Materials and Methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals and housing
All animals were kept under SPF conditions with 12 hours per day light (7.00 h - 19.00 h) and controlled temperature and humidity regimen. Six males and six females of the inbred strains BC/CpbU (BC) (obtained from the Central Laboratory Animal Institute of the Utrecht University, The Netherlands) and LEW/OlaHsd (LEW) (obtained from Harlan, UK) were tested for cholesterol response, as described in the experimental protocol (see below). From each gender and strain two animals were used for reciprocal matings. The F1-hybrids were intercrossed producing an F2 progeny.

Experimental protocol
After weaning, at the age of three weeks, the animals were fed a commercial, pelleted diet (RMH-B®, Hope Farms BV, Woerden, The Netherlands) till the age of seven weeks. The chemical composition of this commercial diet has previously been described. Then, at the age of seven weeks, the commercial diet was supplemented with 5.0% (w/w) olive oil (Reddy, Van de Moortele NV, Oudenbosch, The Netherlands) and 2.0% (w/w) cholesterol (USP, Solvay/Pharmaceuticals BV, Weesp, The Netherlands) during four weeks. This pelleted test-diet had been stored in the freezer until use. The animals had free access to food and water before and during the test-period. At the start and at the end of the test-period, after a 16 hour fast, blood samples were taken by orbital puncture under diethyl-ether anesthesia (between 8.00 and 10.00 h). Serum was collected after centrifugation and stored at -20°C. Following the last blood sampling, the rats were anaesthetised with diethyl-ether, exsanguinated via aorta puncture and the livers and spleens were removed. The tissues were frozen immediately at -70°C.

Chemical analyses
In a total of 192 F2-animals (92 males and 100 females) basal serum cholesterol levels, postdietary liver cholesterol concentrations, and serum phospholipid, cholesterol, aldosterone and corticosterone levels were determined. Lipids were extracted from liver homogenates according to a modification of the method of Abell et al. Liver cholesterol, serum cholesterol and serum phospholipids were measured enzymatically using the appropriate kits supplied by Boehringer Mannheim GmbH (Mannheim, Germany). Serum aldosterone and corticosterone concentrations were determined by radioimmunoassays as previously described.

Genome scan
DNA was isolated from the spleen using a standard procedure. A total of 256 microsatellite (SSLP) markers, polymorphic between the BC and LEW strain, were used for screening of the F2-progeny. These markers were randomly dispersed throughout the rat genome. Primers flanking the microsatellites were obtained from Research Genetics Inc. (MapPairs™, Huntsville, USA). When a microsatellite in BC and LEW differed less than 10 basepairs, the forward primer was 5'-end labelled with [γ-32P]ATP. Twenty nanogram genomic DNA was used for PCR according to manufacturers protocol. Products were separated in standard sequencing gels. When the microsatellites differed more than 10 bp between BC and LEW, 100 ng genomic DNA was used in the PCR reactions. These non-radioactive PCR products were separated in standard agarose gels.
Map construction

Segregation ratio of the genotypes of individual markers was checked by means of the Chi-squared goodness-of-fit test. None of the markers showed significant segregation distortion. The genetic map distance for the markers was computed with JoinMap™, version 3.0. For the establishment of linkage groups, a critical minimal LOD score of 3.0 was used. For calculation of map distances and estimating most likely gene orders, a critical LOD score of 0.05 was used. Recombination frequencies were converted to map distances in centiMorgans using the Kosambi function. Output from JoinMap was converted to figures using the graphics program MapChart.

Statistical analysis

Both for the parental strains and for the F2-intercross rats, all statistical analyses of the measured phenotypes were carried out according to Petrie and Watson using a SPSS PC+ computer program. Two-side probabilities were estimated throughout.

a. Parental strains

The phenotypic characteristics of the BC and LEW rats were checked for normality using the Kolmogorov-Smirnov one-sample test. All results within groups were found to be normally distributed. Student's one sample t test for paired data was used to evaluate changes with time within groups. The significance of the differences between groups was calculated by a two-way analysis of variance (ANOVA) with strain and gender as main factors. Homogeneity of the variances was tested by Bartlett's test. For some phenotypes the variances had to be equalized by transformation (i.e. logarithmic) of the data. After transformation the variances were similar and the transformed within-group data still were normally distributed. For the serum and liver parameters the two-way ANOVA was performed with body weight as covariate, because there were significant differences in body weight between the two strains.

If the ANOVA showed significant effects the group means were further compared with the unpaired Student's t test. These tests were performed with pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was tested using a F test. To take into account the greater probability of a type I error due to multiple comparisons, the level of significance for the Student's t tests was pre-set at P<0.05/[times a group is used for a comparison] instead of P<0.05, according to Bonferroni's adaptation. In all other cases, the probability of a type I error <0.05 was taken as the criterion of significance.

b. F2-animals

Within each gender, all traits were normally distributed (Kolmogorov-Smirnov one-sample test). Gender appeared to have a significant influence on the phenotypes (unpaired Student's t test, p<0.05), except on final body weight and baseline serum cholesterol level. For the genetic analysis of the gender-influenced traits in the combined male and female population, these phenotypes were first normalized in each gender. The measured levels were subtracted by the mean established for that gender and then divided by the standard deviation of that gender. Within the combined male and female population the (transformed) variables were normally distributed according to the Kolmogorov-Smirnov one-sample test.

QTL analysis

The location of the QTLs affecting the measured (transformed) quantitative traits were determined using the interval-mapping module of the MapQTL computer package (version 4.0). QTL analysis was also performed by MQM-mapping on the MapQTL computer.
program. QTL-likelihood plots were produced by using the markers that flank the LOD score peak of identified QTLs in the interval-mapping method, as cofactors for QTL mapping on the MapQTL computer program.

For each trait and chromosome the LOD score thresholds were calculated by permutation analysis\(^1\) in order to achieve the chromosome-wide significance levels of 5%. Based on the paper of Lander and Kruglyak\(^2\) an association was assumed suggestive when the LOD score was between 57.6% and 100% of the LOD score threshold for significance.

If a DNA marker and the trait of interest are segregating independently, the values of the trait will be equally distributed among the homozygote and heterozygote genotypes. All data within genotype groups were found to be normally distributed by using the Kolmogorov-Smirnov one-sample test. For each group (females and males, females or males), co-segregation of phenotypes with alleles at marker loci was evaluated by comparing the values between different genotypes via one-way ANOVA with or without body weight as co-variate. For serum and liver parameters body weight was used as co-variate, since in F\(_2\) rats there were between some of these parameters and body weight weak, but significant linear associations (Table I). Pearson’s linear correlation coefficients (\(r\)) were calculated; significance was assessed by a two-tailed test. In the ANOVA tests, homogeneity of variances was tested (Bartlett's test). When necessary, the variances were equalized by logarithmic or logistic transformation of the data.\(^8\) After transformation the within-group data were still normally distributed.

**Interactions**

Selected two-locus pairs (i.e. the loci that were flanking a candidate gene) were tested for epistatic interactions with a two-way ANOVA with body weight as a co-variate.

**References**

9. SPSS Inc. *SPSS/PC+™ 4.0, Base Manual for the IBM PC/XTAT and PS/2V (Release 4.0).* 1990; SPSS Inc., Chicago, IL USA.
Table I. Associations between body weight and serum or liver parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pearson's linear correlation</th>
<th>F2 rats</th>
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<tbody>
<tr>
<td></td>
<td>Males (n = 92)</td>
<td>Females (n = 100)</td>
</tr>
<tr>
<td>Baseline serum cholesterol(^a)</td>
<td>( r ) 0.2202</td>
<td>-0.1306</td>
</tr>
<tr>
<td></td>
<td>p-value 0.036(^c)</td>
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<tr>
<td>Postdietary serum cholesterol(^b)</td>
<td>( r ) 0.0873</td>
<td>0.1421</td>
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<tr>
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<td>p-value 0.408</td>
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<td>Postdietary serum phospholipid(^b)</td>
<td>( r ) 0.0035</td>
<td>0.2104</td>
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<tr>
<td></td>
<td>p-value 0.973</td>
<td>0.036</td>
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<tr>
<td>Postdietary liver cholesterol(^b)</td>
<td>( r ) 0.3061</td>
<td>0.1432</td>
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<td>p-value 0.003</td>
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<td>Postdietary serum aldosterone(^b)</td>
<td>( r ) -0.1228</td>
<td>-0.2465</td>
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<td>p-value 0.252</td>
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<tr>
<td>Postdietary serum corticosterone(^b)</td>
<td>( r ) -0.1135</td>
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<td></td>
<td>p-value 0.287</td>
<td>0.041</td>
</tr>
</tbody>
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\(^a\) Association with initial (Day 0) body weight.
\(^b\) Association with final (Day 28) body weight.
\(^c\) Significant associations (\( P < 0.05 \)) are indicated in bold characters.
Fig. I  The genetic map (left) and LOD score plot (right) for initial body weight in the (LEW x BC)F₂-intercross. The thick horizontal line represent the threshold value of the LOD score considered as significant for linkage. (A) Rat chromosome 7 (RNO7), females. (B) Rat chromosome 15 (RNO15), males plus females.
Fig. II The genetic map (left) and LOD score plot (right) for postdietary serum cholesterol level in the (LEW x BC)F2-intercross. The thick horizontal line represent the threshold value of the LOD score considered as significant for linkage. (A) Rat chromosome 2 (RNO2), males plus females. (B) Rat chromosome 16 (RNO16), males plus females.
Fig. III  The genetic map (left) and LOD score plot (right) for postdietary serum phospholipid level in the (LEW x BC)F₂-intercross. The thick horizontal line represents the threshold value of the LOD score considered as significant for linkage. Rat chromosome 11 (RNO11); 1 = males plus females, 2 = females.
Fig. IV The genetic map (left) and LOD score plot (right) for postdietary serum aldosterone level in the (LEW x BC)F2-intercross. The thick horizontal line represents the threshold value of the LOD score considered as significant for linkage. (A) Rat chromosome 1 (RNO1), females. (B) Rat chromosome 18 (RNO18), females.