MODULATION OF AGGRESSION IN MALE MICE: INFLUENCE OF CAGE CLEANING REGIME AND SCENT MARKS

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Abstract *Animal Welfare 2000, 9: 281-295*

Group housing of male laboratory mice often leads to welfare problems due to aggressive behaviour. From a welfare perspective, individual housing is not a preferred solution to these problems – and so we sought other ways of reducing aggression between male mice. Aggression peaks after disturbances such as cage cleaning. Transfer of olfactory cues during cage cleaning procedures has been repeatedly proposed as a means of reducing these peaks in aggression. In this study, the aggression-modulating properties of olfactory cues were studied by investigating the effects of their source and distribution on aggression after cage cleaning in groups of male BALB/c mice. The physiological effects of aggression on individuals within a group were also monitored.

Our results indicated that neither kinship nor distribution of urine marks affected aggression. Olfactory cues from nesting and bedding material, however, affected aggression to a marked degree: transfer of nesting material reduced aggression significantly, while transfer of sawdust containing urine and faeces seemed to intensify aggression. None of the physiological data revealed any differences between dominant and subordinate animals, nor any correlations with aggressiveness, except that dominant animals gained weight more rapidly than subordinate ones. We conclude that the transfer of nesting material will reduce aggression, or at least slow down its development, and thus aid the reduction of social tension due to cage cleaning.

Keywords: *aggression, animal welfare, kinship, mice, nesting material, olfactory cues*

Introduction
Social versus individual housing
In their natural habitat, male mice will usually form territories in which unfamiliar males are not tolerated, while familiar subordinate males are tolerated to a certain extent (Crowcroft 1966; Mackintosh 1970, 1973). Laboratory mice are often housed in single-sex groups of 3–10 animals, and form – mainly despotic – dominance relationships (Poole & Morgan 1973,

1976; Mondragón *et al* 1987). Evidence exists that tolerance of subordinate males is usually highest in high density groups, preventing dominant mice from forming individual territories. This phenomenon is known as the crowding effect (Van Oortmerssen 1971; Busser *et al* 1974; Hurst *et al* 1993). In many cases, depending on strain and age and after an initial period of fighting to establish the hierarchy, the animals live in harmonious social groups (Bisazza 1981; Brain & Parmigiani 1990). In other cases, however, frequent fighting may occur. Aggression may reach such high levels that individual animals are wounded badly (Van Oortmerssen 1971; Bisazza 1981; Van Loo unpublished data). In these cases, an excessive form of aggression has arisen in which some animals will repeatedly attack certain individuals causing severe stress and physical injuries (Brain 1990). To prevent further deleterious effects, these mice are housed individually in most laboratory animal facilities. Individual housing, however, has frequently been shown to be stressful for mice. Detrimental effects of individual housing include both behavioural and physiological abnormalities usually referred to as 'isolation stress' or 'isolation syndrome' (eg Chance & Mackintosh [1962]; Ader & Friedman [1964]; Hatch *et al* [1965]; Barrett & Stockman [1966]; Gärtner [1968]; Baer [1971]; Brain [1975]; Haseman *et al* [1994]). Although 'isolation' is a term often used in this context, it is worth noting that in the majority of cases 'individual housing' – with visual, olfactory and/or acoustic information about conspecifics – is the more appropriate term. In a recent study examining the preference of subordinate mice for the presence of their dominant cage mate, we found that subordinate mice preferred to dwell in close proximity to the dominant animal rather than alone (Van Loo & Baumans 1998). Similar results have been reported in rats (Gärtner 1968). These results indicate that group housing should be preferred over individual housing, even if this may lead to aggression between the mice. Group housing is also recommended by the 'Berlin Report' (O'Donoghue 1993), by the Council of Europe (1997) and by the Rodent Refinement Working Party (1998). The results and recommendations mentioned above imply that other solutions should be sought to prevent the development of excessive aggression in group-housed male mice.

Aggression after cage cleaning

It is known that aggression between male mice peaks after disturbances (Rodent Refinement Working Party 1998). A common and rather drastic disturbance that all groups of laboratory mice undergo is cage cleaning. Cage cleaning is a necessary routine procedure in laboratory animal facilities which leads to a multitude of novel environmental stimuli that may temporarily disrupt the social hierarchy of the animals in the cage (Rodent Refinement Working Party 1998). Indeed, studies with wild mice reveal that mice depend to a large extent on the use of olfactory stimulation for their social communication. They mark their territory with urine and other glandular substances and may thus communicate with other males to recognize one another and advertise social status (Ropartz 1977; Brown 1985; Hurst 1990, 1993; Hurst *et al* 1993). Both source and distribution of olfactory cues are of importance in this respect (Bishop & Chevins 1987; Hurst *et al* 1993). Furthermore, evidence exists that, through olfactory cues, kin recognition can affect social interactions and, more specifically, aggression between male mice (Kareem & Barnard 1982, 1986; Kareem 1983).

The controversy

To inhibit aggression as a consequence of cage cleaning, several procedures have been practised, all of which are based on the assumption that olfactory cues are of major importance. The most widely used methods are the transfer of a handful of dirty sawdust to the clean cage, and the partial or complete replacement of sawdust in the dirty cage. These,

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or similar, methods of cage cleaning are recommended by O'Donoghue (1993). However, controversy exists, as to whether transfer of these olfactory cues induces a decrease or increase in aggression. Even within the same institute, experiences differ. McGregor *et al* (1991) observed aggression to be lower when cages were partially cleaned; whereas – although at a later stage – Gray and Hurst (1995) showed that complete removal of all olfactory cues induced the least aggression, while partial cleaning of the cage elicited most aggression. Evidence in favour of the transfer of olfactory cues through sawdust is mainly based on the personal experiences of animal caretakers and researchers. Although these experiences are extremely valuable, the evidence is incomplete and has not been tested experimentally. For example, there are no suggestions as to how or how much sawdust should be transferred, or whether it should contain urine and faeces. The Rodent Refinement Working Party (1998) states that this is clearly an area where more research is needed.

In an attempt to clarify the controversies that exist with respect to cage cleaning and aggression, and possibly to modify existing recommendations on cage cleaning, we studied the effect of different cage cleaning regimes on aggression between male mice by transferring olfactory cues from various origins. In addition, we studied the effect of different distributions of urine marks on aggression. To investigate any effects of kin recognition on aggression, the study included groups consisting of full siblings as well as groups consisting of male mice mixed at weaning.

Animals and methods

The protocol for this experiment was peer-reviewed for scientific and ethical value, and approved by the Animal Experiments Committee of Utrecht University. Decapitation is a legal form of euthanasia for small rodents in The Netherlands, and its use in this study was considered to be methodologically justified (see, *Physiology*).

Animals and husbandry

Thirty-six male mice, *Mus musculus*, of the BALB/cAnNCrlBR strain were used. The mice were housed in groups of three in 375cm² wire-topped Macrolon® type II cages (Techniplast, Milan, Italy) provided with 50g of sawdust (Lignocel® ¾; Rettenmaier & Söhne, Ellwangen-Holzmühle, Germany) and three Kleenex tissues (Kimberly-Clark Corporation®, Ede, The Netherlands) as nesting material. Tap water and food pellets (RMH-B®, Hope Farms, Woerden, The Netherlands) were provided *ad libitum*. The animal room had a controlled photoperiod (lights on between 0700h and 01900h), temperature (23–24 °C), relative humidity (60 ± 5%), and ventilation (18–20 air changes h⁻¹).

Six groups consisted of mice that were full siblings, and six groups consisted of mixed weanlings (age at weaning: 3 weeks). At the start of the experiment, the mice were 4 weeks old. The animals were individually marked on the tail with a black waterproof marker. The mark was renewed weekly.

Procedure and data collection I: effect of cage cleaning

Cages were cleaned weekly in one of the following three ways:

i)   Clean: clean cage with clean sawdust and clean nesting material.
ii) Sawdust: as in i), but an additional 5–10 g sawdust containing both urine and faeces was transferred from the dirty cage.
iii) Nest: clean cage with clean sawdust, but nesting material was transferred from the dirty cage.

In total, each cage was cleaned nine times. Every group was alternately subjected to each of the three cleaning procedures, in a previously established randomized block procedure. In this way, each group was subjected to each of the cage cleaning procedures three times in a period of 9 weeks. Prior to cage cleaning, food and water were weighed and refreshed and animals were weighed and checked for wounds. Immediately after cage cleaning, the behaviour of the animals was recorded on videotape (Panasonic AG-6024-E; Matsushita Electric Industrial Co Ltd, Osaka, Japan) for a period of 1h. Due to restrictions in the experimental set-up, the number of cages cleaned and videotaped simultaneously was limited to four. To minimize the influence of time of day on behaviour, the order of cage cleaning and recording was altered weekly according to a previously established randomization procedure using SPSS for MS Windows, Release 6.1 (SPSS Inc, Chicago, USA).

Procedure and data collection II: effect of distribution of urine marks

At the age of 12 weeks, one of the subordinate animals of each group was removed and euthanased (for details see, *Physiology*). The remaining 12 pairs were left undisturbed for 3 weeks (except for routine cleaning once weekly) to enable dominance relationships to be re-established. On the day of testing, the mice of each couple were placed in Macrolon® type II cages separated from each other by a wire mesh. The floors of the cages were covered with plasticized filter paper (Benchkote Plus Reel®; Whatman Scientific Ltd, Maidstone, UK). After the animals had roamed freely in the cage for 30min, they were kept separated in Macrolon® type I (Techniplast, Milan, Italy) cages for 5min. The filter papers were examined with UV lighting and urine drops were marked and counted. The animals of each pair were then allowed to interact for 10min in a test arena with a floor covered with one of the following filter papers:

i)   Both: filter paper containing the urine of both the dominant and the subordinate animal.
ii)   Dominant: filter paper containing the urine of the dominant animal only.
iii)   Subordinate: filter paper containing the urine of the subordinate animal only.
iv)   Blank: filter paper containing no urine.

Each filter paper was covered with a thin layer of clean sawdust.

During these 10min interactions, the behaviour of the animals was videotaped. The animals were then returned to their home cage. This procedure was repeated daily for 4 days according to a randomized block design.

Behavioural analysis

Latency until first agonistic encounter, frequency and duration of agonistic encounters and the number of escalated encounters (fights) were scored from videotape. Behaviours interpreted as agonistic included several offensive behaviours such as vigorous sniffing of head, tail or genitals of the opponent, tail rattling, chasing, biting and fighting, and several defensive behaviours such as adopting upright and sideways defensive postures, fleeing and active defence. The identities of the males involved in an encounter were also noted. A male was said to initiate an agonistic encounter when it showed the first agonistic behaviour in an interaction. A male was said to win an encounter when its opponent showed submissive behaviour terminating the agonistic encounter.

Dominant, subdominant and subordinate status were allocated to animals that initiated and won, respectively, most, intermediate, and fewest numbers of encounters.

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Physiology

At the age of 12 weeks (one subordinate animal group^{-1}) or 16 weeks (the two remaining animals group^{-1}), the animals were euthanased between 1000h and 1100h by decapitation. This method was chosen to enable blood collection without contamination by anaesthetic compounds. Trunk blood was collected in ice-cooled 1.5ml Eppendorf® reaction tubes (Netheler-Hinz-GmbH, Hamburg, Germany) containing 50iu heparin ml^{-1} blood. Testes were removed and weighed; the adrenals were removed, fixed in buffered formaldehyde (4%) and weighed.

Blood was centrifuged (3000rpm for 25min at 20°C) and plasma stored at -20°C until assayed. Testosterone concentration was measured using a solid phase ^{125}I radioimmuno-assay (CAC® Total Testosterone TKTT; Diagnostic Products Corporation, Los Angeles, USA).

Adrenals were processed through increasing concentrations of ethanol, cleared in xylene and infiltrated with liquid paraffin. They were then embedded in paraffin and sliced in 3μm sections. Sections were stained with Haematoxylin and Eosin (Merck KgaA, Darmstadt, Germany). For each mouse, the size of the adrenal cortex was quantified using an automatic image analyser (IBAS 2000®; Kontron, Munich, Germany).

Statistical analyses

All behavioural data (with the exception of escalated fights) were transformed logarithmically in order to better conform to a normal distribution. Behavioural data, as well as data on body weight, and food and water intake were analysed using a multivariate analysis of variance for repeated measures with multiple comparisons. The Bonferroni correction was applied where necessary. Data on escalated fights were analysed using the non-parametric Friedman test.

All physiological data (food and water intake, body and organ weights, testosterone levels and size of adrenal cortex) were correlated to aggressiveness using the Spearman rank order correlation test. The comparisons between post-mortem data for dominant and subordinate mice did not include animals that were euthanased at 12 weeks of age. All the statistical tests were carried out with the aid of SPSS for MS Windows, Release 6.1 (SPSS Inc, Chicago, USA).

The level of individual aggressiveness was used to identify dominant, subdominant and subordinate animals within each group (Figure 1). One group (group 7) showed hardly any aggression in either behavioural test. Group 3 did not show any aggression in the 'effect of urine marks' test (Figure 1, bottom). As a result, the hierarchies in these groups could not be reliably established. Consequently, when comparisons were made between dominant, subdominant and subordinate mice, these two groups were omitted from the further analyses.

Results

No statistically significant differences were found between siblings and mixed weanlings for any of the behavioural or physiological results (Table 1). Further analyses were thus carried out without taking this factor into account.

Effects of differential cage cleaning

The effects of differential cage cleaning on latency until first agonistic encounter, frequency and (mean) duration of agonistic encounters are presented in Figure 2.
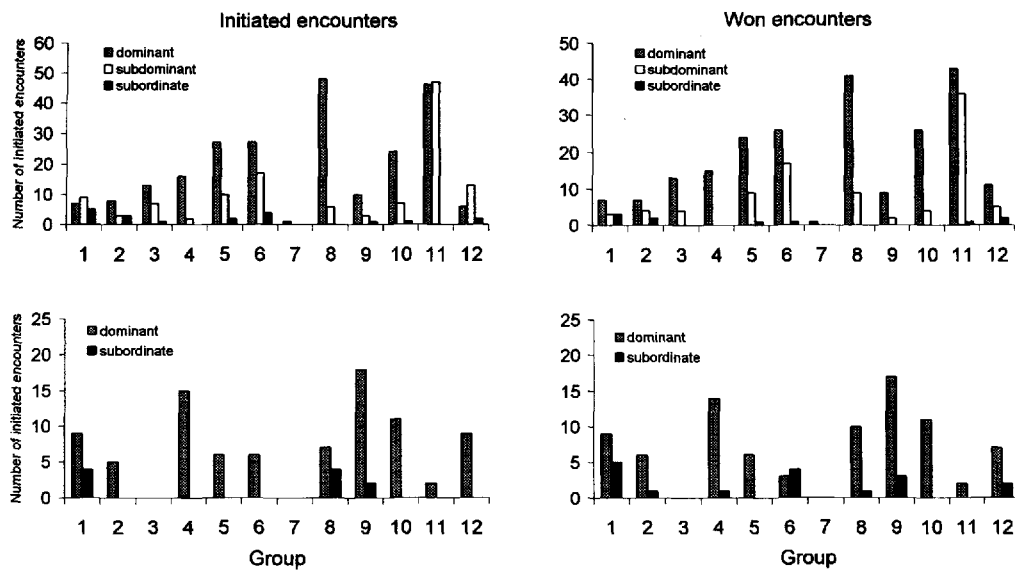
Figure 1   Cumulative number of encounters initiated (left) and won (right) per group for each mouse after cage cleaning (top) and in the effect of urine marks experiment (bottom). Groups 1–6 are mixed weanlings; groups 7–12 are full siblings.

Table 1   Mean (± SEM) behavioural and physiological responses of siblings vs mixed weanlings. Behavioural data are grouped for time and treatment, physiological data are grouped for hierarchy.

		Siblings	Mixed weanlings
Agonistic behaviour	Latency (s)	1657.1 ± 414.2	1718.3 ± 167.3
	Frequency (h^{-1})	3.4 ± 1.3	2.8 ± 0.6
	Duration (s h^{-1})	40.4 ± 15.7	31.7 ± 6.4
	Mean encounter duration (s)	7.2 ± 1.7	7.1 ± 0.5
Physiology	Testes (mg)	189.3 ± 3.0	183.4 ± 8.3
	Adrenal cortex (μm)	0.29 ± 0.009	0.31 ± 0.008
	Testosterone (ng ml^{-1})	6.8 ± 2.2	8.6 ± 2.8

Latency until the first agonistic encounter was significantly influenced by the type of cage cleaning ($P < 0.05$, Figure 2, top left). Multiple comparisons indicated that this was mainly due to a difference between 'nest' and 'sawdust' (Bonferroni $\alpha = 0.017$, $P_{sawdust-nest} = 0.026$). Type of cage cleaning also had a significant effect on frequency of agonistic encounters in the hour following cage cleaning ($P < 0.01$; Figure 2, bottom left). Multiple comparisons indicated that this was caused mainly by the smaller number of agonistic encounters when the nest is transferred to the clean cage (Bonferroni $\alpha = 0.017$, $P_{clean-nest} = 0.007$, $P_{sawdust-nest} = 0.008$). The duration of agonistic encounters in the first hour after cage cleaning was also significantly influenced by the type of cage cleaning ($P < 0.01$; Figure 2, top right). Multiple comparisons indicated that this was caused mainly by a difference between 'nest' and 'sawdust' and – to a lesser extent – by a difference between 'clean' and 'nest' (Bonferroni $\alpha = 0.017$, $P_{sawdust-nest} = 0.006$, $P_{clean-nest} = 0.032$). The mean duration encounter^{-1} (Figure 2,
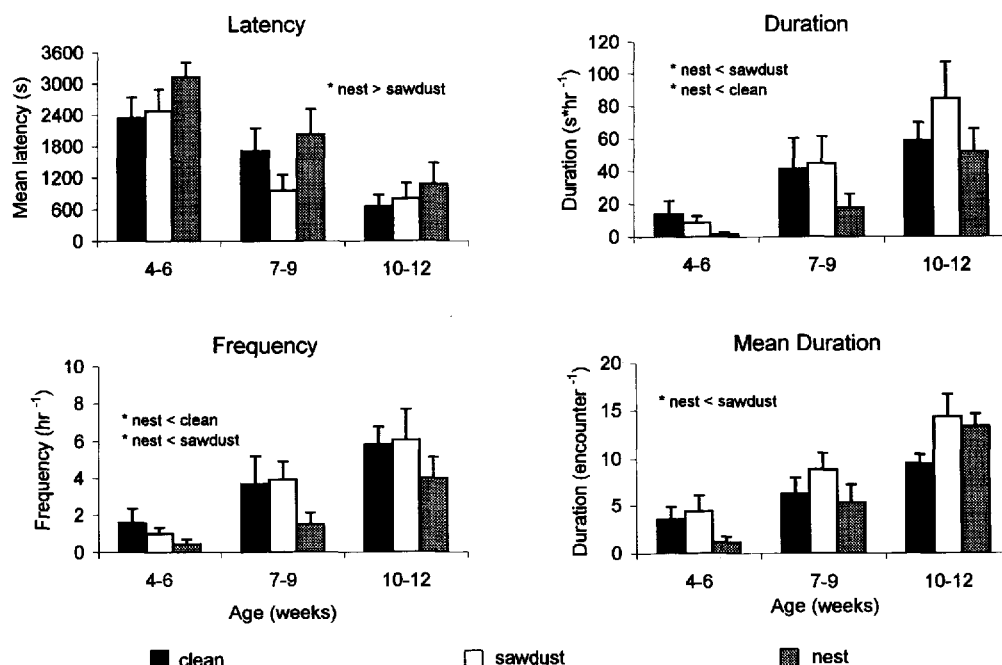
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Figure 2   Mean (± SEM) latency until first agonistic encounter (top left), frequency (bottom left), duration (top right) and mean duration encounter^{-1} (bottom right) of agonistic encounters during the first hour after cage cleaning for three different cleaning procedures and three age categories of mice. Significant differences ($P < 0.05$) between cleaning procedures are indicated by *.

bottom right) was similarly influenced by the type of cage cleaning, as is frequency and duration ($P < 0.05$), with the difference between 'nest' and 'sawdust' as the main contrast effect (Bonferroni $\alpha = 0.017$, $P_{sawdust-nest} = 0.012$).

Figure 2 also shows that both frequency and duration of agonistic encounters increased, and latency until first agonistic encounter decreased with age ($P < 0.001$). Older mice not only show *more* agonistic encounters, the encounters also last longer with increasing age ($P < 0.001$). All time effects can be explained by a linear effect.

Aggressive behaviour that escalated into fights did not occur very frequently (Figure 3). In total, 38 fights were observed in 108 hours of observation. The majority of fights occurred when dirty sawdust had been transferred to the clean cage (23 out of 38 times).

Effects of urine marks

Both dominant and subordinate mice mainly urinated in the half of the cage which was near the divider – and thus nearest to their cage mate ($P < 0.001$; Table 2). The number and position of urine spots did not differ between dominant and subordinate mice.

Table 2   **Mean (± SEM) number of urine spots at the back of the cage or near the divider for dominant and subordinate mice.**

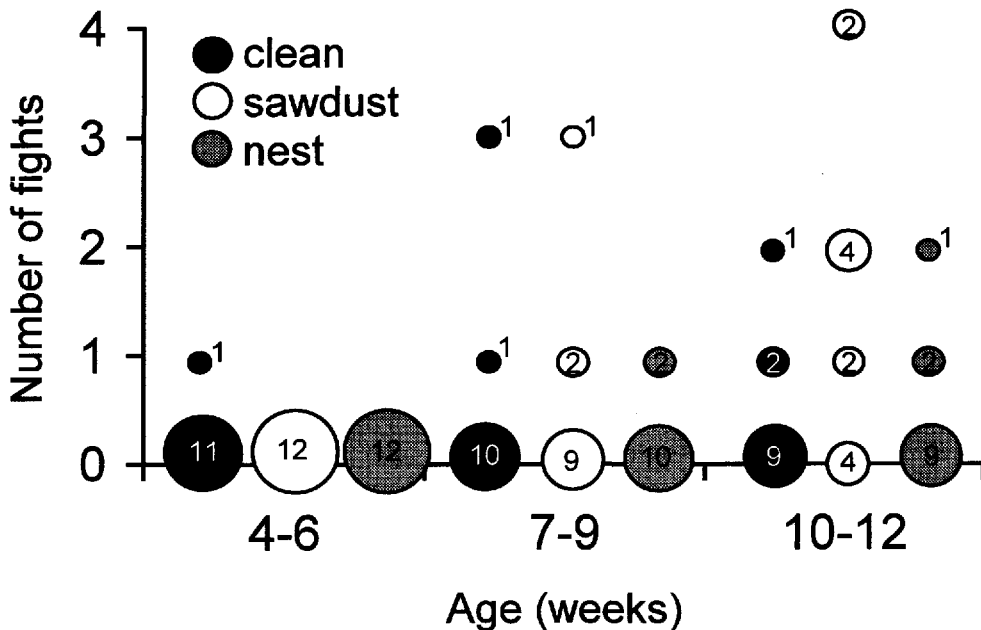|	Dominant		Subordinate	
Day	Back	Divider	Back	Divider
1	18.8 ± 4.1	51.8 ± 6.7	21.2 ± 2.8	38.9 ± 6.3
2	13.8 ± 2.9	48.2 ± 4.9	15.2 ± 3.8	45.0 ± 6.8
3	18.0 ± 3.8	48.5 ± 7.5	20.9 ± 3.1	48.7 ± 6.6
4	18.8 ± 3.6	46.6 ± 5.1	16.9 ± 3.0	43.0 ± 5.2

Figure 3   **Number of fights during the first hour after cage cleaning for three different cleaning procedures and three age categories. The sizes of the circles and their numbers correspond to the number of groups involved.**

No differences were found in frequency or (mean) duration of agonistic encounters, nor in latency until first agonistic encounter when behaviour of mice was compared with both, either or none of their urine marks present in the test arena.

Physiology and histology

The animals that were classified as dominant at the end of part I of the experiment, differed significantly in weight gain from those that were classified as subdominant or subordinate ($P < 0.001$; Figure 4): the dominant mice were initially the lightest mice, and gained weight more rapidly.
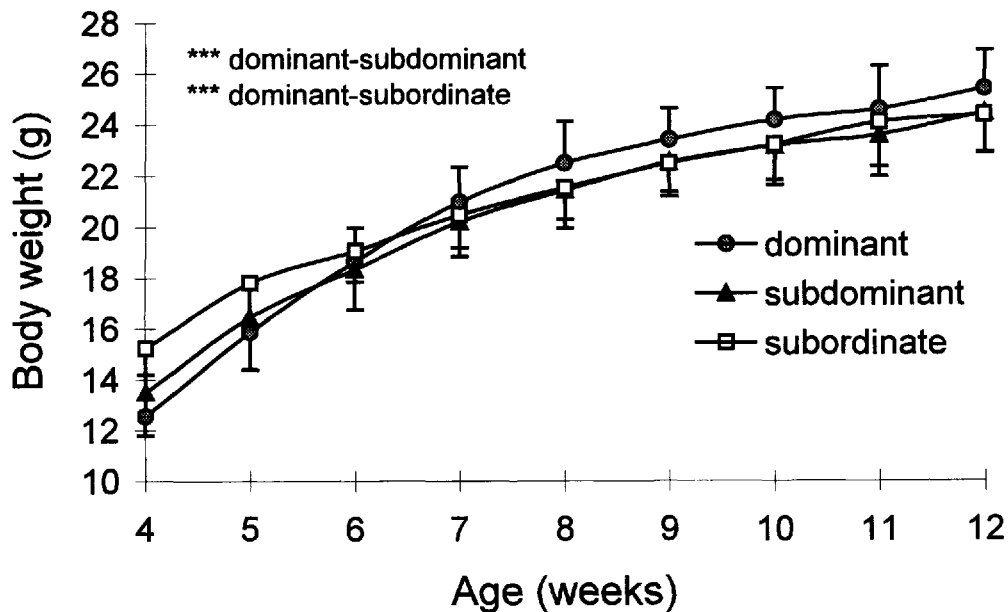
Figure 4 Mean (± SEM) body weight of dominant (n = 12), subdominant (n = 12) and subordinate mice (n = 12) at 4–12 weeks of age. Significant ($P <$ 0.001) differences in weight gain are indicated by ***.

Testes weight, testosterone levels, and size of the adrenal cortex did not differ significantly between dominant and subordinate mice, nor were there significant correlations between these measures and aggression (Table 3). Weight of the adrenals had to be discarded from the analysis due to a technical failure during weighing.

Table 3 Physiological variables (mean ± SEM) of dominant and subordinate mice and their correlation (r_s) with aggression. (ns – not significant.)

	Dominant	Subordinate	Correlation with aggression
Testes (mg)	187.9 ± 4.0	190.3 ± 3.5	$r_s = -0.1396$, ns
Adrenal cortex (µm)	305.5 ± 10.9	298.1 ± 7.6	$r_s = 0.0915$, ns
Testosterone (ng ml^{-1}) (median)	12.5 ± 3.3 (1.14)	5.4 ± 2.7 (1.24)	$r_s = 0.0519$,ns

Discussion
Kinship
Neither behavioural nor physiological data yielded any difference between groups consisting of full siblings and groups consisting of mice mixed at weaning. Male mice reach puberty at 5–7 weeks of age, and are not sexually mature until the age of 8–10 weeks (Baumans 1999). At the age of weaning (ie 3 weeks), the mice in this experiment were still in the pre-adolescent phase and mixed weanlings thus became familiarized with one other before maturation. Profound effects of familiarity on social interactions and aggression between mice have been shown repeatedly (Lagerspetz & Sandnabba 1982; Kareem 1983; Winslow & Miczek 1984; Hurst 1990, 1993). Although kinship has also been shown to affect social interactions between male mice (Kareem 1983; Kareem & Barnard 1986), the degree of

familiarity in the present experiment may have concealed any effects of relatedness. Indeed, Kareem and Barnard (1982) found that differences between non-siblings, half-siblings and full siblings disappeared when animals had prior experience with one another. Another explanation for the lack of differences between siblings and non-siblings in the present experiment could be that mice of the BALB/c strain are not able to discriminate between kin and non-kin. Studies demonstrating the ability of mice to discriminate between kin and non-kin have all been performed with either outbred or random bred strains (Hayashi & Kimura 1983; Kareem 1983; Kareem & Barnard 1986). Recently, some evidence has become available that in inbred strains, such as the BALB/c strain, the urinary component responsible for kin recognition is similar between families, making a distinction between kin and non-kin very difficult (Nevison *et al* 2000).

Physiology and histology

In this study, several physiological parameters that are known to be influenced by aggression, hierarchy or social stress were measured and correlated to the level of aggression. Post-mortem data on testes weight, adrenal cortex size and testosterone level, however, did not reveal any significant differences between subordinate and dominant mice, nor did they correlate significantly with aggression. It is worth noting that the mean testosterone level of dominant mice was higher than the mean testosterone level of subordinate mice. This is in accordance with Bishop and Chevins (1988) who also found higher, though not significant, levels of testosterone in dominant mice. Although a circadian variation in testosterone release has been shown in BALB/c mice, testosterone is known to be emitted in a pulsatile pattern (Lucas & Eleftheriou 1980). This may account for the large variation in measurements within the group of mice studied here, thus obscuring any possible differences between dominant and subordinate mice. Testes weight, on the other hand, is not susceptible to large experimental variation, so any significant difference between dominant and subordinate mice should have been revealed. Testes weight in both mice and rats has been reported not to differ between dominant and subordinate animals (Bishop & Chevins 1988; Dijkstra *et al* 1992). Others, however, have found that testes of dominant mice are heavier than those of subordinate mice (Brain & Benton 1983). Adrenal gland measurements are often used in studies involving social stress. Once again, the results are inconclusive. Some studies report that dominant mice have lighter adrenals, whereas others find no differences between dominant and subordinate mice (Bishop & Chevins [1988]; for a review, see Brain & Benton [1983]).

During this experiment, body weight was measured. Mice that were classified as dominant on the basis of their behaviour were initially slightly lighter than the subordinates, but gained significantly more weight during the course of the experiment. Jeppesen and Hansen (1985) found similar results. Mainardi *et al* (1977) proposed that lightweight individuals have a slightly better chance of becoming dominant in a social situation, while Bartos and Brain (1994) provided evidence that, after the social hierarchy stabilizes, dominant mice weigh more than subordinate ones.

Behaviour after cage cleaning

Agonistic behaviour differed substantially between groups throughout the experiment (Figure 1). Animals that showed higher levels of agonistic behaviour in the first weeks of the experiment continued to do so towards the end of the experiment. Several authors have proposed that aggression and social behaviour are part of behavioural strategies that may differ between strains and individuals (Bisazza 1982; Benus *et al* 1990; Sluyter *et al* 1995).

Despite this large variability in agonistic behaviour between groups, a significant effect of cage cleaning on agonistic behaviour was found. Animals whose cages were cleaned with transfer of nesting material showed lower levels of agonistic behaviour and higher latencies to first agonistic encounters than those whose cages were cleaned either completely or with transfer of sawdust (Figure 2). This effect was particularly clear when cages cleaned with transfer of sawdust were compared to cages cleaned with transfer of nesting material. Although escalations in aggression did not occur very often (Figure 3), most fights took place when dirty sawdust was transferred. This is in accordance with Gray and Hurst (1995) who found that if increasing amounts of material (whether sawdust, a marking block or the cage itself) remained soiled during cleaning, aggression after cage cleaning was higher. The latter is, however, not in accordance with the profound effects of transfer of nesting material compared to complete cleaning in the present study.

The origin of olfactory cues

As already mentioned (see, *Introduction*), olfactory communication between male mice depends for a large part on urine marks (Ropartz 1977; Brown 1985; Hurst 1990, 1993; Hurst *et al* 1993). Mice, however, have a large number of other, glandular sources of secretions, such as salivary glands, plantar glands and the preputial gland. The secretions from these glands are especially important in controlling sexual and aggressive behaviours (Brown 1985; Rodent Refinement Working Party 1998). Plantar glands are used for recognition and toleration of individuals or colony members (Brown 1985). Although some contradictory results have been found, the overall consensus appears to be that the urine of male mice has aggression-eliciting potencies (Mugford 1972; Stoddart 1980; Brown 1985). On the other hand, evidence has been provided for the existence of aggression-inhibiting pheromones in mice. Jones and Nowell (1975), for example, report that home-cage odours of both group-housed and isolated males contain a factor which inhibits aggression. A fact of major importance to the present study is that mice will keep their nests clean of urine and faeces (Blom *et al* 1993). Olfactory cues present in the nest would thus derive from the secretions of the plantar and other body glands. If these glands are used to enable recognition of group members and have no aggression-eliciting components, as might be the case with urinary marks, this would explain why the transfer of nesting material had aggression-inhibitory effects in the present study.

Possible explanations of the controversy

Transfer of sawdust seemed to have aggression-eliciting effects in the present study, although these results were not significant. The large variety of effects that are reported in the literature and by animal caretakers might be explained in different ways. First, urine marks may contain pheromones not only from the preputial glands (potentially aggression-eliciting pheromones) but also from the coagulating glands (potentially aggression-inhibiting pheromones; Jones and Nowell [1975]; Stoddart [1980]). Whether the ultimate effect of urine marks is aggression-inhibiting or eliciting or has no effect would thus depend on the relative composition of the marks. Second (see, *Introduction*), the properties that would make 'a handful of sawdust' a useful tool in inhibiting aggression after cage cleaning are not well documented. This 'handful', may consist of sawdust containing urine and faeces of the dominant mouse only, of one or more subordinate animals, or of all animals, each of which might have different effects on aggression. In other cases, the sawdust might have been taken from the nesting quarters. Its effect would then be comparable to the effect of the transfer of nesting material in the present study.

The *distribution* of scent marks might also be of importance. It has frequently been shown that dominant mice have a markedly different urinary marking pattern than subordinate ones (Desjardins *et al* 1973; Hurst 1990). Dominant mice mark an area with numerous small streaks and spots, while subordinate mice deposit their urine in a few large pools. This distribution of the scent marks in 'a handful of sawdust' may be mixed up after transfer, while the distribution of scent marks in the nesting material would be relatively undisturbed. In part II of our study, we did not find any evidence to support the theory that either the odour donor (ie dominant or subordinate animal) or the distribution of urine marks influences aggression. No differences were found in aggression when the urine marks of dominant, subordinate, both or neither mouse were transferred to a clean cage. This is not in accordance with Hurst (1993) who found that the presence of familiar subordinate's urine provoked more aggression in dominant mice, while the presence of its own urine did not affect aggression compared to a control situation with no urine present. Close scrutiny of the urine marks on the filter paper in the present study revealed that the urine marks of subordinate mice were not distributed as would be expected according to Hurst (1993) and Desjardins *et al* (1973). We found that both the dominant and the subordinate mouse of each pair deposited many small spots and a few larger pools of urine.

Animal welfare implications

The most important and conclusive finding in this study was that the transfer of nesting material significantly reduced aggression between male mice after cage cleaning. Overall, no extreme aggression was observed, and none of the animals suffered physical injuries. In other words, the animals lived in a rather stable environment and no measures to reduce aggression would be necessary were this the general case in laboratory animal facilities.

Whether the transfer of nesting material would help to reduce aggression once it has already reached extreme levels, remains an issue for further research. If, however, transfer of nesting material was put into practice as part of a routine cleaning procedure, this could help to keep aggression at an acceptable level – or at least significantly slow down its development. In turn, this would enable male laboratory mice to live in harmony for longer periods of time.

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