**Buccal micronucleus assay as a useful tool to evaluate the stress-associated genomic damage in shelter dogs and cats: new perspectives in animal welfare**

Journal of Veterinary Behavior

# Buccal Micronucleus Assay as a useful tool to evaluate the stress-associated genomic damage in shelter dogs and cats: new perspectives in animal welfare

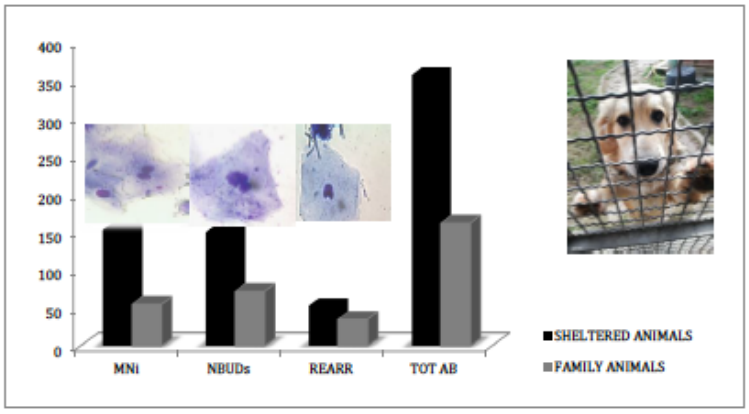
--Manuscript Draft--

| | |
|---|---|
| Manuscript Number: | JVEB-D-21-00024R2 |
| Article Type: | Research Paper |
| Keywords: | Genomic Damage; Nuclear Buds; Mammals; welfare; Companion Animals |
| Corresponding Author: | Alfredo Santovito<br>University of Turin<br>Turin, Piemonte ITALY |
| First Author: | Alfredo Santovito |
| Order of Authors: | Alfredo Santovito |
| | Martina BUGLISI |
| | Chiara SCIANDRA |
| | Manuel SCARFO' |
| Abstract: | Highlights<br><br>1. Group-housing can increase stress levels and contribute to physiological problems<br><br>2. We evaluated, by MNi assay, the level of genomic damage in shelter cats and dogs<br><br>3. We recruited 30 shelter cats and dogs and 30 family cats and dogs used as control<br><br>4. Significant differences in the MNi frequency were found between the two groups<br><br>5. The ethotest confirms the increased levels of aberrations in stressed animals |
| Suggested Reviewers: | P.A. Pesavento<br>University of California Davis School of Veterinary Medicine<br>papesavento@ucdavis.edu<br>This author has dealt with problems concerning the health of animals in shelters |
| | This author has dealt with problems concerning ani This author has dealt with problems concerning ani<br>Texas Tech University<br>a.protopopova@ttu.edu<br>This author has dealt with issues concerning the health and the welfare of animals in shelters |
| Response to Reviewers: | |

**Highlights**

1. Group-housing can increase stress levels and contribute to physiological problems
2. We evaluated, by MNi assay, the level of genomic damage in shelter cats and dogs
3. We recruited 30 shelter cats and dogs and 30 family cats and dogs used as control
4. Significant differences in the MNi frequency were found between the two groups
5. The ethotest confirms the increased levels of aberrations in stressed animals

**Graphical Abstract**



# Title: Buccal Micronucleus Assay as a useful tool to evaluate the stress-associated genomic damage in shelter dogs and cats: new perspectives in animal welfare

**Running Head: Micronuclei frequency in shelter dogs and cats**

**Authors: Alfredo SANTOVITO, Martina BUGLISI, Chiara SCIANDRA, Manuel SCARFO'**

University of Turin, Department of Life Sciences and Systems Biology, Via Accademia Albertina n. 13, 10123 Torino (Italy)

*Corresponding Author:

Alfredo SANTOVITO, University of Turin,

Department of Life Sciences and Systems Biology

Via Accademia Albertina n. 13, 10123 – Torino (Italy)

Tel.: +39-0116704554; Fax: +39-0116704508;

E-mail: alfredo.santovito@unito.it

*Abstract*

Shelters play a key role in saving animals from straying. However, the space restrictions, the lack of resources and the high animal turnover can increase stress levels and the rate of infectious diseases in cats and dogs. The aim of this study is to evaluate, through the buccal micronucleus assay, the level of genomic damage in shelter cats and dogs with respect to that observed in family cats and dogs. The hypothesis is that stressful environmental conditions, such as those potentially present in shelters, can affect the level of genomic damage. Study population included thirty healthy mixed breed cats and dogs with a minimum two-year presence in a shelter. The control group consisted of thirty healthy cats and dogs living in a home environment, using age/sex matching. The micronucleus assay was performed on one thousand exfoliated buccal cells per subject. Significant differences were found between shelter and family cats and dogs in terms of micronuclei frequency, indicating that a condition of stress found in sheltered animals may increase the levels of genomic damage. The ethotest confirms the increased levels of total aberrations in both stressed shelter cats and dogs. Conversely, no significant differences in the level of genomic damage were found between the sexes, as well as no correlation was found between age and the frequencies of micronuclei. In conclusion, we provided evidence of a possible correlation between physiological stress conditions and increased levels of genomic damage in a sample of sheltered cats and dogs. The results of our study also suggest that the buccal micronucleus assay, also considering the relatively low cost of laboratory procedure and its non-invasiveness, could be potential additional tool that, combined with the ethotest, may be able to provide a more comprehensive picture of the health status of animals living in communities.

**Keywords**: Genomic Damage; Nuclear Buds; Mammals; Welfare; Companion Animals

## 1. Introduction

Shelters play a key role in saving animals from straying. However, living in shelter can contribute to the development of various welfare-related problems for animals by causing less adoptability and, consequently, complicating the management of shelters (Wells et al., 2002; Lord et al., 2013; Kubesova et al., 2017). Since the animal welfare in shelters is both an ethical and an economical issue, it is important to better understand and evaluate it in order to improve the service provided by shelters (Normando et al., 2006).

It is generally accepted that many animal shelters can be potentially stressful places for animals, mainly due to space restrictions, lack of resources and high animal turnover (Kessler and Turner, 1999; Wells et al., 2002).

In addition, euthanasia on cats and dogs in shelters is forbidden in Italy. As a consequence, this "no-kill policy" extends their stay in shelters, increasing the number of animals housed (Anderson et al., 2015; Righi et al., 2019).

Undoubtedly, arriving at a shelter can be extremely stressful and even traumatic for an animal. Losing an emotional bond, changing daily routines and being placed in a different environment full of new and unusual stimuli are all conditions that result in minimal possibilities of interaction with conspecifics and humans (Hennessy et al., 2001; Coppola et al., 2006). The lack of social interaction, the limited possibility of movement, the minimal control over the surrounding environment and the unpredictable noise levels can make living in a shelter a stressful condition, particularly, for extremely social animals such as dogs (Beerda et al., 2000; Wells et al., 2002; Taylor et al., 2007; Titulaer et al., 2013). For example, it was observed that staying in a shelter can induce behavioural changes in dogs as well as significantly modify their behaviour (Wells and Hepper, 2000). An increased frequency of auto-grooming, circling, eating faeces, paw lifting, standing upright, digging, whining, and scratching are all examples of behavioural changes (Beerda et al., 1999).

Shelters can represent a stressful environment for cats as well. Indeed, approximately 80% of Swedish shelters have experienced abnormal behaviours in sheltered cats, such as fearfulness, aggression, feeding disorders and inappropriate elimination behaviours (Eriksson et al., 2009). Moreover, as Gourkow et al. (2014) observed, sheltered cats display several behavioural problems, such as crawling, freezing, feeling startled and retreating from humans – all signs of a poor welfare. It was found that these behaviors reduced their resistance to upper respiratory tract infections (Gourkow et al., 2013). Upper respiratory diseases represent the primary health issue reported in cats during their stay in shelters, supporting the hypothesis that behavioural elements and activities could be related to a poor health status (Gourkow et al., 2013).

The present work aims to assess the level of genomic damage in buccal mucosa cells of both shelter and family cats and dogs by the buccal micronucleus assay. The tested hypothesis was that physiological stress conditions, like those potentially present in some shelters, could affect the levels of genomic damage in terms of increased frequencies of micronuclei (MNi), nuclear buds (NBUDs) and other nuclear rearrangements.

Buccal micronucleus assay is one of the most widely non-invasive techniques used to measure genetic damage in human and animal population studies (Lazalde-Ramos et al., 2017; Benvindo-Souza et al., 2019; Borges et al., 2019). MNi are chromosome fragments or whole chromosomes that fail to segregate properly during mitosis which appear in interphase as small additional nuclei. NBUDs are the result of elimination processes from cells of amplified DNA and/or excess chromosomes (Fenech et al., 2011). It has been observed that the natural MNi frequency varies between certain limits (ranging from 3 to 23 MNi per 1000 cells) in different human populations. However, no frequency data is present in literature with regard to the prevalence of micronuclei in mammals like cats and dogs. In this scenario, the further purpose of our work was to evaluate, in buccal cells of these two mammals, the background level of genomic damage in terms of micronuclei and nuclear buds frequencies.

## 2. Materials and Methods

### 2.1. Subjects

The study population included thirty healthy mixed breed cats and thirty healthy mixed breed dogs, randomly sampled with a minimum two-year stay in a shelter, time that we consider sufficient for genomic damage to occur. Although data regarding the average permanence of animals in shelters where we sampled were not available, in Italy, it is estimated that 41 % of dogs in shelter are represented by adult dogs (over 4 years old) with almost no chance of being adopted (Dalla Villa et al., 2013).

As control groups, we selected healthy house cats (n = 30) and dogs (n = 30), using age/sex matching. All animals belonging to the control group live in an apartment, where they are free to roam. Moreover, all dogs have a minimum of 3-4 daily outings.

Purebred animals were excluded from the sample in order to avoid possible influences of the inbreeding on the level of genomic damage. Shelters were located in Turin, Piedmont, in Northwest Italy. All subjects were fed canned and/or packaged meat or fish food. The state of good health of the animals was confirmed by the veterinarians of the shelter and, as regards the family animals, by the owner.

In order to evaluate the possible influence of the sex on the level of genomic damage, age and sex data were collected. It is well known that drugs and X-rays can alter the level of genomic damage (Santovito et al., 2017). Therefore, we excluded subjects that had contracted acute infections and/or chronic non-infectious diseases and/or were exposure to diagnostic X-rays for a minimum of two years prior to the analysis. The only medication that the sampled subjects received was the flea medication, which is routinely carried out at the entrance to the shelter, and in some cases sporadic drug treatments for intestinal worms.

All animals were treated and housed in compliance with Italian guidelines (available on http://www.aclonlus.org/wp-content/uploads/2014/02/LINEE-GUIDA-LR-34-97.pdf).

Finally, the ethotest was performed in order to assess, among the studied animals, the possible correlation between stress condition and the level of genomic damage.

## 2.2. MNi assay

Exfoliated buccal mucosa cells were collected by gently scraping the mucosa of the inner lining of one or both cheeks with a spatula. Buccal cells were also collected from the inner side of the lower lip and palate. Indeed, the variability in MNi frequency between these areas was found to be minimal for control subjects (Holland et al., 2008). The tip of the spatula was immersed in a fixative solution consisting of methanol/Acetic Acid 3:1, stored at 4 °C prior the analysis. Successively, cells were collected by centrifugation, the supernatant was discarded and the pellet was dissolved in a minimal amount of fixative which was seeded on the slides to detect MNi by conventional staining with 5% Giemsa (pH 6.8) prepared in Sörensen buffer.

Microscopic analysis was performed at 1000X magnification on a light microscope. MNi, NBUDs and other nuclear rearrangements were scored in 1,000 cells with well-preserved cytoplasm per subject according to the established criteria for MNi evaluation (Thomas and Fenech 2011).

## 2.3. Cat Stress Score (CSS) test

A behavioural CSS test was also performed. According to Kessler and Turner (1997), the CCS test is the most widely standardized method for behavioural assessment of stress in cats (Rehnberg et al., 2015; Loberg and Lundmark, 2016). We observed the cats behavior for five minutes, analyzing both their spontaneous and short-term reaction to the sight of a stranger. During this period in fact, the animal has time to react to the sight of a stranger and, thus, it is possible to see its first instinctive reaction. After five minutes, the cat could either change its attitude or keep the same. Successively, the sample was divided in two classes: 1) Class A that included relaxed or weakly

142 tense subjects (subjects with 1-3 score); 2) Class B, that included from very tense to terrified
143 subjects (subjects with 4-7 score). Finally, in order to reduce the risk of bias, all ethotests were
144 performed by the same person.

145

146 2.4. Dog Stress test

147 A dog stress test was performed observing each subjects for 40-50 min, using two of the three steps
148 described in Lucidi et al. (2005). We submitted several tasks for assessment of aggressiveness,
149 temperament, sociability or diffidence and fearfulness. In the first step, Test A, the dogs sample was
150 subdivided into two categories based on two discriminant parameters: A1 corresponding to
151 aggressiveness and A2 corresponding to dominant temperament. In this step, the evaluation of the
152 dogs' responses was based on a binary method (0 or 1): dogs that showed aggressiveness or lack of
153 submissiveness were marked 0 whereas dogs that showed no aggressiveness were marked 1. The
154 second step, Test B, comprised three parts, each evaluating a different behavioural component: B1
155 evaluated the dogs' initiative and how many times they tried to escape from people; B2 examined
156 the dogs' sociability/diffidence; B3 examined fearfulness. In this case, the assessment of the dogs'
157 responses was based on a scoring scale (-1, 0, 1, 2 or 3). Here too, lower ratings correspond to
158 greater stress. As for cats, we subdivided the dogs' sample into two different ethogram classes:
159 class A includes calm subjects with average values greater than 1, whereas class B embraces
160 agitated and/or terrified subjects with average values below than 1.
161 Also in this case, in order to reduce the risk of bias, all ethotests were conducted by the same
162 person.

163

164 *2.3 Statistical Analysis*

Statistical analyses were conducted using the SPSS software (version 24.0, Inc., Chicago, Illinois, USA). Differences in micronuclei frequency between shelter and family cats and dogs, between sexes as well as between animals belonging to different ethogram classes were evaluated by both ANOVA and Kruskal-Wallis tests. The correlation between age and the level of genomic damage was evaluated by regression analysis, whereas multivariate analysis was performed to identify sub-groups according to age and sex score. All *P*-values were two-tailed and the *a priori* level of statistical significance was set at $P<0.05$ for all tests.

## 3. Results

In Table 1 demographic characteristics of groups studied were reported. We sampled sixty cats, subdivided into thirty family cats (mean age 5.60±4.42, fourteen males and sixteen females) and thirty shelter cats (mean age 5.60±4.42, fifteen males and fifteen females). Similarly, for dogs, we sampled sixty subjects subdivided into thirty family dogs (mean age 6.40±3.73, twelve males and eighteen females) and thirty shelter dogs (mean age 5.41±1.64, eighteen males and twelve females). In both species, no significant differences were found between family and shelter subjects in terms of mean age.

In Table 2 results of the statistical evaluation of genomic damage between shelter and family cats and dogs were reported. In Figure 1 some examples of damaged cells observed in our samples were reported. Among family cats, the frequency of MNi, NBUDs and rearrangements were 0.100±0.383, 0.110±0.092, 0.077±0.119, with a frequency of total aberration of 0.287±0.405. Among shelter cats, the frequency of MNi, NBUDs and rearrangements were 0.210±0.209, 0.220±0.183, and 0.087±0.125, with a frequency of total aberration of 0.517±0.373. Significant differences were found between family and shelter cats in terms of MNi ($P<0.001$), NBUDs ($P = 0.010$) and total aberrations ($P = 0.003$).

Among dogs, the frequencies of MNi, NBUDs and rearrangements found in the family group were 0.083±0.095, 0.130±0.154, 0.040±0.068 with a frequency of total aberration of 0.253±0.229, whereas those observed among shelter dogs were 0.300±0.268, 0.280±0.186, 0.090±0.145 with a frequency of total aberration of 0.670±0.399. Significant differences were found between family and shelter dogs in terms of MNi, NBUDs and total aberrations ($P<0.001$).

In both species, no significant differences were found between sexes in terms of MNi, NBUDs, rearrangement and total aberration frequencies (Table 3).

The differences observed in MNi frequency among subjects belonging to different ethogram classes were statistically evaluated (Tables 4 and 5). Among family cats' group, no significant differences emerged among the subjects belonging to different ethogram classes. *Vice versa*, among shelter cats, subjects belonging to ethogram class B showed significant increase in the frequencies of MNi ($P = 0.044$, Anova test), rearrangements ($P = 0.010$, Anova test: $P = 0.005$ Kruskal-Wallis test) and total aberrations ($P = 0.007$ for both Anova and Kruskal-Wallis tests). Also considering the total sample, significant increases in rearrangement ($P = 0.030$, Anova test) and total aberration frequencies ($P = 0.004$, Anova test: $P = 0.016$ Kruskal-Wallis test) were observed among cats belonging to class B (Table 4).

Among dogs, no significant differences were observed between the two classes in both family and shelter subjects, although cats and dogs belonging to class B showed highest levels of genomic damage in both family and shelter animals. However, when the subjects were grouped into a single total sample, dogs belonging to ethogram class B showed significant higher levels of MNi ($P = 0.019$, Anova test: $P = 0.010$ Kruskal-Wallis test), BUDs ($P = 0.007$, Anova test: $P = 0.014$ Kruskal-Wallis test) and total aberrations ($P = 0.011$, Anova test: $P = 0.007$ Kruskal-Wallis test) (Table 5).

Finally, the regression analysis failed ($P>0.05$) to find a significant correlation between age and the frequencies of genomic markers. Similarly, the multivariate analysis did not show significantly any

differences among the six groups according to age and sex (sex-age, $F = 0.191$ for cats and $F$ 0.988 for dogs)

## 4. Discussion

It is known that animal welfare is closely related to the concept of adaptation, which is an intrinsic condition of the animal: during adaptation, the subject who is able to adapt to a new environment, such as the shelter, is in a state of well-being. On the other hand, the one who fails is in a state of stress. In fact, stress is useful only if it is short-lived, because it serves to form the experience of the animal through its motor and vegetative protective reactions. *Vice versa*, a prolonged stress, to which animals living in shelters for several years are subject, could be associated to physiological and genomic alterations, even prolonged over time (Gourkow et al., 2013; Walker et al., 2016).

Domestic cats (*Felis silvestris catus*) and dogs (*Canis lupus familiaris*) are two of the most popular companion animals in Western Countries. In Italy, in 2015, there were an estimated 1,051 authorized shelters housing more than 100,000 dogs and cats (Italian Health Ministry, 2015), whereas, in the U.S., approximately six to eight million cats and dogs enter shelters each year (HSUS, 2014). Shelters provide potentially aversive and stressful social environments, which in combination with the high turnover of animals contribute to the transmission of infectious diseases (Cohn, 2011; Hirsch, 2016). As there is ample evidence to suggest that shelter environment can be stressful and have a negative impact on the welfare of these animals, to measure this impact becomes an important tool and challenge.

One way to determine animal welfare is by assessing how staying at the shelter influences physiology and behavior of the animals. For example, it is known that, when placed into a shelter environment, cats and dogs experience spikes in cortisol levels and increased frequencies of immunological problems (Protopopova, 2016). *Vice versa*, no studies are present in literature assessing the genomic effects of long-term stay in shelters.

241 dogs and compare them with the levels of family cats and dogs.
242 Statistically significant differences were found between shelter and family cats and dogs in terms of
243 MNi, NBUDs and total rearrangements, which indicate that a condition of physiological stress, as
244 can be observed in some shelters, may induce a high level of genomic damage.

245 The relationship between physiological stress and disease development was documented (Bale
246 2005; Fumagalli et al., 2007; Koenig et al., 2011). In particular, chronic stressors was found to be
247 associated with accelerated biological aging (Révész et al., 2014), as well as the stress response was
248 found to influence immune function, with potential consequences for patterns of infection and
249 transmission of disease among and within wildlife, domesticated animals and humans (Hing et al.,
250 2016). This relationship between stress and immune responsiveness appears to be significant.
251 Indeed, when chronic, stress can weaken the immune system, causing disease susceptibility and the
252 development of genomic damage (Gourkow et al., 2013). At genomic level, stress in mice and rats
253 may induce alterations in the expression of hepatic genes, an up-regulation of several markers
254 related to oxidative stress and an increase in apoptotic processes (Depke et al., 2009). Similarly,
255 stress has been shown to influence brain DNA repair genes expression in rats whereas, stress,
256 anxiety and depression have been shown to alter the methylation pattern of DNA in humans.
257 Interestingly, it has been shown that stress caused by trauma increases the level of genomic damage
258 in humans. Indeed, children who have experienced violence have shown a significantly higher level
259 of telomere erosion than their peers (Shalev et al., 2013; Bergholz et al., 2017; Kader et al., 2018).
260 Hence, a possible relationship between stressful conditions and increased frequencies of MNi is not
261 surprising.

262 In humans, higher levels of MNi in peripheral blood lymphocytes and other cell types have been
263 associated, in perspective, with an increased risk of cancer (Bonassi et al., 2011). Similarly, we

cannot rule out a connection between higher levels of MNi and a higher incidence of cancer even in cats and dogs living in shelters as compared to family cats and dogs.

In addition, MNi do not represent only the products of biological errors, but trigger the activation of the immune system related genes through the exposure of DNA fragments, which suggests that the presence of MNi can be perceived by the immune system (Gekara, 2017). MNi also represent a mechanism of elimination of genetic material, such as amplified genes, and contribute to nuclear dynamics and genomic chaos (Ye et al., 2019). The latter represents a process of rapid genomic re-organization that results in the formation of very altered and chaotic genomes (defined by both extreme structural and numerical alterations), some of which can be selected to establish stable genomes (Ye et al., 2019).

In contrast to Santovito et al. (2020), we found no effect of the age on the level of genomic damage neither in dogs nor in cats. It is plausible that the relatively short life expectancy of these two species may mask any possible correlation between age and MNi frequency.

Different markers are used to measure responses to stress in animals, principally the ethotest and analysis of cortisol levels (Hellhammer et al., 2009). In our study, results of the ethotest showed a significant increase of total aberrations among agitated and/or terrified animals (class B) with respect to calm cats and dogs (class A), evidencing a possible relationship between stress condition and increase of the genomic damage. However, we would like to emphasize that the ethotest has not yet been clearly validated against other signs of stress, such as the cortisol level. For example, McCobb et al. (2005) found no correlation between the CSS scores and the corresponding urinary cortisol-to-creatinine ratio, as well as no correlation between CSS and the faecal cortisol metabolites was observed (Rehnberg et al., 2015). This could be probably due to the fact that cortisol levels might not always be an accurate indicator of stress in sheltered animals since the responses in the brain related to stress are caused by several factors and cortisol only affects stress indirectly (Hellhammer et al., 2009; Gourkow et al., 2014). Finally, the ethotest score is subjective

and static, built on behaviours displayed in short intervals of time, that is, as the original method, one minute of observation (Kessler and Turner, 1997).

## 5. Conclusions

In this work we provided evidence of a possible correlation between physiological stress conditions and higher levels of genomic damage in a sample of sheltered cats and dogs.

In literature, stress in sheltered animals has been assessed both qualitatively (behavior analysis) and quantitatively (e.g., cortisol levels, catecholamine levels, heart rate, immune function, etc.) (Pesavento and Murphy, 2014). However, it has been proven that each method exhibits some limitations (Protopopova, 2016). In sight of this, a more complete assessment of shelter animal welfare can be performed by evaluating multiple parameters and proposing new ones (Polgár et al., 2019; Righi et al., 2019). In this perspective, since it has been shown that chronic stress may induce genomic damage (Gourkow et al., 2013), the results of our study suggest that the buccal MNi assay, also considering the relatively low cost of laboratory procedure and its non-invasiveness, could be potential additional tool that, combined with the ethotest, may be able to provide a more comprehensive picture of the health status of animal that live in communities.

## Disclosure of Interest

The Authors declare that they have no conflicts of interest for this article.

## Acknowledgements

This study was financed by University of Turin with local 2015-2018 grants. The authors would like to thank all veterinary and shelter volunteers who allowed us access to shelters and participated

312 in the collection of buccal samples and that offer valuable work useful to improve animal welfare.
313 We would also like to thank the Professor Sonia Slaviero for her contribution in revising English.

314

315 **Availability of data**

316 The authors declare their willingness to provide, upon request, detailed data relating to this work.

317

318 **Ethical statements**

319 The permission for the study was obtained from the Local Ethics Committee and from the
320 veterinarians in chief of the shelters.

321 Moreover, the handlers of each dog and cat in the study agreed to take the buccal epithelial sample
322 from their dog/cat.

323

## References


Anderson, K. A., Brandt, J. C., Lord, L. K., Miles, E. A., 2013. Euthanasia in animal shelters: Management's perspective on staff reactions and support programs. Anthrozoös, 26(4), 569-578.

Bale T. L., 2005. Sensitivity to stress: dysregulation of CRF pathways and disease development. Horm Behav. 48(1), 1-10.

Beerda, B., Schilder, M. B., Bernadina, W., Van Hooff, J. A., De Vries, H. W., Mol, J. A., 1999. Chronic stress in dogs subjected to social and spatial restriction. II. Hormonal and immunological responses. Physiol. Behav. 66(2), 243-254.

Beerda, B., Schilder, M. B., Van Hooff, J. A., De Vries, H .W., Mol, J. A., 2000. Behavioral and hormonal indicators of enduring environmental stress in dogs. Anim. Welf. 9, 49-62.

Benvindo-Souza, M., Borges, R. E., Pacheco, S. M., de Souza Santos, L. R., 2019. Genotoxicological analyses of insectivorous bats (Mammalia: Chiroptera) in central Brazil: The oral epithelium as an indicator of environmental quality. Environ. Pollut. 245, 504-509.

Bergholz, L. M., Subic-Wrana, C., Heylmann, D., Beutel, M. E., Wiltink, J., Kaina, B., 2017. DNA damage in lymphcytes of patients suffering from complex traumatization. DNA Repair 521, 03-109.

Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26, 93-100.

Borges, R. E., de Souza Santos, L. R., Benvindo-Souza, M., Modesto, R. S., Assis, R. A., de Oliveira, C., 2019. Genotoxic Evaluation in Tadpoles Associated with Agriculture in the Central Cerrado, Brazil Arch. Environ. Contam. Toxicol. 77(1), 22-28

Cohn, L. A., 2011. Feline Respiratory Disease Complex. Vet. Clin. N. Am. –Small 41(6), 1273-1289.

Coppola, C. L., Grandin, T., Enns, R. M., 2006. Human interaction and cortisol: Can human contact reduce stress for shelter dogs? Physiol. Behav. 87, 537-541.

Dalla Villa, P., Barnard, S., Di Fede, E., Podaliri, M., Candeloro, L., Di Nardo, A., Siracusa, C., Serpell, J. A. 2013. Behavioural and physiological responses of shelter dogs to long-term confinement. Vet. It. 49(2), 231-241.

Depke, M., Steil, L., Domanska, G., Völker, U., Schütt, C., Kiank, C., 2009. Altered hepatic mRNA expression of immune response and apoptosis-associated genes after acute and chronic psychological stress in mice. Mol. Immunol. 46(15), 3018-3028.

Eriksson, P., Loberg, J., Andersson, M., 2009. A survey of cat shelters in Sweden. Anim. Welf. 18(3), 283-288.

Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J. W., Parry, J., Thomas, P., 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26, 125-132.

Fumagalli, F., Molteni, R., Racagni, G., Riva M. A., 2007. Stress during development: Impact on neuroplasticity and relevance to psychopathology. Prog. Neurobiol. 81(4), 197-217.

Gekara, N. O., 2017. DNA damage-induced immune response: Micronuclei provide key platform. J. Cell. Biol. 216, 2999-3001.

Gourkow, N., Lawson, J. H., Hamon, S. C., Phillips, C. J. 2013. Descriptive epidemiology of upper respiratory disease and associated risk factors in cats in an animal shelter in coastal western Canada. Can. Vet. J. 54(2), 132.

Gourkow, N., La Voy, A., Dean, G. A., Phillips, C. J., 2014. Associations of behaviour with secretory immunoglobulin A and cortisol in domestic cats during their first week in an animal shelter. Appl. Anim. Behav. Scie. 150, 55-64.

Hellhammer, D. H., Wüst, S., Kudielka, B. M., 2009. Salivary cortisol as a biomarker in stress research. Psychoneuroendocrinology 34(2), 163

Hennessy, M. B., Voith, V. L., Mazzei, S. J., Buttram, J., Miller, D. D., Linden, F., 2001. Behavior and cortisol levels of dogs in a public animal shelter, and an exploration of the ability of these measures to predict problem behavior after adoption. Appl. Anim. Behav. Scie.

73,217-233.

Hing, S., Narayan, E. J., Thompson, R. C. A., Godfrey S. S., 2016. The relationship between physiological stress and wildlife disease: consequences for health and conservation. Wild Res. 43(1), 51-60.

Hirsch, E. N., 2016. Feline Stress. Methodological considerations for non-invasive assessment of cats housed in groups and singly. PhD thesis, Swedish University of Agricultural Sciences, Skara. Available on https://pub.epsilon.slu.se/13682/1/hirsch_en_160927.pdf. Accessed on 10-29-2019.

Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., Fenech, M., 2008. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutat. Res. 659(1-2), 93-108.

Humane Society of the United States (HSUS), 2014. Annual Report 2014. Available on: https://www.humanesociety.org/sites/default/files/docs/2014-hsus-annual-report.pdf. Accessed on 11-10-2019.

Italian Health Minister, 2015. Cani e Rifugi. Available on: http://www.salute.gov.it/portale/temi/p2_6.jsp?lingua=italiano&id=3093&area=cani&menu=abbandono). Accessed on 11-10-2019.

Kader, F., Ghai, M., Maharaj, L., 2018. The effects of DNA methylation on human psycology. Behav. Brain. Res. 346, 47-65.

Kessler, M. R., Turner, D. C., 1997. Stress and adaptation of cats (Felis silvestris catus) housed singly, in pairs and in groups in boarding catteries. Anim. Welf. 6(3), 243-254.

Kessler, M. R., Turner, D. C., 1999. Effects of density and cage size on stress in domestic cats (Felis silvestris catus) housed in animal shelters and boarding catteries. Anim. Welf. 8(3), 259-267.

Koenig J. I., Walker C. D., Romeo R. D., Lupien S. J., 2011. Effects of stress across the lifespan. Stress 14(5), 475-80

Kubesova, K., Voslarova, E., Vecerek, V., Vucinic, M., 2017. Investigating Some of the Factors that Affect the Selection of Shelter Cats by Adopters in the Czech Republic. Anthrozoös 30(4), 623-633.

Lazalde-Ramos, B. P., Zamora-Pérez, A. L., Sosa-Macías, M., Galaviz-Hernández, C., Zúñiga-González, G. M., 2017. Micronculei and nuclear anomalies in Mexico's indigenous population. Salud Publica Mex. 59, 532-539.

Loberg, J. M., Lundmark, F., 2016. The effect of space on behaviour in large groups of domestic cats kept indoors. Appl. Anim. Behav. Scie. 182, 23-29.

Lord, E., Widmar, N. O., Litster, A., 2014. Economic impacts of adoption and fundraising strategies in animal shelters. Prev. Vet. Med. 113(4), 423-429.

Lucidi, P., Bernabo, N., Panunzi, M., Dalla Villa, P., Mattioli, M., 2005. Ethotest: A new model to identify (shelter) dogs' skills as service animals or adoptable pets. Appl. Anim. Behav. Scie. 95(1-2), 103-122.

McCobb, E. C., Patronek, G. J., Marder, A., Dinnage, J. D., Stone, M. S., 2005. Assessment of stress levels among cats in four animal shelters. J. Am. Vet. Med. Ass. 226(4), 548-555.

Normando, S., Stefanini, C., Meers, L., Adamelli, S., Coultis, D., Bono, G., 2006. Some factors influencing adoption of sheltered dogs. Anthrozoös 19(3), 211-224

Pesavento, P. A., Murphy, B. G., 2014. Common and Emerging Infecious Diseases in the Animal Shelter. Vet. Pathol. 51(2), 478-491.

Polgár, Z., Blackwell, E. J., Rooney, N. J., 2019. Assessing the welfare of kennelled dogs - A review of animal-based measures. Appl.Anim. Behav. Scie. 213, 1-13.

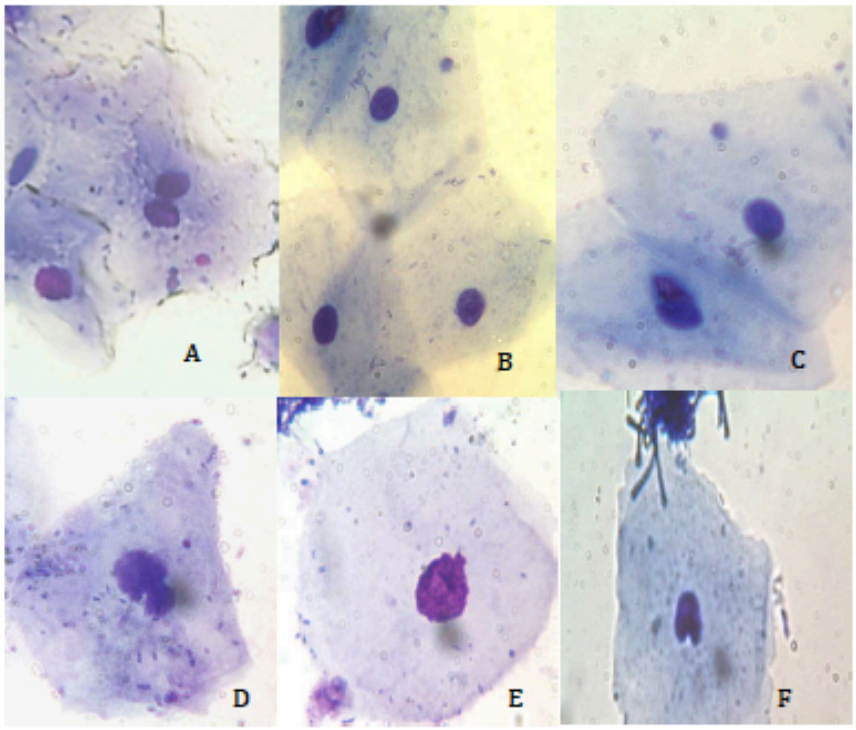
Protopopova, A., 2016. Effects of sheltering on physiology, immune function, behavior, and the welfare of dogs. Physiol. Behav. 159, 95-103.

Rehnberg, L. K., Robert, K. A., Watson, S. J., Peters, R. A., 2015. The effects of social interaction and environmental enrichment on the space use, behaviour and stress of owned housecats

427 facing a novel environment. Appl. Anim. Behav. Scie. 169, 51-61.
428 Révész, D., Verhoeven, J. E., Milaneschi, Y., de Geus, E. J. C. N., Wolkowitz O. M., Penninx, B.
429 W. H., 2014. Dysregulated physiological stress systems and accelerated cellular aging.
430 Neurobiol. Aging 35(6), 1422-1430.
431 Righi, C., Menchetti, L., Orlandi, R., Moscati, L., Mancini, S., Diverio, S., 2019. Welfare
432 Assessment in Shelter Dogs by Using Physiological and Immunological Parameters.
433 Animals (Basel) 9(6), 340.
434 Santovito, A., Delsoglio, M., Manitta, E., Picco, G., Meschiati, G., Chiarizio, M., Gendusa, C.,
435 Cervella, P., 2017. Association of GSTT1 Null, XPD 751 CC and XPC 939 CC Genotypes
436 With Increased Levels of Genomic Damage Among Hospital Pathologists. Biomarkers
437 22(6), 557-565.
438 Santovito, A., Gendusa, C., 2020. Micronuclei Frequency in Peripheral Blood Lymphocytes of
439 Healthy Subjects Living in Turin (North-Italy): Contribution of Body Mass Index, Age and
440 Sex. Ann. Hum. Biol. 47(1), 48-54.
441 Shalev, I., Moffitt, T. E., Sugden, K., Williams, B., Houts, R. M., Danese, A., Mill, J., Arsenault, L.,
442 Caspi, A., 2013. Exposure to violence during childhood is associated with telomere erosion
443 from 5 to 10 years of age: a longitudinal study. Mol. Psychiatry 18(5), 576-581
444 Taylor, K. D., Mills, D. S. M., 2007. The effect of the kennel environment on canine welfare: A
445 critical review of experimental studies. Anim. Welf. 16, 435-447.
446 Thomas, P., Fenech, M., 2011. Buccal micronucleus cytome assay. Methods Mol. Biol. 682,235-
447 248.
448 Titulaer, M., Blackwell, E. J., Mendl, M., Casey, R. A., 2013. Cross sectional study comparing
449 behavioural, cognitive and physiological indicators of welfare between short and long term
450 kennelled domestic dogs. Appl. Anim. Behav. Scie. 147, 149-158.
451 Vojtkovská, V., Voslářová, E., Večerek, V., 2020. Methods of Assessment of the Welfare of Shelter
452 Cats: A review. Animal 10, 1527.

453 Walker, J. K., Dale, A. R., D'Eath, R. B., Wemelsfelder, F., 2016. Qualitative Behaviour Assessment
454 of dogs in the shelter and home environment and relationship with quantitative behaviour
455 assessment and physiological responses. Appl. Anim. Behav. Scie. 184, 97-108.
456 Wells, D. L., Hepper, P. G., 2000. Prevalence of behavior problems reported by owners of dogs
457 purchased from animal rescue. Appl. Anim. Behav. Scie. 69, 55-65.
458 Wells, D. L., Graham, L., Hepper, P. G., 2002. The influence of auditory stimulation on the
459 behavior of dogs housed in a rescue shelter. Anim. Welf. 11, 385-393.
460 Ye, C.J., Sharpe, Z., Alemara, S., Mackenzie, S., Liu, G., Abdallah, B., Horne, S., Regan, S., Heng,
461 H.H., 2019. Micronuclei and Genome Chaos: Changing the System Inheritance. Genes
462 10(5), 366.

Figure 1 – Examples of damaged cells observed in our samples




A) Binucleated cell with micronucleus; B) and C) mononucleated cells with micronucleus; D), E) nuclear buds; F) identation. These last two aberrations were included in the Rearrangement category.

Table 1 - General characteristics of the studied samples

| | Cats | Dogs |
|---|---|---|
| **Family** | | |
| N | 30 | 30 |
| Age (mean±SD) | 5.60±4.42 | 6.40±3.73 |
| Males | 14 | 12 |
| Females | 16 | 18 |
| **Shelter** | | |
| N | 30 | 30 |
| Age (mean±SD) | 5.23±4.43 | 5.41±1.64 |
| Males | 15 | 18 |
| Females | 15 | 12 |

N = number of studied subjects;
S.D. = Standard Deviation

Table 2 – Statistical evaluation of genomic damage between Shelter and Family cats and dogs

| | N | N Cells | MNi N (Mean±DS %) | NBUDs N (Mean±SD %) | REAR N (Mean±SD %) | TOTAL ABERRATIONS N (Mean±SD %) |
|---|---|---|---|---|---|---|
| **CATS** | | | | | | |
| **Family** | 30 | 30,000 | 30 (0,100±0.383) | 33 (0.110±0.092) | 23 (0.077±0.119) | 86 (0.287±0.405) |
| **Shelter** | 30 | 30,000 | 63 (0.210±0.209)* | 66 (0.220±0.183)** | 26 (0.087±0.125) | 155 (0.517±0.373)*** |
| **DOGS** | | | | | | |
| **Family** | 30 | 30,000 | 25 (0.083±0.095) | 39 (0.130±0.154) | 12 (0.040±0.068) | 76 (0.253±0.229) |
| **Shelter** | 30 | 30,000 | 90 (0.300±0.268)* | 84 (0.280±0.186)* | 27 (0.090±0.145) | 201 (0.670±0.399)* |

N = number of studied subjects; N Cells = Number of Analyzed Cells; S.D. = Standard Deviation; MNi = micronuclei; NBUDs = nuclear buds; REAR = rearrangements;.
* $P<0.001$ (Kruskal-Wallis and ANOVA tests) and $P = 0.029$ (Multivariate analysis) with respect to family group.
** $P = 0.010$; *** $P = 0.003$ (Kruskal-Wallis and ANOVA test) with respect to family group.

Table 3

Click here to access/download;Table;renamed_3ec39.docx

Table 3 – Evaluation of the level of genomic damage according to sex

| | N | N Cells | MNi N (Mean±DS %) | NBUDs N (Mean±SD %) | REAR N (Mean±SD %) | TOTAL ABERRATIONS N (Mean±SD %) |
|---|---|---|---|---|---|---|
| **CATS** | | | | | | |
| **Males** | 29 | 29,000 | 42 (0.145±0.198) | 51 (0.176±0.133) | 32 (0.110±0.147) | 125 (0.431±0.377) |
| **Females** | 31 | 31,000 | 51 (0.165±0.392) | 48 (0.155±0.173) | 17 (0.055±0.085) | 116 (0.374±0.403) |
| **DOGS** | | | | | | |
| **Males** | 30 | 30,000 | 70 (0.233 ±0.275) | 67 (0.223±0275.) | 20 (0.067 ±0124.) | 157 (0.523 ±0.436) |
| **Females** | 30 | 30,000 | 45 (0.150±0.161) | 56 (0.187±0.183) | 19 (0.063±0.107) | 120 (0.400±0.322) |

N = number of studied subjects; N Cells = Number of Analyzed Cells; S.D. = Standard Deviation; MNi = micronuclei; NBUDs = nuclear buds; REAR = rearrangements

Table 4

Click here to access/download;Table;renamed_48b69.docx

Table 4 – Evaluation of genomic damage among different ethogram classes in cats

| | N | N Cells | MNi N (Mean±DS %) | NBUDs N (Mean±DS %) | REAR N (Mean±DS %) | TOTAL ABERRATIONS N (Mean±DS %) |
|---|---|---|---|---|---|---|
| **FAMILY CATS** | | | | | | |
| ETHOGRAM CLASS A | 15 | 15,000 | 6 (0.040±0.083) | 13 (0.087±0.074) | 10 (0.067±0.072) | 29 (0.193±0.096) |
| ETHOGRAM CLASS B | 15 | 15,000 | 24 (0.160±0.538) | 20 (0.133±0.105) | 13 (0.087±0.155) | 57 (0.380±0.558) |
| **SHELTER CATS** | | | | | | |
| ETHOGRAM CLASS A | 12 | 12,000 | 14 (0.117±0.134) | 20 (0.167±0.107) | 2 (0.017±0.039) | 36 (0.300±0.252) |
| ETHOGRAM CLASS B | 18 | 18,000 | 49 (0.272±0.230) [a] | 46 (0.256±0.215) | 24 (0.133±0.141) *, [b] | 119 (0.661±0.376) **, [c] |
| **TOTALS** | | | | | | |
| ETHOGRAM CLASS A | 27 | 27,000 | 20 (0.074±0.113) | 33 (0.122±0.097) | 12 (0.044±0.064) | 65 (0.241±0.187) |
| ETHOGRAM CLASS B | 33 | 33,000 | 73 (0.221±0.397) | 66 (0.200±0.182) | 37 (0.112±0.147) [d] | 90 (0.533±0.481)***, [e] |

N = number of studied subjects; N Cells: Number of analysed cells; S.D. = Standard Deviation; MNi = micronuclei; NBUDs = nuclear buds; REAR = rearrangements

Class A = calm subjects; Class B = agitated and/or terrified subjects

[a] $P = 0.044$; [b] $P = 0.010$ ; [c] $P = 0.007$; [d] $P = 0.030$; [e] $P = 0.004$ (compared with class A, ANOVA test)

* $P = 0.005$; ** $P = 0.007$; *** $P = 0.016$ (compared with class A, Kruskal-Wallis)

Table 5 – Evaluation of genomic damage among different ethogram classes in dogs

| | N | N Cells | MNi N (Mean±DS %) | BUDs N (Mean±DS %) | REAR N (Mean±DS %) | TOTAL ABERRATIONS N (Mean±DS %) |
|---|---|---|---|---|---|---|
| **FAMILY DOGS** | | | | | | |
| ETHOGRAM CLASS A | 18 | 18,000 | 11 (0.061±0.078) | 17 (0.094±0.135) | 9 (0.050±0.079) | 37 (0.206±0.215) |
| ETHOGRAM CLASS B | 12 | 12,000 | 14 (0.117±0.111) | 22 (0.183±0.170) | 3 (0.025±0.045) | 39 (0.325±0.238) |
| **SHELTER DOGS** | | | | | | |
| ETHOGRAM CLASS A | 11 | 11,000 | 22 (0.200±0.195) | 23 (0.209±0.202) | 10 (0.091±0.104) | 55 (0.500±0.344) |
| ETHOGRAM CLASS B | 19 | 19,000 | 68 (0.358±0.291) | 61 (0.321±0.169) | 17(0.089±0.166) | 146 (0.768±0.404) |
| **TOTALS** | | | | | | |
| ETHOGRAM CLASS A | 29 | 29,000 | 33 (0.114±0.148) | 40 (0.138±0.170) | 19 (0.066±0.090) | 92 (0.317±0.302) |
| ETHOGRAM CLASS B | 31 | 31,000 | 82 (0.265±0.264)*,[a] | 83 (0.268±0.180)**, [b] | 20 (0.065±0.136) | 185 (0.597±0.409)***, [c] |

N = number of studied subjects; N Cells = Number of analysed cells; S.D. = Standard Deviation; MNi = micronuclei; NBUDs = Nuclear Buds; REAR = rearrangements

Class A = calm subjects; Class B = agitated and/or terrified subjects

[a] $P = 0.019$; [b] $P = 0.007$; [c] $P = 0.011$ (compared with class A, ANOVA)

* $P = 0.010$; ** $P = 0.004$; *** $P = 0.007$ (compared with class A, Kruskal-Wallis test)