Analysis of fighting-associated wounds causing death of young male CD-1 mice in carcinogenicity studies.

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Summary
Early death of CD-1 mice due to fighting-associated wounds was analysed using information gathered from the control groups of twenty mouse carcinogenicity studies, all deaths occurring within the first 50 weeks of the studies being reviewed. Of the 1453 mice in each sex, 101 (6.95 %) male and 69 (4.75%) female decedents with a statistical significance (p=0.016), in favour of females, were recorded during the first 50 weeks of study. The hazard ratio for gender was found to be 1.45. Analysis of factors contributing to death revealed that 26 (25.7%) males had integumentary wounds, 27 (26.7%), males exhibited neoplastic lesions, and 48 (47.5%) males had other changes. In females, the figures were 11 (15.9%), 28 (40.6%), and 30 (43.4%), respectively. A high proportion of the observed lesions such as ulceration, abscess-formation and granulomatous inflammation of the skin, subcutaneous tissues or muscle, were considered to be fighting-associated wounds. Of the neoplastic causes of death, haematopoietic tumours were the most common, followed by osteosarcoma, and some skin or mammary tumours. One of the most common non-tumour factors contributing to death was kidney diseases (nephropathy and glomerulonephritis), followed by urinary obstruction of males. Some animals died from trauma/fracture, dosing accidents, unknown reasons or were also sacrificed due to poor clinical condition. From the mortality analysis of fighting-associated factors contributing to death, there was no significant statistical difference (p values, males=0.55, females=0.94) between single and multiple housed animals. In the hazard ratio analysis between single and multiple housing, multiple housed males have 1.32 times the risk of death when compared with single housed males, whereas this figure in females were found to be 1.05. In conclusion, housing density, especially in males, had an impact on survival; however, it could not be attributed solely to fighting-associated integumentary lesions.

Introduction
As a result of modern animal husbandry methods and strict genetic and microbiological controls, sporadic deaths in untreated mice have been greatly reduced. However, even though the numbers are limited, sporadic deaths still occur and cause problems in the animal experimentation. Information on causes of death and/or factors influencing decedents, and mortalities in laboratory rodents, have been reviewed by several authors *(Haseman et al, 1994; Ettlin et al, 1994; Rao et al, 1990; Maita et al, 1988; Glaister, 1986).* Inflammation of the integumentary system is one of the main contributing factors to death in young mice, to a greater extent in males *(Glaister, 1986).* Some findings such as ulceration, abscess-formation, and pyogranulomatous inflammation of the skin, subcutis or muscle have been known to be caused by aggressive behaviour in multiple-housed male mice *(Percy & Barthold, 1993; Faccini et al, 1990).* Although, there are many reasons to primarily cause skin inflammatory lesions including infection of virus or bacteria, infestation with parasites, and even also behaviour-associated skin problems *(Percy & Barthold, 1993)* and also skin infections are frequently accompanied by pruritus with self-excoriation, it would be worth conducting a survey of how many mice die from fighting-associated wounds and estimating how much the multiple housing contributes to the deaths, especially those due to fighting-associated wounds. Thus those wounds due to fighting, which could be associated with death, were analysed statistically in respect of sex and housing.

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Materials and Methods

Animals

Male and female CD-1 mice obtained from Charles River (UK) Limited were maintained as control animals for carcinogenicity studies at Huntingdon Life Sciences. At the estimated age of 5-6 weeks, one to four mice were placed at random in suspended solid bottom polycarbonate housing with sawdust or woodchip bedding or in stainless steel wire cages. Animal room temperature and relative humidity were generally maintained at 18 to 23ºC and 38 to 68 % respectively during the study. Artificial light was set to give 12 hours continuous light and 12 hours continuous dark per 24 hours. Filtered air was ducted into the animal room and extracted to provide approximately 15 air changes per hour. All mice had free access to tap water bottles with sipper tubes and ground SDS Rat and Mouse No. 1 modified maintenance diet (SDS Special Diets, Witham, UK). Drinking water and diet were routinely subjected to chemical analysis to monitor possible influences on the study. Food hoppers and water bottles were changed daily or up to every two days. An acclimatisation period of 14 days was allowed between arrival/allocation to test groups and the start of treatment. During this period a review of animal health was undertaken by a veterinary officer.

Histopathology

All decedent animals were necropsied completely according to GLP compliant Standard Operating Procedures (SOPs). Samples of all the tissues were preserved in 10 % Neutral Buffered Formalin (except eyes, which were preserved in Davidson's fluid, and testis/epididymides, which were initially fixed in Bouin's solution and then transferred to 70% industrial methylated spirits). In addition, samples of any macroscopically abnormal tissue, (all nodules and tissue masses) were routinely preserved, along with samples of adjacent tissues where appropriate. All tissues were embedded in paraffin wax and sections cut at 4-5 micrometers were stained with haematoxylin and eosin. For bilateral organs, sections of both the left and right organ were examined. The initial examination was under-taken by the study pathologist, the results of which were then subjected to a routine peer review by a second pathologist. The diagnoses reported represent the consensus opinions of both pathologists. All macroscopic and microscopic findings were presented by an automated data collection system and entered into Xybion, a pathology software system.

Study design

This report is based on 20 mouse carcinogenicity studies conducted at Huntingdon Life Sciences during the period 1990-2002. Information was gathered from control groups (a total 1453 male and 1453 female mice). Each control group consisted of at least 50 males and 50 females. All deaths occurring within the first 50 weeks of each study were reviewed by studying data from clinical, post-mortem and histopathological findings, and were ascribed a factor or factors contributing to death whenever possible. For the multiple causes of death, only a single dominant contributing factor was assigned to a single animal but secondary cause of death was shown as well in the table (Table 3). The day of death were recorded in each case.

Statistical analysis

Survival analyses were performed on males and females separately. Survival curves between males and females were compared. Also, comparison was made between single and multiple housed groups to assess the effects of housing numbers on aggression behaviour-associated factors. For each data set, separate Kaplan-Meier survival curves *(Kaplan & Meier, 1958)* were produced to show the survival distributions for males and females through time. The number of animal deaths for males and females were compared using a Log-rank test *(Mantle, 1966)*. Data from each data set were subsequently fitted to a Cox's proportional hazards model *(Collett, 1994)*, where gender and study were treated as factors in the model; the study was included to take into account potential differences that may exist between studies. For each data set in turn, a log-minus-log plot was produced to visually check the proportional hazards assumption. To quantify the effect of size on mortality between sexes and also between multiple and single housing, a hazard ratio statistic was obtained along with 95% confidence limits *(Armitage et al, 1999)*. All statistical analyses were performed in SAS 8.2 *(SAS Institute, 1999)*.

Results

Mortality

The percent survival of males and females are shown in Figs. 1 and 2. From 1453 mice in each sex, 101 (6.95 %) and 69 (4.75%) decedents were recorded before 50 weeks of study in males and females, respectively. There was statistical evidence to suggest that mortality rates were significantly greater for male mice (p=0.016 for up to 50 weeks study) when compared with female mice. The log-minus-log plot showed approximately parallel lines between both sexes to suggest that the proportionally hazard assumption could be assumed. The hazard ratio for gender was found to be 1.45 (1.10 males, 2.01 females) in the mice up to 50 weeks of study. Of the decedents up to week 50 of study, 26 (25.7%) males had integumentary wounds, 27 (26.7%) males exhibited neoplastic lesions and 48 (47.5%) males had other changes. In females, these figures for integumentary wounds, neoplastic, and others were 11 (15.9%), 28 (40.6%), and 30 (43.4%), respectively

Data concerning the factors contributing to death of all decedents occurring before week 50 were collected and are presented in Tables 1, 2, 3 and Figs 4, 5.

Integumentary wounds

A high proportion of the factors contributing to death were due to lesions including ulceration (7 males, 1 female), abscessation (7 males, 4 females) and pyogranulomatous inflammation (12 males, 4 females) in the skin. These lesions were seen more in males and were distributed along the head, face, neck, or lower abdomen; some of these lesions were seen from the beginning of the studies. Muscular abscessation was noted only in 2 females.

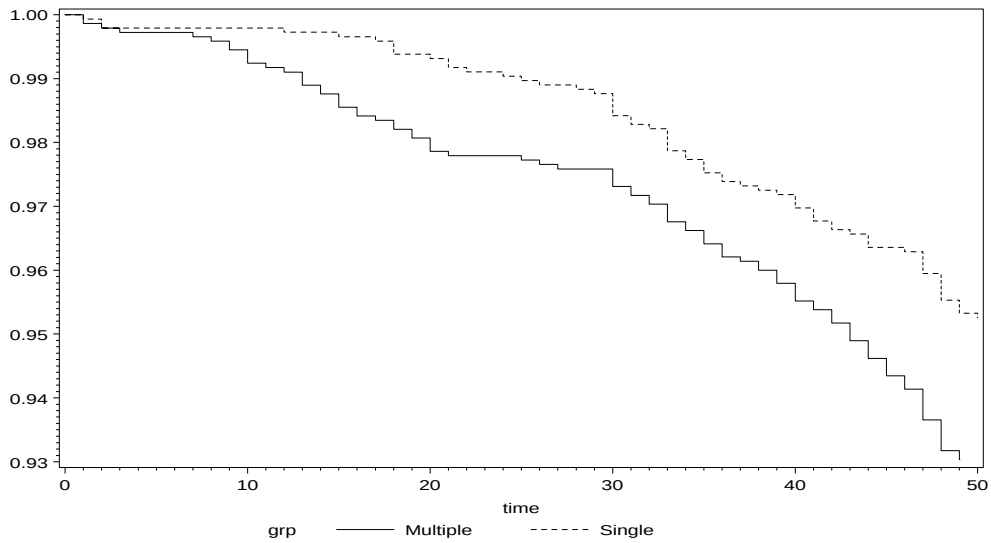

Fig. 1. Kaplan Meier survival curves for male and female CD-1mice up to week 50 of study. There was a significant difference in probability of survival between sexes in week 50 (p=0.016). The Hazard ratio was found to be 1.45 (1.10, 2.01).

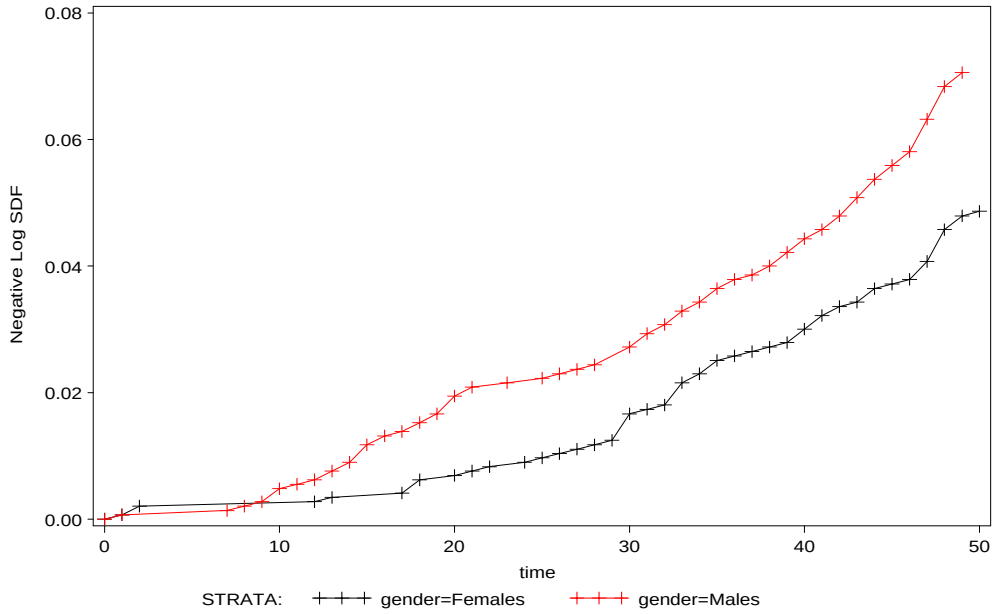

Fig. 2. Log-minus-log plot for males and females for mortality up to and including Week 50. The log-minus-log plot showed approximately parallel lines between both sexes.

Analysis of fighting-associated contributory factors to death

From the mortality analysis for fighting- associated factors contributing to death between single and multiple housed animals, there was no evidence to suggest that mortality rates were significantly greater in multiple housed animals than in single housed animals in both sexes (Tables 4, 5) (Figs. 4, 5). However, males were more likely to be affected by housing density (males, p=0.55) (females, p=0.94). In the hazard ratio analysis between single and multiple housing, males were found to be 1.32 (0.56, 3.09), whereas females were found to be 1.05 (0.28, 3.91).

Neoplastic findings

Malignant lymphoma was the most frequent cause of death. Two histiocytic sarcomas and two myeloid leukaemias were found in both sexes. A single case of bronchiolo-alveolar adenoma in the lung and a single case of hepatocellular adenoma in the male were reported but were considered to be incidental tumours. In the females, two osteosarcomas of the femur were seen. In the female mammary gland, a single case of adenocarcinoma and a single case of carcinosarcoma were observed. In addition, a single case of mammary adenocarcinoma was found together with myeloid leukaemia in a female which was found dead. Since myeloid leukaemia was more likely to cause death, this tumour is excluded from the figures. One trichoepithelioma was seen in a female decedent mouse.

Other findings

A wide spectrum of specific factors contributory to death was identified. The most frequently seen were kidney lesions, nephropathy (seen in 3 males and 6 females) and glomerulonephritis (4 males, 4 females). Nine males died from urinary tract obstruction, but this was not seen in any females. These mice died suddenly without any obvious adverse clinical signs being noted. Two females revealed haemorrhagic ovarian cysts. There were five cases of cardiomyopathy and a single case of arteritis in males. A

Table 1. Details of studies used in this survey.

Study	Route of administra-tion	Animals per cage	Number of animals		No. of sporadic deaths before 50 weeks		Type of mortalty			
							Found dead		Killed humane reasons	
			male	female	male	female	male	female	male	female
A	d	1	120	120	4	2	1	1	3	1
B	o	1	120	120	3	3	0	0	3	3
C	o	1	112	112	9	12	3	8	6	4
D	o	1	60	60	5	2	3	0	2	2
	Sub-total		412	412	21	19	7	9	14	10
E	d	2	50	50	8	4	1	2	7	2
F	d	2	50	50	5	4	5	2	0	2
G	d	2	60	60	4	2	2	1	2	1
H	d	2	50	50	4	0	2	0	2	0
I	d	2	50	50	3	1	3	1	0	0
J	d	2	50	50	7	2	4	2	3	0
K	d	2	112	112	7	7	1	4	6	3
L	d	2	50	50	2	3	0	0	2	3
M	o	2	50	50	3	5	0	3	3	2
	Sub-total		522	522	43	28	18	25	25	13
N	d	3	51	51	9	2	3	1	6	1
	Sub-total		51	51	9	2	3	1	6	1
O	d	4	72	72	8	6	6	5	2	1
P	d	4	68	68	3	3	2	2	1	1
Q	d	4	52	52	5	5	3	1	2	4
R	o	4	104	104	8	3	2	2	6	1
S	o	4	120	120	4	1	2	0	2	1
T	d	4	52	52	0	2	0	0	0	2
	Sub-total		468	468	28	20	15	10	13	10
		Total	1453	1453	101	69	43	35	58	34

total control mice, male,1453 and female, 1453; o,oral gavage; d, dietary administration

single female decedent showed liver necrosis and one male exhibited hepatic angiectasis. In some animals, trauma/fracture (3 males and 1 female) or dosing accident (in oral gavage studies) (1 male) were recorded as major factors contributing to death. Six mice of each sex were sacrificed due to poor clinical condition. A number of animals (15 males and 10 females) were found dead and did not show any significant clinical or histological evidence of cause.

Discussion

There were several types of mortality such as accidental death, death during blood collection, cannibalism in multiple housing, found dead, killed for humane reasons and killed in extremis. Predetermi-

Table 2. Profiles of factors contributory to death expressed by time of death in the male CD-1 mouse.

Time of death (weeks of study)		1 –10	11-20	21-30	31-40	41-50	Total
Number of deaths		10 (0.69) [a]	20 (1.38)	9 (0.62)	26 (1.79)	36 (2.48)	101 (6.95)
Integumentary wounds	ulceration-skin	1	1	1	2	2	7
	abscessation-skin	1	3		2	1	7
	pyogranulomatous inflam.-skin/subcutis	1	3	3	5		12
	Sub-total	3	7	4	9	3	26 (25.7%)
Neoplastic							
Lymphoid/ multicentric	malignant lymphoma	1	2	2	6	12	23
	histiocytic sarcoma					2	2
	myeloid leukaemia				1	1	2
Lungs	bronchiolo-alveolar adenoma					(1)[i]	
Liver	hepatocellular adenoma				(1)[i]		
	Sub-total	1	2	2	7	15	27 (26.7%)
Others							
Urogenital	nephropathy		1		1	1	3
	glomerulonephritis	1				3	4
	urinary tract obstruction				5	4	9
Cardiovascular	cardiomyopathy		2		1	2	5
	arteritis				1		1
Gastrointestinal	hepatic angiectasis					1	1
Others	trauma/fracture		2	1			3
	dosing accident	1					1
	poor clinical condition	1			2	3	6
	unknown	3	6	2	1	3	15
	Sub-total	6	11	3	11	17	48 (47.5%)

a, % based on total mice; total male control mice, 1453; i, incidental tumour

ned terminations, including killed for humane reasons and killed in extremis, are important to avoid or reduce autolysis, and to avoid undue suffering. Under the experimental conditions of carcinogenicity studies, it is often difficult to identify a precise cause of death for all decedent animals, as some of the intercurrent deaths are predetermined deaths, instead of death due to natural causes. The mortality curve is thus a composite of morbidity and mortality rather than a true survival curve *(Glaister, 1986)*. In general, male and female mortality patterns are different and mortality profiles were reported to be *controversial (Rae, 1999; Toseland & White, 1994; Chandra & Frith, 1992; Maita et al, 1988; Glaister, 1986; Homburger, et al, 1975)*. Urinary tract obstruction occurred occasionally only in male mice and played an important role in a high proportion of male decedents, which is consistent with previous reports *(Percy & Barthold, 1993; Bendle & Carlton, 1986)*.

It has been proposed that various factors could be involved in behaviour-associated mice disease such as barbering amongst the mates, nasal alopecia due to mechanical abrasion, trichotillomania *(Thorn-*

Table 3. Profiles of factors contributory to death expressed by time of death in the female CD-1 mouse.

Time of death (weeks of study)		1 –10	11-20	21-30	31-40	41-50	Total
Number of deaths		3 (0.21) [a]	7 (0.48)	13 (0.84)	21 (1.45)	25 (1.72)	69 (4.75)
Integumentary wounds	ulceration-skin					1	1
	abscessation-skin			2	1	1	4
	pyogranulomatous inflam.-skin/subcutis		2	1		1	4
	abscessation-muscle		1		1		2
	Sub-total	0	3	3	2	3	11 (15.9%)
Neoplatic							
Lymphoid/ multicentric	malignant lymphoma			5	9	5	19
	histiocytic sarcoma					2	2
	myeloid leukaemia				1	1	2
Bone	osteosarcoma			1		1	2
Mammary gland	adenocarcinoma				1(1)[i]		1
	carcinosarcoma				1		1
Skin	trichoepithelioma			1			1
	Sub-total	0	0	7	12	9	28 (40.6%)
Others							
Urogenital	nephropathy			1	2	3	6
	glomerulonephritis		1	1		2	4
	haemorrhagic ovarian cysts					2	2
Gastrointestinal	liver necrosis					1	1
Others	trauma/fracture		1				1
	poor clinical condition	1		1	1	3	6
	unknown	2	2		4	2	10
	Sub-total	3	4	3	7	13	30 (43.4%)

a, % based on total mice; total female control mice, 1453; i, incidental tumour

burg et al, 1973), and also fighting. Also it is possible that single housed animals have self-induced skin lesions *(Percy & Barthold, 1993; Litterst, 1974)*, therefore fighting-associated wounds do not necessarily mean that these lesions resulted from fighting. Also multiple housing does not exclusively mean that animals have fighting-associated skin wounds. As only major cause of death was determined in this study, it could be argued that there were probably some animals that did not included as a fighting-associated factors to contributing death that died from other major causes of death rather than fighting-associated death but with minor fighting-related skin lesions. It is possible that some animals with fighting-associated skin findings but still alive up to 50 weeks of study. As this analysis was confined to the decedents due to the possible fighting-associated wounds, those animals were excluded from the figures.

There were findings such as ulceration, abscessation and pyogranulomatous inflammation of the skin, subcutis, or muscle. Fighting has been pointed out in multiple housed animals as a causative factors, *(Faccini et al, 1990; Glaister, 1986);* aggressive

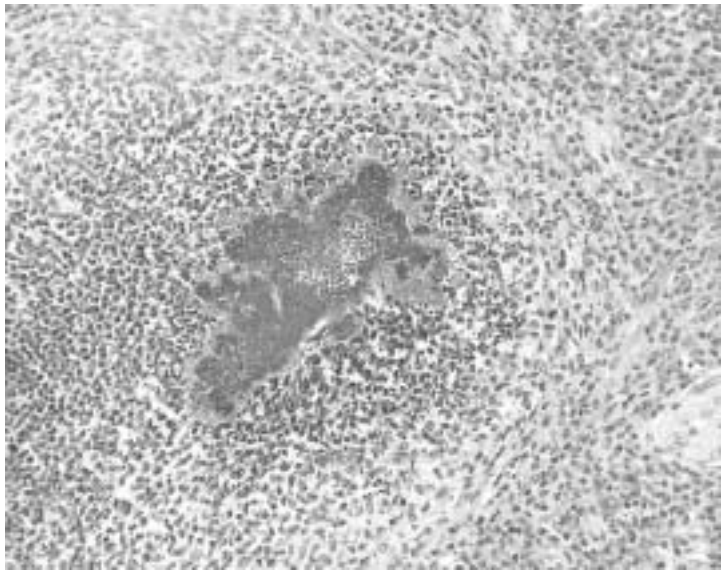

Fig. 3. Pyogranulomatous inflammation of the subcutis occurred in male CD-1 mice. Note chronic active focal suppurative lesions. x200, H&E

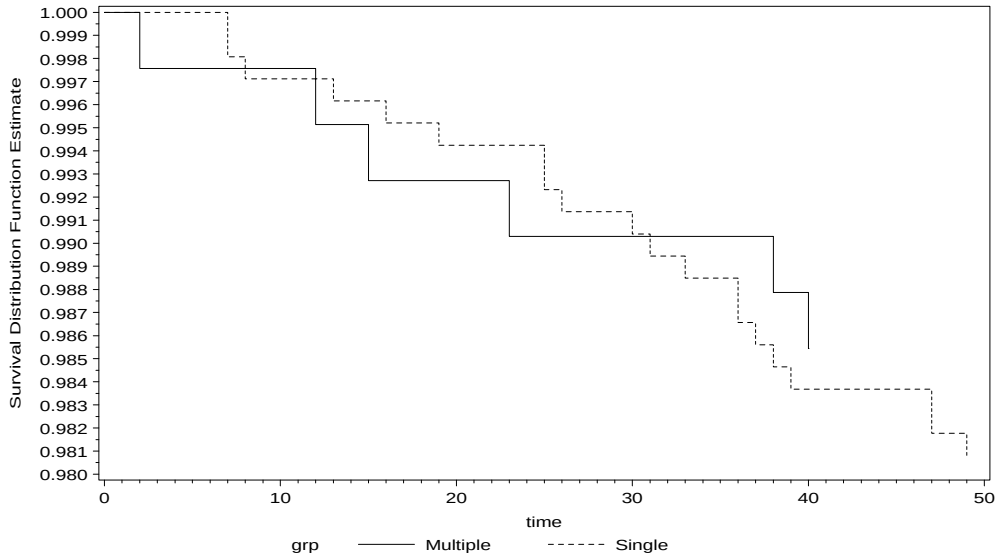

Fig. 4. Kaplan Meier survival curves for single and multiple housed animals for fighting-associated factors contributing to death in the male CD-1 mice up to week 50 of study. There was no significant difference between single and multiple housing.

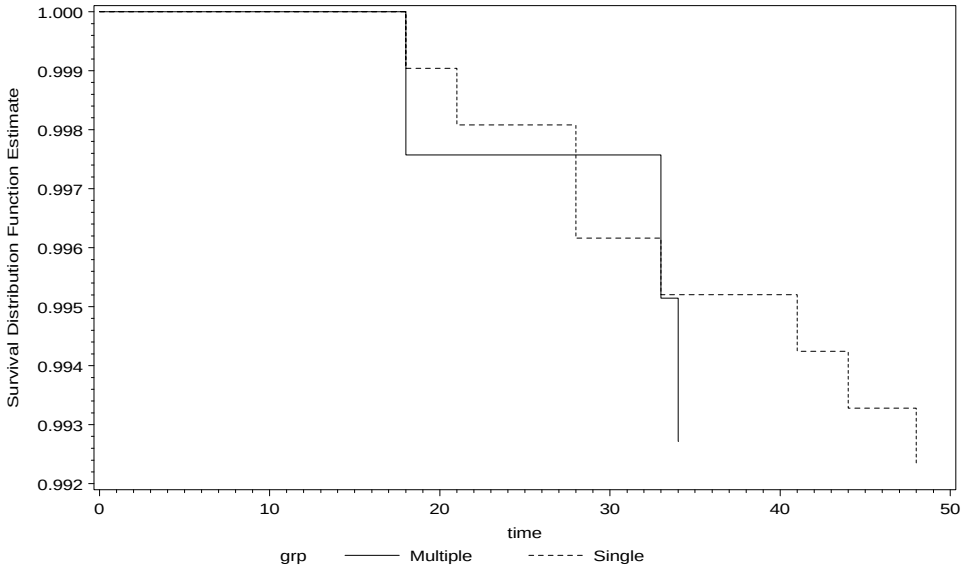

Fig. 5. Kaplan Meier survival curves for single and multiple housed animals for fighting-associated factors contributing to death in the female CD-1 mice up to week 50 of study. There was no significant difference between single housing and multiple housing.

behaviour amongst cage mates produces abrasions or ulcerative wounds. Traumatic wounds sometimes lead to pyogranulomatous inflammation, which is considered to be a sporadic secondary infection, such as Staphylococcal or Staphylococcal abscesses or Corynebacterium infection, which is not necessarily contagious. Wounds range from superficial healing scabs to deep-rooted purulent inflammation, often with abscess formation and involvement of deep structures in muscle *(Percy & Barthold, 1993)*.

It has been shown that aggression of male mice within groups is induced by the social conflict *(Fano et al, 2001; Brain, 1997; Brain, 1990; Benton & Brain, 1981)*. Social stress of mice impacts on immunosuppressive glucocorticoids, lowered antibody production, enhanced tumour growth, higher blood pressure and high incidence of kidney disease *(Cacho et al, 2003; Fano et al, 2001; Brayton & Brain, 1974)*. Those stressors causing physiological alteration may be associated with latent infections *(Avitsur et al, 2001)*. Fighting in male mouse amongst the mates leading to wounded animals may have effects on physiological research parameters *(Kaliste-Korhonen & Eskola, 2000)*.

In conclusion, there was no greater difference in incidence of fighting-associated factors contributory to death between single housing and multiple housing of between 2 to 4 per cage in both sexes, but it was more likely to be in the males. From the hazard ratio analysis, it could be suggested that the risk of death increased in multiple housing compared to single housing but it tended to be more probable in male animals than in females. It is also suggested that fighting behaviour is partly, but not exclusively, associated with higher male mortalities, this could not be ascribed as the single cause.

Acknowledgments

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Table 4. Possible influence of number of mice per cage on fighting-associated factors contributing to death in male CD-1 mice.

Number of animals per cage	1	2	3	4
		522	51	468
Lesions Total no. of mice	**412**			
		1041[a]		
ulceration-skin		5 (0.96)		1 (0.21)
	1 (0.24)	**6 (0.58)**		
abscess-skin		2 (0.38)		4 (0.85)
	1 (0.24)	**6 (0.58)**		
Pyogranulomatous inflam.-skin/subcutis		3 (0.57)	1 (2.00)	4 (0.85)
	4 (0.97)	**8 (0.77)**		
Total		10 (1.92)	1 (2.00)	9 (1.92)
	6 (1.46)	**20 (1.91)**		
***P* values**		*p*=0.55		
Hazard ratio (confidential limits)		1.32 (0.56, 3.09).		

a, combined, multiple housing; b, % based on total mice

Table 5. Possible influence of number of mice per cage on fighting-associated factors contributing to death in female CD-1 mice.

Number of animals per cage	1	2	3	4
		522	51	468
Lesions Total no. of mice	**412**			
		1041[a]		
ulceration-skin				1 (0.21)
		1 (0.01)		
abscess-skin		1 (0.19)		2 (0.43)
	1 (0.24)	**3 (0.29)**		
pyogranulomatous inflam.-skin/subcutis		2 (0.38)		2 (0.43)
		4 (0.38)		
abscess-muscle				
	2 (0.49)			
Total		3 (0.57)		5 (1.07)
	3 (0.73)	**8 (0.77)**		
***P* values**		*p*=0.94		
Hazard ratio (confidential limits)		1.05 (0.28, 3.91).		

a, combined, multiple housing; b, % based on total mice

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