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ScienceDirect
Regulatory Toxicology and Pharmacology 48 (2007) 184–193
Regulatory Toxicology and Pharmacology
www.elsevier.com/locate/yrtph

Effects of housing condition on experimental outcome in a reproduction toxicity study

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Received 10 July 2006
Available online 28 March 2007

Abstract

In most toxicity studies single housing is still preferred, as social stress is believed to have an effect on experimental outcome through interaction with the toxic compound or by increasing variation. There are also arguments that single housing will have a similar effect.

In this study the qualitative and quantitative effects of single- and social housing of rats has been investigated on immune- and endocrine responses, histopathology and body- and organ weights in a one-generation endocrine disrupter study (OECD 415) in rats exposed to tetrabromobisphenol A (TBBPA). The results of this study show that differences in parameters between the housing conditions were rarely noted. Striking results of the study are that in several parameters significant differences were noted in the un-dosed control group in single versus group housed animals, meaning that TBBPA can mask or enhance housing effects, or vice versa. In one case single housing altered the effect of the toxic compound. Depending on the endpoints of the study, the type of housing condition must be taken into consideration as findings like these could have great implications for the interpretation and validity of results from toxicological assays and the number of animals needed to detect significant effects of toxic compounds.

Keywords: Housing condition; Social stress; Experimental outcome; Rats; Reproduction toxicity study; Brominated flame retardant; Immune responses; Endocrine responses; Histopathology; Organ weight

1. Introduction

One of the oldest principles in biomedical research is to guarantee comparability and reproducibility of results (Weihe, 1993), within and between laboratories (Roe, 1994; Gur and Waner, 1993). This is especially true for testing for regulatory purposes. The use of historical control data is common practice in comparing and interpreting research results (Gur and Waner, 1993), but can be limited due to variation in results (Roe, 1994). Among others (see Roe, 1994; Gur and Waner, 1993; Carakostas and Banerjee, 1990; Öbrink and Rehbinder, 2000) experimental conditions are believed to affect experimental results (Everitt and Foster, 2004). This has led to standardized protocols, such as the "OECD Guidelines for the Testing of Chemicals", in which those conditions have been implemented that should minimize variation in experimental conditions and consequently in experimental results (Van Zutphen et al., 2001). Housing conditions are believed to have an important environmental influence on rodent research results (Everitt and Foster, 2004). In general, standardization of housing conditions has led to stimulatory poor conditions, as cages only contain features such as food, water, bedding and (sometimes) litter material (Olsson et al., 2003).

An example of such a standardized experimental condition is the single housing of rats in statutory required animal testing (see "OECD Guidelines for the Testing of

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doi:10.1016/j.yrtph.2007.03.004

Chemicals''). A major reason to withhold rats from social housing in toxicity studies is the concern for 'social stress'. This is believed to have a profound impact on various parameters in a qualitative or quantitative manner (Stefanski et al., 2001; Nyska et al., 2002; Sharp et al., 2002,2003; Weinreich et al., 1996) through interaction with the toxic compound or by increasing the variation. This seems contradictory, as the rat is a gregarious species. From this point of view it can be assumed that withholding rats from social contact could equally well have an effect on various parameters. Whereas the lack of social contact in single housed rats has been associated with affective disorders such as major depression, stress-induced behavioral changes and negative stress related physiological effects such as reduced growth rate and adrenal hyper- and hypotrophy (Westenbroek et al., 2003a,b, 2005), the presence of social contact has not (Hutchinson et al., 2005).

Other reasons to withhold rats from social housing are in most cases based on arguments of individual food- and water intake measurements. However, several authors have described alternative ways to measure individual food- and water intake. Good predictions can be made based on parameters such as energy balance, linear growth (body and bone length) and body composition (all components including body mass, total body water, fat, protein and ash). For adequate information we would like to refer to Cortright et al. (1997a,b), Dickinson et al. (2001), Skalicky et al. (2001) and Swithers and Davidson (2005).

In an ongoing toxicity study that investigated the modification of the hazard profile of tetrabromobisphenol A (TBBPA) animals were supposed to be single housed to allow recording of individual food consumption and to avoid bias from hierarchical stress as effects of hierarchical stress were anticipated to interact with the effect of TBBPA. TBBPA is a brominated flame retardant that has a high annual production volume (Alaee et al., 2003). It is applied in many consumer products to improve fire safety, particularly of printed wiring boards in electronic devices, and also of polymers, such as polystyrene, resins and adhesives. Emissions, which are likely to come mainly from additive use in these materials, can contaminate indoor environment. In this way, TBBPA forms a source for human exposure, possibly together with food, thus explaining the presence of TBBPA in human blood (Hagmar et al., 2000; Watanabe and Sakai, 2003; Thomsen et al., 2005). Although toxicity and teratogenicity of TBBPA appear to be low (Darnerud, 2003), concern for potential endocrine activity of TBBPA has been suggested by its activities related to the thyroid hormone system, as observed in bioassays (Meerts et al., 1999; Hamers et al., 2006), and related to the estrogen system, as observed in a uterotrophic assay (Kitamura et al., 2005). Furthermore, persistent lipophilic polyhalogenated aromatic hydrocarbons are known to affect the immune system (Ross et al., 1996). These concerns necessitated the modification of the hazard profile of this compound, with regard to ongoing risk assessment for human health. For this purpose a repeated oral dose one-generation reproduction study in rats (OECD 415, 1983), enhanced for endocrine- and immunological endpoints, has been conducted. The offspring was analyzed to detect developmental effects, which can be anticipated from endocrine disruption, particularly of the thyroid hormone system (Boelaert and Franklyn, 2005). Focus of analysis was on endocrine organs, and on organs directly related to endocrine functioning (e.g. liver and its metabolism). General toxicological parameters, as defined in the OECD 415 protocol (1983), were addressed with respect to comparability with similar studies, but also since many organs were considered specific targets of endocrine functions (e.g. reproductive organs).

The offspring was supposed to be single housed. For practical reasons this was not feasible and therefore males of all dosing-groups were housed socially and females of all dosing-groups were single housed. In order to investigate the qualitative and/or quantitative effects of housing condition on the experimental parameters, an additional investigation has been performed within and parallel to the above described original study. Additionally single housed males and socially housed females were added. The effect of housing condition in the context of the overall response of the animal to experimental procedures will be presented. It was hypothesized that the experimental outcome of single- and socially housed animals would show different effects of TBBPA. The effect of 'social' stress or (di)stress due the lack of social contact in context to the findings will shortly be discussed on the basis of literature.

2. Animals, materials and methods

2.1. Study design

2.1.1. Experimental set-up; parental generation

Eighty male and 80 female 4 weeks old SPF Wistar outbred rats (HsdCpb:WU) were purchased from Harlan (Horst, The Netherlands) delivered by van in filter boxes. The animals were housed in small unisex groups (2–5 animals per cage) of mixed weight ranges. This parental generation (P-generation) was exposed to the brominated flame retardant tetrabromobisphenol A (TBBPA), which was obtained as a technical mixture of various producers added to a commercial pelleted diet without soy (ABDiets, RMH, Woerden, The Netherlands). The experimental protocol followed the OECD 415 guideline (OECD 415, 1983), with the exception of dose-group arrangement: the animals were distributed via the benchmark approach (Slob, 2002; Woutersen et al., 2001) among eight dosing-groups (0, 3, 10, 30, 100, 300, 1000 and 3000 mg/kg body weight, when calculating with daily feed consumptions in the range of 40–150 g/kg body weight, depending on stage of life) with 10 males and 10 females in each group. Compared to the original protocol (OECD 415, 1983), the animals were assigned to more dosing-groups with less animals in each group, this to improve the assessment of dose–response relations. For males, exposure started after approximately 1 week of acclimatization at 5 weeks of age and lasted for minimal 70 days until mating. Exposure of females started at 9 weeks of age, which is approximately at least 14 days before mating, and continued during pregnancy and lactation (see Fig. 1). For mating, animals were housed in pairs, avoiding sibling mating. Pregnant females were single housed. The parental animals were euthanized after mating (males) or after weaning (females).

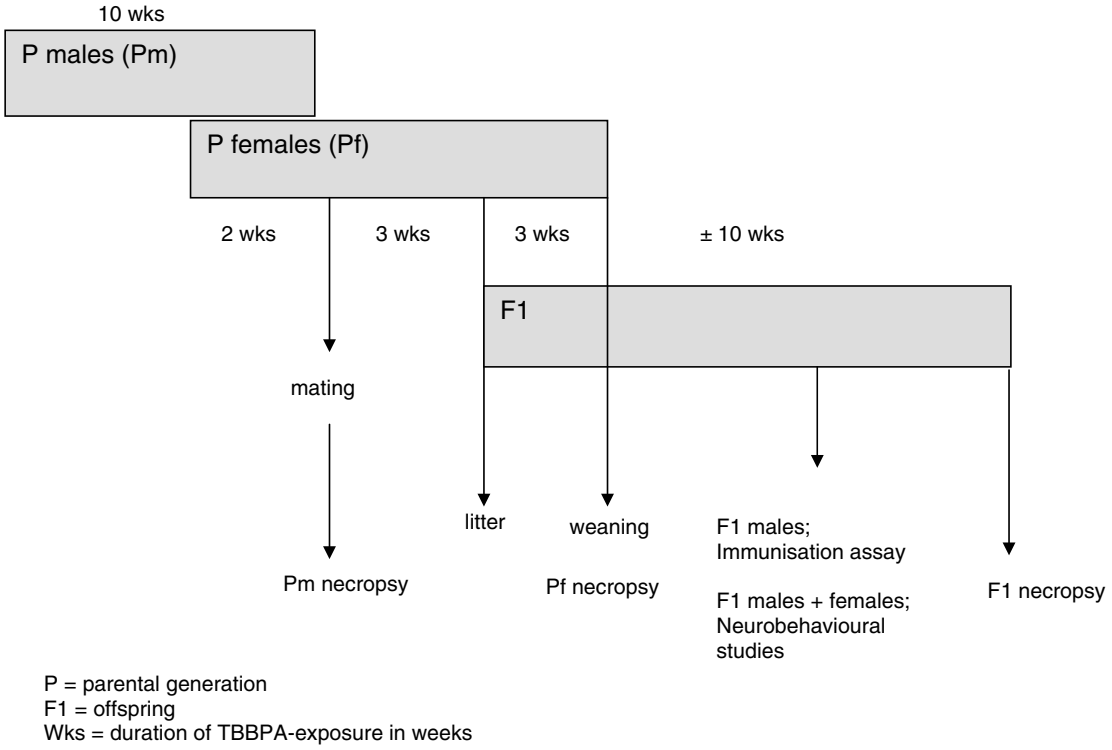

Fig. 1. Experimental set-up: start and duration of TBBPA-exposure per generation and experimental procedures performed per generation in time.

2.1.2. Experimental set-up; F1-generation (offspring)

At the time of weaning (pups 3 weeks of age) the litters were separated by gender and implanted with a microchip transponder for identification. Exposure of the offspring to TBBPA continued in the same way and to the same dose as their respective parents till the end of the study (necropsy), but at least for 9 weeks (see Fig. 1). The F1 followed the experimental protocol as described for the parental generation (8 dosing-groups, 10 males and 10 females per group; OECD 415, 1983) enhanced for endocrine and immunological endpoints following Toyoda et al. (2000) and Andrews et al. (2001), as effects of the toxic compound on these parameters were expected.

To asses the effect of TBBPA on immune parameters, 47 male animals were, at the time of weaning, randomly assigned to an immunization assay to test the immune response to Sheep Red Blood Cells (SRBC) (see details later). The remaining males and all females (10 animals per gender per dose-group) were used in neurobehavioral studies (not reported here) and/or necropsy at the end of the study.

The original study supposed animals to be single housed to allow recording of individual food consumption and to avoid bias from hierarchical stress in the experimental results. For practical reasons this was not feasible and therefore males of all dosing-groups were housed socially in Macrolon type IV cages (dimensions $480 \times 375 \times 210$ mm with a floor area of 1500 cm^2; Tecniplast, Italy) (2–5 animals per cage, depending on the neurobehavioral studies the animals were involved in). Females of all dosing-groups were single housed in Macrolon type III H cages (dimensions $425 \times 266 \times 185$ mm with a floor area of 800 cm^2; Tecniplast, Italy).

In order to investigate the qualitative and/ or quantitative effects of housing condition on the experimental parameters and therefore on the experimental results, an additional investigation has been performed within and parallel to the above described original study. Additionally single housed males and socially housed females (2–5 animals per cage) of exposure-groups 0, 300 and 3000 were added (10 animals per gender per exposure-group). These were surplus animals from the F1 and had therefore the same history as the animals in the main (original) study.

All experiments have been approved by the institutional Animal Ethical Review Committee, according to Dutch legislation.

2.2. Housing and care

Throughout all studies at all stages, animals of both generations were housed in temperature controlled rooms (21 ± 2 °C with 50–60% relative humidity, air flow rate 0.5 m/s) under a 12 h/12 h light–dark regimen (fluorescent lighting from 7.00 to 19.00) with a radio on as background-noise. All animals were visually inspected on a daily basis for general condition and possible clinical abnormalities. Autoclaved chopped wood bedding (Technilab, The Netherlands) was changed weekly and at that time the animals were weighed and submitted to in-hand clinical observations. All animals received food and tap water ad libitum and were held under SPF conditions.

2.3. Immunization assay

Persistent lipophilic polyhalogenated aromatic hydrocarbons are known to affect the immune system (Ross et al., 1996). To investigate the effect on the immune system an immunization assay has been performed. Forty-seven male animals were randomly assigned to this immunization assay at the time of weaning. Also within this immunization assay the effect of housing condition on the immune parameters were investigated. For the original study 32 males (four animals per dose-group; eight dose-groups in total) were housed socially (two animals per Macrolon type IV cage (Tecniplast, Italy). For the additional study 15 males (five animals per dose-group; three dose-groups in total) were single housed in Macrolon type III cages (Tecniplast). This set-up has provided the investigation of effect of housing condition on immune parameters. At the age of 11 weeks the rats were injected intraperitoneally with a 20% suspension of sheep red blood cells (SRBC), prepared from defibrinated sheep blood (OXOID) according to Van Loveren et al. (1991). A booster with the same volume of the same preparation was given after a 14-day interval.

2.4. Necropsy

At the end of the treatment period, at the age of approximately 16 weeks, five male (among which the animals used in the immunization assay) and five female animals per exposure group per housing condition were necropsied. The animals were euthanized by exsanguination from the abdominal aorta under O_2/N_2O/isoflurane-anaesthesia.

The males used in the immunization assay were necropsied on the first necropsy days in order to allow for clustered immunological analysis. From these animals, blood was collected for hematological- and immunological parameters. On the following necropsy days all other necropsies were performed in a targeted manner to avoid clustering of animals from single exposure-groups and housing conditions. Females were necropsied on the first day of diestrous, which was determined on the basis of daily vaginal smears in the preceding week.

During necropsy, the following organs and tissues were dissected for further (bio)chemical or histopathological analysis: reproductive organs (ovaries, uterus, mammary glands, testis, seminal vesicles with coagulation glands), immunological organs (popliteal lymph nodes, spleen, thymus), circulatory and respiratory systems (heart, lungs), digestive system (liver, pancreas, stomach, duodenum, colon, ileum, jejunum, caecum), urinary system (kidneys, urinary bladder), nervous system (brain, sciatic nerve) and the endocrine system (pituitary, thyroid glands including parathyroids, adrenals), skin, bone marrow (from femur) and blood (plasma).

All organs were weighed directly after dissection or after fixation (thyroid glands, pituitary). Defined parts of the liver were snap frozen in liquid nitrogen and stored at −80 °C, plasma aliquots were stored at −20 °C, a sample of whole blood and part of the spleen were freshly analyzed, and all remaining dissected organs were fixed in standard formalin for further histopathological processing.

In the above mentioned collected tissues, the following endpoints for this study were addressed: cellular composition (blood, bone marrow), immunological subpopulations and NK activity (spleen), immunoglobulins, thyroid hormones, clinical plasma components (alkaline phosphatase activity (ALP), alanine acetyltransferase activity (ALAT), total protein, creatinine, cholesterol, albumin, glucose and urea), TBBPA kinetics (liver) and histopathology of multiple organs (see Section 3).

2.5. Immunology/haematology

For hematology one femur shaft was syringed with 4 ml ICD (Impulse Cytophotometer) solution, containing 0.322% trisodium-citrate-dihydrate, 0.34% sodium-dihydrogenphosphate-dihydrate, 0.387% disodium-hydrogenphosphate-dihydrate, 0.117% citric-acid-monohydrate, 0.365% dextrose, 0.496% sodiumchloride in demineralized water, pH 7.4, at 20 °C. The resulting cell suspension and the collected EDTA blood were kept at 4 °C until automated analysis in an ADVIA120 (Bayer) within 4 h. Analysis of the spleen subpopulations (T-lymphocytes (CD3), T-helper (CD4), cytotoxic T-cells (CD8), Natural Killer cells (NK, CD161a) and B-lymphocytes (CD45RA), was carried out on fixed cell suspensions prepared of approximately one-third of the organ with a FACSCALIBUR (B&D) in two separate runs. Numbers of these subpopulations were calculated from the ratios determined by FACS (fluorescent activated cell sorter) analysis, cell counting with the Coulter Counter Z2, and weights of spleen parts. The NK-function test was essentially performed as described previously (De Jong et al., 1980), with ^{51}Cr-labeled YAC lymphoma cells as targets.

2.6. Histology

After fixation, organs sampled for histopathology were dehydrated in an automated manner and paraffinized and embedded according to RIVM SOPs (compatible with Bahnemann et al., 1995). Sections of 4 μm were stained with haematoxylin and eosin in an automated way. Microscopical observations were made by comparison of control and exposed samples. Scoring of suspected effects was done without knowledge of treatment, or by morphometrical methods. These included on-screen measurements of distances (e.g. for endometrium thickness) or automated quantification of colors or color intensity (e.g. for TSH immunohistochemistry), all on digital projections. AnalySIS (Soft Imaging System, Münster, Germany) was the software used for morphometry.

2.7. TH analysis

Total concentrations of circulating thyroid hormones thyroxine T4 and T3 were determined in plasma with a standard radioimmunoassay, validated for rats and mice, as described by Friedrichsen et al. (2003).

2.8. Data analysis

Effects of housing condition on the experimental outcome of 92 parameters were investigated in this study. This number includes the histopathology of several tissues, but the arbitrary scores of these parameters were not included in the data-analysis. The remaining parameters were analyzed as follows. Effects of the brominated flame retardant on the parameters were analyzed by dose–response modeling and estimation of the Critical Effect Dose (CED; Woutersen et al., 2001; Slob, 2002) by use of the statistical package Possible Risk Obtained from Animal Studies (PROAST, version 01).

Before further analysis, a Levene's test of homogeneity of variance and a Kolmogorov–Smirnov test of normality was run for all parameters. The majority of the parameters demonstrated normality and equal variances between groups, which made parametric tests preferable. The significance of the differences between groups was calculated by means of Analysis of Variance (ANOVA). Gender, housing condition and dose-group were taken as fixed factors and age as covariate for all parameters, except for organ weights. If gender differences appeared, the ANOVA was performed per gender.

Age and body weight differed between the experimental groups as a result of the experimental design. These factors are known to influence organ weights (Bratt et al., 2001; Gur and Waner, 1993). As we were not interested in the effect of body weight on organ weights and to minimize the effect of age and body weight, in order to investigate the effect of housing condition, we adjusted the organ weights by using the linear correlation coefficients as described in Trieb et al. (1976). As potential effects of endocrine disruption may confound the conclusions of organ sizes, when expressed relative to body weight (Schärer, 1977), the absolute organ weights were also taken into the ANOVA. No differences were found between the two approaches on experimental outcome. The given *P*-values in Section 3 are based on the adjusted organ weights.

When an effect of the brominated flame retardant was revealed by dose–response modeling, estimation of the Critical Effect Dose or in the analysis of variance, the ANOVA was performed within the dose-group on housing condition and gender. Otherwise the ANOVA was performed on all dose-groups pooled together.

A rejection-criterion of 0.05 was set for all statistical tests. When the analyses of variance showed statistically significant effects, the group means were further compared with the unpaired Student's *T*-test or with the Bonferroni post hoc test. Based on results of previous experiments no direction could be assumed of the qualitative and/or quantitative nature of these effects, therefore all statistics are two-tailed. The Statistical Package for the Social Sciences (SPSS, version 12.0) was used for all statistical calculations of significance of differences between the groups.

In order to compare the variance due to single housing versus social housing, an *F*-ratio test (Wolfram, 2003) on the within-group variance was performed per parameter per gender. The within-group variance of some parameters was equal for both housing conditions, which has resulted in 76 tests. A rejection-criterion of 0.05 was set for the tests, but since this is the given alpha for individual comparisons the α-value had to be lowered to avoid spurious positives. The Bonferroni correction is a multiple-comparison correction used when several statistical tests are being performed simultaneously. Based on the 76 tests and the Bonferroni correction the rejection-criteria were set at 0.0006579.

3. Results

3.1. In life results

At the time of necropsy, values (mean ± SD) of age and body weight were calculated. The females were slightly older than the males ($P < 0.064$). Within gender significant age differences were found between the housing conditions. Socially housed males (mean days of age ± SD; 119 ± 8.6) were older than single housed males (109 ± 9.11) ($P < 0.0001$), while the single housed females (121 ± 9.1) were older than the socially housed ones (114 ± 10.27) ($P < 0.017$).

Body weight was significantly different between genders (see Fig. 2). Males were heavier than females ($P < 0.0001$). Housing condition had an effect on the body weight of females: the single housed females were heavier than the socially housed ones ($P < 0.002$).

3.2. Pathology

All organ weights showed significant differences for gender. Therefore all organ weights were analyzed separately for males and females. Significant housing effects, independent of exposure, were found on the thymuses and the prostates of males. Socially housed males had heavier prostates ($P < 0.0001$) and lighter thymuses ($P < 0.004$) than single housed males. For females, housing condition showed a trend on brain weight ($P < 0.067$) and uterus weight ($P < 0.022$). Both organs were heavier for the socially housed animals.

Significant housing effects were also noted for the liver in the un-dosed control group for both males (Fig. 3) and females (Fig. 4). Single housed males ($P < 0.059$) and females ($P < 0.05$) had heavier livers than socially housed animals.

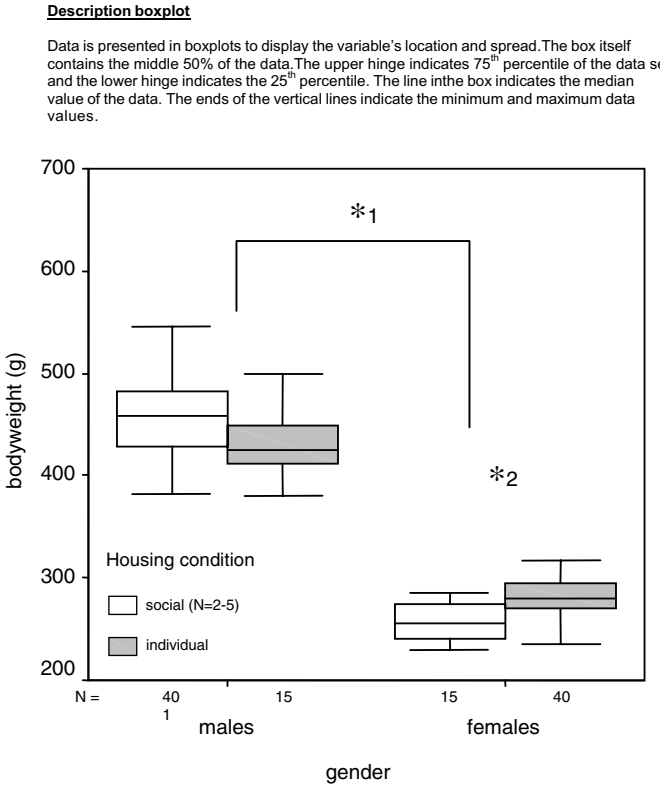

Fig. 2. The effect of housing condition on absolute body weight. *1 Significant difference ($P < 0.05$) based on gender irrespective of housing condition, *2 significant difference ($P < 0.05$) based on housing condition between the socially and single housed female rats. *N*, number of animals.

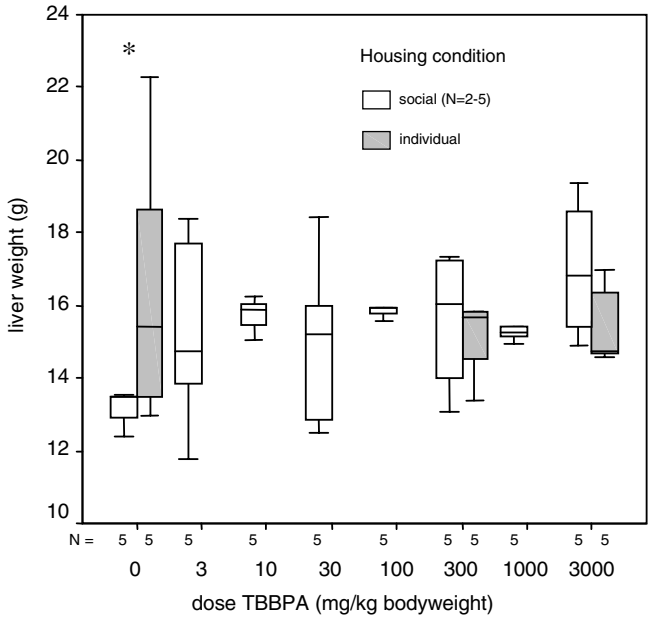

Fig. 3. The effect of housing condition on the absolute weight of male livers. *Significant difference ($P < 0.05$) based on housing condition between the social and single housed animals in the un-dosed control groups. *N*, number of animals.

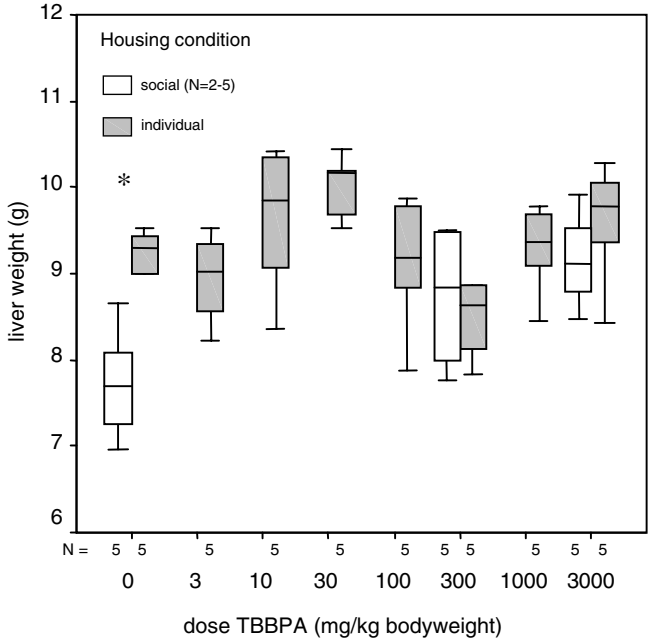

Fig. 4. The effect of housing condition on the absolute weight of female livers. *Significant difference ($P < 0.05$) based on housing condition between the social and single housed animals in the un-dosed control groups. *N*, number of animals.

3.3. Histopathology

Histopathological analysis revealed no observed effects of housing condition on the prostate, in contrast to pathological analysis. Furthermore, no observed effects of housing condition were found on the histopathology of the skin, other reproductive organs, immunological organs, circulatory and respiratory systems, digestive system, urinary system, nervous system or endocrine system except for the thyroid.

In the thyroid, blind scoring of follicle size and cell activation (an arbitrary combined score of cell size, cell vacuolization and nuclear size) revealed changes in cell activation due to TBBPA exposure in combination with housing condition for both males and females. In all un-dosed control groups the thyroid gland contained well-filled follicles, cubic epithelial cells, medium-sized nuclei and moderate vacuolization. The effect of TBBPA was expressed as a change towards cylindrical morphology of the follicle lining cells and enlarged nuclei in the highest dose-groups. In addition, single housed animals had more pronounced changes in follicle morphology and extensive cellular vacuolization compared to socially housed animals (see Fig. 5 for results in the males).

3.4. Hematology and Immunology

Housing effects were found on the absolute number of large unstained cells, relative distribution width (RDW) of the erythrocytes and on reticulocytes. The absolute number of large unstained cells ($P < 0.036$) and the percentage of reticulocytes ($P < 0.028$) were significantly higher for the single housed animals. The socially housed animals had a significantly higher RDW ($P < 0.007$).

The differential white blood cells in bone marrow were counted. No effects of housing condition were found on the leukocytes. Only a trend ($P < 0.069$) of housing was found on the percentage of CD161A of the total of spleen cells. CD161A was higher in single housed males.

No effect of housing was seen on NK-cell activity and on the increase of IgM. A strong significant ($P < 0.001$) difference between housing conditions was present on IgG in which the single housed males had higher levels of IgG than socially housed males.

3.5. Clinical chemistry of plasma

Significant effects of gender were found on albumin ($P < 0.0001$), alanine acetyltransferase activity ($P < 0.019$), glucose ($P < 0.0001$) and on the total of proteins ($P < 0.004$). Except for albumin, males had significant higher levels of the aforementioned parameters. Housing condition had no effect on the clinical chemistry of plasma.

3.6. Endocrine parameters

The endocrine parameter thyroxine (T4) has been analyzed separately for gender for the reason that males had significantly higher levels ($P < 0.002$) than females. Within gender no effect of housing condition was found.

Housing condition had no effect on triiodothyronine (T3) in males. In the un-dosed control group socially housed females had significantly higher levels of T3 ($P < 0.001$) compared to single housed females (see Fig. 6).

3.7. Within-group variance

The *F*-ratio test, without the Bonferroni correction, revealed significant differences for the males on spleen ($P < 0.0001$), thymus ($P < 0.011$), reticulocytes ($P < 0.009$), lymphocytes in bone marrow ($P < 0.041$), eosinophils in bone marrow ($P < 0.029$). In these parameters the within-group variance of single housed males was significantly larger than that of the socially housed males. Significantly larger within-group variances for the socially housed males were found on the percentages of CD3 ($P < 0.031$), CD4 ($P < 0.036$) and CD8 ($P < 0.0001$) and on MCHC ($P < 0.002$), neutrophils ($P < 0.001$) and on the prostate (dorsal) ($P < 0.0001$). Significant differences on the within-group variances of females were found on the kidneys ($P < 0.041$) and alanine acetyltransferase activity ($P < 0.0001$), in which the within-group variance

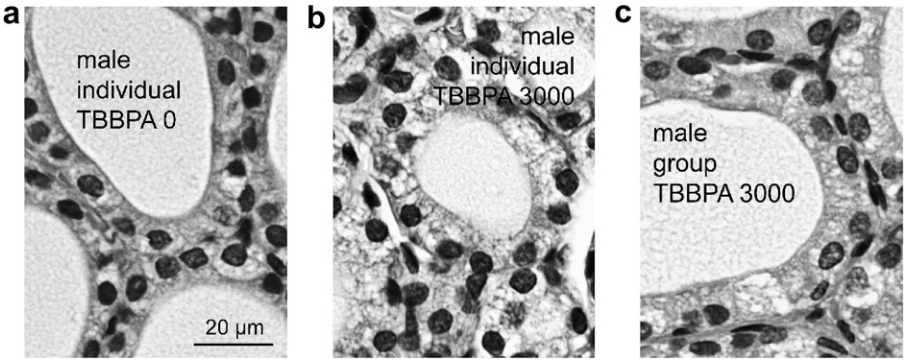

Fig. 5. Histopathology of the thyroid gland. (a) Well-filled follicles, cubic epithelial cells, medium-sized nuclei, moderate vacuolization. (b) Small thin follicles, cylindrical epithelial cells, enlarged nuclei, extensive vacuolization. (c) Normal follicles containing colloid, cylindrical epithelial cells, enlarged nuclei, less extensive vacuolization.

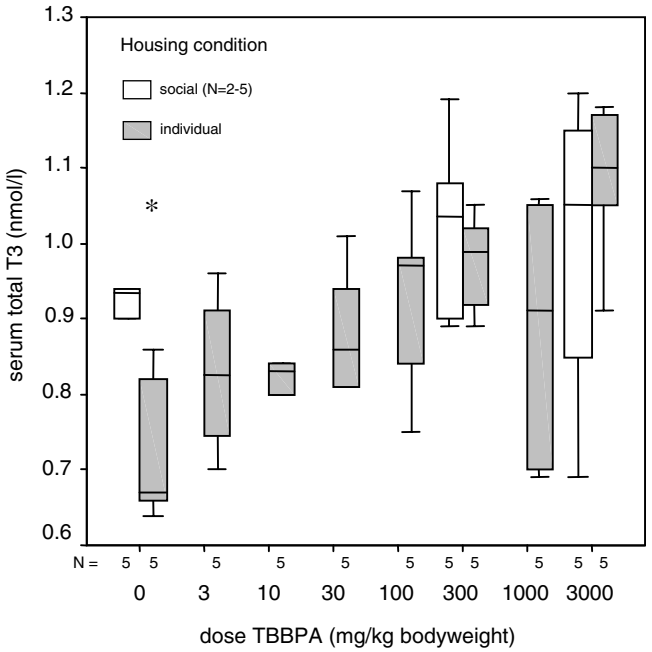

Fig. 6. The effect of housing condition on serum total T3 in females in the un-dosed control group. *Significant difference ($P < 0.05$) based on housing condition between the social and single housed animals in the un-dosed control groups. *N*, number of animals.

of single housed animals was larger. Socially housed females had larger within-group variances on the weight of heart ($P < 0.039$) and ovaries ($P < 0.044$).

After the Bonferroni correction the significant differences on spleen, prostate, percentage of CD8 and alanine acetyltransferase activity remained the same.

All results are summarized in Table 1 by means of the direction of the effect.

Table 1
Overview of the direction of significant effects

Housing effect	Direction
Males	
Thymus weight	S > S-H
Prostate weight	S < S-H
Liver weight (0-group)	S > S-H
Large unstained cells	S > S-H
RDW (absolute)	S < S-H
Reticulocytes	S > S-H
% CD161A	S > S-H
IgG	S > S-H
Females	
Body weight	S > S-H
Brain weight	S < S-H
Uterus weight	S < S-H
Liver weight (0-group)	S > S-H
T3 (0-group)	S < S-H
Gender effect	
All organ weights	M > F
Body weight	M < F
Albumin	M > F
ALAT	M > F
Glucose	M > F
Total of proteins	M > F
T4	M > F
Interaction effect	
M + F thyroid tissue (↑ 0–3000 exposure)	S > S-H
Within-group variance (after Bonferroni correction)	
Males	
Spleen weight	S > S-H
Prostate	S < S-H
% CD8	S < S-H
Females	
ALAT	S > S-H

S, single housed; S-H, social housed (2–5 animals per cage); M, males; F, females.

4. Discussion

In most toxicity studies single housing is still the preferred housing condition, as social housing is believed to have an effect on the experimental outcome in a qualitative and/or quantitative manner through interaction with the toxic compound or by increasing the variation due to social stress. However, there are also arguments that single housing will have a similar effect on experimental outcome (among others: Van der Harst, 2003; Von Frijtag et al., 2000, 2002; Benefiel and Greenough, 1998) due to stress caused by the lack of social contact. Therefore, in this study the qualitative and quantitative effects of single- and social housing were investigated according to a study protocol based on the OECD 415 guideline (OECD, 1983), enhanced for endocrine- and immunological endpoints for the offspring. It was hypothesized that the experimental outcome of single- and socially housed animals would show different effects of the brominated flame retardant tetrabromobisphenol A (TBBPA).

The results in this study confirmed our hypothesis only partly. Only in 12% of the 92 tested parameters statistically significant effects were observed for housing condition, and 5% of the parameters showed statistically significant effects on the within-group variance. In 3% of the cases housing condition had a significant effect in the un-dosed control groups and in 2% housing interacted with gender. In one case housing condition interacted with TBBPA. More prominent were gender differences in 28% of the cases. As a result of the experimental design, age in days differed per gender and within gender per housing condition. Body weight differed per gender and within gender effects of housing condition were present.

Differences in body weight between genders occur, even between animals of the same sex and the same age (Hurst et al., 1996). This is also the case in this study in which males are heavier than females. Among females body weight differed between the housing conditions. According to Tamashiro and colleagues (2004) the magnitude and direction of the difference in body weight is dependent on the intensity, duration of exposure and sensitivity to a stressor, in this case TBBPA or housing condition. It is not likely that the intensity and duration of exposure to

TBBPA underlie the difference in body weight of the females between the housing conditions, as no dose-dependent effects of TBBPA have been found on body weight and the difference is absent for males. It is more likely that females are more sensitive to certain stressors, which could be the housing condition itself in this case. In studies of Westenbroek and colleagues (2003a,b, 2005) similar results have been found.

Despite the adjustments for body weight and age, gender differences were present for all organ weights, which is partly in line with the findings of Gur and Waner (1993). Differences due to housing condition were expected on adrenal gland and thymus weight, as changes in these parameters are the direct consequence of activation of the hypothalamic–hypophysis–adrenal axis (Selye in Klein et al., 1992). Among males significant housing effects were indeed found on the thymus weight. The thymuses of socially housed males were lighter, which might identify (di)stress, as in general stress decreases thymus weights. This is not confirmed by adrenal gland weight, as no differences in this parameter between the housing conditions appeared. Care should be taken when interpreting thymus weight, as thymus weight is dependent of many factors and highly variable as a result of normal biological variation (Haley, 2003).

In females housing effects were present on the weight of brain and uterus. Both organs were heavier in the socially housed animals. This is in line with the results of a study of Benloucif et al. (1995) in which socially housed rats had increased brain weights compared to individually housed controls. Housing laboratory rodents in socially/enriched or single/impoverished environments alter many aspects of their cerebral anatomy (Katz and Davies, 1984). In a study of Katz and Davies (1984) socially enriched housing was associated with significant increase in the weight and length of the cerebrum, thicker occipital cortices and increased relative numbers of neurons and oligodendrocytes compared to their single housed impoverished controls. Results of our neurobehavioral studies (not presented here) confirm these findings. Conclusively, housing female rats socially can result in better-developed and therefore heavier brains.

It is difficult to explain the differences in uterus weight between the housing conditions. Females were scheduled to be sacrificed at the first day of diestrous. After necropsy measurements on uterus, vagina and endometrium thickness revealed that females were necropsied at all different stages of the estrus cycle, although the number of females per different stage was evenly distributed over housing condition and dose-group. Therefore, the stage of the estrus cycle cannot be the explanation for differences in uterus weight between socially and individually housed female rats. As yet the differences on the development of the uterus remain unexplained.

Derivatives of TBBPA, such as lipophilic polyhalogenated aromatic hydrocarbons, are known to affect the immune system. For this reason immunological endpoints have been added to the original experimental protocol (OECD 415, 1983). Many of these immunological parameters have been analyzed in protocols investigating stress. These studies are of interest for this investigation, as differences in experimental outcome between the housing conditions are hypothesized to be due to 'social' stress or the absence of social contact. Differences in basal and stress-induced levels of peripheral blood leukocytes in laboratory rats can be expected, due to the type of stressor (Stefanski and Grüner, 2006; Hurst et al., 1999). Lower numbers of CD4, CD8, B cells and NK levels were found in (psycho)socially stressed animals (Stefanski and Grüner, 2006). On the other hand, Baldwin et al. (1997) found no differences in immune and endocrine parameters, i.e. antibodies, peripheral blood leukocytes and plasma corticosterone, in differently stressed rats after immunization with SRBC. Klein et al. (1992) also found no differences in NK-cell activity, splenic reactivity and antibodies (IgG) in a model of chronic social stress in rats. The results of Baldwin et al. (1997) and Klein et al. (1992) are in line with our findings, except that in our study single housed males show higher levels of IgG than socially housed males.

Significant differences based on housing condition were observed in T3 for females and on liver weight of males and females. Striking is that the effect of housing condition is only observed in the un-dosed control groups. This might suggest that for these parameters the brominated flame retardant masks or enhances the subtle effects of housing condition or that, depending on the parameter, single- or social housing masks the effect of the toxic compound.

In the thyroid changes in cell activation were revealed due to TBBPA exposure in combination with housing condition for both males and females. In the highest dose-group the effect of single housing is stronger, as the histopathology of these animals differentiates more severely from the normal histopathology. So in this case housing condition interacts with the toxic compound and single housing alters the effect of the toxic compound. An explanation for this could be that element concentration, in this study TBBPA-concentration, in animal organs and tissues can change due to inner- (physiological state of the animal) and external conditions under which the animals are kept (Uchino et al., 1990). These changes might be more severe in single housed animals and therefore alter the effect of TBBPA.

Differences in the variance of results between single- and social housing are present, but not as obvious as predicted. No distinct difference can be made in variation between single- or group housing as the results are evenly distributed over both housing conditions. So social housing does not necessarily mean an increased variation and therefore an increased number of animals needed to detect significant differences related to the treatment given. This is in line with a study of Mering et al. (2001).

In conclusion, the results of this study show that there is no reason to withhold rats from social housing based on the arguments of increased variance. The differences in

the investigated parameters between the housing conditions are absent, small or of minor importance, because the experimental outcome does not exceed the normal biological variation. One obvious result is that housing condition can have quantitative and qualitative effects on parameters in which single housing alters the effect of the toxic compound.

As models for hazard identification should be specific, relevant and sensitive, findings like these could have great implications for the interpretation and validity of results from toxicological assays. We recommend to seriously reconsider standardized protocols in which animals are to be single housed. Housing conditions should be based on experimental objectives/data rather than on tradition. The choice of housing condition in a research protocol should be regarded in the context of the overall response of the animal to experimental procedures (Smith and Corrow, 2005).

Acknowledgments

This study has been conducted parallel to and within the tiered screening program of the FIRE project, which aims at the toxicological characterization of the potent and environmentally relevant brominated flame retardants. This project is focused on endocrine disrupting and immunological effects, in view of risk assessment for human and wildlife health. The authors acknowledge support by the European Commission under the project FIRE (QLRT-2001-00596). The authors are solely responsible for the contents of this paper, which does not necessarily represent the opinion of the European Community. The Community is not responsible for any use that might be made of the data.

We thank Dr. Klaus Rothenbacher of BSEF for facilitating the technical mixture of TBBPA.

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