



Page Proof Instructions and Queries

Journal Title: Laboratory Animals (LAN)

Article Number: 793450

Thank you for choosing to publish with us. This is your final opportunity to ensure your article will be accurate at publication. Please review your proof carefully and respond to the queries using the circled tools in the image below, which are available by clicking “Comment” from the right-side menu in Adobe Reader DC.*

Please use *only* the tools circled in the image, as edits via other tools/methods can be lost during file conversion. For comments, questions, or formatting requests, please use . Please do *not* use comment bubbles/sticky notes .



*If you do not see these tools, please ensure you have opened this file with Adobe Reader DC, available for free at get.adobe.com/reader or by going to Help > Check for Updates within other versions of Reader. For more detailed instructions, please see us.sagepub.com/ReaderXProofs.

No.	Query
	Please note, only ORCID iDs validated prior to acceptance will be authorized for publication; we are unable to add or amend ORCID iDs at this stage.
	Please confirm that all author information, including names, affiliations, sequence, and contact details, is correct.
	Please review the entire document for typographical errors, mathematical errors, and any other necessary corrections; check headings, tables, and figures.
	Please confirm that the Funding and Conflict of Interest statements are accurate.
	Please ensure that you have obtained and enclosed all necessary permissions for the reproduction of artistic works, (e.g. illustrations, photographs, charts, maps, other visual material, etc.) not owned by yourself. Please refer to your publishing agreement for further information.
	Please note that this proof represents your final opportunity to review your article prior to publication, so please do send all of your changes now.
AQ: 1	Please supply page nos for Gouveia and Hurst 2017

Non-aversive photographic measurement method for subcutaneous tumours in nude mice

Agustina Resasco¹ , Ana C Carranza Martin^{1,2}, Miguel A Ayala¹,
Silvina L Diaz³ and Cecilia Carbone¹

Abstract

We have developed a new method for the measurement of subcutaneous tumour volume which consists in taking photographs of mice in their home cages, to refine the standard method of measurement with calipers. We consider this new method to be non-aversive, as it may be more compatible with mice behavioural preferences and, therefore, improve their welfare. Photographs are captured when mice voluntarily go into an acrylic tube containing graph paper that is later used as a scale. Tumour volumes measured with the caliper and the non-aversive photographic method were compared to those obtained by water displacement volume and weight. Behavioural and physiological changes were evaluated to assess animal welfare. Significant differences were found between measurements obtained with the caliper and the non-aversive photographic method, v. the reference volume acquired by water displacement ($P < 0.001$). Nevertheless, there was good consistency for these measurements when tumours were measured repeatedly, with all Intra-Class Correlation Coefficients above 0.95. Mice on which the non-aversive photographic method was employed were significantly less reluctant to establish contact with the experimenter ($P < 0.001$) and behaved less anxiously in a modified-Novely Suppressed Feeding test. Particularly, statistically significant differences were found in connection with the latency to eat an almond piece ($P < 0.05$), the frequency of grooming ($P < 0.001$) and the frequency of defecation ($P < 0.001$). Corticosterone concentration in faeces and blood glucose were determined and no significant changes were found. Therefore, we propose the non-aversive photographic method to measure subcutaneous tumours as a way to refine methodologies in the field of experimental oncology.

Keywords

Welfare, tumour model, non-aversive, mice, refinement

Date received: 24 January 2018; accepted: 13 July 2018

Growing tumours in athymic (commonly referred to as ‘nude’) mice has been an important part of preclinical anticancer drug testing since they were first described in 1969.¹ Despite certain limitations, the growth of subcutaneous tumours in nude mice is still a commonly used tool in cancer-drug development.¹

Even though the standard method for subcutaneous tumour measurement is through the use of calipers,² which are inexpensive, fast and easy to use, these measurements have been described as inaccurate, as their variability can sometimes reach 25%.³ New measurement techniques have been described, but these are laborious and dependent upon physical restraint or the use of anaesthetic agents.^{3,4,5,6}

Beside animal welfare considerations, the stress produced by immobilization modifies both the animals’ behaviour and physiology⁷ and, thus, experimental

¹Laboratorio de Animales de Experimentación, Facultad de Cs Veterinarias, UNLP, Argentina

²Instituto de Genética Veterinaria (CONICET-UNLP), Facultad de Cs Veterinarias, UNLP, Argentina

³Instituto de Biología Celular y Neurociencias Prof. E. De Robertis (UBA-CONICET), Argentina

Corresponding author:

Agustina Resasco, Laboratorio de Animales de Experimentación, Facultad de Cs Veterinarias, UNLP, Calle 60 y 118 s/n, La Plata (1900), Buenos Aires, Argentina.
Email: agustinaresasco@gmail.com

results can be affected. While the effect of stress over tumour volume is not clear yet, handling mice has been shown to increase tumour volume^{8,9} and metastases.¹⁰ Controversially, in this last article, the authors also describe an increase of the natural killer (NK) cells in situ, which mediates the immune anti-tumour response. Increased activity of NK cells in animals reared in enriched environments, which are considered to be eustress, along with decreased tumour volume have also been reported.¹¹ The effect of stress over immunity and therefore over tumour development, seems to be affected by the perceived controllability and intensity of the stressor.¹²

It is well known that mice prefer to have their bodies, and especially their vibrissae, in contact with another surface, a property called thigmotaxis.¹³ Because of this property, the use of tunnels to handle this species minimizes anxiety responses, especially when they get used to the procedure.⁷ This habituation is not detected when mice are manipulated by their tail, where the repetition of the procedure does not minimize negative responses to human contact.⁷

We compared the accuracy of the measurements taken with a caliper to those obtained with a less stressful technique that we have called 'non-aversive photographic measurement method', which is described here for the first time. The effect of both methods on the mice physiology and behaviour were also evaluated in order to understand the impact that both methods have on their welfare.

Materials and methods

Animals and husbandry

Fifty-six, female and male, 4–6-week-old, specific pathogen free (SPF) nude mice (stock N:NIH(S)-*Fox1^{nu}*) were acquired from the Animal Facility at the Faculty of Veterinary Sciences of La Plata National University, Argentina (LAE-FCV-UNLP). To avoid confounded effects of tail handling with caliper measurement, mice were cup handled¹⁴ and manipulated by the same experimenter throughout the study. Animals were housed at the LAE facility, in Lab Products Super Mouse 750TM (480 cm²) individually ventilated cages (34 air changes per hour in positive pressure mode) with aspen shavings as bedding (Guillermo N Cabañas, Ciudad Autónoma de Buenos Aires, Argentina) and tissue paper as nesting material. Food pellets (Cooperación, Alicooper S.A., Santa Fe, Argentina) and water were administered *ad libitum*. All supplies were sterilized and cages were changed once a week. Animals were kept at a room temperature of 22 ± 2°C and a 12:12 dark: light cycle (lights on at 7 a.m.).

This study was approved by the FCV-UNLP IACUC under protocol number 42-1-14T and conducted in compliance with the Guide for the Care and Use of Laboratory Animals¹⁵ and the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition).¹⁶

Cell line and tumour transplant

A549 cells obtained from a solid tumour grown in different mice were used. Tumour pieces were fragmented into 2 mm³ chunks and transferred into a drop of Minimum Essential Medium (MEM) cell culture media before subcutaneous implantation in the right or left thoracic area of nude mice using a trocar.¹⁷

For the inoculation, animals were anaesthetized with ketamine/xylazine (100–10 mg/kg, i.p., respectively) with no previous fast, and room temperature was kept at a minimum of 25°C to prevent hypothermia. Intraoperative analgesia was provided by xylazine (administered for the anaesthesia) and 1% lidocaine at the site of the incision. As it was a minor procedure, a single dose of 10 mg/kg of tramadol was administered subcutaneously before the animals woke up as post-operative analgesia and they were closely monitored in order to avoid unnecessary pain. Surgery was aseptically performed under a clean bench with sterilized surgical instruments and materials and, as clipping was not needed for this strain, only povidone-iodine was employed as skin antiseptic. A small incision (approximately 0.4 cm) was made in the lateral abdominal area and a subcutaneous tunnel was produced with the trocar before implanting the tumour piece. Special attention was given in order to place the tumour in the middle of the lateral area of each mouse, as it is important for the non-aversive photographic method to have the whole surface of the tumour facing the camera. Because only a small incision was made, wound edges remained close together and no stitches were needed. The total length of the procedure for each animal was less than 5 min. After the surgery, mice were kept on a heating pillow until recovery, and oxygen was administered when necessary. Animals were supervised every 8 hours for 5 days after the procedure and then daily until the end of the experiment. Observations included body condition scoring, surgical wound complications (presence of inflammation, burst releasing fluids, pus), mice general appearance and behaviour (ocular or nasal discharges due to lack of grooming, presence of mice sleeping outside the nest, nest quality, increased aggression, engaging in normal behavioural activities), behavioural changes due to tumour development (abnormal gait or impaired locomotion, increased grooming or nociception at the site of the tumour),

skin general appearance and tumour characteristics and size. No deviations from normality were observed in any of the inoculated animals.

Experimental design

In order to avoid variability and reduce animal anxiety, the same experimenter (AR) carried out all the procedures so that complete blinding for treatment was not possible. To overcome this problem, tumour-volume recording was made by another researcher (ACCM) and AR did not have access to previously registered data on tumour volumes. Moreover, a blinded observer (ACCM) was designated to perform the behavioural observations.

Experiment 1. The aim of this experiment was to evaluate whether the new method would be valid to measure subcutaneous tumours. Thus, intra-observer variation with the caliper and the non-aversive photographic method was quantified as well as the time needed to take each measurement. In addition, the effect of each method on mice was evaluated by means of biochemical and behavioural parameters.

Twenty tumour-transplanted, female, nude mice were housed in groups of 5 and kept for 3 weeks to allow the tumours to grow. On the first day of the following 2 weeks, i.e. weeks 4 and 5, measurements were obtained with the caliper in half of the mice and with the non-aversive photographic method on the other half. Immediately after these measurements were taken, blood samples were collected on week 4 to analyse glucose levels, and, on week 5, the Novelty-Suppressed Feeding (NFS) test was conducted. On week 6, intra-observer variation was evaluated measuring the tumours with both methods on 2 consecutive days (days 1 and 2 of week 6). Subsequently, mice were euthanized by cervical dislocation and tumours were dissected to be weighted and determine their volume by water displacement.

Experiment 2. Our purpose was to evaluate whether any of the methods induced variations in behaviour towards the experimenter over time, as well as in faecal corticosterone. Twenty female and 16 male mice were housed in groups of 2 animals per cage for 4 weeks. Tumour volume was measured with the caliper in half of the cages and with the non-aversive photographic method in the other half. To identify each mouse, the tumour transplant was made either on the left or on the right side. Every week, tumour measurements were performed on days 1 and 2. Behavioural observations were registered on day 2 immediately after tumour measurement. Cage change took place

after behavioural observation. On day 3 of each week all faecal boli were collected from the cages.

Tumour-volume measurement

Tumour volume was calculated by the use of the modified ellipsoid formula $(\text{length} \times \text{width}^2)/2$.¹⁸ Tumour measurements were made with a caliper or with the non-aversive photographic method, as needed. The time that was required to take each determination was recorded with a stopwatch. After euthanasia, tumour weight and water displacement data were registered. Water displacement was determined by the difference in distilled water volume at room temperature measured in a test tube after introducing the tumours. Results were expressed in cm^3 .

For the non-aversive photographic measurement method, a rectangular section of a transparent acrylic tube which was 12 cm long, 4 cm wide and 5 cm tall was used (Figure 1(a)). To measure the tumours, graph paper, which occupied half the length of the tube, was used as a scale. The graph paper was inside of the tube, held in place in the middle of the width with an 'L' shape acrylic figure. This apparatus was put inside the animal's home cage (experiment 2) or in another cage with the same characteristics (experiment 1). For experiment 1, animals were introduced one by one by cup handling them into the cage. Inside the cage, the tube was placed in one of the corners adjacent to its longest wall (Figure 1(a)). Mice voluntarily went into the tube through the opposite side of the graph paper as they explored the new object. Most of the times, the animals remained with their head and forelimbs behind the graph paper (Figure 1(b)). When mice had the cranial part of their body behind the graph paper, images were taken by the researcher with the multi-shot function of a NIKON DSLR D3100 camera. The camera was located perpendicular to the longest side of the cage at a distance of 15 cm. Images were then analysed with the Image J software,¹⁹ using the graph paper as scale (Figure 1(c)). When setting the scale in the computer program, the distance taken as reference in the paper was located at the same height as the tumour localization. The scale was calibrated for each picture. Two measurements were taken in each photo: the greatest diameter of the tumour was considered the length and the shortest diameter was considered the width (Figure 1(d)).

Blood collection and glucose determination

Blood samples were taken 5 min after measurements by means of submandibular bleeding using a 21 G needle.²⁰ A single drop was collected (approximately 100 μl) and blood glucose was immediately measured using a compact glucose analyser (ContourTMTS, Bayer).

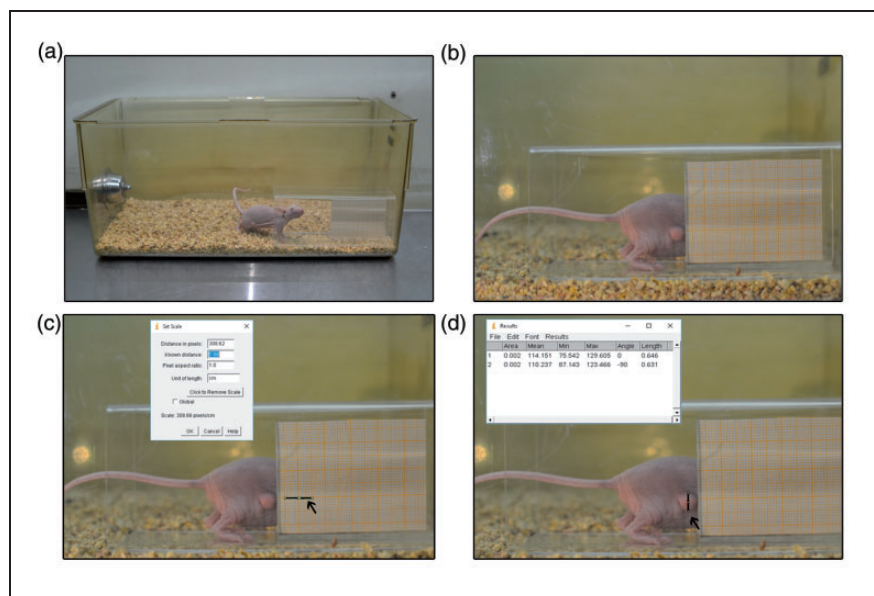


Figure 1. Tumour measurement with the non-aversive method. (a) The apparatus, consisting of an acrylic tube with graph paper inside, is introduced into the mice home cage. (b) The mouse goes into the tube and pictures are taken when the tumour is located next to the graph paper. (c) The scale is set using the graph paper at the same height as the tumour is located. (d) Tumours are measured taking into account their greatest and shortest diameter.

Faecal collection and corticosterone metabolite assay

Faeces were sampled from each cage once per week throughout the study. All faecal boli were collected from the cages 24 hours after the cleaning routine. Samples were frozen at -20°C until processed. Faecal steroid metabolites were extracted according to the method described by Touma et al.²¹ with slight modifications: faeces were homogenized and then 1 ml of 80% methanol was added to 0.1 g of humid faecal pellets. Samples were incubated at room temperature for 120 min in an orbital shaker, then shaken in a vortex for 10 s and finally, centrifuged at c. 500 g for 20 min. The supernatant was frozen at -20°C until analysed.

Corticosterone metabolites were analysed by Enzyme Immunoassay (EIA) as described previously by Munro & Lasley²² with polyclonal antisera and conjugated peroxidase for corticosterone detection (corticosterone CJM006, Department of Population Health and Reproduction, Coralie Munro, UC Davis, CA, USA).

Behavioural assays

Experiment 1. The NSF paradigm is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the centre of a brightly lit arena. For this study, a modified NSF was used.²³ This version of the test does not require food deprivation, as it uses a preferred item (an almond piece) over the regular

food chow. Mice were habituated to the almonds by introducing two almonds per cage in the cleaning cage routine. The day of the test, an almond piece (approximately 1 g) was placed in the centre of an open arena (30 x 40 x 16 cm). Each mouse was placed by cup handling in a corner of the box, and a stopwatch was immediately started. The latency to start eating (defined as the mouse sitting on its haunches and biting the almond with the use of its forepaws) was recorded for a 5-min period. Additionally, while each mouse was in the open field, the frequency of grooming and defecation were recorded. We hypothesized that by using a less aversive method of tumour measurement, mice would be less anxious, and therefore, more willing to venture into the centre of a brightly lit arena to eat a reward while displaying less anxiety-like behaviours.

Experiment 2. The frequency of the voluntary contact of mice with the experimenter was assessed. The evaluation was conducted in the home cage immediately after the second tumour measurement. Both mice from each cage were gently conducted to one side of the cage with a piece of cardboard. Due to cage design, mice were directed underneath the food hopper, so this side of the cage was the least exposed for them. Then, the experimenter placed her hand on the opposite side of the cage and the cardboard was removed. The number of contacts with their head or paws was registered for a 3-minute period. We inferred that by using a less aversive method of tumour measurement, mice would be less anxious and, therefore, more willing to leave the

safer zone in their home cage to explore the experimenter's hand.

Statistical analysis

Animals were randomly assigned to different cages upon arrival at the experimental room by means of a random number sequence generated in Microsoft Excel; in addition, cages were assigned to different treatments and selected for data collection at the different time points in the same modality. Statistical analyses were performed with R 3.3.0, R studio software (2016) and InfoStat (2015). Data was tested for normality using the Shapiro-Wilks normality test. *P*-values below 0.05 were considered significant for all analyses. Results were expressed by means \pm SEM.

To measure intra-observer reliability in experiment 1, the Intra-Class Correlation Coefficient (ICC) was applied. We considered values greater than 0.75 as representative of excellent agreement between the first and the second repetition.²⁴ To compare across methods, the nonparametric Friedman test with Dunn's post hoc test for multiple comparisons were used. Behavioural data was analysed with the non-parametric Kruskal Wallis test. Blood-glucose concentration was evaluated by Analysis of Variance (ANOVA).

In experiment 2, the differences in corticosterone concentration as well as the frequency of voluntary contacts to the experimenter between both methods were analysed on a weekly basis with 2-Way ANOVA. As we did not separate the animals to measure behaviour or to collect faecal samples, the cage was considered the experimental unit. To meet the test assumptions, corticosterone concentration in faeces was logarithmically transformed and the number of contacts with the experimenter was square root transformed. Results from treatments showing significant overall changes were subjected to post-hoc Tukey's test.

Results

Experiment 1

Subcutaneous tumour volumes were measured twice with the classical (caliper) and the proposed non-aversive photographic method. Details of tumour volumes with each method on the first and second repetition can be seen in Table 1. All coefficients showed excellent agreement, as they were all above 0.95. We found significant differences between water displacement volume and measurements with the caliper and the non-aversive photographic method but not within repetitions of each method ($F=9.81$, $P<0.001$). No significant differences were found between weight and water displacement volume.

Table 1. Intra-Class Correlation Coefficients (ICC).

Method and repetition	Mean \pm SEM	ICC
Water displacement volume	0.398 \pm 0.10 cm ³	0.994
Weight	0.391 \pm 0.10 g	
Caliper 1 st	0.556 \pm 0.15 cm ³	0.987
Caliper 2 nd	0.553 \pm 0.15 cm ³	
Non-aversive 1 st	0.614 \pm 0.16 cm ³	0.963
Non-aversive 2 nd	0.609 \pm 0.15 cm ³	

Latency to eat the almond piece in the modified version of the NSF test was significantly shorter for mice in the non-aversive photographic method group compared to those measured with the caliper (Figure 2(a)). Similarly, frequency of grooming (Figure 2(b)) and of defecation (Figure 2(c)) were significantly lower in mice whose tumours were measured with the non-aversive photographic method. *H* values were 3.53 ($P=0.49$) for the NSF test, 8.17 ($P=0.002$) for the frequency of grooming, and 7.94 ($P=0.002$) for the frequency of defecation.

Blood-glucose concentration was slightly higher in the caliper group (142.33 \pm 6.09 mg/dL) compared to the non-aversive photographic method group (130.70 \pm 6.1 mg/dL) but these differences were not statistically significant ($F_{1,18}=1.81$; $P>0.05$). Finally, as expected, the non-aversive photographic method was more time consuming, given that it demanded 71.40 \pm 10.73 s per animal v. 20.22 \pm 1.62 s per animal when the caliper was used. In addition, image processing in the non-aversive photographic method took 98.10 \pm 5.17 s per animal which included scale calibration per image, the measurement of the tumours, adding the data to a database and occasionally, enhancing the image contrast to have a better tumour delimitation.

Experiment 2

The number of voluntary contacts with the experimenter as well as faecal concentrations of corticosterone obtained with both methods are shown in Figure 3. No significant interaction was observed between factors. Voluntary contacts of mice with the experimenter were measured as shown in Figure 3(a). Significant differences were found between treatments ($F_{1,64}=17.53$, $P<0.001$) and among weeks ($F_{3,64}=7.21$, $P<0.001$). From week 2 and until the end of the study, mice that were exposed to the non-aversive photographic method were less reluctant to establish contact with the experimenter than those whose tumours were measured with the caliper. Significant differences in corticosterone concentration were observed across weeks ($F_{3,64}=6.13$, $P<0.001$) but not between methods ($F_{1,64}=0.69$, $P=0.40$) as shown in Figure 3(b).

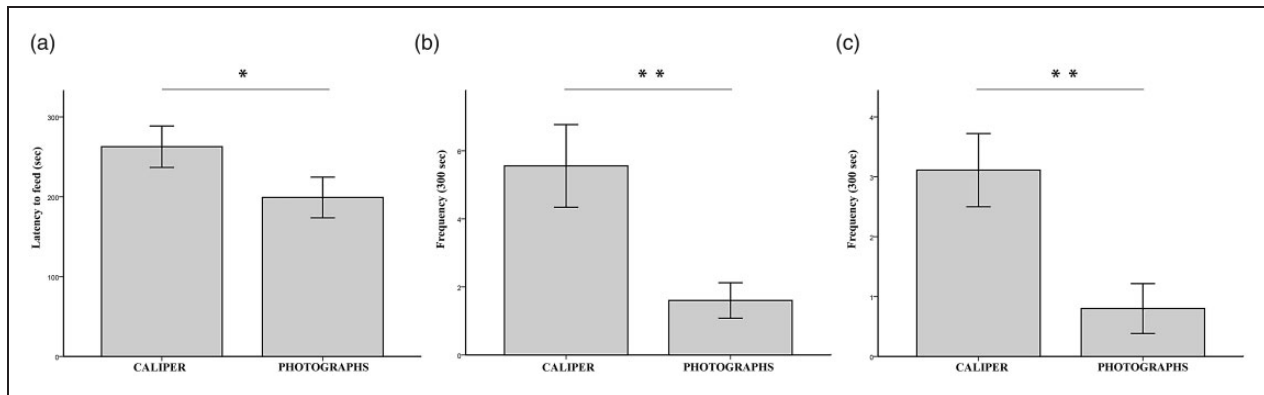


Figure 2. Behavioural output observed immediately after both tumour measurement methods. Anxiety-like behaviour expressed as (a) the latency to eat the almond piece in the modified-novelty suppressed feeding (NSF) test, (b) frequency of grooming and (c) defecation during a 300-s period ($n=10$ mice/group). Kruskal-Wallis test; $*=P<0.05$; $**P<0.01$.

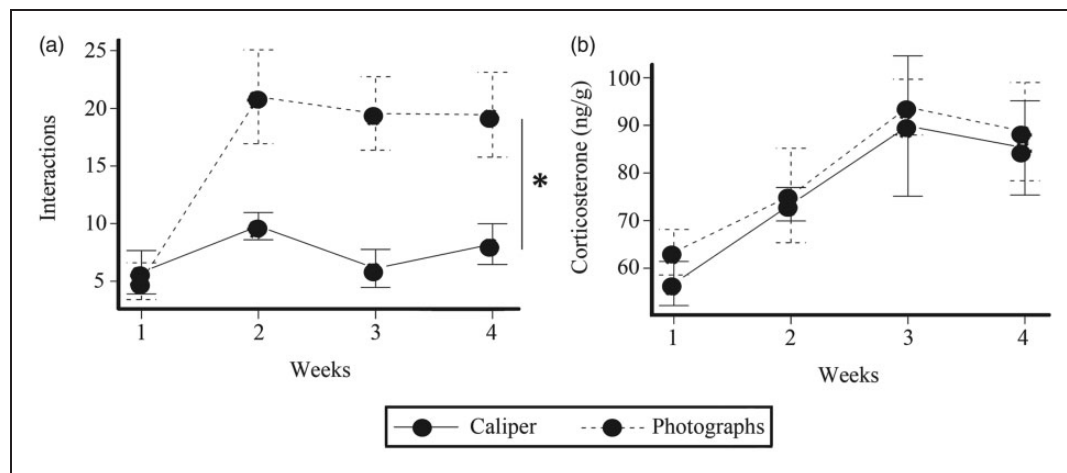


Figure 3. Behavioural and physiological parameters over time in mice subjected to both tumour measurement methods. (a) Temporal sequence of mice-human interaction expressed as number of contacts in a 3-min period ($n=19$ mice/group). (b) Temporal sequence of corticosterone concentration in faeces ($n=19$ mice/group). Two-way Analysis of Variance; $*=P<0.05$; $**P<0.01$.

Discussion

To our knowledge, the non-aversive photographic method has been described here for the first time as a technique to measure subcutaneous tumours that does not require animal immobilization. The need for a method of these characteristics has been recently addressed by Delgado San Martin et al.³ The technique described herein can be done in single- or paired-housed mice in their own home cage, without the need to manipulate animals to record tumour development over time. In this experiment, we identified each mouse by the side on which the tumour was implanted (left or right). Tumour measurement with the non-aversive photographic method in mice housed in groups larger than pairs would also be possible,

as long as the mice are clearly identified. Moreover, even if measurement in the home cage is not possible, reduced anxiety was observed in the NSF test, after mice were cup-handled to another cage where they were presented to the non-aversive photographic method.

As it was expected for well-vascularized and non-calcified tumours, weight yielded the closest value to water displacement volume.² Both the caliper and the non-aversive photographic method produced measurements different from those obtained using the reference method, most likely because the former also considered the skin and subcutaneous tissue.⁵ Even though caliper measurements were closest to water displacement volumes, there was good consistency between repetitions for both techniques, which would make the

non-aversive photographic method acceptable in recording tumour development over time. As the non-aversive photographic method tends to overestimate the size of tumours, pilot studies prior to its implementation are recommended to determine which tumour size is acceptable when setting experimental endpoints. For this purpose, this new method should be compared with modern *in vivo* imaging techniques to overcome the issue of including additional tissues in the determination of tumour volumes. Indeed, this might also aid improving guidelines for mice welfare in cancer research as these do not specify which method they are based on and hence, can have profound implications when setting experimental endpoints.

By means of physiological and behavioural parameters, we evaluated the impact of both methods on mice welfare. The overall interpretation of animal welfare based on objective measurements is challenging given the intrinsic subjectivity of the nature of an animal's affective state.²⁵ Therefore, attention should be placed on the context and the behavioural responses that take place along with physiological data.

Although no significant differences were observed neither in faecal corticosterone nor in blood-glucose concentrations between both methods, the behavioural output for the non-aversive photographic method was drastically different, as mice were less reluctant to establish contact with the experimenter and behaved less anxiously in the NSF test. The short-term stress response to one brief event of restraint may not be visible in corticosterone metabolites over a 24-hour period and thus further studies should analyse long-term changes for this parameter.²⁶ However, the similarity with blood-glucose concentration might hint a similar physiological response between both methods, as sympathetic activation, which rapidly increases circulating glucose, anticipates the hypothalamic-pituitary-adrenal (HPA) response.²⁷ Nevertheless, by focusing on the behavioural data, improved welfare due to a reduction in anxiety for the new method can be hypothesized, as mice in the non-aversive photographic method group ate the almond piece significantly faster in the NSF test, were less reluctant to establish contact with the experimenter and displayed significant less grooming and defecation than mice in the caliper group. Both grooming and defecation are behavioural measures extensively used as anxiety traits.^{28,29,30} We infer that the non-aversive photographic method apparatus did not induce a highly-aversive response, as all the animals decided to enter into the tube. These results are in accordance with Hurst and West³¹, who established that picking up mice by the tail induced aversion and high anxiety, whereas the use of tunnels led to voluntary approach, low anxiety and acceptance of the physical restraint.

Overall, it can be predicted that it will be easier for mice to adapt to this new technique and hence, will produce less variability in experimental variables due to uncontrollable stress exposure. The non-aversive photographic method is also rather inexpensive, as only a transparent acrylic tube and a camera are needed. In our experiment, we used a NIKON DSLR D3100 camera but any good resolution camera can be employed. In addition, even though this technique is not time-consuming (approximately one minute per tumour), we infer that as mice get used to the procedure the time required will be reduced. Image processing time was also short for this new method as it took about 100s to analyse each picture. We did not consider picture-selection time, but a proper training in image shooting can considerably reduce the amount of pictures taken per animal and hence minimize this aspect. Finally, this new technique can also be used in immunodeficient furred mice, provided the area surrounding the tumour is properly shaved.

Altogether, strong behavioural evidences of diminished anxiety responses were found for the non-aversive photographic method in this initial approach, which might help improve mice welfare in cancer research. Nevertheless, the lack of a physiological correlate for these findings highlights the importance of performing further research to characterize mice physiological profile. In particular, with regards to blood corticosterone concentration after the measurements, our experimental design only let us infer a similar association with blood glucose with no effect in the long-term release of this hormone.

Conclusion

As it has been mentioned before, we are describing here for the first time a technique to measure tumour volumes without the need for manipulating the animals and which can also be performed in the mouse's home cage. We also conclude that the non-aversive photographic method is consistent across repetitions. This new technique could be helpful in cancer research, as it could reduce experimental variables related to stress, which negatively influence results.³² As additional benefits, this method is economical and easy to conduct, so it can be easily carried out after a short training. Even though the non-aversive photographic method is slightly more time consuming, mice behaved less anxiously when this new technique was used, which leads us to the conclusion that its implementation can have a positive impact on their welfare. Altogether, the non-aversive photographic method might help to reduce unnecessary stress, particularly in studies that consider the influence of psychoneuroimmunology in cancer development.

Acknowledgements

We would like to thank Dr Vera Baumans and Dr S. Ivan Perez for their thoughtful comments on this article.

Declaration of Conflicting Interests

The authors disclose no potential conflicts of interest.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: AR is funded by a PhD grant from CONICET. ACC received funding from a postdoctoral grant from CONICET. SLD is supported by an Independent Research Career Position from CONICET. CC and MAA have teaching positions at FCV-UNLP. This study was funded by a grant to CC from the Argentine Agency for Promotion of Science and Technology (ANPCyT-PPL2-2011-0008).

ORCID iD

Agustina Resasco  <http://orcid.org/0000-0002-2433-4035>

REFERENCES

- Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. *Eur J Cancer* 2004; 40: 827–836.
- Euhus DM, Hudd C, Laregina MC, et al. Tumor measurement in the nude mouse. *J Surg Oncol* 1986; 31: 229–234.
- Delgado San Martin JA, Worthington P and Yates JW. Non-invasive 3D time-of-flight imaging technique for tumor volume assessment in subcutaneous models. *Lab Anim* 2015; 49: 168–171.
- Ayers GD, McKinley ET, Zhao P, et al. Volume of pre-clinical xenograft tumors is more accurately assessed by ultrasound imaging than manual caliper measurements. *J Ultrasound Med* 2010; 29: 891–901.
- Kerseman V, Cornelissen B, Allen PD, et al. Subcutaneous tumor volume measurement in the awake, manually restrained mouse using MRI. *J Magn Reson Imaging* 2013; 37: 1499–1504.
- Jensen MM, Jørgensen JT, Binderup T, et al. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18 F-FDG-microPET or external caliper. *BMC Med Imaging* 2008; 8: 16.
- Gouveia K and Hurst JL. Reducing mouse anxiety during handling: Effect of experience with handling tunnels. *PLoS One* 2013; 8: e66401.
- Aarstad HJ and Seljelid R. Effects of stress on the growth of a fibrosarcoma in nu/nu and conventional mice. *Scand J Immunol* 1992; 35: 209–215.
- Thaker PH, Han LY, Kamat AA, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat med* 2006; 12: 939–944.
- Brenner GJ, Cohen N, Ader R, et al. Increased pulmonary metastases and natural killer cell activity in mice following handling. *Life Sci* 1990; 47: 1813–1819.
- Benaroya-Milshtein N, Hollander N, Apter A, et al. Stress conditioning in mice: Alterations in immunity and tumor growth. *Stress* 2011; 14: 301–311.
- Sklar LS and Anisman H. Stress and coping factors influence tumor growth. *Science* 1979; 205: 513–515.
- Latham N and Mason G. From house mouse to mouse house: the behavioural biology of free-living *Mus Musculus* and its implications in the laboratory. *Appl Anim Behav Sci* 2004; 86: 261–289.
- Gouveia K and Hurst JL. Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. *Sci Rep* 2017; 7 **[AQ1]**.
- National Research Council. *Guide for the Care and Use of Laboratory Animals*. Washington DC: National Academies Press, 2011.
- Workman P, Twentyman P, Balkwill F, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. *Br J Cancer* 1998; 77: 1–10.
- Thakur MD, Salangsang F, Landman AS, et al. Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. *Nature* 2013; 494: 251–255.
- Tomayko MM and Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 1989; 24: 148–154.
- Schneider CA, Rasband WS and Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012; 9: 671–675.
- Golde WT, Gollobin P and Rodriguez LL. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Lab Anim* 2005; 34: 39–43.
- Touma C, Palme R and Sachser N. Analyzing corticosterone metabolites in fecal samples of mice: a non-invasive technique to monitor stress hormones. *Horm Behav* 2004; 45: 10–22.
- Munro CJ and Lasley BL. Non-radiometric methods for immunoassay of steroid hormones. *Prog Clin Biol Res* 1988; 285: 289–329.
- Koizumi M, Kondo Y, Isaka A, et al. Vitamin C impacts anxiety-like behavior and stress-induced anorexia relative to social environment in SMP30/GNL knockout mice. *Nutr Res* 2016; 36: 1379–1391.
- Fleiss J, Levin B and Cho Paik M. The measurement of interrater agreement. In: Balding DJ, Noel AC, Fisher NI, et al. (eds). *Statistical methods for rates and proportions*. 3rd ed. Hoboken: John Wiley & Sons; 2003, pp 598–626.
- Fraser D. Selecting and combining criteria of animal welfare. In: Kirkwood JK and Hubrecht RC (eds) *Understanding animal welfare: the science in its cultural context*. Oxford: Wiley-Blackwell; 2008, pp. 241–259.
- Cinelli P, Rettich A, Seifert B, et al. Comparative analysis and physiological impact of different tissue biopsy methodologies used for the genotyping of laboratory mice. *Lab Anim* 2007; 41: 174–184.
- Moberg G.P. Biological Response to Stress: Key to Assessment of Animal Well-Being? In: Moberg G.P (ed.) *Animal Stress*. New York: Springer, 1985, pp.27–49.

28. Kalueff AV and Tuohimaa P. Contrasting grooming phenotypes in three mouse strains markedly different in anxiety and activity (129S1, BALB/c and NMRI). *Behav Brain Res* 2005; 160: 1–10.
29. Kalueff AV, Wheaton M and Murphy DL. What's wrong with my mouse model? Advances and strategies in animal modeling of anxiety and depression. *Behav Brain Res* 2007; 179: 1–18.
30. O'Leary TP, Gunn RK and Brown RE. What are we measuring when we test strain differences in anxiety in mice? *Behav Genet* 2013; 43: 34–50.
31. Hurst JL and West RS. Taming anxiety in laboratory mice. *Nat Methods* 2010; 7: 825–826.
32. Poole T. Happy animals make good science. *Lab Anim* 1997; 31: 116–124.

Résumé

Nous avons développé une nouvelle méthode de mesure du volume des tumeurs sous-cutanées qui consiste à photographier les souris dans leur cage, afin d'affiner la méthode standard de mesure avec pieds à coulisse. Nous considérons cette nouvelle méthode comme étant non aversive, car elle peut être plus compatible avec les préférences comportementales des souris et peut, par conséquent, améliorer leur bien-être. Les photographies sont prises lorsque les souris entrent volontairement dans un tube en acrylique contenant du papier millimétré qui est utilisé plus tard comme échelle. Les volumes de la tumeur mesurés avec un pied à coulisse et la **méthode photographique non aversive** ont été comparés à ceux obtenus par déplacement d'eau, volume et poids. Les changements comportementaux et physiologiques ont été évalués afin de déterminer le bien-être des animaux. Des différences significatives ont été observées entre les mesures obtenues avec un pied à coulisse et la **méthode photographique non aversive**, par rapport au volume de référence acquis par déplacement d'eau ($P < 0.001$). Néanmoins, une bonne cohérence de ces mesures a été notée lorsque les tumeurs ont été mesurées à plusieurs reprises, avec des coefficients de corrélation intra-classes tous supérieurs à 0,95. Les souris sur lesquelles la méthode photographique non aversive a été utilisée étaient significativement moins réticentes à établir un contact avec l'expérimentateur ($P < 0.001$) et se comportaient de manière moins anxieuse lors d'un test d'alimentation supprimée par la nouveauté modifié. En particulier, des différences statistiquement significatives ont été observées en ce qui concerne la latence pour manger un morceau d'amande ($P < 0.05$), la fréquence de toilettage ($P < 0.001$) et la fréquence des selles ($P < 0.001$). La concentration de corticostérone dans les fèces et la glycémie ont été déterminées et aucun changement significatif n'a été remarqué. Par conséquent, nous proposons la **méthode photographique non aversive** pour mesurer les tumeurs sous-cutanées comme moyen d'affiner les méthodologies dans le domaine de l'oncologie expérimentale.

Abstract

Wir haben eine neue Methode zur Messung des subkutanen Tumorumfanges entwickelt, die darin besteht, Mäuse in ihren Heimkäfigen zu fotografieren, um die Standardmethode der Messung mit Messschiebern zu verbessern. Wir betrachten diese neue Methode als nicht abschreckend, da sie möglicherweise besser mit den Verhaltenspräferenzen der Mäuse vereinbar ist und somit ihr Wohlbefinden verbessert. Mäuse wurden beim freiwilligen Betreten einer Acrylröhre fotografiert, die mit anschließend als Skala dienendem Millimeterpapier bestückt war. Die mit dem Messschieber und der **nicht-aversiven fotografischen Methode** gemessenen Tumorumfänge wurden mit denen verglichen, die durch Wasserverdrängungsvolumen und -gewicht erhalten wurden. Verhaltens- und physiologische Veränderungen wurden evaluiert, um das Tierwohl zu beurteilen. Es wurden signifikante Unterschiede zwischen den Messungen mit dem Messschieber und der **nicht-aversiven fotografischen Methode** gegenüber dem durch Wasserverdrängung ermittelten Referenzvolumen ($P < 0.001$) festgestellt. Dennoch gab es hier bei wiederholten Messungen dieser Tumore eine gute Übereinstimmung dieser Messungen, wobei alle Intra-Klassen-Korrelationskoeffizienten über 0,95 lagen. Mäuse, bei denen die nicht-aversive fotografische Methode verwendet wurde, waren deutlich weniger scheu, Kontakt mit dem Versuchsleiter herzustellen ($P < 0.001$) und verhielten sich weniger ängstlich in einem modifizierten Novelty Suppressed Feeding Test. Insbesondere wurden statistisch signifikante Unterschiede im Zusammenhang mit der Latenz beim Verzehr eines Mandelstücks ($P < 0.05$), der Häufigkeit der Fellpflege ($P < 0.001$) und der Häufigkeit des Stuhlgangs ($P < 0.001$) festgestellt. Bei der Ermittlung der Corticosteron-Konzentration im Stuhl und im Blutzucker wurden keine signifikanten Veränderungen festgestellt. Daher schlagen wir die **nicht-aversive fotografische Methode** zur Messung von subkutanen Tumoren als Möglichkeit zur Verbesserung der Methodologie in der experimentellen Onkologie vor.

Resumen

Hemos desarrollado un nuevo método para la medición del volumen tumoral subcutáneo que consiste en tomar fotografías de ratones en sus jaulas, para refinar el método estándar de medición con adipómetros. Consideramos que este nuevo método no es aversivo, ya que puede ser más compatible con las preferencias de comportamiento de los ratones y, por lo tanto, mejorar su bienestar. Las fotografías se capturan cuando los ratones entran voluntariamente en un tubo de acrílico que contiene papel cuadriculado que luego se usa como una balanza. Los volúmenes tumorales medidos con el adipómetro y el **método fotográfico no adverso** se compararon con los obtenidos por el volumen y el peso del desplazamiento del agua. Se evaluaron los cambios de comportamiento y fisiológicos para evaluar el bienestar de los animales. Se encontraron diferencias significativas entre las mediciones obtenidas con el adipómetro y el **método fotográfico no adverso**, frente al volumen de referencia adquirido por desplazamiento de agua ($P < 0.001$). Sin embargo, hubo una buena consistencia para estas mediciones cuando los tumores se midieron repetidamente, con todos los Coeficientes de correlación intraclase por encima de 0,95. Los ratones en los que se empleó el método fotográfico no adverso fueron significativamente menos reacios a establecer contacto con el experimentador ($P < 0.001$) y se comportaron con menos ansiedad en un test modificado de alimentación suprimida. En concreto se encontraron diferencias estadísticamente significativas en relación con la latencia para comer una pieza de almendra ($P < 0.05$), la frecuencia de la preparación ($P < 0.001$) y la frecuencia de la defecación ($P < 0.001$). Se determinó la concentración de corticosterona en heces y glucosa en sangre y no se encontraron cambios significativos. Por lo tanto, proponemos el **método fotográfico no adverso** para medir los tumores subcutáneos como una forma de perfeccionar las metodologías en el campo de la oncología experimental.