

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1833701> since 2022-01-19T08:46:03Z

Published version:

DOI:10.1016/j.jveb.2021.09.007

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

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

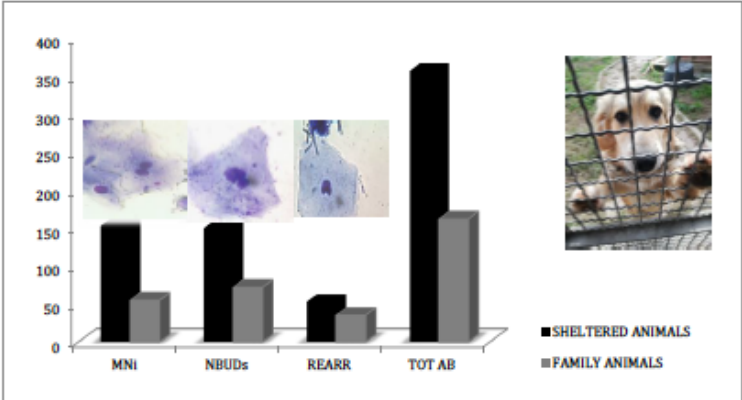
–Manuscript Draft–

| | |
|------------------------|--|
| Manuscript Number: | JVEB-D-21-00024R2 |
| Article Type: | Research Paper |
| Keywords: | Genomic Damage; Nuclear Buds; Mammals; welfare; Companion Animals |
| Corresponding Author: | Alfredo Santovito University of Turin Turin, Piemonte ITALY |
| First Author: | Alfredo Santovito |
| Order of Authors: | Alfredo Santovito Martina BUGLISI Chiara SCIANDRA Manuel SCARFO' |
| Abstract: | Highlights <ol style="list-style-type: none">1. Group-housing can increase stress levels and contribute to physiological problems2. We evaluated, by MNi assay, the level of genomic damage in shelter cats and dogs3. We recruited 30 shelter cats and dogs and 30 family cats and dogs used as control4. Significant differences in the MNi frequency were found between the two groups5. The ethotest confirms the increased levels of aberrations in stressed animals |
| Suggested Reviewers: | P.A. Pesavento University of California Davis School of Veterinary Medicine papesavento@ucdavis.edu 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 Texas Tech University a.protopopova@ttu.edu 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



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

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

4

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

6 University of Turin, Department of Life Sciences and Systems Biology, Via Accademia Albertina

7 n. 13, 10123 Torino (Italy)

8

9 *Corresponding Author:

10 Alfredo SANTOVITO, University of Turin,

11 Department of Life Sciences and Systems Biology

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

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

14 E-mail: alfredo.santovito@unito.it

15

16 *Abstract*

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

37

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

39

40

41

42 **1. Introduction**

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

49 It is generally accepted that many animal shelters can be potentially stressful places for animals,
50 mainly due to space restrictions, lack of resources and high animal turnover (Kessler and Turner,
51 1999; Wells et al., 2002).

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

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

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

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

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

92

93 **2. Materials and Methods**

94 2.1. Subjects

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

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

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

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

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

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

120

121 2.2. MNi assay

122 Exfoliated buccal mucosa cells were collected by gently scraping the mucosa of the inner lining of
123 one or both cheeks with a spatula. Buccal cells were also collected from the inner side of the lower
124 lip and palate. Indeed, the variability in MNi frequency between these areas was found to be
125 minimal for control subjects (Holland et al., 2008). The tip of the spatula was immersed in a
126 fixative solution consisting of methanol/Acetic Acid 3:1, stored at 4 °C prior the analysis.

127 Successively, cells were collected by centrifugation, the supernatant was discarded and the pellet
128 was dissolved in a minimal amount of fixative which was seeded on the slides to detect MNi by
129 conventional staining with 5% Giemsa (pH 6.8) prepared in Sørensen buffer.

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

133

134 2.3. Cat Stress Score (CSS) test

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

141 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

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

172

173 3. Results

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

181 In Table 2 results of the statistical evaluation of genomic damage between shelter and family cats
182 and dogs were reported. In Figure 1 some examples of damaged cells observed in our samples were
183 reported. Among family cats, the frequency of MNi, NBUDs and rearrangements were
184 0.100 ± 0.383 , 0.110 ± 0.092 , 0.077 ± 0.119 , with a frequency of total aberration of 0.287 ± 0.405 .
185 Among shelter cats, the frequency of MNi, NBUDs and rearrangements were 0.210 ± 0.209 ,
186 0.220 ± 0.183 , and 0.087 ± 0.125 , with a frequency of total aberration of 0.517 ± 0.373 . Significant
187 differences were found between family and shelter cats in terms of MNi ($P < 0.001$), NBUDs ($P =$
188 0.010) and total aberrations ($P = 0.003$).

189 Among dogs, the frequencies of MNi, NBUDs and rearrangements found in the family group were
190 0.083 ± 0.095 , 0.130 ± 0.154 , 0.040 ± 0.068 with a frequency of total aberration of 0.253 ± 0.229 ,
191 whereas those observed among shelter dogs were 0.300 ± 0.268 , 0.280 ± 0.186 , 0.090 ± 0.145 with a
192 frequency of total aberration of 0.670 ± 0.399 . Significant differences were found between family
193 and shelter dogs in terms of MNi, NBUDs and total aberrations ($P<0.001$).

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

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

205 Among dogs, no significant differences were observed between the two classes in both family and
206 shelter subjects, although cats and dogs belonging to class B showed highest levels of genomic
207 damage in both family and shelter animals. However, when the subjects were grouped into a single
208 total sample, dogs belonging to ethogram class B showed significant higher levels of MNi ($P =$
209 0.019 , Anova test: $P = 0.010$ Kruskal-Wallis test), BUDs ($P = 0.007$, Anova test: $P = 0.014$
210 Kruskal-Wallis test) and total aberrations ($P = 0.011$, Anova test: $P = 0.007$ Kruskal-Wallis test)
211 (Table 5).

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

215 0.988 for dogs)

216

217 4. Discussion

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

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

234 One way to determine animal welfare is by assessing how staying at the shelter influences
235 physiology and behavior of the animals. For example, it is known that, when placed into a shelter
236 environment, cats and dogs experience spikes in cortisol levels and increased frequencies of
237 immunological problems (Protopopova, 2016). *Vice versa*, no studies are present in literature
238 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

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

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

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

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

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

291

292 **5. Conclusions**

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

295 In literature, stress in sheltered animals has been assessed both qualitatively (behavior analysis) and
296 quantitatively (e.g., cortisol levels, catecholamine levels, heart rate, immune function, etc.)

297 (Pesavento and Murphy, 2014). However, it has been proven that each method exhibits some
298 limitations (Protopopova, 2016). In sight of this, a more complete assessment of shelter animal
299 welfare can be performed by evaluating multiple parameters and proposing new ones (Polgár et al.,
300 2019; Righi et al., 2019). In this perspective, since it has been shown that chronic stress may induce
301 genomic damage (Gourkow et al., 2013), the results of our study suggest that the buccal MNi assay,
302 also considering the relatively low cost of laboratory procedure and its non-invasiveness, could be
303 potential additional tool that, combined with the ethotest, may be able to provide a more
304 comprehensive picture of the health status of animal that live in communities.

305

306 **Disclosure of Interest**

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

308

309 **Acknowledgements**

310 This study was financed by University of Turin with local 2015-2018 grants. The authors would
311 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

324 **References**

- 325 Anderson, K. A., Brandt, J. C., Lord, L. K., Miles, E. A., 2013. Euthanasia in animal shelters:
326 Management's perspective on staff reactions and support programs. *Anthrozoös*, 26(4), 569-
327 578.
- 328 Bale T. L., 2005. Sensitivity to stress: dysregulation of CRF pathways and disease development.
329 *Horm Behav.* 48(1), 1-10.
- 330 Beerda, B., Schilder, M. B., Bernadina, W., Van Hooff, J. A., De Vries, H. W., Mol, J. A., 1999.
331 Chronic stress in dogs subjected to social and spatial restriction. II. Hormonal and
332 immunological responses. *Physiol. Behav.* 66(2), 243-254.
- 333 Beerda, B., Schilder, M. B., Van Hooff, J. A., De Vries, H. W., Mol, J. A., 2000. Behavioral and
334 hormonal indicators of enduring environmental stress in dogs. *Anim. Welf.* 9, 49-62.
- 335 Benvindo-Souza, M., Borges, R. E., Pacheco, S. M., de Souza Santos, L. R., 2019.
336 Genotoxicological analyses of insectivorous bats (Mammalia: Chiroptera) in central Brazil:
337 The oral epithelium as an indicator of environmental quality. *Environ. Pollut.* 245, 504-509.
- 338 Bergholz, L. M., Subic-Wrana, C., Heylmann, D., Beutel, M. E., Wiltink, J., Kaina, B., 2017. DNA
339 damage in lymphocytes of patients suffering from complex traumatization. *DNA Repair* 521,
340 03-109.
- 341 Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011. Micronuclei frequency in peripheral
342 blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis* 26, 93-100.
- 343 Borges, R. E., de Souza Santos, L. R., Benvindo-Souza, M., Modesto, R. S., Assis, R. A., de
344 Oliveira, C., 2019. Genotoxic Evaluation in Tadpoles Associated with Agriculture in the
345 Central Cerrado, Brazil *Arch. Environ. Contam. Toxicol.* 77(1), 22-28
- 346 Cohn, L. A., 2011. Feline Respiratory Disease Complex. *Vet. Clin. N. Am. –Small* 41(6), 1273-
347 1289.
- 348 Coppola, C. L., Grandin, T., Enns, R. M., 2006. Human interaction and cortisol: Can human contact
349 reduce stress for shelter dogs? *Physiol. Behav.* 87, 537-541.

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

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

356 Eriksson, P., Loberg, J., Andersson, M., 2009. A survey of cat shelters in Sweden. *Anim. Welf.*
357 18(3), 283-288.

358 Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J. W., Parry, J., Thomas, P.,
359 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud
360 formation in mammalian and human cells. *Mutagenesis* 26, 125-132.

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

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

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

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

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

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

376 73,217-233.

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

380 Hirsch, E. N., 2016. Feline Stress. Methodological considerations for non-invasive assessment of
381 cats housed in groups and singly. PhD thesis, Swedish University of Agricultural Sciences,
382 Skara. Available on https://pub.epsilon.slu.se/13682/1/hirsch_en_160927.pdf. Accessed on
383 10-29-2019.

384 Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., Fenech,
385 M., 2008. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA
386 damage: the HUMN project perspective on current status and knowledge gaps. *Mutat. Res.*
387 659(1-2), 93-108.

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

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

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

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

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

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

402 Kubesova, K., Voslarova, E., Vecerek, V., Vucinic, M., 2017. Investigating Some of the Factors that
403 Affect the Selection of Shelter Cats by Adopters in the Czech Republic. *Anthrozoös* 30(4),
404 623-633.

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

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

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

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

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

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

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

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

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

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

427 facing a novel environment. *Appl. Anim. Behav. Sci.* 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., Arsenaault, 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. Sci.* 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.

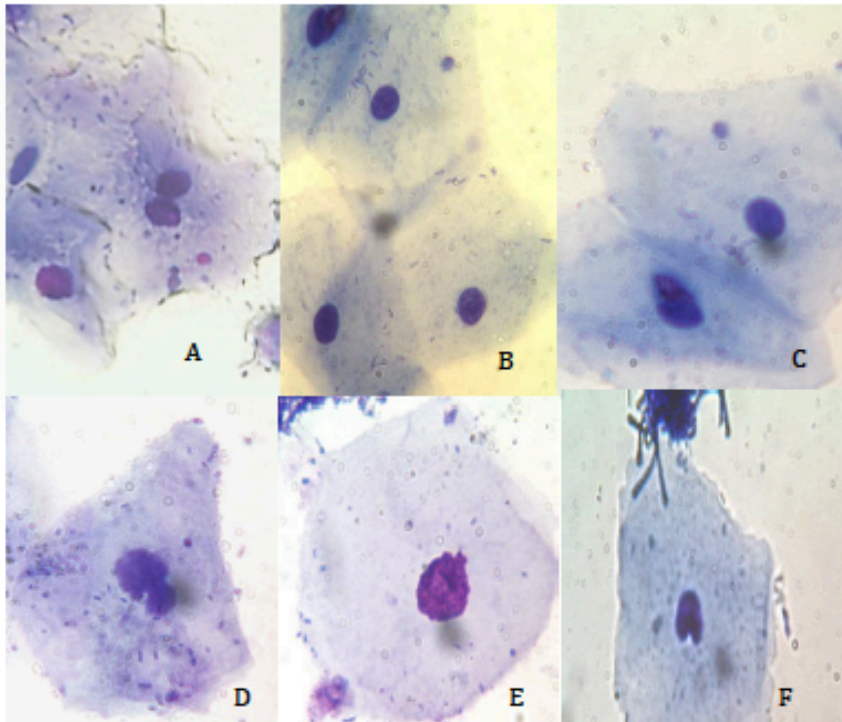
453 Walker, J. K., Dale, A. R., D'Eath, R. B., Wemelsfelder, F., 2016. Qualitative Behaviour Assessment
454 of dogs in the shelter and home environment and relationship with quantitative behaviour
455 assessment and physiological responses. *Appl. Anim. Behav. Scie.* 184, 97-108.

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) idention. 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±SD %) | 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 – Evaluation of the level of genomic damage according to sex

| | N | N Cells | MNi N (Mean±SD %) | 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±0.275.) | 20 (0.067 ±0.124.) | 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 – 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) ^{a, 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

^aP = 0.044; ^bP = 0.010 ; ^cP = 0.007; ^dP = 0.030; ^eP = 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

^aP = 0.019; ^bP = 0.007; ^cP = 0.011 (compared with class A, ANOVA)

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