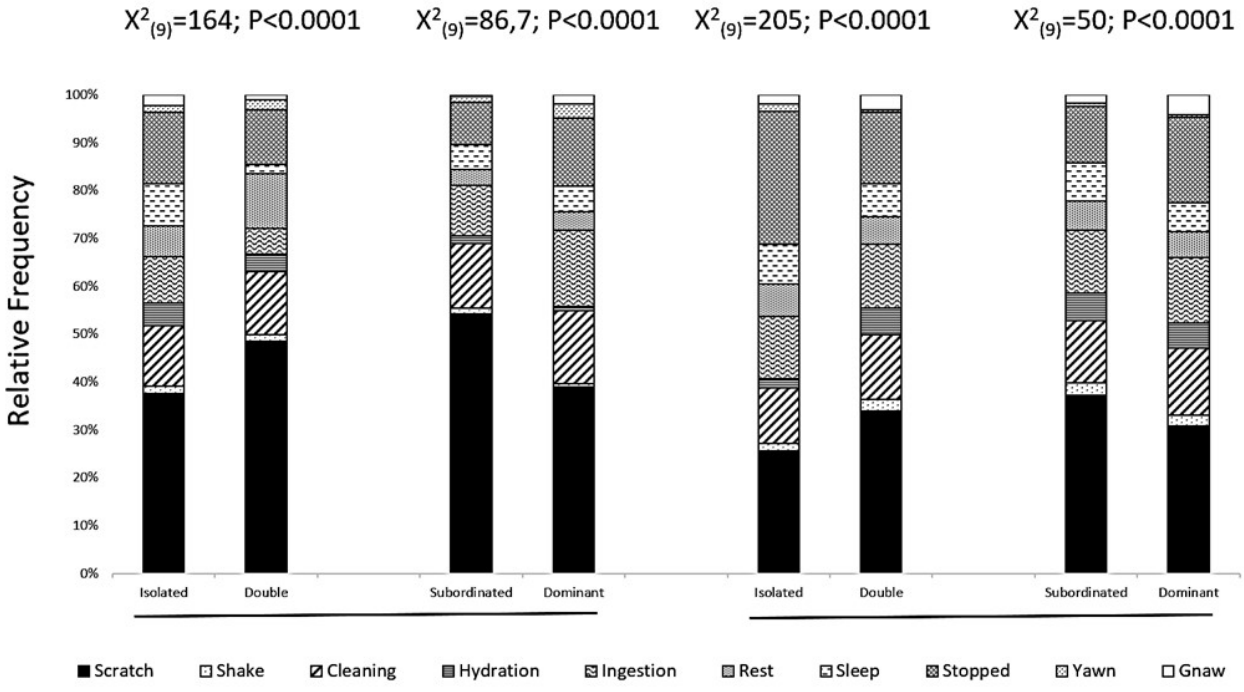

Figure 1. Relative frequency of motor standards for maintenance category. Absolute frequencies were compared between isolated and double animals and between subordinate and dominant animals using the chi-square test, with significantly higher values (P <0.05) accompanied by an asterisk (*).

"sniffing" predominated, with subordinates sniffing more with environmental enrichment (Figure 2). In social interactions the dominant animals were more active, with high levels of acts of "submission" and "grooming" in subordinates, was seen "grooming", and "fights" in the dominant ones. In the enriched environment there was more "pushing" in both and fighting in the dominant ones (Figure 3). The dominant interacted more with the object using it to "lift", "clean", "ingest", and "stop", although the subordinates had significantly more acts, especially "walking", "sniffing", "stop, and "ingest" (Figure 4).

The dominant animals presented the lowest final average weight in both environments, one of them with environmental enrichment (286.0 ± 13.8g) and the other without (277.6 ± 34.3g) when compared to rat kept isolated (313.8 ± 23.3g). and subordinated (306.4 ± 27.3g) without environmental enrichment and isolated (300.9 ± 19g) and subordinated (303.2 ± 5g) with environmental enrichment.

DISCUSSION

The data from the present study emphasize that, in front of the ethical and legal demands of refinement of animal enclosures used in experiments, researchers have used a variety of methods for enrichment of the micro environment. However, it has not been attested, the potential influences of these new elements inserted in the experimental space, which the associated with the macro environment conditions, if they have not involved a rigid planning and previous investigations, may compromise the homogeneity of the sample and, consequently, the interpretation of the results.

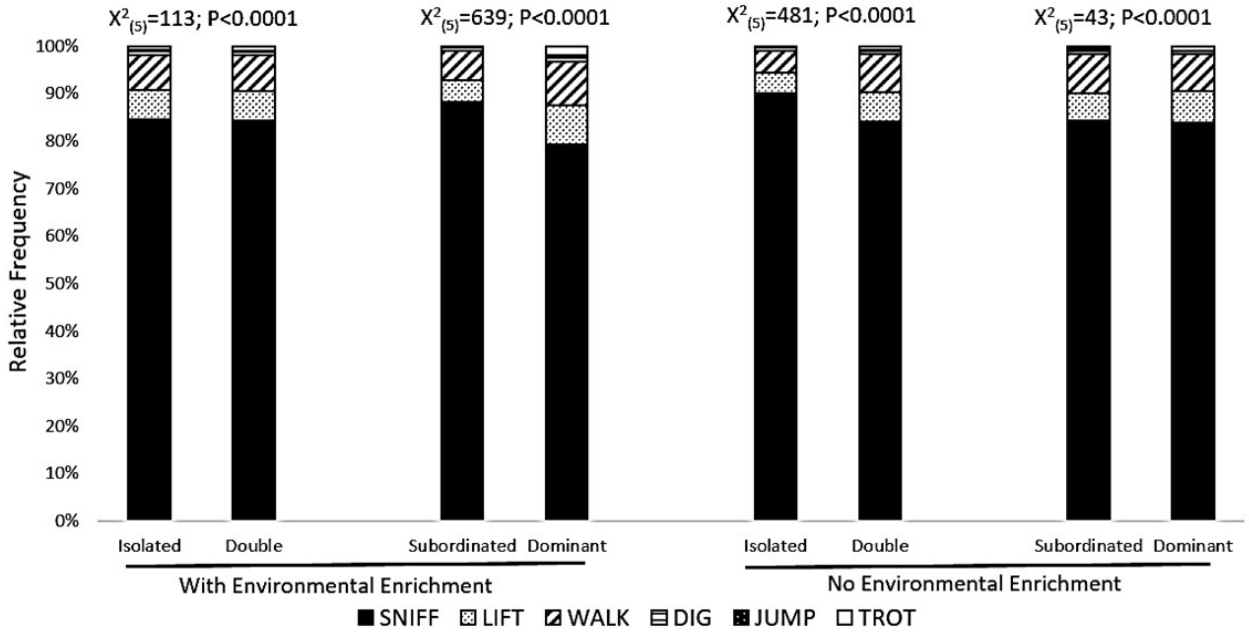

Figure 2. Relative frequency of motor patterns for the cate/gory Exploration. Absolute frequencies were compared between isolated and double animals and between subordinate and dominant animals using the chi-square test, with significantly higher values (P < 0.05) accompanied by an asterisk (*).

Documentary and bibliographical analysis

The characterization of the use of the environmental enrichment by current researches evidenced the existence of a still incipient initiative of the scientific environment to refine its studies and provide better physical and mental conditions for animals associated with the promotion of specific methodologies for common goals. The diversity of objectives and combinations of the environmental enrichment categories promotes the question as to the functionality of this conduct in enabling the comparison between the results of different researches. Once, the analysis of the environmental enrichment used in studies of the same area of activity in a restricted period of time shows a range of possibilities isolating the interventions in their interpretations. Official documents should be considered to support the use of the environmental enrichment in experimental studies. The European Directive (European Directive 2010) states that all animals must have adequate space to express a broad behavioral repertoire. While the England Guide (Research Animals Department 2013) warns of the lack of standardization of the environmental enrichment, and consequent increase in the variation of behavioral and physiological parameters. Some researchers argue that animals in a refined and more complex environment demonstrate a greater variability of answers (Eskola et al. 1999, Tsai et al. 2003), others (Baumans & Van Loo 2013) disagree with this view, arguing that precisely because animals have opportunities to perform their particular behavior more frequently in refined environments and also they enable them to better handle with unexpected events and, thus, have more uniform responses. According to Bayne (2018) the behavioral breadth of the species may help to understand the different conclusions about the gain and uncertainties in providing enrichment for mice in the research.

An important factor to be emphasized in the document recommendations in the

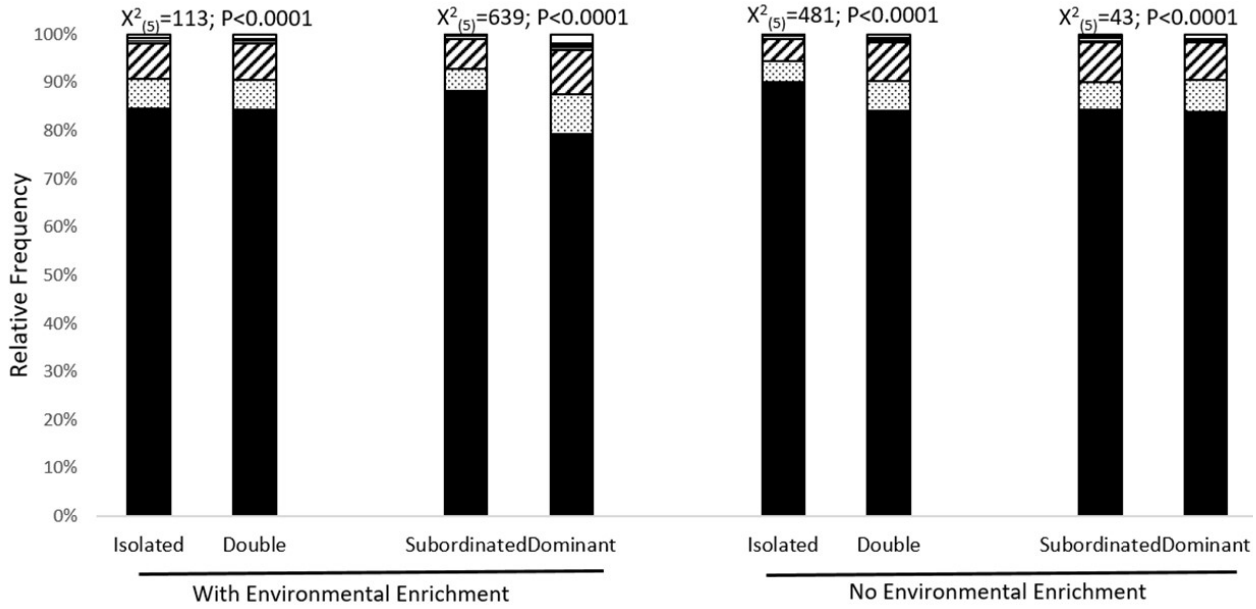

Figure 3. Relative frequency of motor patterns related to the Social Interaction category. Absolute frequencies were compared between isolated and double animals and between subordinate and dominant animals using the chi-square test, with significantly higher values (P < 0.05) accompanied by an asterisk (*).

area, concerns the training of researchers and professionals in the knowledge of experimental models of biology, to ensure proper monitoring of the environmental enrichment (National Research Council 2011). The documents warn of the need for the environmental enrichment programs to be reviewed by Animal Research Ethics Committees, researchers, and veterinarians to ensure their benefits for the animal welfare and for consistency in research objectives. In a more austere posture, the guide from Australia (National Advisory Committee for Laboratory Animal Research 2004) calls for the environmental enrichment to be considered as refinement and aiding in the relief of pain and anguish, and its exemption must be justified. Baumans & Van De Weerd (1996) point out that the environmental enrichment implies attending the animal to improve the animal welfare, so it can not be considered a luxury, but a necessity, suggesting the substitution for the term "environmental refinement". In this context, the institution plays a key role in providing the necessary means and opportunities that contribute to corporate social responsibility. In Brazil, the "Fiocruz animal use manual" (Fundação Oswaldo Cruz 2008) encourages researchers to maintain an environmental enrichment program, both in creation and experimentation. The "Laboratory Animal Care and Procedures Manual of the University of São Paulo" (Neves 2013) refers to the uniformity of responses by pointing out that animals kept in enriched environments can be more physiologically and psychologically stable, ensuring better scientific results. The norms highlight the need for environmental enrichment as refinement demonstrating the influence on the quality of research results, a fact that should be taken into account by researchers in the results of their

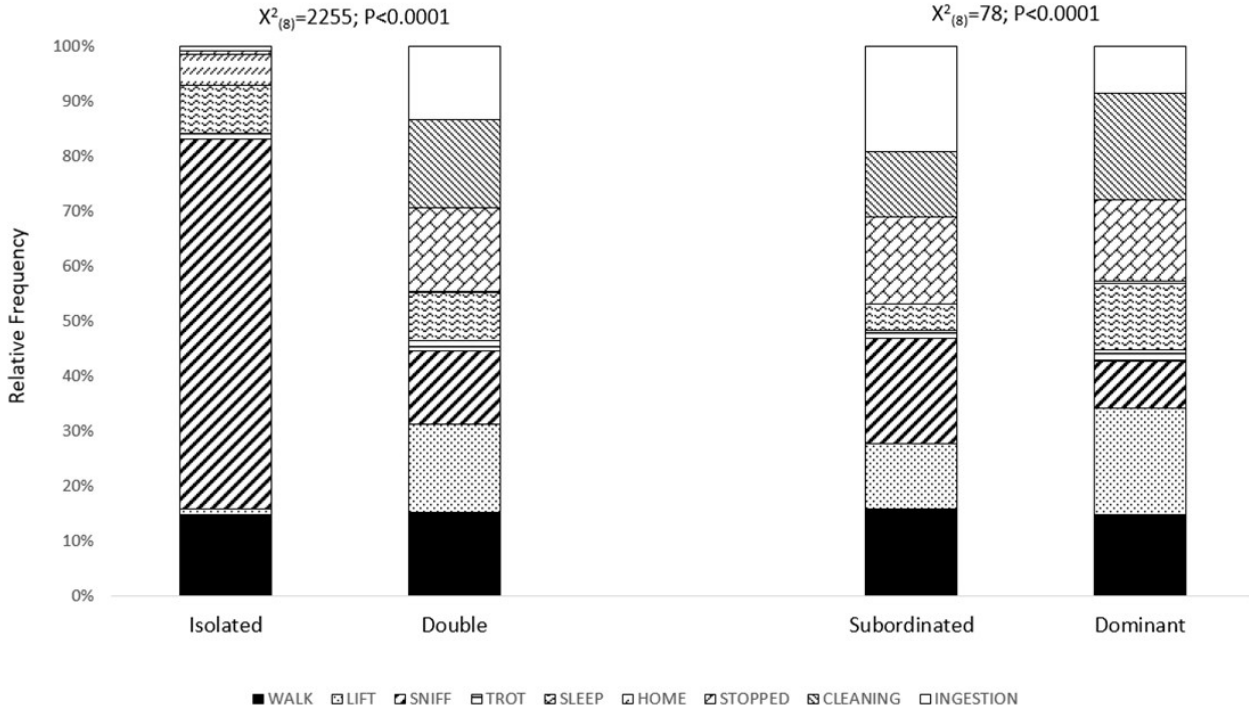

Figure 4. The relative frequency of motor patterns relating to the "Object Interaction category". The absolute frequencies were compared between isolated, double animals, between subordinate, and dominant animals using the chi-square test, with significantly higher values (P < 0.05) followed by an asterisk (*).

scientific data. According to Andersen & Winter (2019), Brazil has adopted an ethical standard and specific rule for animal protection and the promotion of animal welfare, maintaining its representation in international research. The "Brazilian guide for the use of animals for teaching and research activities" is in the final production phase, originated from normative resolutions (RN) no. 12 and no. 25, which to date has already published the introduction and of the norms for the creation and management of some groups as rodents and lagomorphs (CONCEA 2015).

The diversity of methods and greater use of physical and social environment enrichment (Baumans & Van De Weerd 1996) can be inferred from a greater knowledge of this modality of the environmental enrichment, as well as its economic accessibility. However, precisely the increased availability of media contributes to a deficiency in standardization that can make the results of studies incomparable, thus making it difficult to reduce the number of animals, once that studies will be required. It is questionable whether the researchers were concerned with detailed planning as well as a pilot project, taking into account the necessary factors that contemplate the macro, micro environment, and the animal itself, according to the recommendations of RN n.12 and RN n. 25 of the CONCEA (CONCEA 2015). Although there is an increase in the environmental enrichment adherence, the lack of standardization prevents all aspects necessary for the promotion of the animal welfare from being addressed.

Experimental analysis

The data obtained from the experiments attest that behavioral analysis is an efficient parameter to evaluate the influence of the environmental enrichment which used in pilot studies can guide the research planning. The behavioral analysis of the rat, in general, evidenced the positive influence of the social enrichment on the behavior of these animals, as found by Elliott & Grunberg (2005), that registered better habituation performance to new situations. However, it should be considered that social enrichment triggered the emergence of hierarchy, whose imposition of dominance was confirmed by the display of differential behaviors, with the dominant being subordinate easily distinguished, and the higher effectiveness of the social enrichment for the dominant, supporting Galef & Bennett (1999). It was checked that the dominant rat have been responsible for the initiating of the aggressive and reactive interactions. According to Crawley (2007), the hierarchization is expected in groups of confined male mice, with the aggressiveness of the dominant being an adaptive, functional, dynamic, and flexible character, yet structured within certain limits reached by a behavioral pattern limited to social reactions and signals (Mattaraia & Oliveira 2012). Hurst et al. (1996) have verified the existence of three social patterns in rat: dominant, passive subordinates, and aggressive subordinates, and the latter are prone to develop hypertension, whose physiological imbalance, besides compromising the animal welfare, may cause changes in the results of the experiments. Mice can also respond to socialization in an active way by displaying threat, approach or flight; or passive through defensive behaviors or body immobility (Mattaraia & Oliveira 2012). Thus, dominant active animals will be responsible for the attacks, characterized mainly by bites (Takahashi & Blanchard 1982), as evidenced in the present study with the record of gonadal bleeding in a subordinate of the enclosure without environment enrichment. In mice, the maintenance of confined males will commonly result in violent skin lesions, highlighting the

relationship of interaction with the environment and the socialization pattern (Oliveira 2012). Thus, in agreement with Galef & Bennett (1999), it is emphasizing that social enrichment for mice is relevant, but must be associated with the physical enrichment.

Rodents are gregarious animals, so the enclosures should promote contact with specific species (CONCEA 2015). However, social organization and the role of dominant and submissive conditioned at the cost of aggressive behavior should not be neglected (Galef & Bennett 1999), which should be minimized by considering the number of animals per enclosure, compatibility of individuals, availability of refuges and microenvironment zoning systems that allow individual access to food and water. In the other hand, it is possible to improve the conditions of the enclosure with simpler proposals such as by Manser et al. (1998), which through tests of box models and bedding models attested that it is possible to offer enclosures in the form of boxes with closed or open sides, or simply by the inclusion of vertical dividers coupled to the grid of traditional boxes. However, the authors confirmed the preference for dark materials, even at night, closed on the sides, open on the inferior, and lower surface. It occurs because the animals have remained indoors mainly during the day and on the upper surface at night. The efficiency of this model was confirmed, by the authors, by the absence of record of aggression among the co-specifics. The researcher must be aware that hierarchy may influence the behavior and physiology of the animal and that these data must be considered in the analysis of research results.

Through the analysis of the ethogram it was possible to access the environmental enrichment efficiency for the animal welfare through behaviors indicative of natural responses (Barros 2007). The environmental enrichment was effective in containing excess activity in animals kept isolated, which is a relevant fact, especially if these activities are related to repetitive movements or stress. It is noteworthy that the presence of a simple object such as a PVC pipe, used in the present study, provided the option of hiding, being sufficient to improve physical and behavioral conditions. Although in mice it has been attested that they do not preferentially use the PVC pipe because it is closed and poorly ventilated (Marques & Olsson 2007), in mice the object is widely recommended for welfare promotion and used in research with the most diverse. purposes (Patterson-Kane 2001). Galef & Bennett (1999) pointed out that the PVC pipe is indifferent to animals kept isolated, but effectively used by animals arranged in pairs as a refuge for submissive to protect themselves from the onslaughts of the dominant. The authors consider the use of the object in isolated animals to be negative because it restricts the open space of the box. However, in the present study it was corroborated that isolated animals are calmer, reducing food intake and sniffing during exploration, indicative of compulsive and anxious behaviors, suggesting that the possibility of hiding reduces energy expenditure, and consequently promotes greater comfort (Batchelor 1994). In terms of standardization, it is central to the discussion of what is actually providing enrichment, which brings good to animals, not just the insertion of items that may be referred to as "pseudo-enrichments" (Poole 1997).

Another relevant finding was the finding that animals chewed three times less in the presence of the environmental enrichment, reinforcing the containment of anxiety (Batchelor 1994). These results corroborated other studies which have found decreased locomotor activity, hyperactivity, and anxiety (Varty et al. 2000, Weerd et al. 1999, Tomchesson 2004). Weerd et

al. (1999) interpreted the reduction of activity as the development of a better ability to get used, that is, to adapt to the environment and, consequently, the reduction of exploitation, as if learning to control the environment itself.

In front of the presence of the co-specific with environmental enrichment, the subordinates ingested more due to the use of the tube to hide and feed more safely. For animal welfare monitoring, physiological changes such as weight should be observed. Rivera (2010) points out that rodents in the wild use much of their daily routine looking for food, while in the laboratory environment is available in large quantities, which associated with lack of exercise can lead to obesity, which in turn can lead to obesity. lead to alteration of the survey data. The observations of the present study corroborate Tomchesson (2004) who observed in his research with rat that they reduced weight in enriched environments due to metabolic alteration and health improvement, emphasizing the importance of interaction between environment and the social enrichment.

The lack of rigor in maintaining the macro environment in the present study, as expected, may be a finding of the results, emphasizing that although the success of the environmental enrichment is desired, it may have diminished relevance in eliminating low-grade indicative behaviors of the animal welfare, once the macro environment should consist of standardized housing facilities in terms of temperature, humidity, lighting, air quality and noise (Damy et al. 2010). The fact that scratching has not decreased with environmental enrichment in the present study, may be a reflect of the absence of this rigor and inefficiency, especially in controlling these variables. According to Barcelos et al. (2013), scratching represents an immunological expression of physical stress, associated with response to abiotic and biotic factors, thus reinforcing the importance of environmental quality to obtain reliable and reproducible results (Braga 2010) and It becomes necessary for researchers to be aware of these stimuli and indicatives and to report with integrity the exact conditions under which the experiment was conducted, in order to do not make a correct interpretation of unviable results.

Animal welfare implications

The data in the present study attest that the standardization of the environmental enrichment procedures is of paramount importance, as it provides reliable data and consequent reproducibility of studies, as well as favoring the minimization of animals due to the reduction of variation caused by environmental factors. Therefore, in addition to corroborating Fischer et al. 2016 with the adoption of the environmental enrichment as an ethical conduct in animal research, standardization is considered a fundamental procedure in preclinical studies with multidisciplinary approaches involving different laboratories and research groups. Galef & Bennett (1999) recognized in published research four possibilities for enriching the environment: increasing the complexity of the environment, increasing the size of the box, promoting company and promoting intellectual challenges, thereby improving the display of natural behaviors, health, and well-being psychological. However, the authors were skeptical of the evaluations that showed improvement in the welfare of the animals, considering them as subjective and intuitive, without scientific criteria, methodologies, and the success of the intervention depends on the species, sex, and age of the animal. Galef & Bennett (1999) argued that rodents used in experimentation were selected from an interaction of genetics with the environment resulting in docile animals,

a little reactive to humans, and thus likely to increase the degree of the animal welfare under sterile and standardized conditions of the experimental environment. Thus, changes in the environment, especially with the provision of refuges, made animals more dangerous and reactive to humans, making the environmental enrichment as counterproductive. More than 10 years later, laboratory animal science developed ways to attest to preferences and to assess the animal welfare, leading to an understanding of the influence of intervention on the triggering of specific behavior, thus requiring the association between the environmental enrichment and the goal of the search. Consequently, delaying the standardization of the environmental enrichment in the scientific environment, and promoting a range of results that make it difficult to fully compare different researches. However, it is believed that it may be possible to standardize the experimental environment for specific research, one of these proposals being the standardization of the enriched box, as in the past the standardization of the servicing box.

The maintenance box, in addition to safety for the animal, should consider group size, age of animals, if they are family groups, space for movement and relationship of natural behaviors (CONCEA 2015, Van Loo et al. 1996, Meehan & Mench 2007). There has been an evolution in the box models, and from the 1950s to the 1960s the substrate was incorporated and from the 2010s it has invested in the improvement of the physical structure (Feijó 2005). Manser et al. (1998) through preference tests performed with rat proposed to use a dark, closed, and low refuge, whose structure provided for a material easy to handle, clean, sterilize, and not gnawed by the animals, besides promoting efficient monitoring and capture of the animals and involve low production costs. Nowadays, there are alternatives that use modern ventilation systems and individual animal monitoring through microchip. Among the models available in the market, we highlight the Marlau box cited in different surveys (Van Loo et al. 1996, Meehan & Mench 2007) leading to the expectation of a trend towards standardization. First and foremost, the system meets animal welfare better than compared to conventional boxes, since it has a nine times larger area and can accommodate about 12 to 18 rat. The cages have one compartment with food spheres and one with water bottles, plus an upper floor containing a maze. To acquire food, rodents must travel to the upper floor and go through a tunnel. While for hydration should use doors with unilateral movement arranged between the two floors. This enables all animals to have equal access to different environmental enrichment and cognitive stimulation through regular changes in the maze configuration. The cage reduces stressful social interactions as it avoids territorial dominance as animals can enter and exit the maze using two doors on either side. Dominant competition over subordinates may also occur in the Marlau cage, but due to the large area and shelter, it may not impact as much as in conventional cages. Some studies have attested that this box has resulted in better cognitive performances (Fares et al. 2013). Galef & Bennett (1999), opposed to the increase of the maintenance box as a means of promotion of the environmental enrichment, because in addition to effectively large spaces do not correspond to the natural microenvironment of the species, would require a total readjustment of the vivarium generating unnecessary expenses. Therefore, the importance of conducting systematic studies that assess and adjust management issues, animal safety, sanitary conditions, and physical structure is emphasized. It is necessary to consider that

boxes and objects have no corners that can accumulate waste and make cleaning difficult. And also, the demand for a restructuring of the animal houses to accommodate larger boxes, involving the adaptation of support equipment, room, and machinery dimensions, ergonomics for moving and transporting the cages with and without animals, as well as training and increasing the staff of employees. It should be evaluated whether the large size of the box can cause difficulties in handling and monitoring due to the positioning of animals in hard to reach places. It is important to keep in mind for box efficiency that the macro environment should also be standardized and that adaptations of this model for different researches should always be well described so that the standardization protocols can be formatted.

CONCLUSIONS

The present study, in its documental and experimental scope, supports the hypothesis of the urgency and need to standardize the environmental enrichment practices in experimental animals, so that it is possible to promote the animal welfare while ensuring fidelity, reproducibility and comparability of results. Although researchers are mobilizing to fulfill an ethical and legal demand by promoting the environmental enrichment for experimental animals, the diversity of the environmental enrichment in animal responses. Failure to meet the demands of promoting the animal welfare, even in the face of studies that corroborate the effectiveness of the environmental enrichment techniques, denotes the lack of responsibility of the researcher for the research, as it is disregarding the influence of animal maintenance on the quality of scientific experiment results. and there may be changes in them making their scientific value questionable, in other words which corroborates with the phrase of Poole "happy animals make good science". The present study indicates that behavioral analysis is effective in demonstrating the influence of interventions, suggesting that they compose pilot experimental protocols that support research planning. Based on the results presented on the importance of macro and micro environments and the assumption that adequate environmental conditions are minimum requirements for the researcher to obtain reliable and reproducible results, we warn of the urgency of promoting the animal welfare as well as the standardization of the environmental enrichment. In the scientific field it is necessary to pay attention to the responsibility of all those involved in the use of animals in minimizing their suffering and of the researcher in the dissemination of the results. In addition to a complete disclosure of the experimental conditions, the search for a standardization that ensures obtaining of reliable conclusions ensuring research integrity.

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MARTA L. FISCHER[1]
https://orcid.org/0000-0002-1885-0535

GABRIELA S. RODRIGUES[1]
https://orcid.org/0000-0002-0653-0278

WINDY P. AGUERO[1]
https://orcid.org/0000-0003-0348-0807

RAFAEL ZOTZ[1]
https://orcid.org/0000-0001-9610-6843

DAIANE P. SIMÃO-SILVA[1,2]
https://orcid.org/0000-0002-1633-9899

[1]Programa de Pós-Graduação em Bioética, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição, 1155, 80215-901 Curitiba, PR, Brazil

[2]Instituto para Pesquisa do Câncer/IPEC, Departamento Científico, Rua Fortim Atalaia, 1900, 85051-060 Guarapuava, PR, Brazil

Correspondence to: **Daiane Priscila Simão-Silva**
E-mail: dpscientist@gmail.com

Author contributions

Marta L. Fischer: Designed the experiments and analysis; Performed the analysis and wrote the paper. Gabriela S. Rodrigues: Contributed to analysis tools; Performed the analysis and wrote the paper. Windy P. Aguero: Conceived and designed the experiments; Collected the data and contributed in the writing the paper. Rafael Zotz: Designed the experiments and collected the data; contributed in the writing the paper. Daiane P. Simão-Silva: Performed the data and ethical analysis. Wrote the paper.