

The

GERMAN MOUSE CLINIC

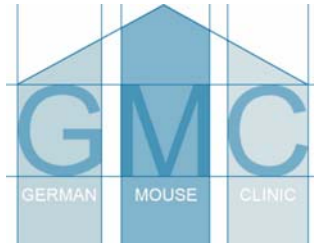
at the Helmholtz Zentrum München
German Research Center for
Environmental Health (GmbH)

Report for C57BL/6NCrI-Chr 7^{C3H/HeNCrI} (Cs7)

Confidential Data

Helmut Fuchs, Valérie Gailus-Durner, Christoph Lengger, Beatrix Naton, Thure Adler, Antonio Aguilar, Lore Becker, Ines Bolle, Markus Brielmeier, Julia Calzada-Wack, Claudia Dalke, Nicole Ehrhardt, Wolfgang Hans, Sabine M. Hölter, Gabriele Hölzlwimmer, Marion Horsch, Anahita Javaheri, Svetoslav Kalaydjiev, Magdalena Kallnik, Eva Kling, Sandra Kunder, Thomas Lisse, Holger Maier, Corinna Mörth, Ilona Moßbrugger, Ildikó Rácz, Birgit Rathkolb, Jan Rozman, Regine Schreiner, Anja Schrewe, Ralf Steinkamp, Johannes Beckers, Heidrun Behrendt, Dirk H. Busch, Jack Favor, Jochen Graw, Gerhard Heldmaier, Heinz Höfler, Boris Ivandic, Thilo Jacob, Hugo Katus, Paulus Kirchhoff, Martin Klingenspor, Thomas Klopstock, Markus Ollert, Leticia Quintanilla-Martinez, Jörg Schmidt, Holger Schulz, Eckhard Wolf, Wolfgang Wurst, Andreas Zimmer, and Martin Hrabé de Angelis

The German Mouse Clinic



The German Mouse Clinic (GMC) was founded January 2002 at the Helmholtz Zentrum München - German Research Center for Environmental Health (GmbH) in Munich to provide an open access platform for standardized mouse phenotyping. The GMC is supported by the National Genome Research Network (NGFN, <http://www.ngfn.de/>) and is a partner of the EUMORPHIA research program (<http://www.eumorphia.org/>).

In the GMC, experts from various fields of mouse genetics, physiology and pathology in close collaboration with clinicians work side by side at one location. We offer a primary phenotypic analysis of mouse consomic animals (more than 240 parameters/mouse) in the areas of allergy, behavior, bone and cartilage, cardiovascular diseases, clinical chemistry, energy metabolism, eye development and vision, immunology, lung function, molecular phenotyping, neurology, nociception, and pathology. Additional screens for host-pathogen interaction can be performed at the GBF Braunschweig. Secondary and tertiary screening for in depth analysis is offered by the different screens and is available on request.

Director

Prof. Dr. Martin Hrabé de Angelis
Institute of Experimental Genetics
Helmholtz Zentrum München
German Research Center for
Environmental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg / München
Tel.: 089-3187-3302
Fax: 089-3187-3500

HelmholtzZentrum münchen
German Research Center for Environmental Health



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1 Summary

1.1 Primary Screening

In a primary screen 64 mice (32 Cs7HH and 32 Cs7HB animals; with respect to chromosome 7, Cs7HH are homozygous C3H and Cs7HB are heterozygous C3H/BL/6) of the C57BL/6NCrl-Chr 7^{C3H/HeNCrl} (Cs7) mouse line have been analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dymorphology, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Cardiovascular Function, Nociception, Lung Function, Energy Metabolism, and Pathology. The screening started on August 21st, 2006. In addition, aged animals have been analyzed by the Pathology Module. These analyses are ongoing. The results are briefly summarized below by screen.

Behavior: Cs7HH mice of both sexes showed a behavioral pattern suggesting reduced social affinity and alterations in **anxiety-related behavior** compared to Cs7HB mice.

Dymorphology, Bone and Cartilage: In the morphological investigation via visual inspection and X-ray analysis, no genotype-specific differences were found. In the dual-energy X-ray absorptiometry (DXA) analysis, only lean mass was significantly increased in female consomic animals compared to controls.

Neurology: All SHIRPA parameters were without pathological findings. No neurological abnormalities were detected. Comparing the forelimb grip strength revealed significantly **increased force** in Cs7HH females.

Clinical Chemistry and Hematology: We only detected subtle differences of unclear relevance in the clinical-chemical parameters. The hematological parameters red blood cell count, hemoglobin levels, hematocrit, as well as mean corpuscular volume were lower whereas mean corpuscular hemoglobin concentration and RDW were higher in Cs7HB mice compared to Cs7HH animals. These small differences could indicate that some of the genes responsible for strain specific differences in the red blood cell count between C57BL/6 and C3H mice are located on chromosome 7.

Immunology: Under the baseline conditions of the primary immunology screen, we found significant differences between Cs7HB and Cs7HH mice concerning the frequencies of CD103 expressing CD8 cells [↑], CD62L expressing CD25+CD4+ cells [↓] and B-cells (CD19+) [↓]. All progenitor cells of leukocyte lineages derive from the hematopoietic stem cell in the bone marrow. The genetic regulation of hematopoietic cell compartments was proposed to be partly linked to the chromosome 7. Thus, the Cs7HB and Cs7HH mice may be a tool in studies on chromosome 7-related genes involved in the regulation of leukocyte proportion.

Cardiovascular: The comparison of the Cs7 consomic to control mice in blood pressure and ECG analysis revealed no effect of the replaced chromosome on cardiovascular function in the basal conditions of the primary screen. Only a subtle difference in Q amplitude was found.

Lung Function: Female Cs7HH mice showed a significantly higher specific tidal volume, specific minute ventilation, minute ventilation and mean inspiratory flow rate. However, breathing rate and respiratory timing were not affected. According to our studies, there are no indications for an association of lung function to Chromosome 7 (Reinhard *et al.*, 2002/2005). Overall, differences in females are small and are unlikely to reflect a clear phenotype.

In the screens **Allergy**, **Eye**, **Nociception**, and **Energy Metabolism**, no genotype-specific differences could be found.

1.2 Recommendations for Secondary Screening

Behavior: If the mouse provider wants to follow up the observations made here, we suggest measurement of stress hormones in these mice, particularly corticosterone.

Immunology: We propose to examine the TGF-beta level in the serum of the mice in order to test the hypothesis that differences in the expression of CD103 and in the frequencies of CD25 CD4 cells are related to differences in the level of TGF-beta. Furthermore, analysis of lymphoid organs like bone-marrow and spleen could reveal, at which developmental stage differences in the B-cell compartment occur.

Pathology: The Pathology Screen has performed a comprehensive analysis of aged animals. Since the evaluation has not been finished yet, the results will be submitted separately. A brief overview is given in Table 30, Chapter 3.13.

Please contact Valérie Gailus-Durner to discuss further steps and details.

2 General Part

2.1 Known Phenotypes

C57BL/6N is resistant to dystrophic cardiac calcification (DCC) whereas C3H/HeNcrI carries the susceptibility locus *Dyscalc1* that was identified on chromosome 7 by quantitative trait locus (QTL) analysis (Ivandic *et al.*, 1996; Ivandic *et al.*, 2001). Therefore, the consomic mice present with calcification in response to myocardial injury.

All further findings which will be shown in this report we consider as new.

2.2 Expected Phenotypes

The provider expects multifarious phenotypes because many traits are determined by QTLs on chromosome 7.

2.3 Mice

2.3.1 Number and kind of mice

The mouse line C57BL/6NcrI-Chr 7^{C3H/HeNcrI} (Cs7) is consomic for chromosome 7. Chromosome 7 was transferred by repeated backcrossing from inbred C3H/HeNcrI (donor) into the C57BL/6NcrI (recipient) background.

With respect to chromosome 7,
Cs7HH are homozygous C3H
Cs7HB are heterozygous C3H/BL/6.

Table 1: Cs7 mice provided for analysis.	
Numbers in brackets indicate animals which were kept in reserve.	
Genotype / Sex	Number of Animals
C3H/C3H (Cs7HH) females	16
C3H/C3H (Cs7HH) males	16 (+4)
C3H/BL/6 (Cs7HB) females	16 (+4)
C3H/BL/6 (Cs7HB) males	16 (+ 4), 3 died* (replaced)

* These mice died after blood withdrawal.

2.3.2 Housing conditions

In the GMC mice are housed in type II polycarbonate cages in individually ventilated caging (IVC) systems (VentiRack Bioscreen TM, Biozone, Margate, UK) on wood fiber (Altromin, Lage, Germany). The IVCs operate with positive

pressure. Mice are transferred in weekly intervals to new cages with forceps in Laminar Flow Class II changing stations. Mice are fed with irradiated standard rodent high energy breeding diet (Altromin 1314) and given semidemineralized filtered (0.2 μm) water *ad libitum*. Light is adjusted to a 12h/12h light/dark cycle; temperature and relative humidity are regulated to $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$, respectively. In specified modules husbandry conditions are adjusted according to the experiment requirements (See corresponding sections). All people attending the facility completely change their garment (jackets and trousers autoclaved) and shoes and wear caps and masks before entering the GMC (Brielmeier *et al.*, 2002).

Outbred 8-week-old male SPF Swiss mice are used as sentinels and kept on a mixture of new bedding and aliquots of soiled bedding (50:50) from all cages of the IVC rack. In addition, the sentinels were also exposed to soiled air from all “upstream” cages of the IVC rack. Health monitoring is carried out by on-site examination of the sentinel mice by certified laboratories according to FELASA recommendations (www.felasa.org).

Mice are kept according to the German laws. Tests were carried out by authority of the Regierung von Oberbayern.

2.4 Workflow

2.4.1 Standardized workflow for the primary screen in the German Mouse Clinic

Mouse consomic animals entering the GMC are examined in a primary screen according to the following standard workflow (Fig. 1, Gailus-Durner, Fuchs *et al.*, 2005). Analyzed parameters are listed in Table 2.

After the mice arrive at the GMC, they are acclimatized in the new environment for one week. The males then start in the Behavior Screen. There they stay for three weeks. Directly after the behavior tests, the anatomical inspection of the Dymorphology Screen is performed. In the next week, the Neurology Screen is applied. One week later the mice go through the tests of the Eye Screen. When the mice were 12 weeks old, blood is taken, and samples are distributed to the blood-based screens for Clinical Chemistry, Immunology, and Allergy. One week later, the animals are tested in the Nociceptive Screen. One week later the mice were passed to the Cardiovascular Screen wherein the mice stay two weeks. In parallel, 10 consomic animals (five males / five females) and 10 controls (five males / five females) leave the animal facility for the Lung Function Analysis, which for technical reasons is located elsewhere. These animals are, for hygienic reasons, not allowed to re-enter the German Mouse Clinic. The females go directly to Pathology. The males are used to freeze organs for future molecular phenotyping on request (remaining organs from those animals are analyzed by the Pathology).

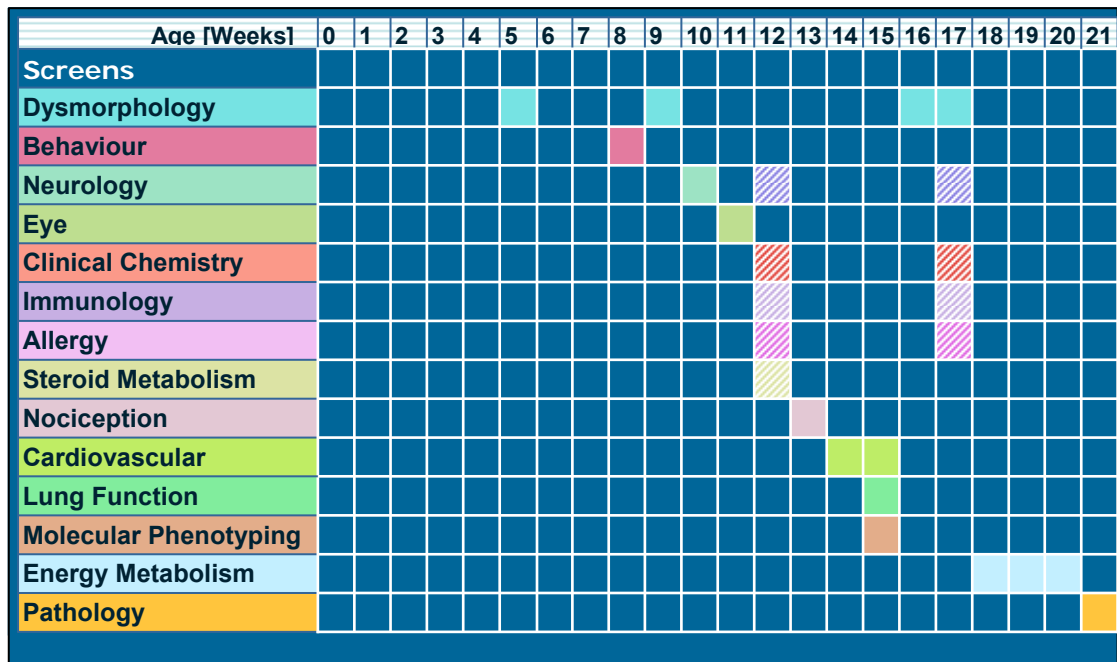



Figure 1: Workflow of the primary screen
 Explanation below,  Analysis of blood-based parameters.

All other animals go through the bone and cartilage tests of the Dysmorphology Screen. Five weeks after testing of the first blood sample, a second sample is taken to confirm the findings. Then the mice stay three weeks in the Metabolic Screen. After completion of the primary screen all animals are analyzed macro- and microscopically in the Pathology.

The screening of female animals starts one week later and follows the same workflow (with the exception of Molecular Phenotyping sampling). Deviations from our Standard operation procedure (SOP) are listed below; please take the specific number of analyzed animals from the sections of the applied screen.

2.4.2 Applied screens

The GMC standard workflow for the primary screen as described above was applied to analyze the Cs7 mutant mouse line. As more than the demanded number of 60 animals (15 mice per sex per genotype) had been delivered, the screen Lung Function and Molecular Phenotyping could be provided with one additional mouse of each sex and genotype. Some parameters measured in the blood based screens could not be determined in all animals, as it was not possible to get the needed amount of blood from these animals. A few animals died during the primary screen after blood withdrawal and thus could not be analyzed for all parameters.

2.4.3 Quality Assurance

The Quality Assurance as part of the Quality Management at the GMC consists of the following elements: standardized analyses via Standard Operating Procedures (SOP) and validation of analysis protocols by different institutions within the EUMORPHIA program, standardized data and project management supported by the central database system MausDB (Maier *et al.*, 2008) and the GMC coordination tool CoordDB as well as Quality Control and continuous training of the staff.

Coordination of the GMC's operations

The GMC management team (Core Facility) coordinates the scientific issues, logistics and administration of the GMC. The coordination software tool CoordDB supports the GMC management team in handling the incoming phenotyping requests and managing the complex phenotyping workflow of the primary and secondary screening. Besides the operational business activities the management team organizes the expansion of the screening services in collaboration with its partners. Additionally, the management arranges regular training of the staff members and the clinic's quality assurance.

Standardized Operation Procedures (SOP) and Validation of Protocols

The GMC developed a set of SOPs which cover all steps from mouse import and handling to phenotyping and data analysis. These SOPs are strictly followed during the whole screening process in the GMC and all procedures are documented.

The GMC is one of the major partners of the EUMODIC consortium that emerged from the EUMORPHIA program (Brown *et al.*, 2005), a consortium for the selection, establishment, and standardization of phenotyping protocols for mice as models for human diseases and for mouse husbandry. Cross-validation of protocols by EUMORPHIA is performed by the different institutions. A collection of the protocols (EMPreSS) is posted on the EUMORPHIA web site (<http://www.eumorphia.org/EMPreSS/>; Mallon *et al.*, 2008).

Central Database System

Another tool for quality assessment is the central database system which ensures full traceability of samples and documentation of all data. All mouse data is entered into the system (e.g. date of birth, sex, cage) and all screening results linked to the corresponding SOP as well as any changes of the mouse conditions are immediately put in.

Quality Control

In addition to routinely screen-specific quality control tests, control animals of selected strains (e.g. C57BL/6J and C3HeB/FeJ) are analyzed through the standard protocol for all phenotypes at regular intervals. This data is reviewed by the coordination team.

A tissue archive has been established for the storage of tail and blood plasma samples taken from all mice that have ever been analyzed in the GMC. The tail clips can be used for post-hoc genotyping in case of doubtful genotype information. The sanitary status of every mouse completing the screening can be tested by means of these plasma samples.

Continuous Training

Regularly specific training courses are held at the GMC. Specialists are invited to give lectures and to offer practical training at special days. Staff training is documented and maintained by the management team.

2.5 Statistical Analysis of Data

If not otherwise stated, data of males and females was analyzed separately comparing consomic and control data using a Student's t-test or ANOVA. Tables summarizing the data will show mean \pm standard error of the mean. Significant differences are indicated stepwise from 0.05, 0.02, 0.01, 0.001 to 0.0001. Raw data will be available on request.

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Abbreviations and Wording

DCC	dystrophic cardiac calcification
GMC	German Mouse Clinic
IVC	individually ventilated cage
Cs7HH	Chr 7: homozygous C3H
Cs7HB	Chr 7: heterozygous C3H/BL/6
FELASA	F ederation of E uropean L aboratory A nimal S cience A ssociations, 25 Shaftesbury Avenue, London W1D 7EG, UK, www.felasa.org

Table 2: Primary Screen at GMC

Screens	Goal	Methods
Dysmorphology, Bone and Cartilage	morphological analysis of body, skeleton, bone and cartilage	morphological observation, bone densitometry, X-ray, micro-computed tomography
Behavior	locomotor, exploratory, emotional and social behavior, object recognition memory	modified hole board
Neurology	assessment of muscle, spinocerebellar, sensory, and autonomic function	modified SHIRPA protocol
Eye	assessment of morphological alterations of the eye	funduscopy laser interference biometry slit lamp biomicroscopy histological analysis
Clinical Chemistry and Hematology	determination of clinical-chemical and hematological parameters in blood	blood autoanalyzer, ABC-animal blood counter
Immunology	analysis of peripheral blood samples for immunological parameters	flow cytometry, Multiplex Bead Array
Allergy	analysis of total plasma IgE	ELISA
Nociception	detection of altered pain response	hot plate assay
Cardiovascular	assessment of functional cardio-vascular parameters	non-invasive tail-cuff blood pressure measurement, surface limb ECG
Lung Function	assessment of alterations in breathing patterns	whole body plethysmography (Buxco®)
Molecular Phenotyping	RNA expression profiling	DNA-chip technology
Energy Metabolism	measurement of altered body weight regulation, body temperature and energy balance	bomb calorimetry
Pathology	microscopic and macroscopic examination	histology, immunochemistry

3 Specific part

3.1 Behavior Screen

3.1.1 Introduction

Genetic studies in the mouse are important for the elucidation of molecular pathways underlying behavior. The goal of this endeavor is not only the identification of genes that control brain function and influence behavior, but also understanding of genetic factors involved in human psychiatric disorders (Tarrantino & Bucan, 2000; Bucan & Abel, 2002). These disorders are associated with quantitative phenotypes called “intermediate traits” or endophenotypes, some of which, in contrast to the full complex disorder, can readily be modeled in mice. These traits are risk factors which are considered to be closer to the genetic etiology than the full syndrome. Examples are anxiety in depression, prepulse inhibition and working memory deficits in schizophrenia, and social interaction deficits in autism and schizophrenia (Seong *et al.*, 2002; Gottesman & Gould, 2003; Inoue & Lupski, 2003).

In the attempt to efficiently screen for candidate endophenotypes within a limited time frame, we use the modified Hole Board (mHB) test as primary screen in the behavioral phenotyping module of the GMC. This test allows the comprehensive analysis of a range of parameters known to be indicative of behavioral dimensions such as locomotor activity, exploratory behavior, arousal, emotionality, memory and social affinity in a single short test (See Ohi *et al.*, 2001).

3.1.2 Summary

Cs7HH mice of both sexes showed a behavioral pattern suggesting reduced social affinity and alterations in anxiety-related behavior compared to Cs7HB mice.

3.1.3 Mice

Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype. They entered the laboratory at the age of six weeks, were given two weeks for acclimatization and were tested at the age of eight weeks. Three days before testing, an object (metal cube) was placed into the home cage and removed one day before testing. In this screen, 30 female mice (15 Cs7HB, 15 Cs7HH) and 28 male mice (13 Cs7HB, 15 Cs7HH) were available for analysis.

3.1.4 Material and Methods

The modified Hole Board test was carried out according to the procedures described by Ohi *et al.*, 2001. The test apparatus consisted of a test arena (100 x 50 cm), in the middle of which a board (60 x 20 x 2 cm) with 23 holes (1.5 x 0.5 cm) staggered in three lines with all holes covered by movable lids was placed, thus representing the central area of the test arena as an open

field. The area around the board was divided into 12 similarly sized quadrants by lines taped onto the floor of the box (See Ohl *et al.*, 2001). Both box and board were made of dark grey PVC. All lids were closed before the start of a trial. For each trial, an unfamiliar object (a blue plastic tube lid, similar in size to the metal cube) and the familiar object (metal cube) were placed into the test arena with a distance of 2 cm between them. The illumination levels were set at approximately 150 lux in the corners and 200 lux in the middle of the test arena.

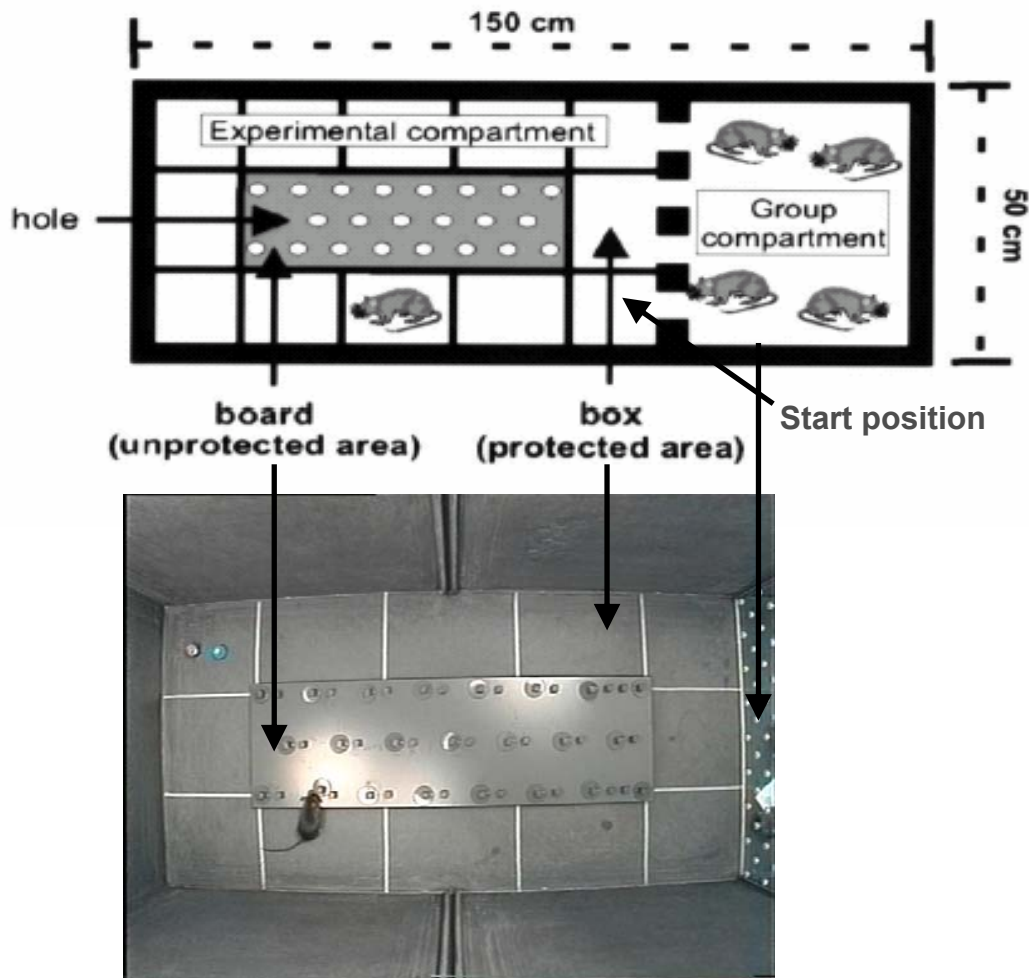


Figure 2: Test arena for modified Hole Board test.

For testing, each animal was placed individually into the test arena and allowed to explore it freely for 5 min. The animals were always placed into the test arena in the same corner next to the partition, facing the board diagonally. The two objects were placed in the corner quadrant diametrical to the starting point. During the 5 min trial, the animal's behavior was recorded by a trained observer with a hand-held computer. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen). Additionally, a camera was mounted 1.20 m above the center of the test arena, and the animal's track was videotaped and its locomotor path analyzed with a video-tracking system (Ethovision 2.3, Noldus, Wageningen). After each trial, the test arena was cleaned carefully with a disinfectant.

Data were statistically analyzed using SPSS software (SPSS Inc, Chicago, USA). The chosen level of significance was $p < 0.05$.

3.1.5 Results

Behavioral analysis of spontaneous activity in a novel environment, as measured by the **modified Hole Board** test, revealed for locomotor activity that Cs7HH mice started the exploration of the mHB later than Cs7HB mice as indicated by increased latency to first line crossing (Tab. 4). Although only as a *tendency*, the genotype differentially affected both sexes in the frequency of turns (Table 5) as well as in the angular velocity (Table 5): while Cs7HH females did not differ from Cs7HB females, Cs7HH males made less turns and exhibited reduced angular velocity compared to Cs7HB males.

Concerning anxiety-related behavior, the maximum duration of stay on board was enhanced in Cs7HH mice of both sexes (Table 5), while the average time spent on board was enhanced only in Cs7HH females compared to Cs7HB females, whereas males showed the opposite trend (Table 4). Exploration of the holes on the board showed the same sex-specific trends in direction, but this effect was only a *tendency* (hole exploration frequency, Table 4).

Regarding social affinity, Cs7HH mice of both sexes spent less time at the partition than Cs7HB mice (group contact total duration, Table 4), while the number of approaches as well as the latency to the first visit at the partition did not differ (group contact frequency and latency, Table 4).

There were no genotype effects on any other observed parameter (Table 3).

3.1.6 Discussion

The primary behavioral observation in the modified Hole Board as a novel environment demonstrated only a subtle effect of the transfer of chromosome 7 from C3H/HeNCrI into C57BL/6NCrI background on spontaneous forward locomotor activity. This conclusion is based on the observation that although Cs7HH mice of both sexes started to explore the novel environment later and Cs7HH males additionally with reduced turning behavior (reduced turn frequency and angular velocity), firstly, the sex-specific effect of the chromosome transfer was a *tendency* only and, secondly, the general locomotion-related parameters line crossing frequency, total distance travelled and speed of movement remained unchanged.

On the other hand, Cs7HH mice of both sexes exhibited reduced social behavior indicated by less time spent at the partition. In addition, Cs7HH mice, particularly Cs7HH females, spent more time in the centre of the mHB – namely on the board – suggesting reduced anxiety-related behavior. Whether the alterations in social and anxiety-related behavior are related or reflect two phenotypes can not be decided from this data set, but we speculate that they are related.

Table 3: Evaluation of the behavioral phenotype	
Behaviors which are considered affected in consomic animals due to the pattern of significantly altered parameters are marked in red.	
Behavior	Measured parameters
Forward locomotor activity	Line crossings (latency), Total distance traveled
Vertical exploratory behavior	Rearings in the box (number, latency), Rearings on the board (number)
Speed of movement	Mean and maximum velocity
Immobility	Time spent immobile
Risk assessment	Stretched attends
Anxiety-related behavior	Latency until first board entry, Time spent on board (females), Maximum duration of board entry, Board entries
Horizontal exploratory behavior	Hole exploration, object exploration (obi);
Grooming behavior	Latency to grooming, Time spent grooming, Number of groomings
Defecation	Latency to defecation, Number of boli
Social affinity	Group contacts (latency), Time spent at partition
Familiar object exploration	Latency to obj. expl., Time spent in obj. expl., Number of obj. expl.
Unfamiliar object exploration	Latency to obj. expl., Time spent in obj. expl., Number of obj. expl.

Taken together, Cs7HH mice of both sexes showed subtle alterations in locomotion and reductions in anxiety-related behavior and social affinity in the mHB. Interestingly, QTL were mapped on mouse chromosome 7 that control AVP and CRF-R1 transcript abundance, which may indicate loci that coordinate regulation of the CRF system (Garlow *et al.*, 2005). Hormones of the hypothalamic-pituitary-adrenal axis are known to be involved in stress responses, anxiety-related and social behaviors (for reviews see Summers, 2001 and File & Seth, 2003).

If the mouse provider wanted to follow up the observations made here, we suggest measurement of stress hormones in these mice, particularly corticosterone.

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Table 4: Results of behavioral observation in the modified Hole Board test

Data are presented as mean \pm standard error of the mean.

Yellow indicate significant alterations only in one sex.

Parameter	Cs7HB		Cs7HH		Male + Female		ANOVA		
	Male	Female	Male	Female	Cs7HB	Cs7HH	sex	genotype	Interaction
	(n=13)	(n=15)	(n=15)	(n=15)	(n=28)	(n=30)			
Line crossing [frequency]	142.23 \pm 6.21	133.93 \pm 5.55	128.64 \pm 6.85	129.27 \pm 4.32	137.79 \pm 4.14	128.97 \pm 3.92	n.s.	n.s.	n.s.
Line crossing [latency]	1.07 \pm 0.21	0.97 \pm 0.05	1.52 \pm 0.23	1.71 \pm 0.37	1.01 \pm 0.1	1.62 \pm 0.22	n.s.	p<0.05	n.s.
Rearings in box [frequency]	45.92 \pm 3.01	37.33 \pm 2.08	38.29 \pm 3.52	35.47 \pm 2.08	41.32 \pm 1.94	36.83 \pm 1.99	p<0.05	n.s.	n.s.
Rearings in box [latency]	19.57 \pm 3.63	26.32 \pm 4.49	28.02 \pm 4.22	25.23 \pm 3.18	23.19 \pm 2.96	26.58 \pm 2.58	n.s.	n.s.	n.s.
Hole exploration [frequency]	40.15 \pm 4.16	43.27 \pm 3.66	34.21 \pm 2.96	50.67 \pm 2.99	41.82 \pm 2.72	42.72 \pm 2.58	p<0.01	n.s.	p = 0.06
Hole exploration [latency]	30.42 \pm 5.79	21.4 \pm 4.04	30.84 \pm 7.12	19.51 \pm 4.66	25.59 \pm 3.49	24.98 \pm 4.26	n.s.	n.s.	n.s.
Hole visit [frequency]	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	n.s.	n.s.	n.s.
Hole visit [latency]	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	n.s.	n.s.	n.s.
Board entry [frequency]	10.08 \pm 1.33	10.73 \pm 1.16	9.14 \pm 1.68	10.6 \pm 0.98	10.43 \pm 0.86	9.9 \pm 0.95	n.s.	n.s.	n.s.

Table 4: Results of behavioral observation in the modified Hole Board testData are presented as mean \pm standard error of the mean.

Yellow indicate significant alterations only in one sex.

Parameter	Cs7HB		Cs7HH		Male + Female		ANOVA		
	Male	Female	Male	Female	Cs7HB	Cs7HH	sex	genotype	Interaction
	(n=13)	(n=15)	(n=15)	(n=15)	(n=28)	(n=30)			
Board entry [latency]	74.19 \pm 10.46	56 \pm 8.67	84.39 \pm 18.19	69.18 \pm 10.53	64.45 \pm 6.82	76.52 \pm 10.24	n.s.	n.s.	n.s.
Board entry [total duration %]	8.5 \pm 1.21	8.68 \pm 0.65	7.48 \pm 1.18	11.88 \pm 1.1	8.6 \pm 0.65	9.76 \pm 0.89	p<0.05	n.s.	p<0.05
Rearing on board [frequency]	2.15 \pm 0.61	1 \pm 0.26	1.43 \pm 0.75	2.6 \pm 0.92	1.54 \pm 0.33	2.03 \pm 0.6	n.s.	n.s.	n.s.
Rearing on board [latency]	227.01 \pm 19.26	225.38 \pm 21.38	250.96 \pm 20.88	210.61 \pm 21.29	226.14 \pm 14.27	230.09 \pm 15.15	n.s.	n.s.	n.s.
Risk assessment [frequency]	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	n.s.	n.s.	n.s.
Risk assessment [latency]	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	n.s.	n.s.	n.s.
Group contact [frequency]	15.31 \pm 1.24	14.8 \pm 1.04	16.43 \pm 1.19	12.33 \pm 0.9	15.04 \pm 0.79	14.31 \pm 0.82	p<0.05	n.s.	n.s.
Group contact [latency]	9.82 \pm 2.45	10.93 \pm 2.53	7.86 \pm 2.11	13.41 \pm 2.51	10.41 \pm 1.74	10.73 \pm 1.7	n.s.	n.s.	n.s.
Group contact [total duration %]	8.84 \pm 0.83	9.03 \pm 0.83	8.07 \pm 0.88	6.1 \pm 0.58	8.94 \pm 0.58	7.05 \pm 0.54	n.s.	p<0.05	n.s.

Table 4: Results of behavioral observation in the modified Hole Board testData are presented as mean \pm standard error of the mean.

Yellow indicate significant alterations only in one sex.

Parameter	Cs7HB		Cs7HH		Male + Female		ANOVA		
	Male	Female	Male	Female	Cs7HB	Cs7HH	sex	genotype	Interaction
	(n=13)	(n=15)	(n=15)	(n=15)	(n=28)	(n=30)			
Grooming [frequency]	1.46 \pm 0.39	1 \pm 0.34	1.21 \pm 0.24	0.53 \pm 0.22	1.21 \pm 0.25	0.86 \pm 0.17	n.s.	n.s.	n.s.
Grooming [latency]	198.32 \pm 23.35	226.39 \pm 21.14	207.29 \pm 17.88	270.61 \pm 14.97	213.36 \pm 15.62	240.04 \pm 12.86	p<0.05	n.s.	n.s.
Grooming [total duration %]	1.55 \pm 0.41	0.92 \pm 0.4	1.65 \pm 0.47	0.48 \pm 0.3	1.21 \pm 0.29	1.04 \pm 0.29	p<0.05	n.s.	n.s.
Defecation [frequency]	0.85 \pm 0.32	0.53 \pm 0.13	0.71 \pm 0.27	0.6 \pm 0.19	0.68 \pm 0.16	0.66 \pm 0.16	n.s.	n.s.	n.s.
Defecation [latency]	180.13 \pm 38.54	153.17 \pm 37.75	176.76 \pm 39.51	163.52 \pm 39.01	165.69 \pm 26.63	169.91 \pm 27.3	n.s.	n.s.	n.s.
Unfamiliar object exploration [frequency]	7.23 \pm 0.58	8.8 \pm 0.84	6.79 \pm 0.6	7.47 \pm 0.77	8.07 \pm 0.54	7.14 \pm 0.49	n.s.	n.s.	n.s.
Familiar object exploration [frequency]	7.92 \pm 0.64	7.8 \pm 0.77	7.86 \pm 0.64	8.6 \pm 0.81	7.86 \pm 0.5	8.24 \pm 0.51	n.s.	n.s.	n.s.
Unfamiliar object exploration [latency]	23.88 \pm 3.48	26.74 \pm 6.86	29.81 \pm 6.98	22.7 \pm 2.89	25.41 \pm 3.96	26.13 \pm 3.68	n.s.	n.s.	n.s.

Table 4: Results of behavioral observation in the modified Hole Board testData are presented as mean \pm standard error of the mean.

Yellow indicate significant alterations only in one sex.

Parameter	Cs7HB		Cs7HH		Male + Female		ANOVA		
	Male	Female	Male	Female	Cs7HB	Cs7HH	sex	genotype	Interaction
	(n=13)	(n=15)	(n=15)	(n=15)	(n=28)	(n=30)			
Familiar object exploration [latency]	19.93 \pm 2.24	28.52 \pm 8.74	22.23 \pm 5.15	21.16 \pm 2.59	24.53 \pm 4.79	21.68 \pm 2.77	n.s.	n.s.	n.s.
Unfamiliar object exploration [total duration %]	1.32 \pm 0.11	1.84 \pm 0.2	1.44 \pm 0.23	1.85 \pm 0.28	1.6 \pm 0.13	1.66 \pm 0.18	p<0.05	n.s.	n.s.
Familiar object exploration [total duration %]	1.01 \pm 0.11	1.14 \pm 0.14	0.95 \pm 0.08	1.32 \pm 0.15	1.08 \pm 0.09	1.14 \pm 0.09	p<0.05	n.s.	n.s.
Object Index	0.14 \pm 0.04	0.22 \pm 0.07	0.15 \pm 0.06	0.13 \pm 0.06	0.18 \pm 0.04	0.14 \pm 0.04	n.s.	n.s.	n.s.

Table 5: Video-tracking results regarding locomotor behavior

Data are presented as mean \pm standard error of mean.

Yellow indicate significant alterations only in one sex.

Parameter	Cs7HB		Cs7HH		Male + Female		ANOVA		
	Male	Female	Male	Female	Cs7HB	Cs7HH	sex	genotype	Interaction
	(n=13)	(n=15)	(n=15)	(n=15)	(n=28)	(n=30)			
Total Distance Moved [cm]	3562.32 \pm 121.78	3261.02 \pm 158.19	3207.57 \pm 153.1	3358.83 \pm 94.13	3400.91 \pm 104.15	3285.81 \pm 88.03	n.s.	n.s.	n.s.
Mean Velocity [cm/sec]	21.14 \pm 0.51	21.15 \pm 0.47	20.4 \pm 0.6	20.84 \pm 0.35	21.15 \pm 0.34	20.62 \pm 0.34	n.s.	n.s.	n.s.
Maximum Velocity [cm/sec]	71.95 \pm 1.48	74 \pm 1.86	73.57 \pm 2.44	71.16 \pm 1.21	73.05 \pm 1.21	72.32 \pm 1.33	n.s.	n.s.	n.s.
Turns [Frequency]	1788.69 \pm 32.82	1634 \pm 81.78	1639.64 \pm 50.96	1705.47 \pm 37.67	1705.82 \pm 47.96	1673.69 \pm 31.43	n.s.	n.s.	p=0.05
Mean Turn Angle [degrees]	26.9 \pm 0.72	24.07 \pm 0.57	26.07 \pm 0.36	25.15 \pm 0.73	25.38 \pm 0.52	25.6 \pm 0.42	p<0.01	n.s.	n.s.
Angular Velocity [degrees/sec.]	191.11 \pm 5.6	169.19 \pm 5.18	172.91 \pm 2.07	168.8 \pm 5.18	179.36 \pm 4.28	170.78 \pm 2.84	p<0.01	p=0.06	p=0.07
Absolute Meander [degrees/sec.]	19.4 \pm 0.58	17.4 \pm 0.48	19.14 \pm 0.38	18.42 \pm 0.6	18.33 \pm 0.41	18.77 \pm 0.36	p<0.01	n.s.	n.s.
Board entry [maximum duration. sec.]	6.32 \pm 0.57	6.72 \pm 0.51	7.01 \pm 0.74	10.01 \pm 1.17	6.53 \pm 0.37	8.56 \pm 0.75	p<0.05	p<0.05	n.s.
Mean distance to wall [cm]	7.17 \pm 0.21	7.15 \pm 0.25	7.11 \pm 0.27	7.79 \pm 0.19	7.16 \pm 0.16	7.46 \pm 0.17	n.s.	n.s.	n.s.
Mean distance to board [cm]	8.53 \pm 0.16	8.64 \pm 0.2	8.57 \pm 0.22	8.2 \pm 0.1	8.59 \pm 0.13	8.38 \pm 0.12	n.s.	n.s.	n.s.

3.2 Dysmorphology, Bone and Cartilage

3.2.1 Introduction

In the Dysmorphology, Bone and Cartilage Screen of the German Mouse Clinic mice are analyzed for morphological abnormalities in different organ systems with special focus on bone and cartilage development and homeostasis. The aim of the screen is to establish mouse models for human skeletal diseases like osteoporosis (McLean & Olsen, 2001; Rosen *et al.*, 2001), scoliosis (Giampietro *et al.*, 2003), limb defects (Mariani & Martin, 2003), osteogenesis imperfecta (Chipman *et al.*, 1993) or osteoarthritis (Abe *et al.*, 2006). We adapted the successful dysmorphological screening protocol from the Munich ENU-Mutagenesis Screen (Hrabé de Angelis *et al.* 2000) for use in the German Mouse Clinic. The nomenclature of the parameters was adapted according to the Mammalian Phenotype Ontology wording (www.informatics.jax.org/searches/MP_form.shtml). Further tests for defects in bone development and homeostasis were taken over from human diagnosis, and were adapted for the use in mice analysis. Such tests include: X-ray analysis, bone densitometry, and, in special cases, micro computed tomography. Detailed protocols for screening for bone and cartilage phenotypes in mice are described in Fuchs *et al.* (2006).

3.2.2 Summary

In the morphological investigation via visual inspection and X-ray analysis, no genotype-specific differences were found. In the dual-energy X-ray absorptiometry (DXA) analysis, only lean mass was significantly increased in female consomic animals compared to controls.

3.2.3 Mice

Thirty male (15 Cs7HB, 15 Cs7HH) and 30 female (15 Cs7HB, 15 Cs7HH) mice were analyzed by morphological inspection at the age of 9 weeks. 16-week-old consomic animals (20 animals) and controls (20 animals) entered the bone density and X-ray analysis.

3.2.4 Material and Methods

The Dysmorphology, Bone and Cartilage module of the German Mouse Clinic analyzed the mice in different phases:

1. At the age of five weeks, i.e. when the mice entered the facility, the general physical condition and health were checked,
2. at the age of nine weeks, a morphological observation as a whole-body checkup was performed; and
3. at the age of 16 to 17 weeks, X-ray analysis and bone densitometry were performed.

Morphological Observation

The animals were screened using the protocol for morphological analysis from Fuchs *et al.* (2000) as adapted for the German Mouse Clinic.

Using a clickbox (supplied by the MRC Institute of Hearing Research, Nottingham, UK) we tested the mice's ability to hear a sound of 20 kHz. The reaction of the animals was classified into six categories (0=no reaction at all, 1=no Preyer reflex, 2= retarded reaction, 3= normal reaction, 4= strong reaction, 5= particularly strong reaction).

X-ray Images

Equipment: Faxitron X-ray Model MX-20 (Specimen Radiography System, Illinois, USA),

NTB Digital X-ray Scanner EZ 40 (NTB GmbH, Diepholz, Germany),

Quality control: Calibration of the system is done in monthly intervals,

Settings: Voltage 25 kV, integration time 40 ms,

Procedure: The anesthetized mouse was fixed on an X-ray-permeable plate and placed in the machine. Using iX-Pect software supplied by the manufacturer of the X-ray scanner, the image was taken and analyzed. Analysis was done qualitatively by visual inspection of the images as well as quantitatively by using the ruler tool of iX-Pect software.

Bone density analysis

Equipment: pDEXA Sabre X-ray Bone Densitometer (Norland Medical Systems. Inc., Basingstoke, Hampshire, UK; distributed by Stratec Medizintechnik GmbH, Pforzheim, Germany),

Quality control: Calibration of the system was done in daily intervals using the QC and the QA phantoms delivered by the manufacturer. Results from the quality control were recorded by the system.

Settings: Scan speed 20 mm/s, Resolution 0.5 mm x 1.0 mm, HAW 0.020

Procedure: After anesthesia, the weight and length of the mouse were recorded, and the mouse was placed in the analyzer. After a scout scan, the area of interest was optimized and the measure scan started.

Data-analysis: For analysis of the data, regions have to be defined. The standard analysis comprises a whole body analysis as well as a whole body analysis excluding the skull.

Statistical analysis of data

Analysis of quantitative data sets was carried out using StatView software package (SAS Corporation).

3.2.5 Results and Discussion

Sixty animals of Cs7 consomic strain were analyzed in the Dysmorphology, Bone and Cartilage module of the German Mouse Clinic. In the morphological investigation via visual inspection and X-ray analysis, no genotype-specific differences were found (Tables 6 and 7). In the Clickbox test (Table 8) to test the hearing ability of the mice, we observed a normal reaction in consomic animals and controls. In the bone densitometry using DXA analysis (Table 9),

only lean mass was significantly increased in female Cs7HH animals compared to Cs7HB controls. The sex differences we observed are common in many mouse strains, and thus are not abnormal (unpublished data).

3.2.6 References

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Abbreviations

BMC	bone mineral content
BMD	bone mineral density
DXA	dual-energy X-ray absorptiometry
μ CT	micro computed tomography
pQCT	peripheral quantitative computed tomography
pBMD	partial bone mineral density (excluding skull)
sBMD	specific bone mineral density

Table 6: Results from the morphological inspection (nine-week old mice)

Parameter	Male		Female	
	Cs7HB	Cs7HH	Cs7HB	Cs7HH
Growth				
normal	15	15	15	15
Weight				
normal	15	15	15	15
Body size				
normal	15	15	15	15
Eyes				
normal	15	15	15	15
Coat hair growth				
normal	15	15	15	15
Coat hair texture				
normal	15	15	15	15
Hair follicle structure / orientation				
normal	15	15	15	15
Skin pigmentation				
normal	15	15	15	15
Skin texture / condition				
normal	15	15	15	15
Vibrissae				
normal	15	15	15	15
Limbs				
normal	15	15	15	15
Digits				
normal	15	15	14	15
one digit missing	-	-	1	-
Tail				
normal	15	15	15	15
Teeth				
normal	15	13	15	15
maxillary ones bright (nearly white)	-	2	-	-
Ear morphology				
normal	15	15	15	15
Musculature				
normal	15	15	15	15
Seizures / epilepsy				
no	15	15	15	15
Motor capabilities / coordination				
normal	15	15	15	15
Movement				
normal	15	15	15	15
Feeding / drinking behavior				

normal	15	15	15	15
Respiratory system				
normal	15	15	15	15
Reproductive system				
normal	15	15	15	15
Other abnormalities				
no	15	15	15	15
Animals analyzed	15	15	15	15

Table 7: Results from the X-ray analysis (16-week old mice)

Parameter	Male		Female	
	Cs7HB	Cs7HH	Cs7HB	Cs7HH
Skull shape				
normal	10	10	10	10
Mandibles				
normal	10	10	10	10
Maxilla				
normal	10	10	10	10
Teeth				
normal	10	10	10	10
Orbit				
normal	10	10	10	10
Number of cervical vertebrae				
normal	10	10	10	10
Number of thoracic vertebrae				
normal	10	10	10	10
Number of lumbar vertebrae				
6	10	10	10	10
Number of pelvic vertebrae				
normal	10	10	10	10
Number of sacral vertebrae				
normal	10	10	10	10
Vertebrae shape				
normal	10	10	10	8
hunchback	-	-	-	2
Number of ribs				
26	10	10	10	10
Rib shape				
normal	10	10	10	10
Scapulas				
normal	10	10	10	10
Clavicle				
normal	10	10	10	10
Pelvis				

normal	10	10	10	10
Femur shape				
normal	10	10	10	10
Tibia				
normal	10	10	10	10
Fibula				
normal	10	10	10	10
Humerus				
normal	10	10	10	10
Ulna				
normal	10	10	10	10
Radius				
normal	10	10	10	10
Number of digits				
normal	10	10	10	10
Completeness of digits				
yes	10	10	10	10
Joints				
normal	9	10	10	10
calcaneus buckled	1	-	-	-
Animals analyzed	10	10	10	10

Table 8: Results from clickbox test (hearing test; nine-week old mice)

Phenotype	Male		Female	
	Cs7HB	Cs7HH	Cs7HB	Cs7HH
0	-	-	-	-
1	-	-	-	1
2	-	2	1	1
3	15	13	14	13
4	-	-	-	-
5	-	-	-	-
Mean Score	3.00	2.87	2.93	2.80

Kruskal-Wallis ANOVA on Ranks: n.s.

0: no reaction at all,
1: very slow reaction,
2: retarded reaction,
3: normal reaction,
4: strong reaction,
5: extremely excited

Table 9: Bone- and weight-related quantitative parameters (17/18-week old mice)
 (data presented as mean \pm standard error of mean)

Parameter	Cs7 HB		Cs7 HH		Cs7HH ~ Cs7HB	Cs7HH ~ Cs7HB	ANOVA		
	Male	Female	Male	Female	Male	Female	<i>p</i> – value genotype	<i>p</i> – value sex	<i>p</i> – value interaction
	(n=10)	(n=10)	(n=10)	(n=10)	<i>p</i> – value	<i>p</i> – value			
BMD [mg/cm²]	58 \pm 1	57 \pm 2	60 \pm 2	57 \pm 2	n.s.	n.s.	n.s.	n.s.	n.s.
pBMD [mg/cm²]	48 \pm 1	46 \pm 2	49 \pm 1	47 \pm 1	n.s.	n.s.	n.s.	n.s.	n.s.
BMC [mg]	655 \pm 27	559 \pm 22	600 \pm 34	589 \pm 26	n.s.	n.s.	n.s.	n.s.	n.s.
Bone Content [%]	2.28 \pm 0.06	2.35 \pm 0.08	2.08 \pm 0.11	2.40 \pm 0.09	n.s.	n.s.	n.s.	< 0.05	n.s.
Body Length [cm]	9.05 \pm 0.05	9.15 \pm 0.08	9.05 \pm 0.05	9.10 \pm 0.07	n.s.	n.s.	n.s.	n.s.	n.s.
Body Weight [g]	28.74 \pm 0.68	23.75 \pm 0.43	28.89 \pm 0.68	24.50 \pm 0.50	n.s.	n.s.	n.s.	< 0.0001	n.s.
Fat mass [units]	6.58 \pm 0.81	3.20 \pm 0.39	5.10 \pm 0.50	2.76 \pm 0.45	n.s.	n.s.	n.s.	< 0.0001	n.s.
Fat Content [units x 100/g]	22.66 \pm 2.60	13.40 \pm 1.48	17.69 \pm 1.61	11.14 \pm 1.74	n.s.	n.s.	n.s.	< 0.001	n.s.
Lean mass [units]	19.03 \pm 0.74	17,58 \pm 0.45	20.79 \pm 0.83	18.91 \uparrow \pm 0.43	n.s.	< 0.05	< 0.05	< 0.05	n.s.
Lean Content [units x 100/g]	66.43 \pm 2.67	74.07 \pm 1.57	71.89 \pm 1.89	77.26 \pm 1.56	n.s.	n.s.	< 0.05	< 0.01	n.s.

3.3 Neurology Screen

3.3.1 Introduction

Neurological dysfunction results in a wide variety of disorders ranging from impaired movement to severe mental illness. Studying the neurobehavioral phenotype of consomic mice is a powerful tool to understand the neural basis of behavior and the pathophysiology of neurological disorders. Comparison of the mouse and human brain transcriptomes shows a good correlation for highly expressed genes in both transcript identity and abundance. Therefore, screening of mice with respect to neurological disorders potentially offers an understanding of etiology and pathogenesis of the human nervous system (Hafezparast *et al.*, 2002).

The primary observation screen is a modification of the Irwin procedure (Irwin, 1968) and was proposed as a rapid, comprehensive and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in a mouse strain (Rogers *et al.*, 1997). Dependant upon results of this primary screen and due to specific questions, additional tests can be carried out for further assessment of neurological functions in a hierarchical way (Schneider *et al.*, 2006).

3.3.2 Summary

Mice were analyzed according to our modified SHIRPA protocol where a battery of behavioral tests is carried out. We analyzed the mice using 23 designed test parameters (See web page: http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html) to detect phenotypic differences between Cs7HH and Cs7HB mice. Each test parameter contributes to an overall assessment in muscle, lower motor neuron, spinocerebellar, sensory and autonomic function. The primary neurological screen is focused on investigating neurological reflexes to determine the neurological functioning of a mouse. Moreover, we measure forelimb grip strength to evaluate muscle function.

All SHIRPA parameters were without pathological findings. No neurological abnormalities were detected. Comparing the forelimb grip strength revealed significantly increased force in Cs7HH females.

3.3.3 Mice

Fifteen 10-week-old male Cs7HH and 15 male Cs7HB mice entered the neurological screen at the beginning of the 37th calendar week 2006. Fifteen 10-week-old female Cs7HH and 15 female Cs7HB mice entered the neurological laboratory one week later. All animals were fed *ad libitum* for a period of one week during their stay in the neurological screen.

3.3.4 Material and Methods

Primary screening 1: modified SHIRPA protocol

Assessment of each animal at age 10 weeks began with observation of undisturbed behavior (*Viewing Jar Behavior*) in a glass cylinder (11 cm in diameter). The mice were then transferred to an arena consisting of a clear Perspex box (420 x 260 x 180 mm) in which a Perspex sheet on the floor is marked with 15 squares. Locomotor activity and motor behavior within this area was observed (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes (*Behavior recorded on or above the arena*). Measurements were completed with the recording of body weight. The last part of the primary screen also involved the analysis of righting reflex, and contact righting reflex. A glass cylinder (35 mm diameter, 135 mm length) was used for testing of the contact righting reflex. Throughout the entire procedure, abnormal behavior, biting, defecation, and vocalization were recorded. Between testing of each mouse, fecal pellets and urination were removed from the viewing jar and arena. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior to testing (Schneider *et al.*, 2006).

Primary screening 2: grip strength

The grip strength meter system determines the fore limb grip strength, i.e. muscle strength of a mouse. The device exploits the tendency of a mouse to grasp a horizontal metal bar while being pulled by its tail. During the trial setup, the mouse grasps a special adjustable grip (2 mm) mounted on a force sensor. The sensor allows measurements of up to 600 Ponds. Five trials were undertaken for each mouse within one minute. The mean value is used to represent the grip strength of a mouse.

All experimental equipment was thoroughly cleaned with Pursept-A and dried prior subsequent tests. Values were presented as means \pm standard error of mean (SEM).

Statistical analysis of the grip strength trial results. Grip strength trial results are compared between genotypes, controlling for the effects of sex and weight, by fitting linear mixed effect models (Pinheiro and Bates, 2000). A linear mixed effect model is a modified analysis of variance/covariance approach allowing for dependencies in the data. In our case, dependencies arise from repeated trials within each mouse. Genotype, sex and weight are modeled as fixed effects. Mouse-specific intercepts are modeled by including the intercept as random effect. Interaction effects are tested for and included in the model if they show a significant contribution. A serial dependency on the trial number can be tested by including the trial number as random effect with an autoregressive correlation structure. Model fitting is performed by the nlme-Package in the open-source statistical software R, a close relative of S-PLUS (The R Project for Statistical Computing, 2004). The p-value for the genotype effect within the specific model found for the data indicates the significance of the statistical test of interest; a confidence interval for the genotype effect can also be extracted.

Secondary Screening: Rotarod test

The TSE-RotaRod 3375 apparatus (Accelerating Model, TSE, Bad Homburg) was used to measure fore limb and hind limb motor coordination, balance and motor learning ability (Jones and Roberts, 1968). The machine was set up in an environment with minimal stimuli such as noise and movement.



Figure 3: The rotarod apparatus

The rotarod device is equipped with a computer controlled motor-driven rotating rod. The unit consists of a rotating spindle and four individual lanes for each mouse (Fig. 3). The software allows pre-programming of session protocols with varying rotational speeds. Infrared beams are used to detect when a mouse falls onto the grids beneath the rotarod.

In general, the mouse is placed perpendicular to the axis of rotation, with head facing the direction of the rotation. All mice were placed on the Rotarod at an accelerating speed from 4 to 40 rpm for 300 sec with 20 min between each trial. In motor coordination testing, mice were given four trials at the accelerating speed at one day. The mean latency to fall off the Rotarod during the trials was recorded and used in subsequent analysis. Before the start of the first trial, mice were weighed.

Statistical analysis of the Rotarod performance results

The Rotarod data contain dependencies, which are more complex than the grip strength data. Repeated measurements arise from four different trials with a break in between. To compare the performance results between genotypes, linear mixed-effect models are fitted, that allow for the dependencies of genotype and trial and for the effects of sex and weight. The latter are modeled as fixed effects. Interaction effects are considered and included in the model, if necessary.

In each model, the parameter of interest is the coefficient of the genotype effect. A significance test or a confidence interval for this coefficient can be extracted from the model fitted.

3.3.5 Parameters

Muscle/lower motor neuron function
Body position, gait, Positional passivity, tail elevation, grip strength, defecation
Spinocerebellar function
Body position, gait, righting reflex, tail elevation, grip strength
Sensory function
Transfer arousal, touch escape, gait, pinna reflex, righting reflex
Autonomic function
Palpebral closure, defecation, lacrimation
Neurological reflexes
Righting reflex (pons), contact righting reflex, pinna reflex
General appearance
Body weight, body position, transfer arousal, touch escape, vocalization, positional passivity, aggression, spontaneous activity, locomotor activity, skin color

3.3.6 Results

All **SHIRPA parameters** were without significant findings besides the increased body weight of Cs7HH mice with females being more affected. No neurological abnormalities were detected.

Comparing the **forelimb grip strength** revealed increased force in Cs7HH females ($p < 0.05$; Fig. 4).

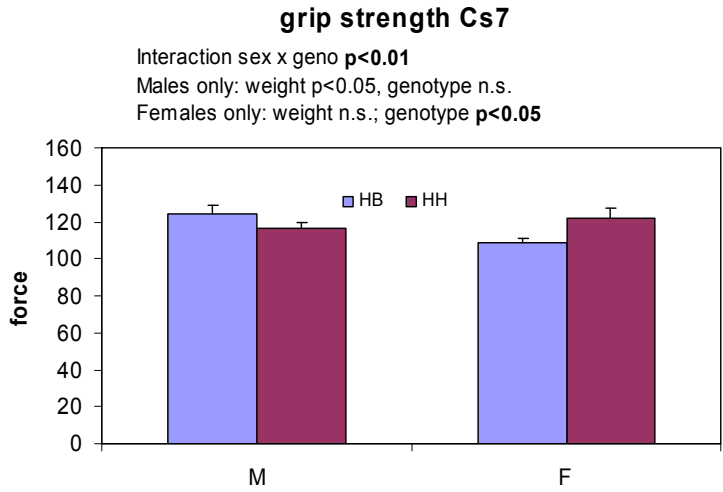


Figure 4: Results from grip strength testing

Plotting the weight of the animals versus the force (“Trial”) shows the weight – dependency of the grip force (Fig. 5).

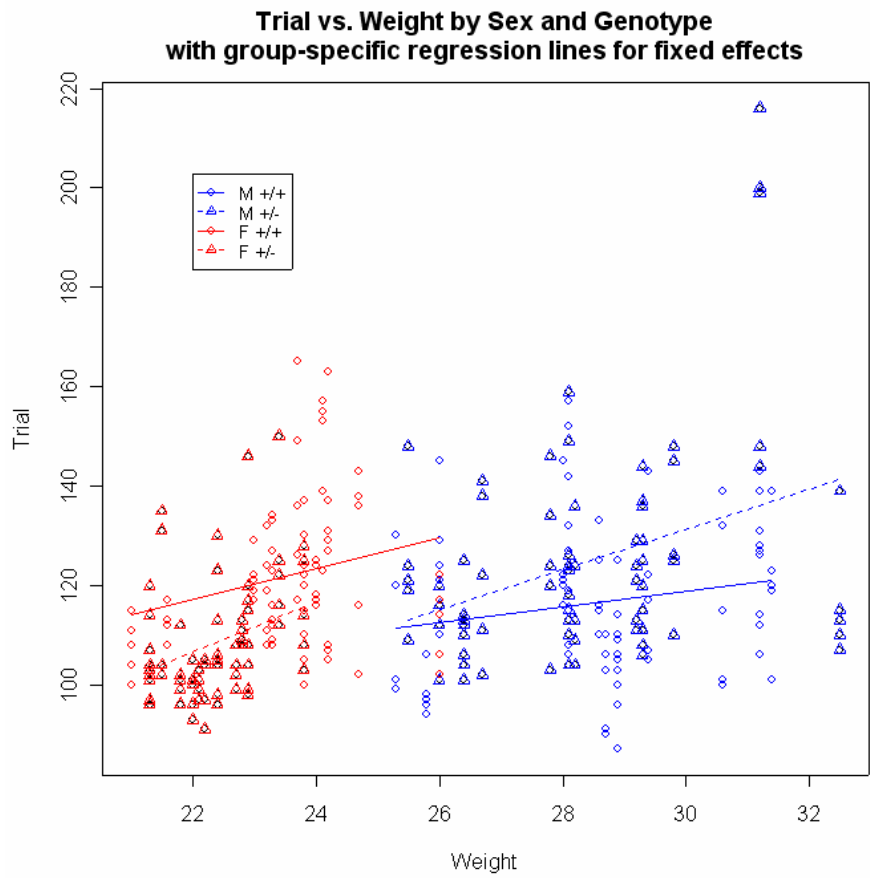


Figure 5: Weight versus force
 (“Trial”; +/+ depicts HH, +/- Cs7HB)

In addition, we tested male Cs7 mice with the **accelerating rotarod**. Mean performance as a measure for motor coordination and balance did not show relevant differences between the genotypes.

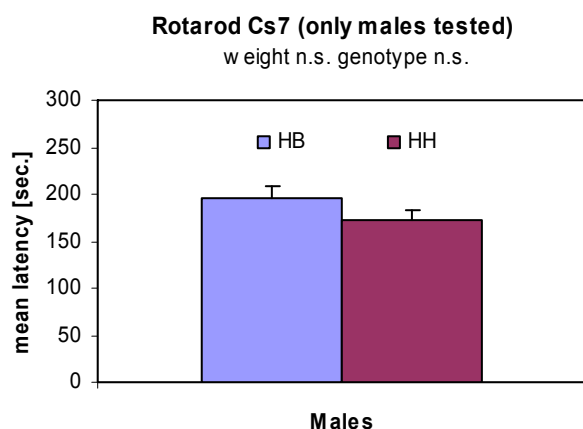


Figure 6: Results from the accelerating rotarod

3.3.7 Discussion

In our primary screen we look especially for neurological dysfunctions. Male and female Cs7 consomic mice of course did not show any neurological abnormalities. However, quantitative analysis of grip strength and rotarod performance allows also detection of non-pathological alterations.

C57BL/6 and C3H mice differ in several parameters (Schneider *et al*, 2006). For example grip strength was described to be higher in C3H than in C57BL/6 (Grubb *et al.*, 2004) whereas rotarod performance is better in C57BL/6 (Brooks *et al.*, 2004; McFayden *et al.*, 2003). However these differences are often a consequence of the altered body weight (Maurissen *et al*, 2003; McFayden *et al.*, 2003). For grip force we often observe increased force in heavier animals as we also observe here (Fig. 3).

Cs7HB as well as Cs7HH mice of both sexes show this dependency but in female mice there seem to be an additional genotype-effect. Since it is difficult to evaluate clearly if the weight has a linear effect on force, a small difference in grip force as observed here should not be over-interpreted. But the slope of the group-specific regression lines might be different.

In the rotarod test the slightly heavier Cs7HH mice showed also a slightly decreased latency.

Taken together, the replacement of Chromosome 7 in C57BL/6NCrI mice with the respective one from C3H/HeNCrI does result in some alterations although the control animals still harbor one copy of chromosome 7. Consomic strains allow for the analysis of complex traits and Chromosome 7 has been described to determine phenotypes of the immune system, the skeleton, the

cardiovascular system as well as tumorigenesis (provider info). C3H and C57BL/6 also differ in several behavioral and neurological characteristics but the impact of chromosome 7 has not been analyzed in-dept so far.

At the moment the differences found here are regarded to be mainly caused by the different weight of the animals, but we cannot exclude other strain effects. And one might speculate if the differences would be more pronounced when comparing the Cs7 mice with pure C57BL/6NCrl chromosome 7.

3.3.8 References

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Abbreviations

SHIRPA **S**mithKline Beecham Pharmaceuticals, **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit, **I**mperial College School of Medicine at St Mary's **R**oyal London Hospital, St Bartholomew's and the Royal London School of Medicine **P**henotype **A**ssessment
http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html

Table 10: Recording of body weight							
Data are presented as mean ± standard error of mean.							
Parameter	Male			Female			both
	Cs7HB (n=15)	Cs7HH (n=15)	p-value	Cs7HB (n=15)	Cs7HH (n=15)	p-value	p-value
Body Weight [g]	26.4±0.5	26.8±0.4	<i>n.s.</i>	22.4±0.2	23.6±0.3	<0.01	<0.05

Table 11: Behavior recorded in viewing jar							
Statistical analysis: chi-squared test; significance p<0.05							
Parameter	Male			Female			both
	Cs7HB (n=15)	Cs7HH (n=15)	p-value	Cs7HB (n=15)	Cs7HH (n=15)	p-value	p-value
Body Position							
Inactive	0	0		0	0		
Active	15	15		15	15		
Excessive Activity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Tremor							
Absent	15	15		15	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Palpebral closure							
Eyes open	15	15		15	15		
Eyes closed	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Coat Appearance							
Tidy and well groomed	15	15		15	15		
Irregularities	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Whiskers							
Present	10	15		13	13		
Absent	5	0	<i>n.s.</i>	2	2	<i>n.s.</i>	<i>n.s.</i>
Lacrimation							
Absent	15	15		15	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Defecation							
Present	15	15		15	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>

Table 12: Recording of locomotor activity and behavior in the arena

Statistical analysis: chi-squared test; significance $p < 0.05$. Locomotor activity data are shown as mean (\pm SEM)

Parameter	Male			Female			both
	Cs7HB (n=15)	Cs7HH (n=15)	<i>p</i> -value	Cs7HB (n=15)	Cs7HH (n=15)	<i>p</i> -value	<i>p</i> -value
Transfer arousal							
Extended freeze	0	0		0	0		
Brief freeze	15	15		10	10		
Immediate movement	0	0	<i>n.s.</i>	5	5	<i>n.s.</i>	<i>n.s.</i>
Locomotor activity	26.9 \pm 3.1	31.5 \pm 2.5	<i>n.s.</i>	33.0 \pm 1.8	29.5 \pm 1.7	<i>n.s.</i>	<i>n.s.</i>
Gait							
Fluid movement	15	15		15	15		
Lack Fluidity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Tail Elevation							
Dragging							
Horizontally extension	0	0		0	0		
	12	8		9	7		
Elevated/Straub tail	3	7	<i>n.s.</i>	6	8	<i>n.s.</i>	<i>n.s.</i>
Touch Escape							
No response	0	0		0	0		
Response to touch	15	15		13	15		
Flees prior to touch	0	0	<i>n.s.</i>	2	0	<i>n.s.</i>	<i>n.s.</i>
Positional Passivity							
Struggles when held by tail	15	15		15	15		
No struggle	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>

Table 13: Behavior recorded in or above the arenaStatistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female			both
	Cs7HB (n=15)	Cs7HH (n=15)	<i>p</i> - <i>value</i>	Cs7HB (n=15)	Cs7HH (n=15)	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>
Skin color							
Blanched	0	0		0	0		
Pink	15	15		15	15		
Bright deep red	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Trunk curl							
Absent	0	0		0	0		
Present	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>	<i>n.s.</i>
Limb Grasping							
Absent	15	15		15	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Pinna Reflex							
Present	15	15		15	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Corneal Reflex							
Present	15	15		15	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Righting Reflex							
Rights itself	15	15		15	15		
Fails to right when re- leased	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Contact Righting							
Present	15	15		15	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Evidence of biting							
None	14	15		15	15		
Biting in response to handling	1	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Vocalization							
None	13	8		10	8		
Vocal	2	7	<i>n.s.</i>	5	7	<i>n.s.</i>	<i>n.s.</i>

3.4 Eye Screen

3.4.1 Introduction

In the primary screen, different methods were employed to analyze the eyes of consomic mouse line in comparison to their control littermates. Mice were examined for anterior segment abnormalities by slit lamp biomicroscopy (Favor, 1983), as well as for posterior segment abnormalities by funduscopy. The axial eye length was measured by laser interference biometry (LIB; Puk *et al.*, 2006). If required, the retinal function can be tested with a high throughput electroretinography (ERG; Dalke *et al.*, 2004) in a secondary screen.

In humans blindness is caused by several different ocular diseases. Among these, the cataracts are responsible for half of all cases (Johnson and Foster, 2003). The retinal disorders cover a broad variety of clinical symptoms and many different genes are involved in the corresponding pathological conditions in humans. The two most important groups are retinitis pigmentosa (RP) and age-related-macular-degeneration (ARMD; for recent reviews, see Rivolta *et al.*, 2002 and Stone *et al.*, 2001). Mouse models are appropriate tools to understand the genetic and biochemical mechanisms of ocular disorders. There is a rapid increasing number of mouse consomic animals available suffering from various types of eye diseases (for recent reviews see Graw, 2003 and Dalke & Graw, 2005).

3.4.2 Summary

No genotype-specific differences between Cs7HH and Cs7HB mice were detected.

3.4.3 Mice

Thirty Cs7HB (15 male, 15 female) and 30 Cs7HH mice (15 male, 15 female) entered the Eye Screen at the age of 11 weeks. Mice were first examined by slitlamp biomicroscopy and funduscopy, on the following day the laser interference biometry was performed. Mice were kept under standard laboratory conditions with food and water *ad libitum*. When the mice were killed for pathological examinations (Pathology Screen, 3.12.2), the eyes of some mice were fixed for histological analysis in the eye screen.

3.4.4 Materials and Methods

Funduscopy (Ophthalmoscopy): The posterior parts of both eyes were examined by funduscopy. After pupil dilation with one drop of atropine (1%), the mouse is grasped firmly in one hand and clinically evaluated using a head-worn indirect ophthalmoscope (Sigma 150 K, Heine Optotechnik, Herrsching, Germany) in conjunction with a condensing lens (90D lens, Volk, Mentor, OH, USA) mounted between the ophthalmoscope and the eye.

Slit Lamp Biomicroscopy: Mice were examined biomicroscopically for eye abnormalities as previously described (Favor, 1983). Briefly, pupils were dilated with a 1% atropine solution applied to the eyes at least 10 min prior to examination. Both eyes of the mice were examined by slit lamp biomicroscopy (Zeiss SLM30) at 48x magnification with a narrow beam slit lamp illumination at 25-30° angle from the direction of observation. Observed phenotypic variants of the eyes were carefully documented.

Laser Interference Biometry (LIB) was performed using the “AC Master” (Meditec, Carl Zeiss, Jena, Germany) equipped with a new technique, optical low coherence interferometry (OLCI), adapted for short measurement distances (Schmucker and Schaeffel, 2004). Mice were anaesthetized with 137 mg Ketamine and 6.6 mg Xylazine per kg body weight and placed in front of the ACMaster.

Histology: Eyes were fixed 24 hours in Davidson solution, dehydrated and embedded in plastic medium. Transverse 2 µm sections were cut with an ultramicrotome, stained with methylene blue and basic fuchsin and evaluated with a light microscope.

Statistical Analysis: Laser interference biometry data were statistically analyzed using MS-Excel. Differences between mouse groups were evaluated with the Student’s t-test. Statistical significance was set at p<0.05. Data are presented as mean values ± standard error of the mean (SEM).

3.4.5 Parameters

Funduscopy
(qualitative) abnormalities of the retinal fundus and optic disc, vessel alterations and development disorders
Slit lamp biomicroscopy
(qualitative) abnormalities of lens and cornea like opacity and development disorders
Laser Interference Biometry (LIB)
axial eye length abnormalities
Histology
(qualitative) retinal lamination and morphology of cell layers and lens
Morphology
(qualitative) like size and degree of closure

3.4.6 Results and Discussion

All Cs7 mice were examined by **funduscopy**. Only minor, unilateral alterations of the fundus were detected in three mice. No abnormalities associated with the chromosome replacement were detected (Table 14).

A total of 60 mice were examined ophthalmologically by **slit lamp biomicroscopy** (Table 15). No anterior segment phenotype was shown to be associated with the replacement of Chromosome 7.

To conclude, no genotype-specific differences between Cs7HH and Cs7HB mice were detected in the Eye screen.

3.4.7 References

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Abbreviations

n.s. not significant
NAD no abnormality detected

Table 14: Results from Funduscopy				
Genotype	NAD	White dot (unilateral)	Cloudy patch (unilateral)	White streak (unilateral)
male Cs7HH (n=15)	15			
female Cs7HH (n=15)	15			
male Cs7HB (n=15)	13	1	1	
female Cs7HB (n=15)	14			1

Table 15: Results from Slit Lamp Biomicroscopy				
Genotype	NAD	Nuclear opacity	Posterior capsule opacity	Microphthalmia/ Anophthalmia
male Cs7HH (n=15)	15			
female Cs7HH (n=15)	15			
male Cs7HB (n=15)	14			1
female Cs7HB (n=15)	14			1

3.5 Clinical Chemistry and Hematology

3.5.1 Introduction

The aim of the Clinical-Chemical Screen is the detection of hematological changes, defects of various organ systems, and changes in metabolic pathways and electrolyte homeostasis by means of suitable laboratory diagnostic tools. Since most inherited metabolic disorders are known to lead directly or indirectly, via altered organ functions, to changes in the parameters investigated, this screening process provides a comprehensive investigation of clinical phenotypes with counterparts in humans and animal species (Rathkolb *et al.*, 2000). The methods used are routine procedures, allowing the appropriate screen of large numbers of mice for a broad spectrum of clinical-chemical and hematological parameters (Champy *et al.*, 2004; Hough *et al.*, 2002).

3.5.2 Summary

In the primary clinical-chemical screen, thirty (15 males / 15 females) Cs7HB control mice and thirty (15 males / 15 females) Cs7HH consomic mice were analyzed. Twenty different clinical-chemical parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we measured ten basic hematological parameters. We only detected subtle differences of unclear relevance in the clinical-chemical parameters. The hematological parameters red blood cell count, hemoglobin levels, hematocrit, as well as mean corpuscular volume were lower whereas mean corpuscular hemoglobin concentration and RDW were higher in Cs7HB control mice compared to Cs7HH consomic animals. These small differences could indicate that some of the genes responsible for strain specific differences in the red blood cell count between C57BL/6 and C3H mice are located on chromosome 7.

3.5.3 Mice

Fifteen 13-week-old control Cs7HB males and fifteen 13-week-old consomic Cs7HH males entered the clinical-chemical screen at the beginning of the 29th calendar week 2006. Fifteen 14-week-old control females and fifteen 14-week-old consomic females entered the clinical-chemical screen one week later.

3.5.4 Materials and Methods

Blood Withdrawal and Storage

The Clinical-chemical Screen of the German Mouse Clinic routinely analyzed the mice at 13 weeks of age. A blood sample was taken from an ether-anesthetized mouse by puncturing the retro-orbital sinus with a non-heparinized capillary (0.8 mm in diameter; Laborteam K&K; Munich, Germany; Art.No. 1.28.13.1.2). The time for sample taking was recorded in a work list. Blood was collected in a heparinized tube (Li-heparin, KABE; Nümbrecht, Germany; Art.No. 078028). An additional smaller sample was col-

lected (using the same capillary) in an EDTA-coated tube (KABE, Art.No 078035). Each tube was immediately inverted five times to achieve a homogeneous distribution of the anticoagulant.

The Li-heparin-coated tubes were stored in a rack at room temperature for two hours. Afterwards, cells and plasma were separated by a centrifugation step (10 min, 4656 x g; Biofuge, Heraeus; Hanau, Germany). Plasma was distributed between the Immunology Screen (30 µl), the Allergy Screen (30 µl), the Clinical-Chemical Screen (130 µl) and the Steroid Screen (residual), while the cell pellet was given to the Immunology Screen for FACS-analysis. The plasma sample for the clinical chemical analysis was transferred into an Eppendorf tube and diluted 1:2 with aqua dest. The solution was mixed for a few seconds (Vortex genie, Scientific Industries, New York, America) to prevent clotting and then centrifuged again for 10 min at 4656 x g. Additionally the Clinical-Chemical Screen received the EDTA-blood sample for hematological investigations.

Clinical Chemistry

The screen was performed using an Olympus AU 400 autoanalyzer and adapted reagents from Olympus (Hamburg, Germany) and Roche (Mannheim, Germany). In the primary screen, 20 different parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes.

Hematology

A volume of 50 µl EDTA-blood was used to measure basic hematological parameters with a blood analyzer, which has been carefully validated for the analysis of mouse blood (ABC-Blutbild-Analyzer, Scil Animal Care Company GmbH; Viernheim, Germany). Number and size of red blood cells, white blood cells, and platelets are measured by electrical impedance and hemoglobin by spectrophotometry. Mean corpuscular volume (MCV), mean platelet volume (MPV) and red blood cell distribution width (RDW) are calculated directly from the cell volume measurements. The hematocrit (HCT) is assessed by multiplying the MCV with the red blood cell count. Mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentrations (MCHC) are calculated from hemoglobin/ red blood cells count (MCH) and hemoglobin/ hematocrit (MCHC), respectively.

Second sample analysis

A second sample, collected from a subgroup of the previously tested mice at the age of 18 weeks to check the reproducibility of these findings could not be tested due to technical problems.

Analysis of Data

Data were statistically analyzed using Excel and Sigma Stat 2.0 with the level of significance set at $p < 0.05$ by an ANOVA test on the influence of genotype and sex and subsequent pairwise comparisons of the means affected by T-test.

3.5.5 Parameters

Proteins and plasma enzyme activities
Alkaline phosphatase (EC 3.1.3.1), α -Amylase (EC 3.2.1.1), Creatine kinase (EC 2.7.3.2), Aspartate-aminotransferase (AST/GOT; EC 2.6.1.1), Alanine-aminotransferase (ALT/GPT; EC 2.6.1.2), Ferritin, Transferrin, Lipase (EC 3.1.1.3), Total protein
Plasma concentrations of specific substrates
Glucose, Cholesterol, Triglycerides, Uric acid, Urea, Creatinine
Plasma concentrations of electrolytes
Potassium, Sodium, Chloride, Calcium, Inorganic phosphate
Basic hematology
White blood cell count (WBC), Red blood cell count (RBC) Hematocrit (HCT), Hemoglobin (HGB), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Red blood cell distribution width (RDW), Platelet count (PLT) and Mean platelet volume (MPV)

3.5.6 Results

Clinical Chemistry

ANOVA revealed no significant genotype related effects on the tested parameters. For some parameters (potassium, total protein, uric acid, CK, lipase) sex-phenotype interactions resulting in significant differences for only one sex could be observed. Sex-related differences were found for many parameters.

Hematology

In the Cs7HH animals, the MCV, haemoglobin and haematocrit were significantly increased while the MCHC and RDW were significantly decreased. Additionally, the RBC was slightly increased in Cs7HH animals, compared to the respective Cs7HB groups.

3.5.7 Discussion

Clinical Chemistry

The alterations in some of the clinical chemical parameters are most likely a finding by chance due to biological variance.

Hematology

The results of the hematological investigations indicate a mild macrocytic blood cell count in consomic animals. The differences found are small and might therefore be findings by chance. However, from evaluating our baseline

data we know that C57BL/6 mice usually have erythrocytes of smaller size and higher number than C3H mice, with the MCHC being situated in a similar range. Haemoglobin values do not show significant differences between the two strains. Since the MCV in the consomic animals resembles typical findings of C3H wild-type mice it might be, that some of the genes responsible for the differences in the red blood cell counts of these two strains are located on chromosome 7. It's interesting to recognize, that the MCV is increased without a simultaneous increase of MCH and reduction of the RBC, suggesting, that these parameters are regulated by an independent locus.

Comparison to baseline data

Most of the values of the parameters tested in consomic and control animals were situated within the ranges normally found in C57BL/6 mice (Hough *et al.*, 2002; Quimby and Loeb, 1999; Kile *et al.*, 2003; own unpublished results) with a few exceptions: Inorganic phosphorus, ferritin and transferrin levels were unusually low in some animals, whereas the plasma urea levels were quite high in some samples. Increased values for CK activity in some individual mice belonging to different groups are most likely an effect of the blood collection procedure, since CK activities are well known to react very sensitive to differences in mouse handling. High glucose values in some of the mice can be due to the genetic background, since C57BL/6 mice have a disposition to develop diabetes. Some parameters of the red blood cell count (MCV, HCT) in the consomic mice showed values that are more like a C3H wild type phenotype.

3.5.8 References

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Table 16: Clinical-chemical parameters at the age of 13 weeks.

Data are presented as mean \pm standard error of mean.

Parameter	Cs7HH (A)			Cs7HB (B)			A~B	A~B	A~B
	Male	Female		Male	Female		Male	Female	All
	(n=15)	(n=15)	<i>p</i> -value	(n=15)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Sodium [mmol/l]	149.2 \pm 0.43	149.6 \pm 0.45	n.s.	149.2 \pm 0.33	148.9 \pm 0.47	n.s.	n.s.	n.s.	n.s.
Potassium [mmol/l]	4.16 \pm 0.07	4.33 \pm 0.05	p<0.05	4.28 \pm 0.05	4.13 \pm 0.05	n.s.	n.s.	p<0.01	n.s.
Calcium [mmol/l]	2.35 \pm 0.02	2.32 \pm 0.03	n.s.	2.31 \pm 0.03	2.28 \pm 0.03	n.s.	n.s.	n.s.	n.s.
Chloride [mmol/l]	109.1 \pm 0.17	107.9 \pm 0.39	p<0.05	109.6 \pm 0.33	107.9 \pm 0.42	p<0.01	n.s.	n.s.	n.s.
Inorganic Phosphate [mmol/l]	1.51 \pm 0.04	1.77 \pm 0.05	p<0.001	1.49 \pm 0.05	1.77 \pm 0.05	p<0.001	n.s.	n.s.	n.s.
Total Protein [g/dl]	5.25 \pm 0.07	5.44 \pm 0.05	p<0.05	5.21 \pm 0.05	5.28 \pm 0.06	n.s.	n.s.	p<0.05	n.s.
Creatinine [mg/dl]	0.348 \pm 0.008	0.343 \pm 0.006	n.s.	0.346 \pm 0.006	0.345 \pm 0.005	n.s.	n.s.	n.s.	n.s.
Urea [mg/dl]	55.5 \pm 2.2	51.5 \pm 1.78	n.s.	58.8 \pm 2.16	50.5 \pm 2.88	p<0.05	n.s.	n.s.	n.s.
Uric acid [mg/dl]	1.27 \pm 0.09	2.17 \pm 0.09	p<0.001	2.04 \pm 0.18	2.07 \pm 0.1	n.s.	p<0.001	n.s.	n.s.
Cholesterol [mg/dl]	95.4 \pm 6.33	72.6 \pm 2.72	p<0.01	89 \pm 5.34	72.7 \pm 3.11	p<0.05	n.s.	n.s.	n.s.
Triglyceride [mg/dl]	70 \pm 5.8	60 \pm 3.4	n.s.	85 \pm 7.1	65 \pm 5.4	p<0.05	n.s.	n.s.	n.s.
Creatine Kinase [U/l]	184.4 \pm 34.34	147.4 \pm 34.34	n.s.	339.2 \pm 64.25	141.8 \pm 33.71	p<0.05	p<0.05	n.s.	n.s.
Alanine-Amino-transferase (ALT) [U/l]	16.9 \pm 2.06	16.1 \pm 2.55	n.s.	31.1 \pm 6.69	15.3 \pm 2.08	p<0.05	n.s.	n.s.	n.s.
Aspartate-Aminotransferase (AST) [U/l]	24.4 \pm 1.49	24.5 \pm 1.52	n.s.	37.9 \pm 8.81	23.7 \pm 1.46	n.s.	n.s.	n.s.	n.s.
Alkaline Phosphatase [U/l]	85.7 \pm 2.94	110.7 \pm 2.26	p<0.001	82.3 \pm 4.07	109.9 \pm 2.83	p<0.001	n.s.	n.s.	n.s.
α -Amylase [U/l]	2163 \pm 94.4	1721 \pm 31.3	p<0.001	2346 \pm 137.3	1701 \pm 40.7	p<0.001	n.s.	n.s.	n.s.
Glucose [mg/dl]	162.3 \pm 6.8	155.5 \pm 5.1	n.s.	158.4 \pm 9.1	160.1 \pm 7.8	n.s.	n.s.	n.s.	n.s.
Ferritin [ng/ml]	26.1 \pm 1.86	29.1 \pm 3.03	n.s.	26.6 \pm 1.06	24.9 \pm 1.16	n.s.	n.s.	n.s.	n.s.
Transferrin [mg/dl]	111.5 \pm 1.02	119.5 \pm 1.52	p<0.001	112.5 \pm 1.1	116.3 \pm 1.13	p<0.05	n.s.	n.s.	n.s.
Lipase [U/l]	28.1 \pm 1.55	65.6 \pm 2.68	p<0.001	32.3 \pm 2.2	57 \pm 2.14	p<0.001	n.s.	p<0.05	n.s.

Table 17: Hematological parameters at the age of 13 weeks.

Data are presented as mean \pm standard error of mean.

Parameter	Cs7HH (A)			Cs7HB (B)			A~B	A~B	A~B
	Male	Female		Male	Female		Male	Female	All
	(n=15)	(n=15)	<i>p</i> -value	(n=15)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
White blood cell count [10 ³ /μl]	5.4 \pm 0.34	4.8 \pm 0.28	n.s.	5.3 \pm 0.31	4.3 \pm 0.32	p<0.05	n.s.	n.s.	n.s.
Red blood cell count [10 ³ /μl]	10 \pm 0.09	9.8 \pm 0.11	n.s.	9.8 \pm 0.08	9.6 \pm 0.09	n.s.	n.s.	n.s.	p<0.05
Platelet count [10 ³ /μl]	866 \pm 38.1	757 \pm 23.7	p<0.05	913 \pm 45.1	789 \pm 27	p<0.05	n.s.	n.s.	n.s.
Hemoglobin [g/dl]	15.2 \pm 0.16	15.5 \pm 0.17	n.s.	15 \pm 0.14	15 \pm 0.09	n.s.	n.s.	p<0.05	p=0.01
Hematocrit [%]	50.3 \pm 0.4	49.8 \pm 0.44	n.s.	47.6 \pm 0.4	47.3 \pm 0.4	n.s.	p<0.001	p<0.001	p<0.001
Mean corpuscular volume [fl]	50.5 \pm 0.36	50.8 \pm 0.44	n.s.	48.5 \pm 0.26	49.5 \pm 0.5	n.s.	p<0.001	n.s.	p<0.001
Mean corpuscular hemoglobin [pg]	15.3 \pm 0.11	15.8 \pm 0.09	p<0.001	15.3 \pm 0.08	15.7 \pm 0.12	p<0.01	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	30.3 \pm 0.18	31.2 \pm 0.18	p<0.001	31.4 \pm 0.19	31.8 \pm 0.15	n.s.	p<0.001	p<0.05	p<0.001
Red blood cell distribution width [% of MCV]	13.2 \pm 0.09	12.7 \pm 0.09	p<0.001	13.5 \pm 0.08	13.4 \pm 0.1	n.s.	p<0.05	p<0.001	p<0.001
Mean Platelet Volume [fl]	5.29 \pm 0.05	5.28 \pm 0.05	n.s.	5.27 \pm 0.03	5.36 \pm 0.04	n.s.	n.s.	n.s.	n.s.

3.6 Immunology Screen

3.6.1 Introduction

Mouse models have been a primary source of information for understanding the intricate mechanisms of the immune system (Blüethmann and Ohashi, 1994; Mak *et al.*, 2001; Fischer 2002; Rogner and Avner, 2003). The Immunology Screen at the GMC was set up to conduct a broad immunological phenotyping of mouse consomic lines with the intention of identifying distinct gene functions, which play key roles in the immune defenses of the organism through a complex network of cellular and soluble components (Janeway *et al.*, 2004). In primary screen we measure leukocyte populations in peripheral blood and immunoglobulin levels in blood plasma.

The proportions of leukocyte populations in peripheral blood are genetically regulated (eg. Mice: Chen and Harrison 2002; Men: Hall, *et al.*, 2000,). As a consequence, inbred strains differ in the frequency of leukocyte subsets in the lymphoid organs and in peripheral blood. Moreover, several CD antigens are restricted to specific mouse strains (e.g. Carlyle *et al.*, 2006) or interstrain differences occur concerning the level of expression of certain CD antigens (e.g. Haegel and Ceredig 2005). Strain specific differences in the immune response are further reflected in different susceptibilities to infectious agents (e.g. Medina *et al.*, 2001).

In individual mice, the number of circulating leukocytes and the proportions of subpopulations show daily rhythmic variations (Yellon and Tran 1992) and depend further on homeostatic proliferation and/or retraction (Freitas and Rocha 2000), as well as on activation through environmental and/or microbial factors (e.g. Grewal *et al.*, 1997), which might be related to subtle behavioral characteristics (e.g. Kim *et al.* 1999). Furthermore, sex-dependent factors are documented to influence the immune status (Krzych *et al.*, 1978) and have an impact on infection susceptibility (Pasche *et al.*, 2005).

The levels of Ig classes and IgG isotypes are characteristic of a special inbred mouse strains and seem to underlie genetic control mechanisms (Sant'Anna *et al.*, 1985).

3.6.2 Summary

All progenitor cells of leukocyte lineages derive from the hematopoietic stem cell in the bone marrow. The genetic regulation of hematopoietic cell compartments was proposed to be partly linked to the chromosome 7 (Geiger *et al.*, 2001). Thus, the Cs7HB and Cs7HH mice may be a tool in studies on chromosome 7-related genes involved in the regulation of leukocyte proportion.

Under the baseline conditions of the primary immunology screen, we found significant differences between Cs7HB and Cs7HH mice concerning the frequencies of CD103 expressing CD8 cells [↑], CD62L expressing CD25+CD4+ cells [↓] and B-cells (CD19+) [↓].

3.6.3 Mice

We analyzed 30 Cs7HH animals (15 females and 15 males) and twenty-eight (14 females and 14 males) of age- and sex-matched littermate Cs7HB mice.

3.6.4 Material and Methods

Peripheral blood leukocytes (PBLs) were isolated from 500 μ l blood by erythrocyte lysis with NH_4Cl (0.17M) - Tris buffer (pH 7.45) directly in 96-well microtiter plates. After subsequent washing with FACS staining buffer (PBS, 0.5% BSA, 0.02% sodium azide, pH 7.45), PBLs were incubated for 20 min with 1 μ M ethidium monazide bromide (EMA, Molecular Probes, The Netherlands) and Fc block (clone 2.4G2, PharMingen, San Diego, USA). EMA bound to the DNA of dead cells was photocrosslinked by brief light exposure. Cells were then stained with fluorescence-conjugated monoclonal antibodies (PharMingen).

The following main cell populations were analyzed: B-cells (CD19^+ clone 1D3), B1 B-cells ($\text{CD19}^+\text{CD5}^+$, clone 53-7.3), B2 B-cells ($\text{CD19}^+\text{CD5}^-$), T-cells (CD3^+ , clone 145-2C11), CD4^+ T-cells (clone RM4-5), CD8^+ T-cells ($\text{CD8}\alpha$, clone 53-6.7; $\text{CD8}\beta$, clone H35-17.2), γ/δ T-cells (clone GL3), granulocytes (Gr-1^+ , clone RB6-8C5), and NK cells (CD49b^+ , clone DX5). We also analyzed additional subpopulations based on the following surface antigens: IgD (clone 11-26c.2a), B220 (clone RA3-6B2), CD11b (clone M1/70), CD103 (clone 2E7), CD25 (clone PC61), CD62L (clone MEL-14), CD45RA (clone 14.8), Ly-6C (clone AL-21), and CD44 (clone IM7). Data were acquired on a FACS Calibur (Becton Dickinson, San Diego, USA) and were analyzed using FlowJo software (TreeStar Inc, USA). All samples were acquired until a total number of 25,000 cells was reached.

The plasma levels of IgM, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, and IgA were determined by Multiplex Bead Array (Biorad). The presence of rheumatoid factor and anti-DNA antibodies was evaluated by indirect ELISA with rabbit IgG (Sigma-Aldrich, Steinheim, Germany) and calf thymus DNA (Sigma-Aldrich), respectively, as antigens and AP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich). Serum samples from MRL/MpJ-Tnfrsf6^{lpr} mice (Jackson Laboratory, Bar Harbor, USA) were used as positive controls in the autoantibody assays.

3.6.5 Parameters

Flow cytometry
B-cells (CD19 ⁺), B1 B-cells (CD19 ⁺ CD5 ⁺), B2 B-cells (CD19 ⁺ CD5 ⁻), T-cells (CD3 ⁺), CD4 ⁺ T-cells, CD8 ⁺ T-cells, γ/δ T-cells, granulocytes (Gr-1 ⁺), and NK cells (CD49b ⁺). Furthermore, all potential subpopulations which can be identified by co-staining for other surface markers (IgD, B220, CD11b, MHC II, I-A ^k , CD25, CD8 β , CD62L, CD45RA, Ly-6C, CD44) using 6 parameter/5 color flow cytometry were analyzed.
ELISA
IgM, IgG ₁ , IgG _{2a} , IgG _{2b} , IgG ₃ , IgA; anti-DNA antibodies, rheumatoid factor

3.6.6 Results

The analysis of Cs7HH mice in the primary Immunology Screen revealed a statistically significant lower frequency of CD19⁺ cells (B-cells) in male mice and a similar trend in females. All other main lineages (CD4, CD8, γ/δ T-cells, granulocytes) revealed no differences (Table 18). Furthermore, we found a higher CD103 expression on CD8⁺ T-cells in Cs7HB mice and a lower frequency of a CD62L expressing subpopulation of CD25 expressing CD4⁺ T-cells (Table 19).

3.6.7 Discussion

The relevance of these findings is unclear, as the differences are subtle; however, they occur both on females and males. The *cd103* gene is located in chromosome 11 and expression of CD103 on CD8⁺ T-cells is considered to be important mainly for their localization in the gut epithelium (Schon *et al.*, 1999). For CD103 expressing human CD8 T-cells it has been shown, that they have functional traits of regulatory cells (Uss *et al.*, 2006). Besides that CD103 expression is described to be at least partly regulated via TGF-beta (El-Asady *et al.*, 2005), for which its gene is localized on chromosome 7 (Fujii *et al.*, 1986). TGF-beta is functionally closely related to regulatory CD4⁺ T-cells, for which CD25 expression is characteristic. However, CD25 is also expressed on non-regulatory T-cells early upon *in vivo* activation. There are CD62L expressing and no-expressing CD25⁺CD4⁺ regulatory T-cells. Regulatory CD25⁺CD4⁺ T-cells play an important role in the control of autoimmunity (Huber *et al.*, 2004; Sakaguchi 2005).

Little is known about the genetic regulation of the absolute numbers of distinct T-cell subpopulations, which for example depend on homeostatic proliferation and/or retraction, as well as on activation through environmental and/or microbial factors. Our analysis hints to a possible involvement of chromosome 7-related genes in the regulation of peripheral B-cells and perhaps distinct T-cell subsets.

3.6.8 Suggestions

For further analysis it might be interesting to determine the TGF-beta level in the serum of the mice in order to test the hypothesis of whether differences in the expression of CD103 and the frequencies of CD25 and CD62L expressing CD4+CD3+ cells are related to differences in the level of TGF-beta. In order to characterize regulatory CD4+ cells the expression of Foxp3, besides CD25, should be determined. Furthermore, analysis of lymphoid organs like bone marrow and spleen could reveal, at which developmental stage differences in the B-cell compartment eventually are occurring.

3.6.9 References

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Table 18: Basic parameters analyzed in the Immunology Screen.

Data are presented as mean ± standard error of mean.

Parameter	Control (A)			Consomic (B)			A - B	
	Male	Female		Male	Female		Male	Female
	(n=15)	(n=15)	<i>p - value</i>	(n=15)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
CD19+	48.7 ± 3.07	53.9 ± 1.66	n.s.	57.1 ± 1.6	56.3 ± 1.94	n.s.	p<0.05	n.s.
Cd19+CD5+	1.4 ± 0.07	1.28 ± 0.08	n.s.	1.57 ± 0.1	1.15 ± 0.06	p<0.01	n.s.	n.s.
Cd19+CD5-	98.6 ± 0.07	98.72 ± 0.08	n.s.	98.43 ± 0.1	98.85 ± 0.06	p<0.01	n.s.	n.s.
CD4+	11.79 ± 0.46	12.81 ± 0.57	n.s.	10.36 ± 0.54	12.31 ± 0.7	p<0.05	n.s.	n.s.
CD8a+	8.7 ± 0.3	8.18 ± 0.31	n.s.	8.1 ± 0.32	7.7 ± 0.3	n.s.	n.s.	n.s.
gdTCR+	0.13 ± 0.01	0.3 ± 0.02	p<0.001	0.12 ± 0.01	75.05 ± 74.69	n.s.	n.s.	n.s.
GR1++/CD11b+	3.7 ± 0.26	5.3 ± 0.43	p<0.01	4 ± 0.47	5.1 ± 0.46	n.s.	n.s.	n.s.
DX5	5.2 ± 0.81	1.4 ± 0.27	p<0.001	3.7 ± 0.59	1 ± 0.16	p<0.001	n.s.	n.s.
IgG1	549 ± 125.3	447 ± 35.7	n.s.	413 ± 42.8	560 ± 34.5	p<0.05	n.s.	p<0.05
IgG2a	319.3 ± 43.88	240.2 ± 24.48	n.s.	192.1 ± 29.45	307.2 ± 70.38	n.s.	p<0.05	n.s.
IgG2b	511.9 ± 40.3	984.3 ± 68.05	p<0.001	836.3 ± 70.67	1050.7 ± 96.83	n.s.	p<0.001	n.s.
IgG3	189.3 ± 30.32	302.1 ± 69.85	n.s.	207.3 ± 36.18	363.6 ± 54.98	p<0.05	n.s.	n.s.
IgM	1315.1 ± 11.76	1306.4 ± 16.86	n.s.	1342.2 ± 14.99	1300.2 ± 62.36	n.s.	n.s.	n.s.
IgA	1922 ± 271.7	2200 ± 300.8	n.s.	1212 ± 368.9	1602 ± 208.6	n.s.	n.s.	n.s.
Anti- D N A	0.4 ± 0	0.5 ± 0	n.s.	0.5 ± 0	0.5 ± 0	n.s.	n.s.	n.s.
Rheumatoid factor	0.3 ± 0.01	0.3 ± 0.02	p<0.05	0.3 ± 0.02	0.3 ± 0.02	n.s.	n.s.	n.s.

Table 19: frequencies of leukocyte subsets [% of CD8+CD3+, respectively CD4+CD3+ cells]

Data are presented as mean ± standard error of mean.

Parameter	Control (A)			Consomic (B)			A ~ B	
	Male	Female		Male	Female		Male	Female
	(n=15)	(n=14)	<i>p - value</i>	(n=15)	(n=14)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
CD8a+/3+/103+	51.1 ± 1.48	44.9 ± 1.55	p<0.01	47 ± 1.29	40 ± 1.63	p<0.01	p<0.05	p<0.05
CD4+/3+/25+62L++	0.8 ± 0.06	1.23 ± 0.06	p<0.001	1.11 ± 0.07	1.6 ± 0.08	p<0.001	p<0.01	p<0.001

3.7 Allergy Screen

3.7.1 Introduction

The goal of the Allergy screen within the German Mouse Clinic (GMC) is to search animals with IgE alteration or other allergic disorders in order to establish mouse models for allergic diseases and to find new strategies for antiallergic therapy. The increased production of IgE in response to common environmental antigens is the hallmark of atopic diseases in man (Hamelmann *et al.* 1999). Consomic animals with phenotypic alterations in IgE production represent a valuable tool to study and characterize the molecular mechanisms of IgE-mediated allergic hypersensitivity as it does mutant animals (Zhang *et al.* 1997).

3.7.2 Summary

The analysis did not reveal any differences between Cs7HH and Cs7HB mice.

3.7.3 Mice

Two age- and sex-matched group of 30 Cs7HH (15 males, 15 females) and 30 Cs7HB (15 males, 15 female) mice aged 13 weeks were analysed in the Allergy Screen.

3.7.4 Material and Methods

Twelve-week-old male and female mice were screened for alterations in plasma total IgE concentrations. Blood samples were taken from animals by puncturing the retroorbital plexus under isoflurane anesthesia.

Plasma was analyzed for total IgE, using a classical immunoassay isotype-specific sandwich ELISA. In brief, microtiter plates (96-well) were coated with 10 µg/ml anti-mouse-IgE rat monoclonal IgG (clone-PC284, The Binding Site) to detect total IgE. Serum samples were diluted 1:10 and standards for murine IgE (Mouse IgE, k clone C38-2 BD Pharmingen™) were appropriately diluted. As secondary antibodies, biotinylated rat anti-mouse IgE (clone R35-118, BD Pharmingen™) were used followed by incubation with BD OptEIA Reagent Set B (Cat. No. 550534 BD Pharmingen™) Plates were analyzed using a standard micro well ELISA reader at 450 nm. Total murine IgE data are reported in ng/ml, based on a standard curve of purified murine IgE (Alessandrini *et al.*, 2001).

3.7.5 Results

We analyzed the animals at the age of 13 weeks. The analysis of total IgE levels in plasma (mean ± SE) of C3H/C3H (HH) and C3H/BL/6 (HB) mice revealed not statistically significant differences (Table 20). We detected higher mean IgE concentrations in female mice of both groups compared to the males. This difference was statistically significant in both groups and is a

common finding of many inbred strains (Alessandrini *et al.*, 2001; Corteling *et al.*, 2004; Seymour *et al.*, 2002).

In previous studies with QTL mapping in chromosome 7 reported a significant linkage to airway responsiveness (De Sanctis *et al.*, 1999) nevertheless our results did not show alteration in the levels of IgE and the IgE levels fit in to the normal range of C57BL/6

Taken together, under standard screening conditions for primary allergy screen, C3H/C3H (HH) and C3H/BL/6 (HB) mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype.

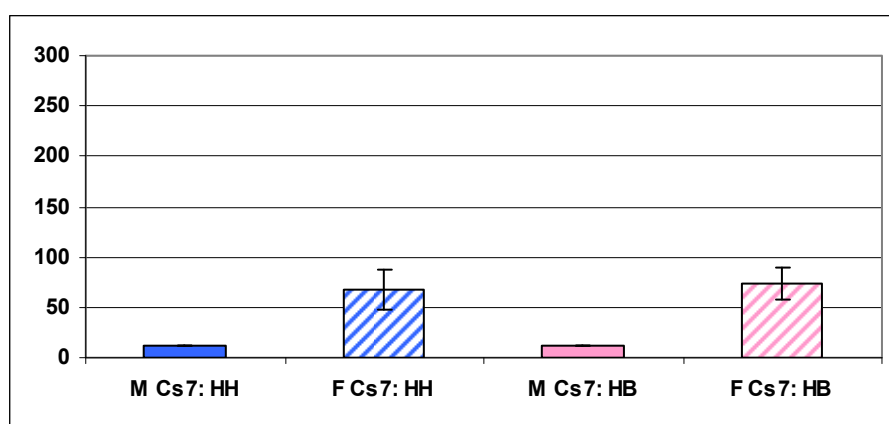


Figure 7: Total plasma IgE in Cs7 mice

Table 20: Total plasma IgE in Cs7 mice (13 weeks old)								
Data are presented as mean \pm standard error of mean.								
	Control (A)			Consomic (B)			A~B	A~B
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=15)	(n=15)		(n=15)	(n=15)		<i>p</i> - value	<i>p</i> - value
Total IgE [ng/ml]	11.7 \pm 0.5	68 \pm 19	<0.05	12.2 \pm 0.5	72.9 \pm 16	<0.05	n.s.	n.s.

Raw data will be available on request.

3.7.6 References

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3.8 Nociceptive Screen

3.8.1 Introduction

Pain is the perception of an aversive or unpleasant sensation that originates from a specific region of the body. The highly subjective nature of pain is one of the factors that make it difficult to define and to treat clinically. Pain is more than a conspicuous sensory experience that warns of danger.

Nociceptors are activated by tissue injury but also by mechanical, thermal, or chemical stimuli. Harmful stimuli applied to the skin or to subcutaneous tissue, activate nociceptors, the peripheral endings of primary sensory neurons whose cell bodies are located in the dorsal root or in the trigeminal ganglia.

A noxious stimulus activates the nociceptor by depolarizing the membrane of the sensory ending. When peripheral tissues are damaged, the sensation of pain in response to subsequent stimuli is enhanced. This phenomenon termed hyperalgesia, may involve a lowering of threshold of the nociceptors or an increase in the magnitude of pain evoked by supra-threshold stimuli. Hyperalgesia can occur both at the site of tissue damage (primary hyperalgesia) and in the surrounding undamaged areas (secondary hyperalgesia; Wall and Melzak, 1984). By means of different inbred mouse strains it could be demonstrated that rodents display large and heritable differences in both nociceptive and analgesic sensitivity (Mogil, 1999; Mogil *et al.*, 1999)

3.8.2 Summary

In the Primary Screen the responsiveness of the intact somatosensory system to thermal pain was tested in the Cs7 mouse line by means of the hot plate test (nociceptive pain). We could not find any differences in pain reactivity between Cs7HH and Cs7HB animals or sex-related differences.

3.8.3 Mice

Thirty Cs7HH (15 male, 15 female), and 30 Cs7HB animals (15 male, 15 female) were tested in our first screen.

3.8.4 Material and Methods

Hot plate test

The mice were placed on a metal surface maintained at $52 \pm 0.2^\circ\text{C}$ (Hot plate system was made by TSE GMBH, Germany; Eddy and Leimbach, 1953). Locomotion of the mouse on the hot plate was constrained by 20 cm high Plexiglas wall to a circular area with a diameter of 28 cm (Fig. 8). Mice remained on the plate until they performed one of three behaviors regarded as indicative of nociception: hind paw lick (h.p. licking), hind paw shake/flutter (h.p. shaking) or jumping.

We evaluated only hind paw but not the front paw responses, because fore paw licking and lifting are components of normal grooming behavior. Each mouse was tested only once since repeated testing leads to profound

changes in response latencies. The latency was recorded to the nearest 0.1 s. To avoid tissue injury 60 s cut-off time was used. The data values are given in seconds.

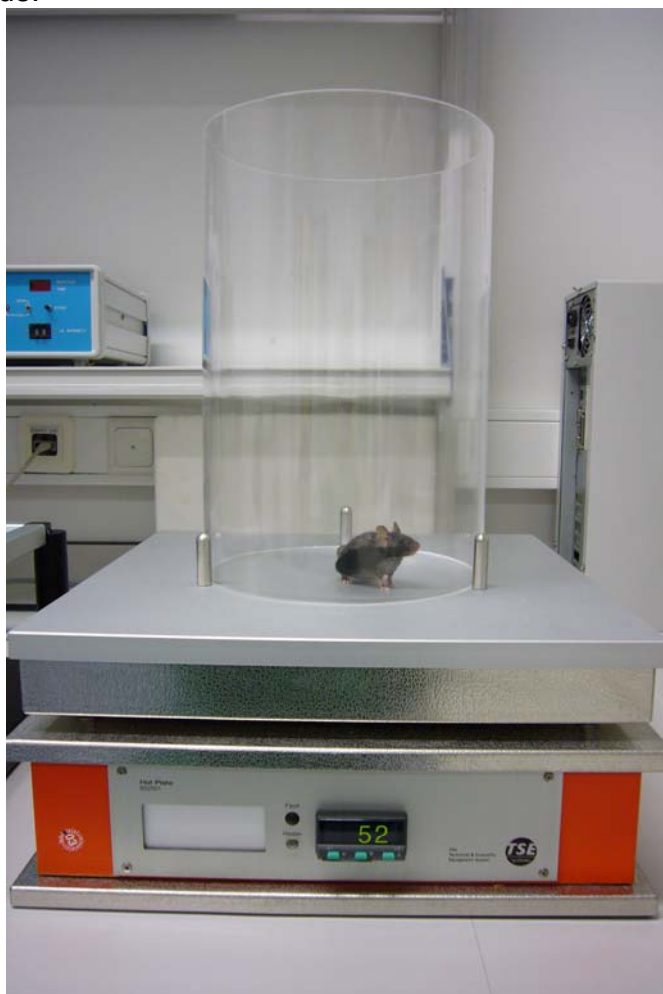


Figure 8: Hot plate system

Statistical analysis

Statistical analysis was performed using a statistical package Statgraphics® (Statistical Graphics Corporation, Rockville, MD). The differences between the groups were compared with ANOVA, LSD test was used as *post hoc*. Statistical significance was assumed at $p < 0.05$.

3.8.5 Parameters

Hind paw licking
Reaction with licking of hind paw to the thermal pain
Hind paw shaking
Reaction with shaking of hind paw to the thermal pain
Jumping
Jumping reaction to the thermal pain

3.8.6 Results and Discussion

The first nociceptive response (the first sign of pain) observed in these mice was 'hind paw shaking'. The Cs7HH and Cs7HB showed this reaction at the same time. The second one was hind paw licking. The Cs7HH mice showed this reaction significantly shorter, but this reaction was not the first sign of pain. The first reaction was the 'shaking', which was made in groups at the same time. The third response was 'jumping'. Again, we found no genotype-related difference. The sexes did not differ either.

We found no significant difference in the pain reactivity between the Cs7HH and Cs7HB mice. The difference, which we found in licking reaction, is only a different behavioral response to escape from the unpleasant situation. Therefore we do not recommend performing additional pain-related studies in these mice.

Until now, no pain-related QTL is known for Chromosome 7. The chronic pain-associated QTL "pain1" is located on Chromosome 15 (Devor *et al.* 2005), the neuropathic pain-associated QTL "pain2" is on Chromosome 2 (Nissenbaum *et al.* 2008). On Chromosome 9 and 10 were found additional QTLs which are associated with inflammatory pain (Wilson *et al.* 2002).

3.8.7 References

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Abbreviations

h.p. hind paw

Table 21: Nociceptive Screen									
Data are presented as mean \pm standard error of mean.									
							ANOVA		
							genotype		sex*genotype
Parameter Latency [s]	HH (B)			HB (A)			A~B	A~B	ANOVA
	Male	Female		Male	Female		Male	Female	
	(n=15)	(n=15)	<i>p-value</i>	(n=15)	(n=15)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
H.p. shaking	11.35 \pm 1.14	11.77 \pm 1.13	n.s.	9.99 \pm 1.14	9.98 \pm 1.136	n.s.	n.s.	n.s.	n.s.
H.p. licking	19.85 \pm 1.58	17.24 \pm 1.6	n.s.	15.08 \pm 1.582	13.8 \pm 1.6	n.s.	0.037	n.s.	n.s.
Jumping	53.9 \pm 2.08	54.36 \pm 2.09	n.s.	56.47 \pm 2.086	58.14 \pm 2.08	n.s.	n.s.	n.s.	n.s.

ANOVA:

SHAKING: genotype: p = 0.172, sex: p = 0.856, genotype*sex: p = 0.847

LICKING: genotype: p = 0.012, sex p = 0.224, genotype*sex: p = 0.675

JUMPING: genotype: p = 0.135, sex p = 0.616, genotype*sex: p = 0.769

3.9 Cardiovascular Screen

3.9.1 Introduction

Blood pressure (BP) analysis provides insights into functions of the vascular system including the regulation of vascular tone and left ventricular pump function. BP is strongly influenced by defects in many organ systems (heart, kidney, lung, liver) and metabolic or (neuro)endocrine pathways. Imbalances in one or, usually several organs and pathways, result in changes of this sensitive global parameter (Krege *et al.*, 1995; Lorenz, 2002; Deschepper *et al.*, 2004).

The ECG measures the electrical activity, rate and rhythm of the heart beat, supplying information about the conductive properties (function of ion channels), the excitable myocardial mass and the propagation of excitation within the heart tissue. Almost all types of cardiac pathologies will eventually cause also distinct ECG changes. Therefore, the ECG provides a comprehensive overview on cardiac function (Doevendans *et al.*, 1998; Ehmke, 2003; Royer *et al.*, 2005).

3.9.2 Summary

The comparison of the Cs7 consomic to control mice in blood pressure and ECG analysis revealed no effect of the replaced chromosome on cardiovascular function in the basal conditions of the primary screen. Only a subtle difference in Q amplitude was found.

3.9.3 Mice

The mice reached the Cardiovascular Screen at the age of 15 weeks, 20 female mice (10 controls, 10 consomic animals) and 20 male mice (10 controls, 10 consomic animals) underwent blood pressure and ECG analysis.

3.9.4 Material and Methods

Tail-cuff blood pressure measurement

Blood pressure was measured in unanesthetized mice with a non-invasive tail-cuff method using the MC4000 Blood Pressure Analysis Systems (Hatteras Instruments Inc., Cary, North Carolina, USA). Four animals were restrained on a pre-warmed metal platform in metal boxes. The tails were looped through a tail-cuff and fixed in a notch containing an optical path with a LED light and a photosensor.



Figure 9: Blood pressure set up

Platform with four measurement slots (left), mouse fixed in a tail-cuff underneath a restrainer box (right).

The blood pulse wave in the tail artery is detected as transformed into an optical pulse signal by measurement of light extinction. Pulse detection, cuff inflation and pressure evaluation are automated by the system software. After five initial inflation runs for habituation, 12 measurement runs are performed for each animal in one session. Runs with movement artifacts are excluded.

After one day of training, in which the animals are habituated to the apparatus and protocol, the measurements are performed on four consecutive days between 8:30 and 11:30 AM.

Surface limb ECG

ECG is performed in anesthetized (isoflurane/pressured air inhalation) mice by use of three metal bracelets that are put on the joints of the feet together with electrode gel. The complete setup is located in a faraday cage. The electrodes are positioned on the front-paws and the left hind-paw, resulting in the bipolar standard limb leads I, II and III and the augmented unipolar leads AVF, AVR, AVL. ECG is recorded for about seven minutes.



Figure 10: ECG-setup

Left: ECG-setup in the faraday cage; right: mouse with bracelet electrode under anesthesia.

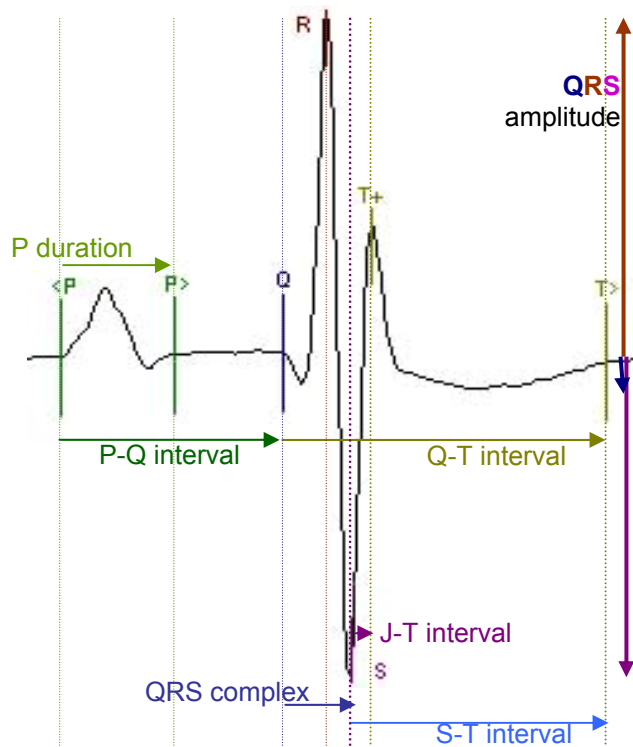


Figure 11: Example of ECG trace with analyzed parameters.

A shape analysis of the ECG traces is performed with the software ECG-auto (EMKA technologies, Paris, France). For each animal, intervals and amplitudes are evaluated from five different sets of averaged beats (usually lead II). The parameter Q-T interval is also corrected for the RR interval. In addition, the recordings are screened for arrhythmias, including supraventricular and ventricular extrasystoles and conduction blockages.

Analysis of data

For blood pressure analysis, at least 20 to 48 individual measurements are pooled to obtain a mean over the four measurement days for each animal. In the quantitative ECG analysis sets of five analyzed beats are averaged for one animal.

The data were analyzed statistically using Statistica. Analysis of variance (ANOVA) tests are used for multi-factorial analysis of sex and genotype. Post hoc analysis for multiple comparisons included a Duncan's Multiple Range Test & Critical Ranges.

3.9.5 Parameters

Blood Pressure Analysis
Systolic Pressure, Diastolic Pressure, Mean Arterial Pressure (MAP), Pulse
ECG Quantitative Analysis
PQ Interval, P-Wave Duration, QRS-Complex Duration, QT Interval, QT _{corrected} Interval, RR Interval, Heart Rate, JT Interval, ST Interval, Q Amplitude, R Amplitude, S Amplitude, QRS Amplitude
ECG Qualitative Analysis
Events of Supraventricular Extrasystoles, Ventricular Extrasystoles, AV I Blockage, AV II Blockage, AV III Blockage, AV Dissociation

3.9.6 Results

Blood pressure analysis (Table 22) revealed no genotype-specific difference. In the **ECG** analysis (Table 23) the only genotype-specific difference was found in Q amplitude reaching significance in the *post hoc* comparison only in females. The Q amplitude was higher in the Cs7HH than in the Cs7HB animals (Fig. 12).

Comparing the results to C3H and C57Bl/6 mice of independent batches that are measured independently for quality control in the primary screen, the Cs7 animals of all groups show a stronger similarity to C57Bl/6 than to C3H mice in most parameters (see Fig. 13 -19 for some examples). However, this is a rather indirect comparison, since in addition to the independent measurement our quality control inbred strains come from a different breeding colony and are maintained under different housing conditions until the testing period in the GMC. This could also be the reason for the generally lower blood pressure measured in the Cs7 compared to inbred strains.

Comparing the Q amplitude to the inbred strains reveals no obvious similarities (Fig. 13). The Cs7HH mice show even higher levels in Q amplitude than the C57Bl/6 mice, while the Cs7HB mice reflect even lower levels than the C3H mice. The origin of these differences is not clear and of unknown relevance.

Under the standard conditions of the primary cardiovascular screen, no specific effect of the chromosome exchange on the basal cardiovascular functions was detectable. All parameters were without pathological findings.

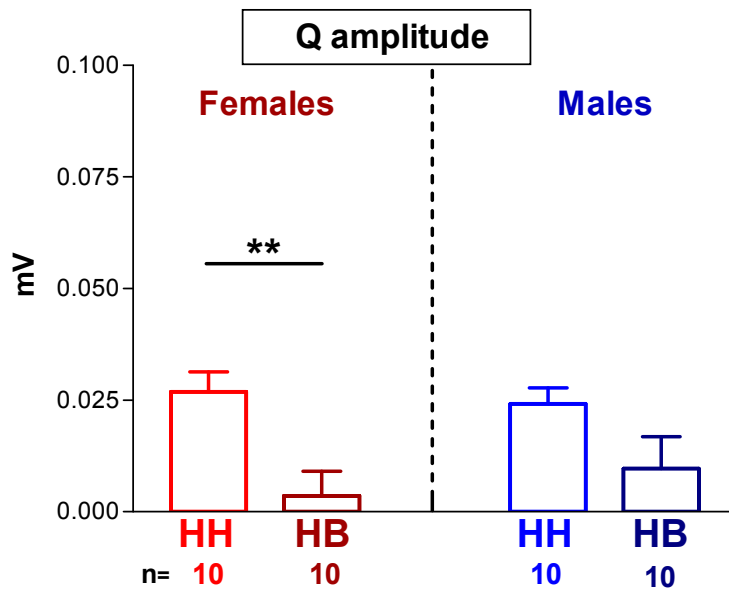


Figure 12: ECG-parameter Q amplitude

A genotype effect was seen as higher Q amplitude levels for Cs7 mice, reaching significance only in females in the post hoc comparison.

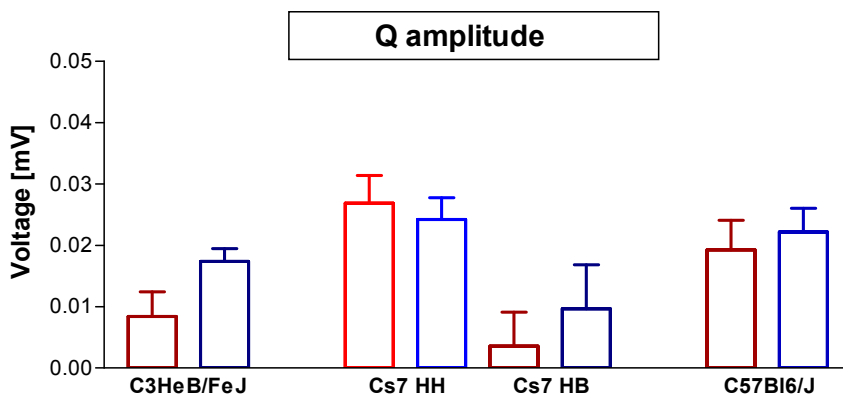


Figure 13: ECG-parameter Q amplitude

Comparison of both Cs7 strains with internal batches of C3H and C57 inbred strains. Cs7HH showed even higher levels than C57, Cs7HB mice even lower levels than C3H mice.

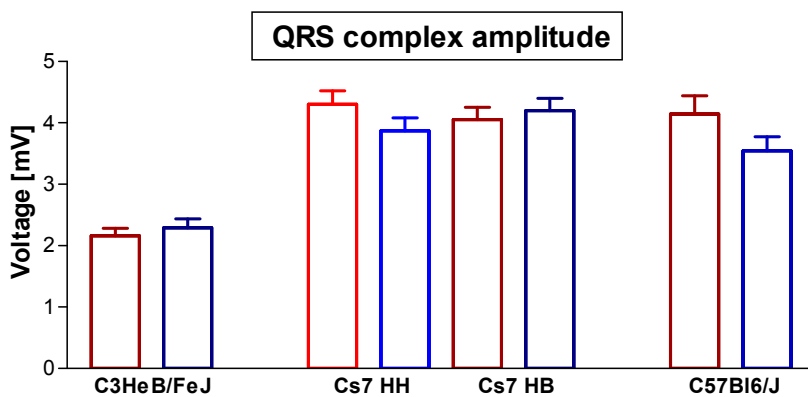


Figure 14: ECG-parameter QRS complex amplitude

Comparison of both Cs7 strains with internal batches of C3H and C57 inbred strains.

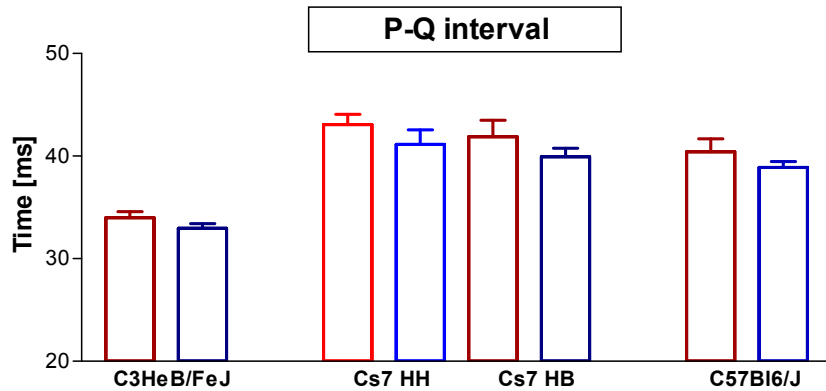


Figure 15: ECG-parameter PQ interval
Comparison of both Cs7 strains with internal batches of C3H and C57 inbred strain

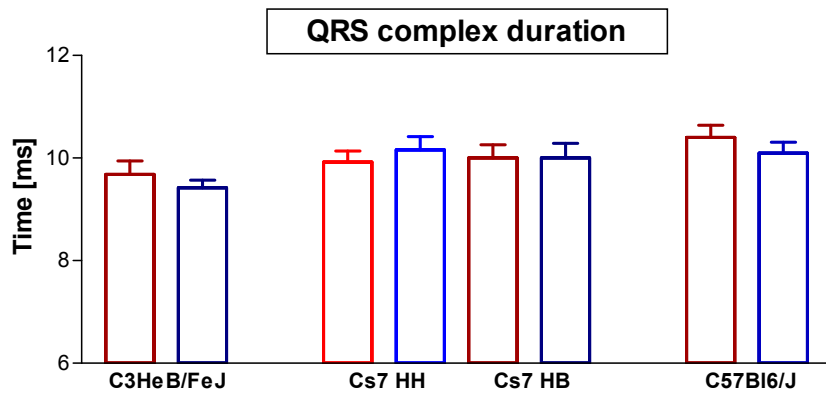


Figure 16: ECG-parameter QRS complex duration
Comparison of both Cs7 strains with internal batches of C3H and C57 inbred strain

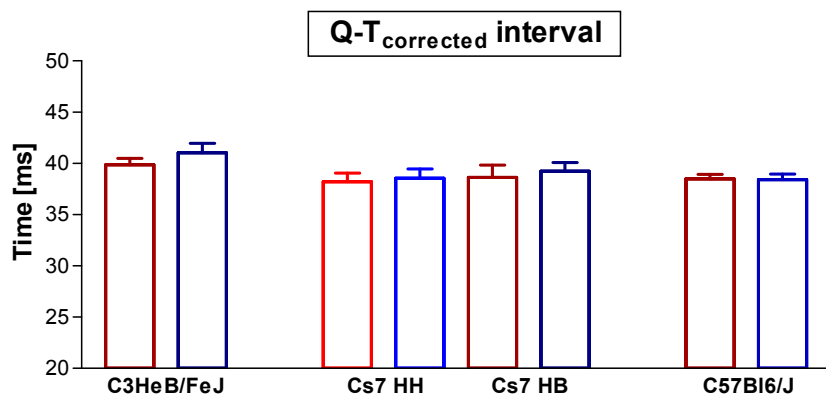


Figure 17: ECG-parameter QT_{corrected} interval
For QT_{corrected} interval the parameter Q-T interval is normalized by heart rate. Comparison of both Cs7 strains with internal batches of C3H and C57 inbred strain

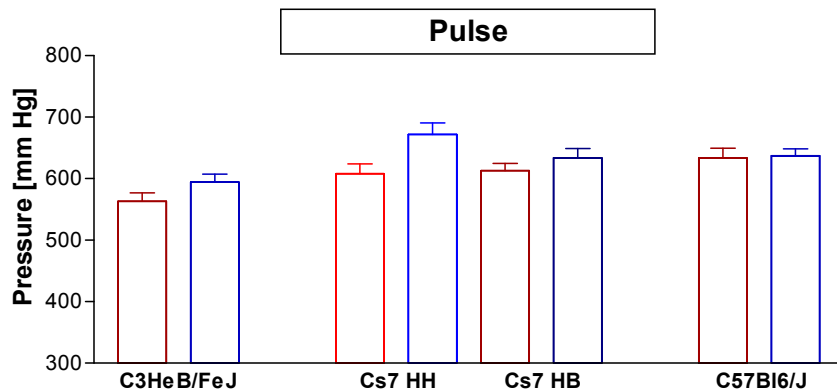


Figure 18: Blood pressure parameter Pulse

Comparison of both Cs7 strains with internal batches of C3H and C57 inbred strain

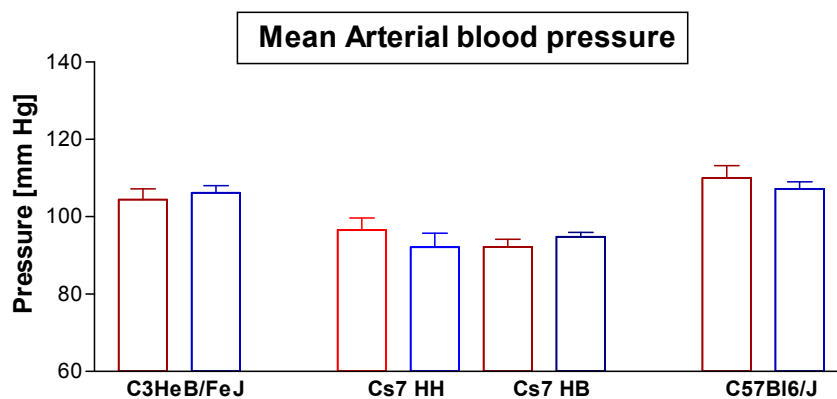


Figure 19: Blood pressure parameter mean arterial pressure

Comparison of both Cs7 strains with internal batches of C3H and C57 inbred strain

3.9.7 References

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Table 22: Blood Pressure Parameters

Data are presented as mean +/- standard error of mean.

Parameter	Control (A)		Mutant (B)		ANOVA			Post hoc test	
	Male	Female	Male	Female	Sex	Genotyp	Interact.	A~B Male	A~B Female
	(n = 10)	(n = 10)	(n = 10)	(n = 10)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Systolic pressure [mm Hg]	104.5 +/- 1.5	100.8 +/- 1.8	102.9 +/- 3.8	105.8 +/- 2.8	n.s.	n.s.	n.s.	n.a.	n.a.
Diastolic pressure [mm Hg]	90.4 +/- 1.2	88.5 +/- 2.0	87.2 +/- 3.7	92.5 +/- 3.3	n.s.	n.s.	n.s.	n.a.	n.a.
Mean arterial pressure [mm Hg]	94.8 +/- 1.2	92.3 +/- 1.9	92.1 +/- 3.7	96.6 +/- 3.1	n.s.	n.s.	n.s.	n.a.	n.a.
Pulse [bpm]	634.0 +/- 15.1	613.3 +/- 11.4	671.9 +/- 18.6	608.2 +/- 15.8	p<0.01	n.s.	n.s.	n.a.	n.a.

Abbreviations

SVES **S**upraventricular **E**xtrasystoles
VES **V**entricular **E**xtrasystoles

Table 23: ECG Parameters

Data are presented as mean +/- standard error of mean.

Parameter	Control (A)						Mutant (B)						ANOVA			Post hoc test	
	Male			Female			Male			Female			Sex	Genotyp	Interact.	A~B Male	A~B Female
	(n = 10)			(n = 10)			(n = 10)			(n = 10)			p - value	p - value	p - value	p - value	p - value
PQ interval [ms]	39.9 +/- 0.8			41.9 +/- 1.6			41.1 +/- 1.4			43.1 +/- 1.0			n.s.	n.s.	n.s.	n.a.	n.a.
P-wave duration [ms]	18.6 +/- 0.3			19.5 +/- 0.3			19.4 +/- 0.7			18.9 +/- 0.3			n.s.	n.s.	n.s.	n.a.	n.a.
QRS-complex duration [ms]	10.0 +/- 0.3			10.0 +/- 0.3			10.2 +/- 0.3			9.9 +/- 0.2			n.s.	n.s.	n.s.	n.a.	n.a.
QT interval [ms]	44.5 +/- 1.3			43.0 +/- 1.4			45.5 +/- 1.6			42.8 +/- 1.0			n.s.	n.s.	n.s.	n.a.	n.a.
QT _{corrected} [ms]	39.2 +/- 0.8			38.6 +/- 1.2			38.6 +/- 0.9			38.2 +/- 0.8			n.s.	n.s.	n.s.	n.a.	n.a.
RR interval [ms]	128.9 +/- 5.0			124.2 +/- 3.8			139.2 +/- 5.1			125.6 +/- 2.5			p<0.05	n.s.	n.s.	n.a.	n.a.
Heart rate [bpm]	472.4 +/- 16.6			488.3 +/- 15.3			438.1 +/- 16.6			482.4 +/- 9.2			p<0.05	n.s.	n.s.	n.a.	n.a.
JT interval [ms]	3.4 +/- 0.2			3.8 +/- 0.5			3.6 +/- 0.2			4.2 +/- 0.5			n.s.	n.s.	n.s.	n.a.	n.a.
ST interval [ms]	34.5 +/- 1.1			33.0 +/- 1.2			35.3 +/- 1.6			32.8 +/- 1.0			n.s.	n.s.	n.s.	n.a.	n.a.
Q amplitude [mV]	0.01 +/- 0.01			0.00 +/- 0.01			0.02 +/- 0.00			0.03 +/- 0.00			n.s.	p<0.01	n.s.	n.s.	p<0.01
R amplitude [mV]	2.94 +/- 0.17			2.93 +/- 0.19			2.72 +/- 0.16			3.13 +/- 0.19			n.s.	n.s.	n.s.	n.a.	n.a.
S amplitude [mV]	-1.26 +/- 0.14			-1.12 +/- 0.09			-1.14 +/- 0.14			-1.17 +/- 0.15			n.s.	n.s.	n.s.	n.a.	n.a.
QRS amplitude [mV]	4.20 +/- 0.20			4.05 +/- 0.20			3.87 +/- 0.22			4.30 +/- 0.22			n.s.	n.s.	n.s.	n.a.	n.a.
Arrhythmias [# of animals]	SVES	VES	other	SVES	VES	other	SVES	VES	other	SVES	VES	other					
	0	2	1	0	1	0	0	0	0	0	1	4					
Regular [# of animals]	8			9			10			6							

3.10 Lung Function Screen

3.10.1 Summary

Neural and mechanical processes that control breathing frequency have been investigated in man for a long time (Mead, 1960; Otis *et al.*, 1959), but only with the availability of mouse inbred strains the contribution of genetic determinants to differential baseline breathing patterns could be elucidated (Tankersley *et al.*, 1997; Tankersley, 1999; Reinhard *et al.*, 2002; Reinhard *et al.*, 2005). By use of genetically engineered mice, candidate genes for human developmental disorders of breathing have been identified (Katz, 2003).

Spontaneous breathing patterns during rest and activity were studied in 15-week-old male and female consomic and wild-type control mice of the Cs7 consomic mouse line. This mouse line was active throughout the whole observation period, so that we could not measure breathing at rest. Therefore, these values and the extent of adaptation from rest to activity are missing which is important for a better assessment of a phenotype.

Female Cs7HH mice showed a significantly higher specific tidal volume, specific minute ventilation, minute ventilation and mean inspiratory flow rate. Notably, these values are situated between values of pure C3H and C57BL/6 mice. However, breathing rate and respiratory timing were not affected. According to our studies, there are no indications for an association of lung function to Chromosome 7 (Reinhard *et al.*, 2002/2005). Overall, differences in females are small and are unlikely to reflect a clear phenotype.

3.10.2 Mice

Male and female Cs7HB and Cs7HH mice were studied at the age of 15 weeks (Table 28). There were no significant differences between Cs7HH and Cs7HB mice in body weight, we detected only common sex differences. Male Cs7HB ($28.6 \pm 0.9\text{g}$) were 14 % heavier than female ($25.0 \pm 0.3\text{g}$) and male Cs7HH (30.3 ± 0.4) were 24 % heavier than females (24.4 ± 0.7).

3.10.3 Material and Methods

Whole Body Plethysmography

A commercially available system from Buxco[®] Electronics (Sharon, Connecticut) was used to assess breathing patterns in unrestrained animals according to the principle described by Drorbaugh and Fenn (1955). It measures the pressure changes which arise from inspiratory and expiratory temperature and humidity fluctuations during breathing (Figs. 20 and 21).

Calibration of the system allows to transform these pressure swings into flow and volume signals so that automated data analysis provides tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF). These data were stored online as mean values at 10 s intervals.



Figure 20: System used at GMC to assess breathing patterns.

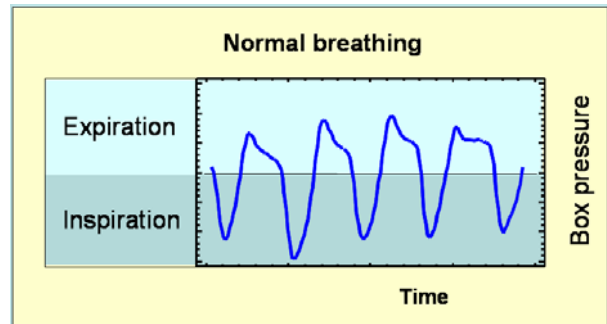


Figure 21: Recorded data used to calculate the breathing parameters.

Measurements were always performed between 8 a.m. and 11 a.m. to account for potential diurnal variations in breathing. The system was set up in a quiet room where temperature and humidity were kept constant throughout the measurements. Before each measurement, the system was calibrated and the actual barometric pressure, temperature, and humidity were supplied to warrant adequate calculations of flow rates and volumes. After placing the animals into the chamber, data recording was immediately started and was continued for 40 min. Mice underwent typical phases during the measuring period. Primarily, the animals were stressed so that the respiratory rate was highest at the beginning. Usually after 5 min. the animals became calmer, they slightly reduced their respiratory rate, and began to explore the chamber and start cleaning themselves – *phase of activity*. Later activity was more and more interrupted by phases of rest or even short periods of snoozing – *resting phase*. Some of the animals even went to *phases of sleep*, which resulted in a further marked decrease in respiratory rate. The frequency histogram of the respiratory rates was determined for each individual, and breathing was analyzed for the above mentioned parameters during the phases of activity and rest. In addition to the directly recorded parameters, mean inspiratory and expiratory flow rates (MEF, MIF) were calculated offline from the ratio of tidal volume and the respective time interval. The relative duration of inspiration (T_i/TT) was determined from the ratio of inspiratory time to total time required for the breathing cycle. Specific tidal volumes and minute ventilations (sTV, sMV) were calculated by relating the absolute values to the body weight of the animal. Furthermore, the mean of all breathing frequencies (mean_f) measured during the 40-minute-period was calculated as a rough and ready parameter to assess whether the duration of rest and activity was similar in all mouse strains.

Statistical Analysis of Data

Statistical analyses were performed using a commercially available statistics package (Statgraphics®, Statistical Graphics Corporation, Rockville, MD). Differences between strains were evaluated by Students t-test. Statistical significance was assumed at $p < 0.05$. Data are presented as mean values \pm standard error of the mean (SEM).

3.10.4 Parameters

Directly recorded data
Tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF).
Calculated data
mean inspiratory flow rates (MEF), expiratory flow rates (MIF), relative duration of inspiration (Ti/TT), specific tidal volumes (sTV), minute ventilations (sMV), mean of all breathing frequencies (mean_f)

3.10.5 Results

Tables 24 and 25 summarize the results obtained for spontaneous breathing under active conditions. Only females exhibited significant differences. Values for specific tidal volume, minute ventilation and mean expiratory flow rate were found to be higher in Cs7HH mice. Interestingly, the values for both – Cs7HH and Cs7HB - mice are situated between values of pure C3H and C57BL/6 mice (Fig. 22, Schulz *et al.*, personal communication).

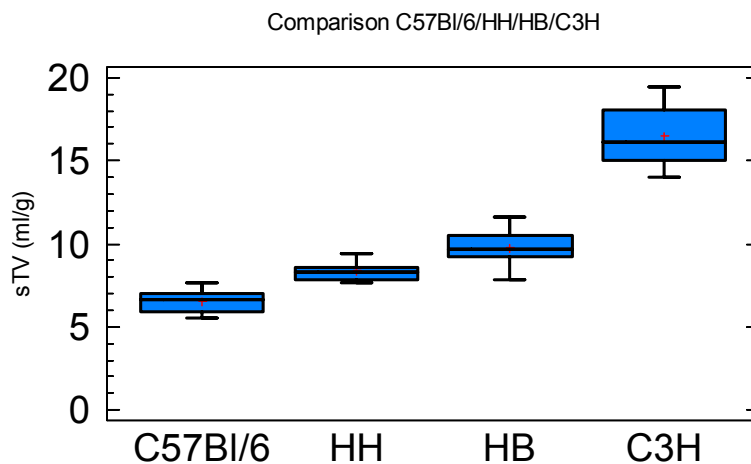


Figure 22: Specific tidal volume at activity

Values of Cs7HH and Cs7HB mice were in between that of C57BL/6 and C3H mice.

3.10.6 Discussion

This mouse line was active throughout the whole observation period, so that we could not measure breathing at rest. Therefore, these values, but also the extent of adaptation from rest to activity is missing which is important for a better assessment of a phenotype.

Female Cs7HH mice showed no differences in breathing rate and timing of breathing but differences related to the depths of breathing, i.e. tidal volume.

According to our previous studies, there are no indications for an association of lung function to Chromosome 7 (Reinhard *et al.*, 2002/2005). Overall, differences in females are small and are unlikely to reflect a clear phenotype.

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Abbreviations

bw	body weight (g)
mean_f	mean of all respiratory rates (1/min)
f	respiratory rate (1/min)
TV	tidal volume (ml)
sTV	specific tidal volume ($\mu\text{l/g}$)
MV	minute ventilation (ml/min)
sMV	specific ventilation (ml/min/g)
Ti	inspiratory time (ms)
Te	expiratory time (ms)
Ti/TT	relative duration of inspiration
PIF	peak inspiratory flow rate (ml/s)
PEF	peak expiratory flow rate (ml/s)
MIF	mean inspiratory flow rate (ml/s)
MEF	mean expiratory flow rate (ml/s).

Raw data are available on demand.

Table 24: Characterization of studied mice

Data are presented as mean \pm standard error of mean.

Parameter	Cs7HB			Cs7HH			HB~HH	HB~HH
	Male	Female		Male	Female		Male	Female
	(n=6)	(n=6)	<i>p</i> - value	(n=6)	(n=6)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Bw [g]	28.6 \pm 0.9	25.0 \pm 0.3	< 0.01	30.3 \pm 0.4	24.4 \pm 0.7	< 0.001	n.s.	n.s.
Age [d]	116.5 \pm 0.2	115.7 \pm 0.3		119.8 \pm 0.2	118.7 \pm 0.2			
Mean_f [1/min]	481.4 \pm 10.6	469.8 \pm 12.5	n.s.	481.8 \pm 14.2	484.4 \pm 11.8	n.s.	n.s.	n.s.

Table 25: Spontaneous breathing pattern of Cs7 mice during activity

Data are presented as mean \pm standard error of mean.

Parameter	Cs7HB			Cs7HH			HB~HH	HB~HH
	Male	Female		Male	Female		Male	Female
	(n=6)	(n=6)	<i>p - value</i>	(n=6)	(n=6)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Activity								
f [1/min]	495.6 \pm 4.6	491.0 \pm 9.2	n.s.	499.5 \pm 5.0	501.2 \pm 6.9	n.s.	n.s.	n.s.
TV [ml]	0.23 \pm 0.01	0.21 \pm 0.01	n.s.	0.23 \pm 0.01	0.24 \pm 0.01	n.s.	n.s.	n.s.
sTV [μl/g]	8.1 \pm 0.3	8.4 \pm 0.3	n.s.	7.5 \pm 0.4	9.8 \pm 0.5	< 0.01	n.s.	< 0.05
MV [ml/min]	112.5 \pm 3.1	101.3 \pm 4.2	n.s.	111.7 \pm 5.5	116.9 \pm 3.5	n.s.	n.s.	< 0.02
sMV [ml/min/g]	3.9 \pm 0.1	4.1 \pm 0.2	n.s.	3.7 \pm 0.2	4.8 \pm 0.2	< 0.01	n.s.	< 0.05
Ti [ms]	44.1 \pm 0.6	41.2 \pm 0.3	< 0.001	42.9 \pm 0.3	42.2 \pm 0.6	n.s.	n.s.	n.s.
Te [ms]	77.0 \pm 1.0	81.2 \pm 2.4	n.s.	77.3 \pm 1.3	77.7 \pm 1.4	n.s.	n.s.	n.s.
Ti/TT	0.36 \pm 0.01	0.34 \pm 0.01	< 0.01	0.36 \pm 0.01	0.35 \pm 0.01	n.s.	n.s.	n.s.
PIF [ml/s]	8.5 \pm 0.2	8.3 \pm 0.3	n.s.	8.5 \pm 0.4	9.2 \pm 0.3	n.s.	n.s.	n.s.
PEF [ml/s]	5.9 \pm 0.4	5.5 \pm 0.2	n.s.	6.1 \pm 0.3	6.3 \pm 0.3	n.s.	n.s.	n.s.
MIF [ml/s]	5.2 \pm 0.1	5.1 \pm 0.1	n.s.	5.2 \pm 0.2	5.6 \pm 0.2	n.s.	n.s.	n.s.
MEF [ml/s]	3.0 \pm 0.1	2.6 \pm 0.1	< 0.02	2.9 \pm 0.1	3.0 \pm 0.1	n.s.	n.s.	< 0.02

3.11 Molecular Phenotyping

3.11.1 Introduction

Comparative genome-wide expression profiling is a powerful tool in the effort to annotate the mouse genome with biological function. The analysis of RNA expression data of mouse lines might support the understanding of the molecular biology of such consomic animals and provide new insights into mammalian gene function. We demonstrated the feasibility to detect transcriptional affected organs employing RNA expression profiling as a tool for molecular phenotyping (Seltmann *et al*, 2005).

In a first discussion **no** organ was selected for analysis.

3.11.2 Methods and Materials

Organ Collection

The molecular phenotyping screen archives organs of consomic mice for subsequent DNA-chip expression profiling analysis. Ten male mice of the Cs7 consomic mouse line were provided to the molecular phenotyping screen.

Table 26: Mutant and control mice stored for expression profiling			
Mouse ID	Genotype	Sex	Date
30051019	Cs7HH	m	20.10.2006
30051057	Cs7HB	m	20.10.2006
30051066	Cs7HB	m	20.10.2006
30051068	Cs7HB	m	20.10.2006
30051011	Cs7HH	m	20.10.2006
30051069	Cs7HB	m	20.10.2006
30051009	Cs7HH	m	20.10.2006
30051007	Cs7HH	m	20.10.2006
30051008	Cs7HH	m	20.10.2006
30051010	Cs7HH	m	20.10.2006
30051060	Cs7HB	m	20.10.2006
30051061	Cs7HB	m	20.10.2006

Organs were collected at the age of 16 weeks. To minimize the influence of circadian rhythm on gene expression, mice were killed between 9 am and 12 am by carbon dioxide asphyxiation. The following 10 organs were collected and archived in liquid nitrogen following our established SOPs (Standard operation protocols): spleen, kidney, testis, liver, heart, lung, thymus, skin / cartilage (outer ear), skeletal muscle and brain. Organs were immediately frozen

and stored in liquid nitrogen until isolation of total RNA. The 100 organ samples collected either may be used for further expression profiling analysis in the GMC or, alternatively may be transferred to the collaborator.

Methods of expression profiling analysis in the GMC

Total RNA is isolated from the selected organs according to the manufacturer's protocol using the RNeasy Midi kits (Qiagen, Germany). For reverse transcription 15µg total RNA of each sample is used and indirectly labeled with Cy3 or Cy5 fluorescent dyes according to a modified TIGR protocol as previously described (Seltmann *et al.*, 2005; Greenwood *et al.*, 2005).

Gene specific DNA probes were PCR amplified from the Lion Bioscience 20k mouse arrayTAG clone set (Heidelberg, Germany) as recently described (Drobyshev *et al.*, 2003). Amplified probes are dissolved in 3xSSC and spotted on aldehyde-coated slides (CEL Associates, Pearland, TX/USA) using the Microgrid TAS II spotter (Genomic Solution Ltd., Huntingdon, UK). Spotted slides are rehydrated, blocked, denatured and dried as recently described (Drobyshev *et al.*, 2003; Beckers *et al.*, 2005; Greenwood *et al.*, 2005). The hybridization mixture is placed on prehybridized microarrays, hybridized at 42°C for 16h and then successively washed in decreasing SSC concentration (3x, 1x, 0.5x and 0.1x) and dried with nitrogen using HS4800 hybstation (Tecan). Slides are scanned with a GenePix 4000A scanner and analyzed using the GenePix Pro3.0 image processing software (Axon Instruments, Burlingame, CA/USA).

Samples of each consomic individual is hybridizes against a pool of wild types. Each sample is analyzed in two experimental replicates including a dye swap experiment. Gene expression data are normalized independently for each DNA chip. Identification of significantly differentially expressed genes is performed using TIGR MultiExperiment Viewer (Mev; Saeed *et al.*, 2003; Tusher *et al.*, 2001).

Selected organs

In a first discussion no organ was selected for analysis. When further examination is considered necessary, expression profiling analysis can be performed using our DNA-chip containing 21,000 probes. Please contact Johannes Beckers, (beckers@gsf.de) to discuss this option.

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3.12 Metabolic Screen

3.12.1 Introduction

The metabolic screening provides a comparative analysis of bioenergetic parameters in mice. Mechanisms which lead to disturbances in body weight regulation and energy metabolism are determined. Hence, the basal energetic demands are monitored during *ad libitum* feeding and under food restricted conditions. In humans unbalanced energy uptake and energy expenditure cause the development of obesity (Spiegelman and Flier, 2001) or anorexia nervosa with severe weight loss (Hebebrand *et al.*, 2003). Some rodent and other species tend to increase activity upon food restriction leading to weight loss when given access to an activity wheel (Exner *et al.*, 2000). Several studies described that fasting in mice results in transient depression of metabolic rate, heart rate, body temperature and locomotor activity (Duffy *et al.*, 1990; Williams *et al.*, 2002). Therefore the primary Metabolic Screening focuses on the determination of food and energy uptake under *ad libitum* conditions and metabolic adaptations during food restriction and serves as the origin for further investigations in the Secondary and Tertiary screening which go into details of energy expenditure and energy storage.

3.12.2 Summary

In the primary metabolic screen 14 animals Cs7HH (7 males/ 7 females) were analyzed. Fourteen Cs7HB control mice (7 males/ 7 females) were available. They were fed under *ad libitum* conditions for two weeks followed by two days of acute fasting. The primary metabolic screen focuses on investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature. The analysis for genotype specific differences revealed no significant results in any of the recorded metabolic parameters.

3.12.3 Mice

Seven adult Cs7HB males and seven adult Cs7HH males entered the Metabolic Screen at the beginning of calendar week 48 in 2006. The females (seven controls and seven consomic animals) entered the metabolic laboratory one week later. The mice were single caged on grid panels (0.5°cm grid hole diameter). They were fed *ad libitum* for a period of 14 days, followed by two days of acute fasting to analyze adaptive responses of metabolism.

3.12.4 Material and Methods

Recorded Data

During the different feeding regimes body weight, food consumption (F_{con}), rectal temperature (T_{re}), daily feces production (Fec), energy uptake (E_{up}), energy content of the feces (E_{fec}), metabolizable energy (E_{met}) and the food assimilation coefficient (F_{ass}) were recorded.

Analysis of Feces

The separation of mice in single cages allowed collection of feces in three day intervals. Samples of lab chow and feces (~1 g) were dried at 60°C for two days, homogenized in a coffee grinder and squeezed to a pill for determination of energy content in a bomb calorimeter (IKA Calorimeter C7000) based on dry measurement principle. Energy uptake is determined as the product of food consumed and the caloric value of the food. To obtain metabolizable energy (E_{met}) the energy content of feces and urine (2% of E_{up} ; Drozd 1975) were subtracted from energy uptake.

Statistical Analysis

All values are presented as means \pm SEM. Two-way-ANOVA (SigmaStat, Jandel Scientific) was used to test for effects of the factors strain and sex (ANCOVA with body mass as covariate). The Fisher test was applied for post hoc multiple comparisons. The Mann-Whitney-Test for paired samples was used to analyze the effect of nutritional status on parameters of energy metabolism.

3.12.5 Parameters

Recorded Data during the different feeding regimes
--

body weight, food consumption (F_{con}), rectal temperature (T_{re}), daily feces production (Fec), energy uptake (E_{up}), energy content of the feces (E_{fec}), metabolizable energy (E_{met}), food assimilation coefficient (F_{ass})
--

3.12.6 Results and Discussion

We could not detect any significant effect of the mutation on energy metabolism parameters. The common sex-specific difference in body mass and rectal body temperature was detected.

Prior to the metabolic screening, no information about effects of the mutation were available. Body mass or other relevant parameters of energy metabolism were not affected by the replacement of chromosome 7.

3.12.7 References

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Abbreviations

F_{con}	Food consumption
T_{re}	rectal temperature
Fec	daily feces production
E_{up}	energy uptake
E_{fec}	energy content of the feces
E_{met}	metabolizable energy
F_{ass}	food assimilation coefficient

Table 27: Metabolic parameters recorded in the primary screen											
Data are presented as mean \pm standard error of mean.											
Parameter	Cs7HB				Cs7HH				2 – Way - ANOVA		
	<i>ad libitum</i>		<i>2 days acute fasting</i>		<i>ad libitum</i>		<i>2 days acute fasting</i>		<i>p</i> <i>genotype</i>	<i>p</i> <i>sex</i>	<i>p</i> <i>interaction</i>
	male (n=7)	female (n=7)	male (n=7)	female (n=7)	male (n=7)	female (n=7)	male (n=7)	female (n=7)	<i>ad libitum</i> <i>food reduced</i>	<i>ad libitum</i> <i>food reduced</i>	
Body weight [g]	29.8 \pm 1.0	24.5 \pm 0.4	24.2 \pm 0.9	19.0 \pm 0.3	30.1 \pm 0.9	25.1 \pm 0.7	24.7 \pm 0.7	19.9 \pm 0.7	n.s. n.s.	<0.001 <0.001	n.s. n.s.
Rectal body temperature [°C]	36.95 \pm 0.14	37.30 \pm 0.07	35.42 \pm 0.21	34.50 \pm 0.73	36.91 \pm 0.18	37.50 \pm 0.09	34.73 \pm 0.44	34.84 \pm 0.46	n.s. n.s.	<0.001 n.s.	n.s. n.s.
Food consumption [g day ⁻¹]	3.9 \pm 0.2	3.7 \pm 0.2	no food for two days		3.8 \pm 0.1	3.7 \pm 0.2	no food for two days		n.s.	n.s.	n.s.
Energy uptake [kJ day ⁻¹]	69.38 \pm 2.75	66.35 \pm 2.95			67.63 \pm 1.67	66.56 \pm 1.06			n.a.	n.a.	n.a.
Energy uptake BW ⁻¹ [kJ g ⁻¹ day ⁻¹]	2.33 \pm 0.07	2.70 \pm 0.11			2.26 \pm 0.07	2.67 \pm 0.10			n.a.	n.a.	n.a.
Feces production [g day ⁻¹]	0.82 \pm 0.04	0.82 \pm 0.03			0.80 \pm 0.03	0.78 \pm 0.05			n.a.	n.a.	n.a.
Energy content feces [kJ g ⁻¹]	15.61 \pm 0.04	15.35 \pm 0.08			15.47 \pm 0.05	15.35 \pm 0.08			n.s.	<0.01	n.s.
Metabolized energy [kJ day ⁻¹]	55.47 \pm 2.28	52.72 \pm 2.45			54.17 \pm 1.36	53.56 \pm 0.10			n.s. n.s. ANCOVA	n.s. n.s. ANCOVA	n.s. n.s. ANCOVA
Metabolized energy [kJ g ⁻¹ day ⁻¹]	1.87 \pm 0.06	2.15 \pm 0.09			1.81 \pm 0.05	2.15 \pm 0.09			n.a.	n.a.	n.a.
Food assimilation coefficient [%]	79.9 \pm 0.5	79.4 \pm 0.3			80.1 \pm 0.5	80.5 \pm 0.9			n.s.	n.s.	n.s.

3.13 Pathology Screen

3.13.1 Summary

The Pathology screen performed a complete morphological analysis with standard stains in the consomic for chromosome 7 (C57BL/6N^{Chr7}C3H/HeNcr1) mouse line (Cs7), created as mouse model for dystrophic cardiac calcification (DCC) (Korff *et al.*, 2006). Subtle myocardial and pericardial changes were detected in a small group of consomic homozygous mice and in a few control mice. Although a specific cardiac phenotype was not found, the results are shown and discussed.

3.13.2 Mice

A total of 37 mice was analyzed macroscopically. Histological analysis was performed in 34 mice: 18 mice homozygous for chromosome 7 (Cs7HH) and 16 control (Cs7HB) mice (Table 28).

Table 28: Cs7 consomic mice and control littermates analyzed.					
Control (Cs7HB)		Consomic (Cs7HH)		Number of Animals	Age [weeks]
Female	Male	Female	Male		
8	8	9	9	34	18-19

3.13.3 Materials and Methods

Primary screen: Mice received in the laboratory of pathology were sacrificed with CO₂. The animals were analyzed macroscopically and weighed (<http://www.geocities.com/virtualbiology/>). The thymus and left lobe of the liver were measured. Blood samples were taken, centrifuged and the serum was saved at -20°C. Tails were preserved at -70°C for further genetic analysis. Following a complete dissection, an x-ray of the complete bone structure was taken, when indicated (Hewlett Packard, Cabinet X-Ray System Faxitron Series). All organs were fixed in 4% buffered formalin and embedded in paraffin for histological examination. Two-µm-thick sections from skin, heart, muscle, lung, brain, cerebellum, thymus, spleen, cervical lymph nodes, thyroid, parathyroid, adrenal gland, stomach, intestine, liver, pancreas, kidney, reproductive organs, and urinary bladder were cut and stained with haematoxylin and eosin (H&E).

Additionally screen: The analysis of the heart was amplified with serial sections in an interval of 100µm, so that a mean of 18 cardiac-sections for animal was revised. Additionally, a section was stained with trichromic Masson's, a special histochemical staining for connective tissue, to detect fibrosis.

3.13.4 Results

Macroscopic examination: The expected extensive lesions like fibrotic scar or necrotic calcification occurring in C3H strain in response to myocardial injury were not observed (Figure 23).



Figure 23: Macroscopic examination of the heart

A: Macroscopic picture of the scar in a C3H mouse 5 days after thermoinjury (arrow). Compare with **B:** The macroscopic picture of a normal heart of a consomic (HH) mouse.

Histological examination: All the organs analyzed appear normal (Table 2). With exception of a small group of mice which developed subtle myocardial and pericardial lesions “spontaneously” (without iatrogenic injury).

Table 29: Overview genotype-specific alterations of Cs7 consomic strain.			
Analyzed:	Alteration	Analyzed:	Alteration
Bodyweight	No	Pancreas	No
Skin	No	Cervical lymph node	No
Musculoskeletal system	No	Thymus	No
Eyes	No	Spleen	No
Cerebrum	No	Thyroid	No
Cerebellum	No	Parathyroid	No
Heart / Arteries	Yes ?	Adrenal gland	No
Trachea	No	Kidneys	No
Lung	No	Urinary bladder	No
Teeth	No	Testes	No
Salivary gland	No	Epididymis	No
Esophagus	No	Funiculus spermaticus	No
Stomach	No	Ovaries	No
Small intestine	No	Uterus	No
Large intestine	No	Vagina	No
Liver	No	Mamma	No

Myocardium: Lesions consisting of necrosis with / or without calcification were found in 6/18 Cs7HH mice and in 1/16 control mice. 33.3% of the Cs7HH mice are affected vs. only 6.25% of the control mice. The difference is not significant. Fischer’s Exact Test p value = 0.090.

Lesions in myocardium	Control (HB)	Consomic (HH)	Total
Number of mice with lesions	1	6	7
Number of mice without lesions	15	12	27
Total	16	18	34

The different lesions found in the Cs7HH mice are shown (Figures 24, 25). Only **one** of the Cs7HH mouse developed severe, multifocal areas of myocardial necrosis, the expected fibrosis was sparse and calcification was absent.

Multifocal calcifications distributed near by coronary vessels were observed in **one** of 18 Cs7HH mouse. The tissue surrounding the vessels was normal.

In **four** of 18 Cs7HH mice focal calcium deposits in the myocardial cells were observed, the lesions were small (probably <10 cells) and apparently isolated. The tissue adjacent to the calcifications showed no reactive changes like necrosis, infiltration or fibrosis.

From the control mouse **one** of 16 was affected, and showed small, focal calcifications (Figure 26).

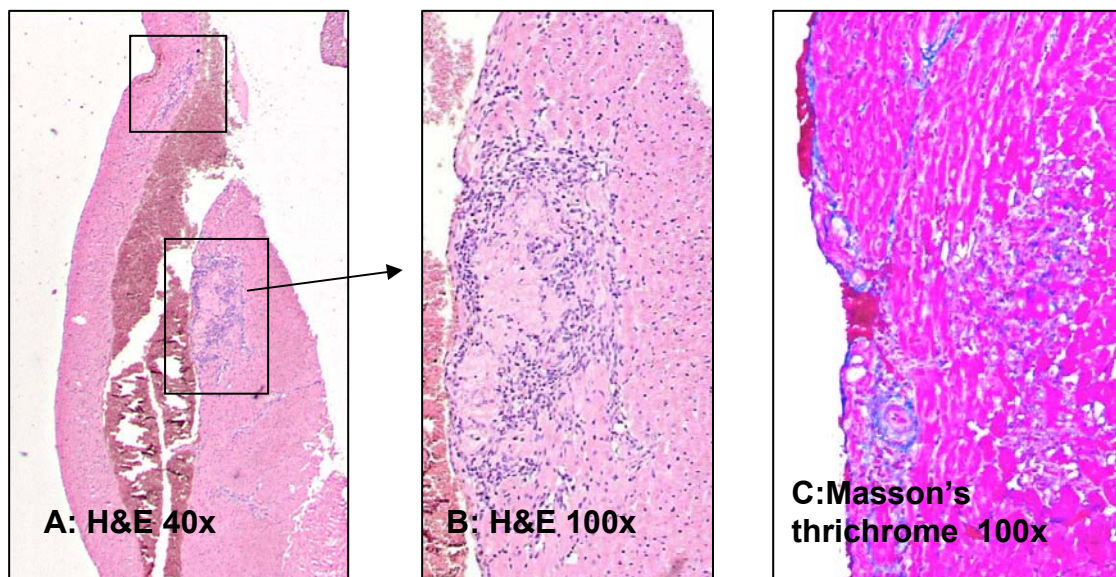


Figure 24: Myocardial necrosis found in a homozygous consomic mouse.

A: Multifocal distribution of the lesions. **B:** High magnification of the necrotic area, delimited by chronic infiltrate (lymphocytes). Calcium deposits are not observed. **C:** Sparse fibrosis present in the necrotic area depicted in blue by Masson's trichrome staining.

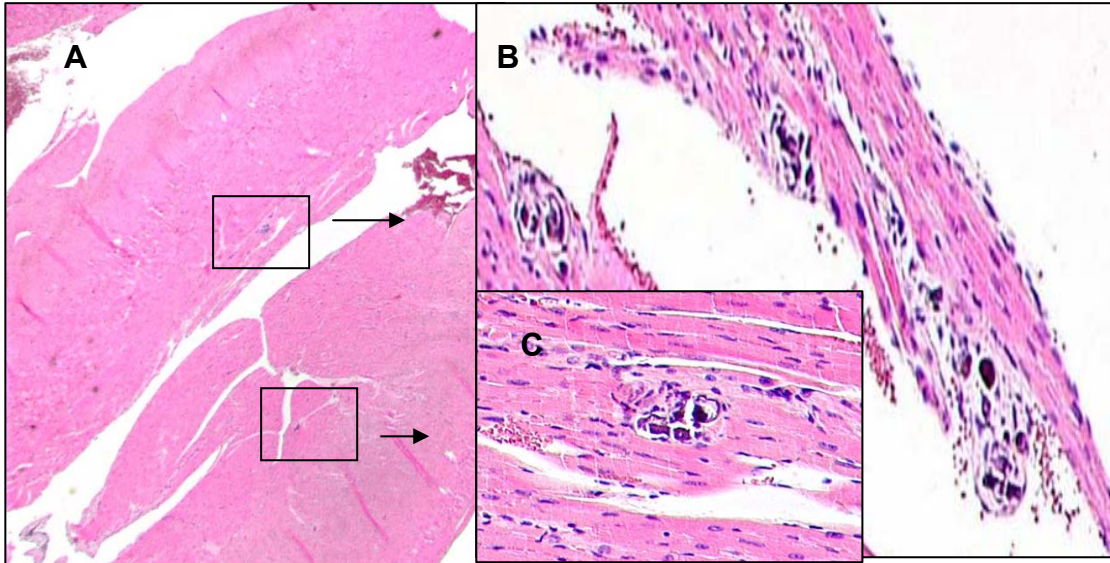


Figure 25: Myocardial calcifications found in a consomic mouse.

A: Shows the multifocal distribution of the lesions H&E 12,5x. **B: C:** High magnification (320x) of the marked areas. H&E staining depicted the calcium deposits as dark-blue spots.

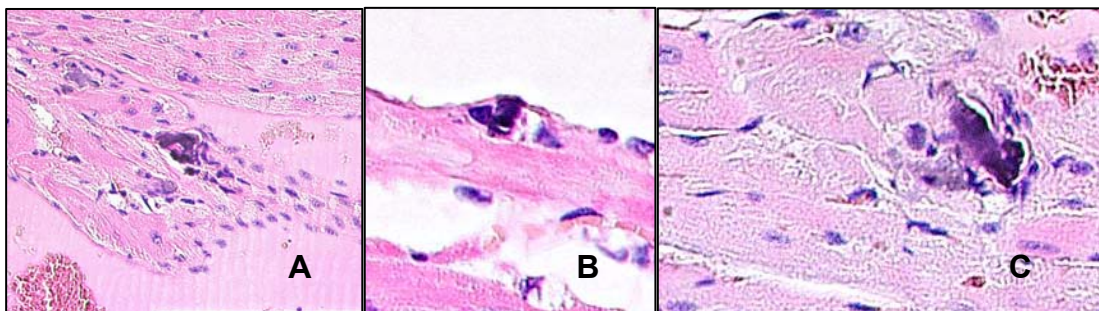


Figure 26: Focal myocardial calcifications

A, B: H&E 320x original magnification of focal myocardial calcifications observed in two homozygous consomic mice. To compare with **C:** H&E 320x magnification of a similar lesion present in a control mouse.

Pericardium.

In two/18 Cs7HH mice and in four/16 control mice an increase of abnormal residual connective tissue in specific areas of the pericardium was observed (Figure 27).

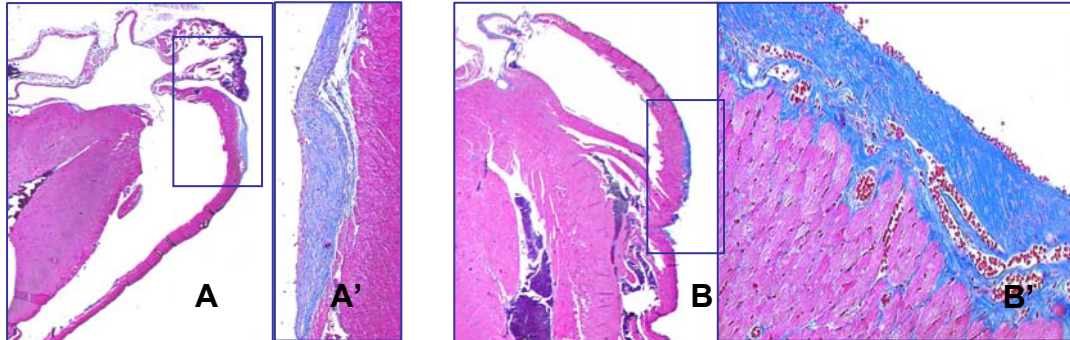


Figure 27: Trichrome Masson's staining of pericardial lesions.

In **A**: The 12.5x magnification shows the pericardial localization of a lesion observed in a Cs7HH consomic mouse. In **B**: The 12.5x magnification displays a similar lesion in a control mouse (marked by boxes). **A'**, **B'**: A high magnification (40x) of the marked boxes to see the strong increase in the number of connective fibers (blue). Calcifications in the fibrotic scar were not observed.

3.13.5 Discussion

In the consomic Cs7 strain, mice homozygous for chromosome 7 (Cs7HH mice) carry the susceptibility locus *Dyscalc1* (Korff *et al.*, 2006). It is present in the C3H strain and determines necrotic calcification occurring in response to myocardial injury. This phenotype is described as **dystrophic cardiac calcification** (DCC; Aherrahrou *et al.*, 2004).

In this mouse model it is expected that the Cs7HH mice develop the DCC phenotype without injury. DCC is characterized by myocardial cell necrosis, calcium deposition, and/or fibrotic changes.

The lesions observed are ambiguous. One can assume that the time point of examination was too early to develop these features (long term effects) Therefore, according with the mouse provider, a secondary screen of aged mice (more than one year) using special stains to detect calcifications was planned and has started recently.

3.13.6 Secondary Screening

Table 30 gives a brief overview on the planned, ongoing and finished experiments.

Table 30: Cs7 consomic mice and control littermates analyzed in secondary screen

Batch	Control (Cs7HB)		Consomic (Cs7HH)		Age [weeks]	Plan / Comment
1)		4			More than 40	Mice as control, with iatrogenic lesions in myocardium (scar with calcifications) Goal: Rx-analyses to see the calcifications in myocardium, as preliminary screen for the histology (<i>finished</i>)
2)			6-8	6-8	More than 40	Goal: identify myocardial lesions with calcifications Methods used: Rx-analysis, Histological analyses of all organs serial cuttings of the heart (18-40 cardiac-sections per animal and heart) performing of special stains (Masson, Mowat, calcium-staining) Electronic microscopy in 2 mice pro group. <i>Finished: the cutting and staining with Mowat and Masson</i> <i>To do: The histological analysis and E.M.</i>
3)	10	10			More than 40	Goal: identify myocardial lesions with calcifications Methods used: Rx-analysis, Histological analyses of all organs serial cuttings of the heart (18-40 cardiac-sections per animal and heart) performing of special stains (Masson, Mowat, calcium-staining) Electronic microscopy in 2 mice per group. <i>Finished: the cutting and staining with Mowat and Masson</i> <i>To do: the histological analysis and E.M.</i>

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Acknowledgements

A large team consisting of scientists, technicians and animal caretakers all contribute to the success of the German Mouse Clinic. We want to thank Reinhard Seeliger, Elfi Holupirek, Susanne Axtner, Miriam Backs, Christine Fürmann, Tamara Halex, Sabine Holthaus, Nadine Kink, Claudia Kloss, Regina Kneuttinger, Kerstin Kutzner, Maria Kugler, Jacqueline Müller, Elenore Samson, Sandra Schädler, Ann-Elisabeth Schwarz, Bettina Sperling, Susanne Wittich, and Claudia Zeller for expert technical help and Daniela Kißling, Manuela Huber, Petra Thalmeier, Sabine Schwarz, and Anica Miedl for the care of the mice.

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Addresses of screeners and modules

Coordinators

Dr. Valérie Gailus-Durner
Dr. Helmut Fuchs
Barbara Ferwagner
Dr. Christoph Lengger
Dr. Beatrix Naton
Prof. Dr. Martin Hrabé de Angelis
Institute of Experimental Genetics
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3613
Fax: 089-3187-3500
Email: gailus@helmholtz-muenchen.de

Behavior Screen

Dr. Sabine M. Hölter
Magdalena Kallnik
Institute of Developmental Genetics
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3674
Fax: 089-3187-3099
Email: hoelter@helmholtz-muenchen.de

Dysmorphology Screen,

Dr. Helmut Fuchs
Dr. Wolfgang Hans
Prof. Dr. Martin Hrabé de Angelis
Institute of Experimental Genetics
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3151
Fax: 089-3187-3500
Email: hfuchs@helmholtz-muenchen.de

Neurology Screen

Dr. Lore Becker
Eva Kling
German Mouse Clinic (GMC)/Neurology
Institute of Experimental Genetics
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3654
Fax: 089-3187-3500
Email: [lore.becker@helmholtz-
muenchen.de](mailto:lore.becker@helmholtz-muenchen.de)

PD Dr. Thomas Klopstock
Friedrich-Baur-Institut,
Neurologische Klinik
Ludwig-Maximilians-Universität München
Ziemssenstraße 1a
D-80336 München
Tel: 089-5160-7474
FAX: 089-5160-7402
Email:
[Thomas.Klopstock@nro.med.uni-
muenchen.de](mailto:Thomas.Klopstock@nro.med.uni-muenchen.de)

Eye Screen

Dr. Claudia Dalke
Institute of Developmental Genetics
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-2910
Fax: 089-3187-2210
Email: dalke@helmholtz-muenchen.de

Clinical-Chemical Screen

Dr. Birgit Rathkolb
Dr. Corinna Mörth
GMC - German Mouse Clinic
Clinical-Chemical Screen
Institute of Experimental Genetics
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3282
Email: [birgit.rathkolb@helmholtz-
muenchen.de](mailto:birgit.rathkolb@helmholtz-muenchen.de)

Prof. Dr. Eckhard Wolf
Institute of Molecular Animal Breeding
and Biotechnology
Genecenter
LMU München
Feodor Lynen-Straße 25
D-81377 München
Tel.: 089-21807-6800
Email: ewolf@lmb.uni-muenchen.de

Immunology Screen

Dr. Thure Adler
Prof. Dr. Dirk Busch
GMC - German Mouse Clinic
Institute for Experimental Genetics
Helmholtz Zentrum München
German Research Center for Environmental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3656
Fax: 089-3187-3500
Email: thure.adler@helmholtz-muenchen.de

Prof. Dr. Dirk Busch
Institute for Medical Microbiology,
Immunology and Hygiene
Technische Universität München (TUM)
Trogerstr. 9
D-81675 München
Tel.: 089-4140-6191
Fax: 089-4140-4139
Email: dirk.busch@lrz.tum.de

Allergy Screen

Anahita Javaheri, MSc
Antonio Aguilar
Prof. Dr. Markus Ollert
Klinik und Poliklinik für Dermatologie
und Allergologie am Biederstein
Technische Universität München (TUM)
Biedersteinerstraße 29
D-80802 München
Tel.: 089-4140-3551 (M.O.)
Tel.: 089-3187-2554 (A.J.)
Fax: 089-4140-3552
Email: ollert@lrz.tum.de

Steroid Screen

Dr. Cornelia Prehn
Prof. Dr. Jurek Adamski
Institute of Experimental Genetics
Helmholtz Zentrum München
German Research Center for Environmental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3231
Fax: 089-3187-3500
Email: prehn@helmholtz-muenchen.de

Nociceptive Screen

Dr. Ildikó Rácz
Laboratory of Molecular Neurobiology
Department of Psychiatry
University of Bonn
Sigmund-Freud-Straße 25
D-53105 Bonn
Tel.: 0228-688-5316
Fax: 0228-688-5301
Email: iracz@uni-bonn.de

Prof. Dr. Andreas Zimmer
Laboratory of Molecular Neurobiology
Department of Psychiatry
University of Bonn
Sigmund-Freud-Straße 25
D-53105 Bonn. Germany
Tel.: 0228-688-5303

Lung Function Screen

Dr. Ines Bolle
Prof. Dr. Holger Schulz
Institute for Inhalation Biology
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-4119
Fax.: 089-3187-2400
Email: schulz@helmholtz-muenchen.de

Molecular Phenotyping

Dr. Marion Horsch
Dr. Johannes Beckers
Institute of Experimental Genetics
Helmholtz Zentrum München
German Research Center for Environ-
mental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3513
Fax: 089-3187-4085
Email: horsch@helmholtz-muenchen.de

Metabolic Screen

Dr. Jan Rozman
Nicole Ehrhardt
Institute of Experimental Genetics
GMC - German Mouse Clinic
Metabolic Screen
Helmholtz Zentrum München
German Research Center for Environmental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3807
Fax: 089-3187-3500
Email: jan.rozman@helmholtz-muenchen.de

Prof. Dr. Martin Klingenspor
Technische Universität München
Nutrition and Food Research Center
Molecular Nutrition
Am Forum 5
D-85350 Freising-Weihenstephan

Cardiovascular Screen

Dr. Anja Schrewe
Institute of Experimental Genetics
GMC - German Mouse Clinic
Cardiovascular Screen
Helmholtz Zentrum München
German Research Center for Environmental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3646
Fax: 089-3187-3500
Email: anja.schrewe@helmholtz-muenchen.de

Dr. Boris Ivandic
Prof. Dr. Hugo Katus
Innere Medizin III
Otto-Meyerhof-Zentrum
Im Neuenheimer Feld 350
D-69120 Heidelberg
Tel.: 06221 - 56-1505
Email: boris_ivandic@med.uni-heidelberg.de

Pathology Screen

Dr. Julia Calzada-Wack
Dr. Gabriele Hölzlwimmer
Dr. Ilona Moßbrugger
PD Dr. Irene Esposito
PD Dr. Leticia Quintanilla-Martinez
Institute of Pathology
Helmholtz Zentrum München
German Research Center for Environmental Health (GmbH)
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3241
Fax 089-3187-3360
Email: irene.esposito@helmholtz-muenchen.de