

CHAPTER IV: Experimental Results

1 Antibody Generation

All the antibodies used in the present thesis are summarized in Table 2, together with further specifications. One of the antibodies was raised in rabbits in Paris by JM Pléau in 1986. He kindly allowed me to make use of two preparations, R-Ab_24, frozen serum without additives, and R-Ab_23, being the same serum stored 1:1 in glycerol. Both preparations had been stored at -20°C from 1986 on. R-Ab_23 was dialyzed (R-Ab_21) and later purified by ammonium sulfate (R-Ab_22). R-Ab_24 was ammonium sulfate-purified (R-Ab_25) in the same way as the other pure serum-preparations (R-Ab_11/_31/_c1) were purified (R-Ab_12/_32/_c2).

Table 2: Synopsis of the Antibody-Characteristics. – Three different rabbit-IgG-antibodies (R-Ab_1x/_2x/_3x) and four different chicken-IgY-antibodies (Ch-Ab_1/_2/_3/_4) were used in this thesis. R-Ab_c1/_c2 were used as a control. All antibodies were generated in our laboratory at Charité (Berlin), apart from R-Ab_23 (received in 50% glycerol) and R-Ab_24 (received as frozen serum) that were a kind gift from JM Pléau (Paris). R-Ab_25 was obtained in Berlin by AS-purification of R-Ab_24, R-Ab_21 and R-Ab_22 were obtained by dialysis against PBS and dialysis + AS-purification of R-Ab_23, respectively. Protein concentrations were obtained by measuring photometric absorption. (Abbreviations: R = rabbit, Ch = chicken, AS = ammonium sulfate, PEG = polyethylenglycol)

Antibody	Specificity	Species	Animal	Source	Ig	Origin	Year of Generation	Purification/ Treatment	Protein concentration
R-Ab_11	Thymulin	Rabbit	K72	Serum	IgG	Berlin	2003	None	not tested
R-Ab_12	Thymulin	Rabbit	K72	Serum	IgG	Berlin	2003	AS	46.70 mg/ml
R-Ab_21	Thymulin	Rabbit	Pléau	Serum	IgG	Paris	1986	Dialysis	17.00 mg/ml
R-Ab_22	Thymulin	Rabbit	Pléau	Serum	IgG	Paris	1986	Dialysis, AS	06.13 mg/ml
R-Ab_23	Thymulin	Rabbit	Pléau	Serum	IgG	Paris	1986	None	43.23 mg/ml
R-Ab_24	Thymulin	Rabbit	Pléau	Serum	IgG	Paris	1986	None	44.60 mg/ml
R-Ab_25	Thymulin	Rabbit	Pléau	Serum	IgG	Paris	1986	AS	19.00 mg/ml
R-Ab_31	Thymulin	Rabbit	K09	Serum	IgG	Berlin	2004	None	14.29 mg/ml
R-Ab_32	Thymulin	Rabbit	K09	Serum	IgG	Berlin	2004	AS	11.84 mg/ml
R-Ab_c1	KLH	Rabbit	K10	Serum	IgG	Berlin	2004	None	13.50 mg/ml
R-Ab_c2	KLH	Rabbit	K10	Serum	IgG	Berlin	2004	AS	08.19 mg/ml
Ch-Ab_1	Thymulin	Chicken	H41	Egg-Yolk	IgY	Berlin	2003	PEG	15.21 mg/ml
Ch-Ab_2	Thymulin	Chicken	H42	Egg-Yolk	IgY	Berlin	2003	PEG	08.00 mg/ml
Ch-Ab_3	Thymulin	Chicken	H32	Egg-Yolk	IgY	Berlin	2002	PEG	10.74 mg/ml
Ch-Ab_4	Thymulin	Chicken	H33	Egg-Yolk	IgY	Berlin	2002	PEG	13.56 mg/ml

2 Antibody Purification

2.1 PEG Precipitation

Chicken egg-yolk was purified in order to separate its antibody content from other proteins and substances. Photometric absorption at 280nm led to estimation of the remaining protein concentrations given in Table 2.

2.2 Ammonium Sulfate-Precipitation

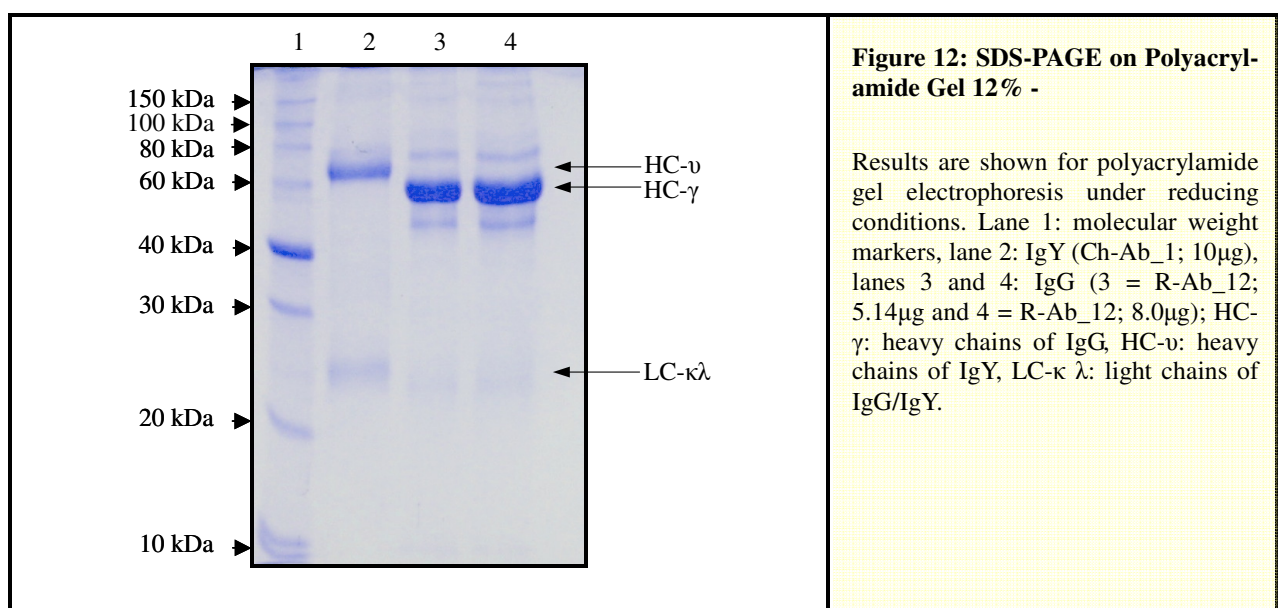
Rabbit serum was purified by ammonium sulfate-precipitation in order to separate its antibody-content from other proteins and substances. Additionally, the antibody R-Ab_22 was obtained by ammonium sulfate-purification of a 1:1 serum-glycerol mixture (R-Ab_23) that had previously been dialyzed against PBS (R-Ab_21). Photospectrometric absorption at 280nm led to estimation of the remaining protein-concentrations shown in Table 2.

2.3 Affinity Chromatography

Affinity chromatography of the antibodies R-Ab_12, Ch-Ab_1 and Ch-Ab_2 was carried out. Severe problems were encountered when elution was performed. Data not shown.

3 Characterization and Comparison of the Antibodies Generated – Assessment of Immunoreactivity

3.1 Polyacrylamide Gel Electrophoresis

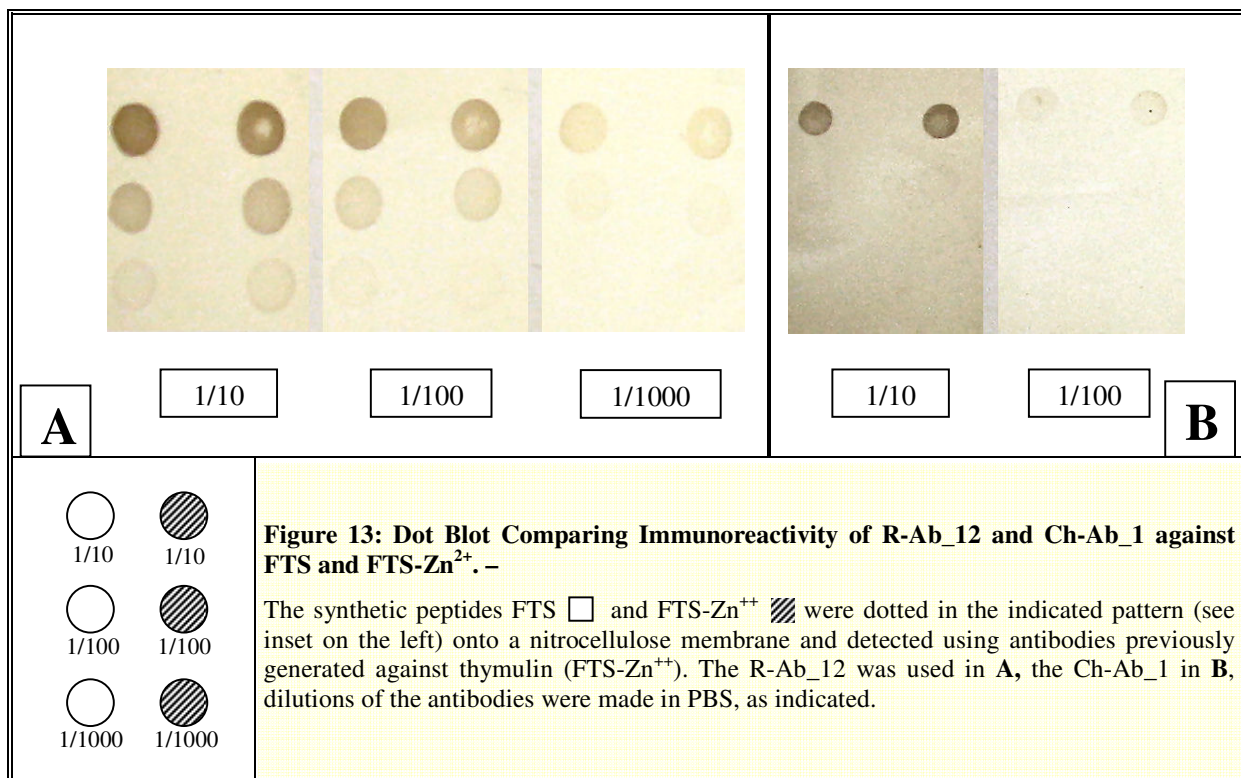


Results are shown in Figure 12. The two main bands in lane 2 represent the heavy chain (HC, molecular weight: 65kDa) and the light chain (LC, molecular weight: 25kDa) of avian IgY. The two main bands in lanes 3 and 4 represent the heavy chain (HC, molecular weight 60kDa) and the light chain (LC, molecular weight: 25kDa) of mammalian IgG. Other bands in both preparations are due to contamination.

3.2 Assessment of Immunoreactivity by Dot Blot

The antibodies obtained following purification were tested by Dot Blot for reactivity against the synthetic peptide FTS in comparison with their reactivity against FTS+Zn²⁺ in an equimolar ratio: the results are shown in Figure 13, exemplified for R-Ab_12 and Ch-Ab_1. No differences in immunoreactivity against FTS and FTS-Zn²⁺ could be detected for IgG-antibodies. Similar but less intense reactions were found for IgY.

Additionally, comparisons between purified and non-purified antibodies were performed: the Dot Blot turned out not to be sufficiently sensitive to allow conclusions (Data not shown).



3.3 ELISA

3.3.1 Optimization of an ELISA for Thymulin

A series of studies were performed to optimize an ELISA for thymulin. To date, existing protocols have not worked satisfactorily, usually revealing insufficient sensitivity to detect circulating levels of thymulin. Changes in parameters like incubation conditions and background blocking conditions led to reproducible and valid results using mammalian antibodies. After introducing a significantly better amplification system, the kit ABC (see figure 14), we set up the final protocol (see Chapter III, 4.3) that allowed further evaluation of all rabbit-antibodies listed in table 2.

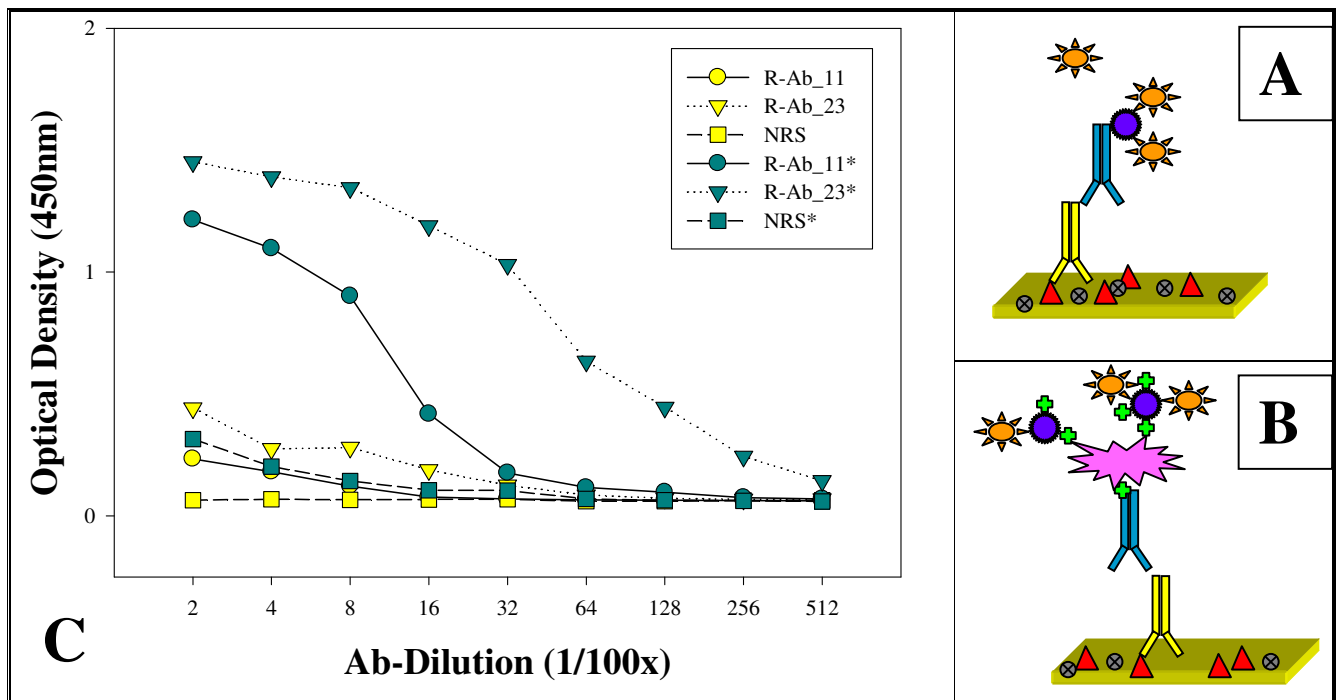


Figure 14: Comparison of Two ELISA Configurations for Detection of Thymulin. – Two antibodies against FTS (R-Ab_11, R-Ab_23) were used following a classic protocol applying a POX-labeled secondary antibody (see inset A), subsequently the same antibodies (*) were used introducing the ABC-kit (avidin-biotin-complex) for further amplification of the response (see inset B). Normal rabbit serum (NRS) was used as control in both cases. Mean values of n=2 are shown in C. Avidin = pink, biotin = green, POX = blue, Thymulin = red, primary antibody = yellow, secondary antibody = turquoise, substrate = orange, blocking substance = grey; - Data plotted with SigmaPlot 4.0. -

Efforts on an ELISA for avian antibodies were given up when strong non-specific interference was encountered. Application of different blocking substances commonly used in ELISA systems (BSA, Gelatin and Casein at varying concentrations) as well as the addition of

detergents to all the buffers used (Tween and Triton X in varying concentrations) were ineffective to improve assay performance. (Data not shown).

3.3.2 Assessment of Immunoreactivity by ELISA

For mammalian antibodies a slightly weaker sensitivity to FTS-Zn²⁺ than against FTS alone was shown by ELISA (see figure 15). All mammalian antibodies were assessed by ELISA. Comparative results are summarized in figure 16.

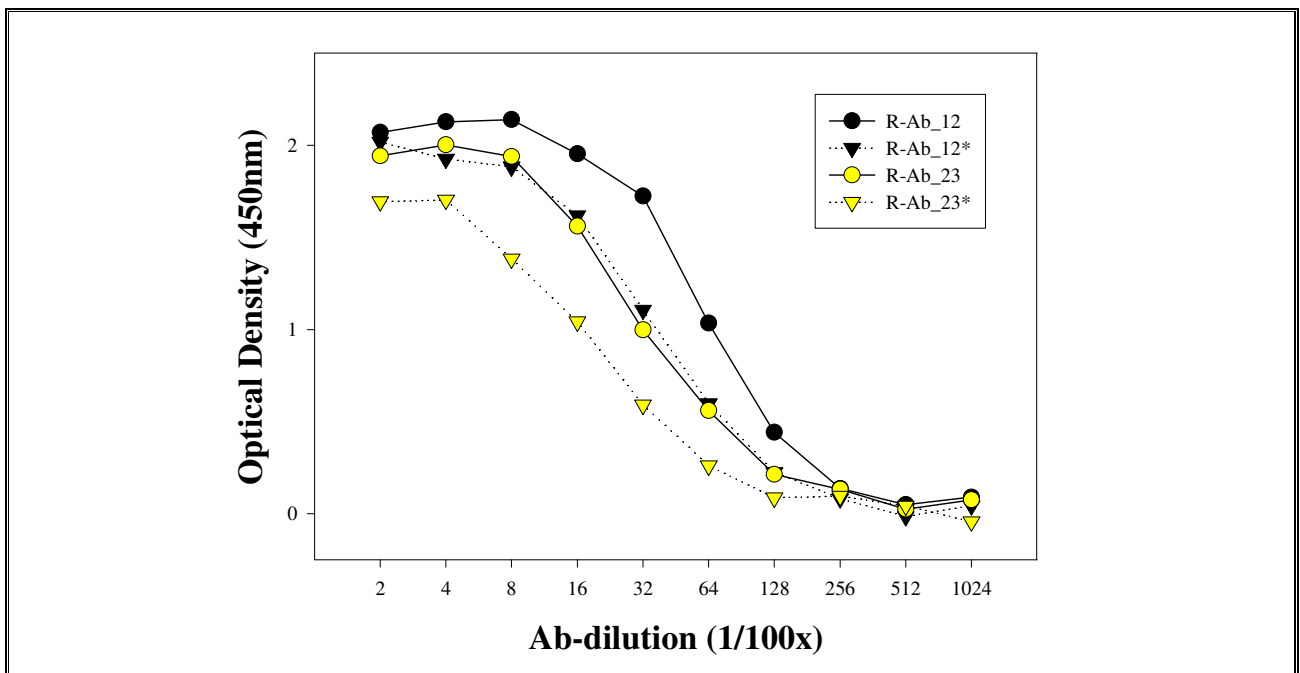


Figure 15: Comparison of Sensitivity of Mammalian Anti-Thymulin-Specific Antibodies (here exemplified by R-Ab_12 and R-Ab_23) against FTS (●) or FTS-Zn²⁺ (▼). - 96-well-ELISA-plates were coated with 100ng FTS alone or 100ng FTS-Zn²⁺ per well and serial dilutions (1:2) of the antibodies were incubated under standard conditions. Optical density was determined with an ELISA-reader. False positive reactions were controlled by measuring signals against non-FTS/FTS-Zn²⁺-containing wells as negative controls. Mean values of n=2 are shown. - Results were plotted with SigmaPlot 4.0 -

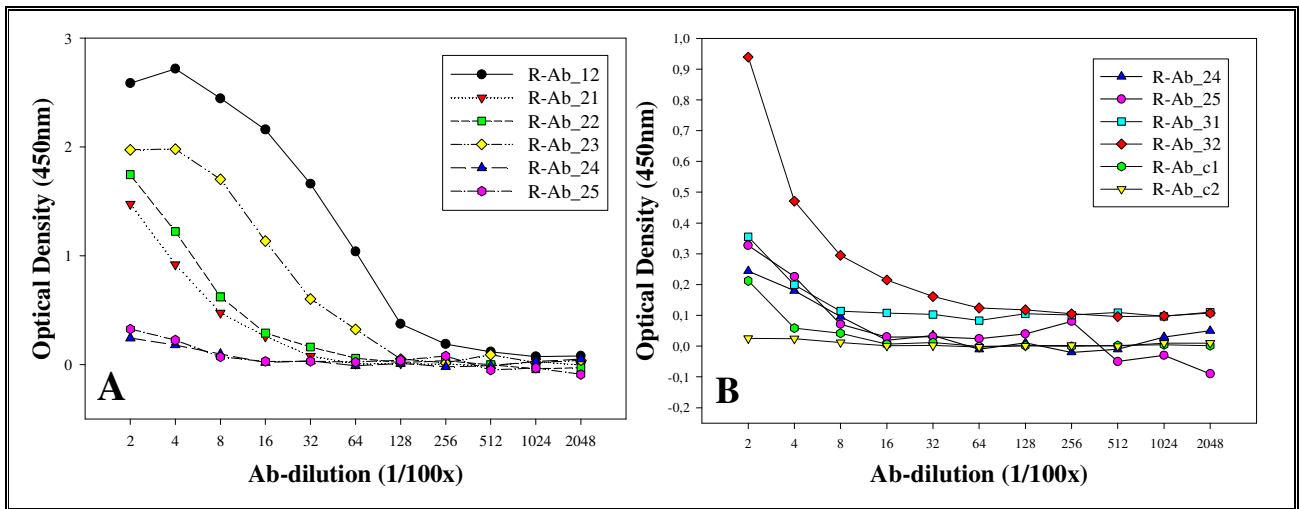


Figure 16: Titration of Selected Mammalian Antibody-Preparations. – Serial 1/2-antibody dilutions were incubated in FTS-Zn²⁺-coated 96-well ELISA plates (100ng/well) in order to compare their sensitivity. Mean values of n=3 are shown. Please note that a different Y-axis scale has been applied in A and B. - Data plotted with SigmaPlot 4.0 -

4 Characterization and Comparison of the Antibodies Generated – Assessment of Biological Activity In Vitro

4.1 In Vitro Quenching

4.1.1 Competitive ELISA

The *in vitro* immunoneutralization capacity, used as an indication for biological activity, is shown in figure 17, exemplified for R-Ab₁₂ and R-Ab₂₃. A quantitative thymulin assay more sensitive than assays based on a similar protocol was obtained with a sensitivity (ED₅₀) of 3560 pg/ml and a detection limit of 620 pg/ml (values approximated from figure 17).

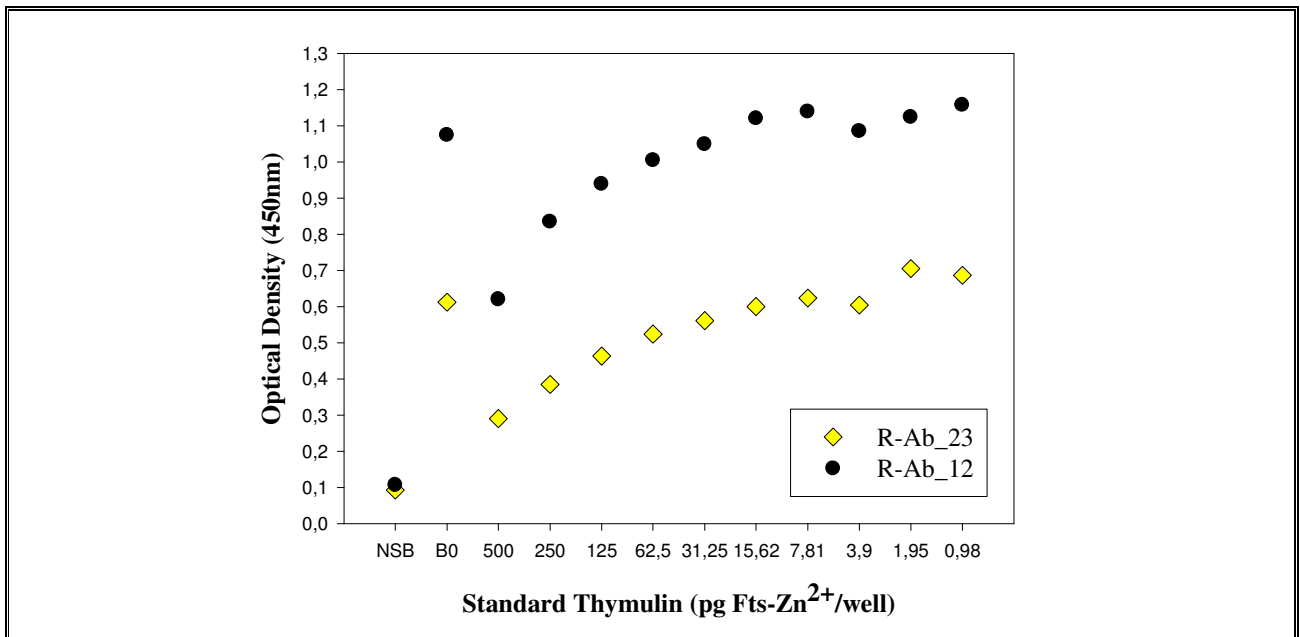


Figure 17: Standard Curve for an Optimized ELISA for FTS. – High-binding 96-well ELISA plates were coated with 50ng FTS-Zn²⁺/well. A synthetic FTS standard serially diluted (1:2 in PBS) was pre-incubated with two different anti-FTS rabbit sera at a 1/4500 (R-Ab_12) and 1/2000 (R-Ab_23) dilution (corresponding to the ED50 of each antibody, calculated from figure 16) in low binding plates and then transferred to the coated plate. (NSB – non-specific binding; B0 – maximum binding). Mean values of 4 experiments are shown. - Data were plotted with SigmaPlot 4.0 -

4.1.2 Sheep Cell Rosette Assay (SCRA) to Detect Remaining FTS

Immunoneutralization *in vitro* was performed and the remaining FTS determined by SCRA. The biological activity of all assessed antibodies is compared in figure 18. For all mammalian anti-thymulin specific antibodies an immunoneutralization of at least 90% was achieved. In the case of avian antibodies the quenching was less successful (80%). The anti-KLH control antibodies did not neutralize any thymulin (see figure 18).

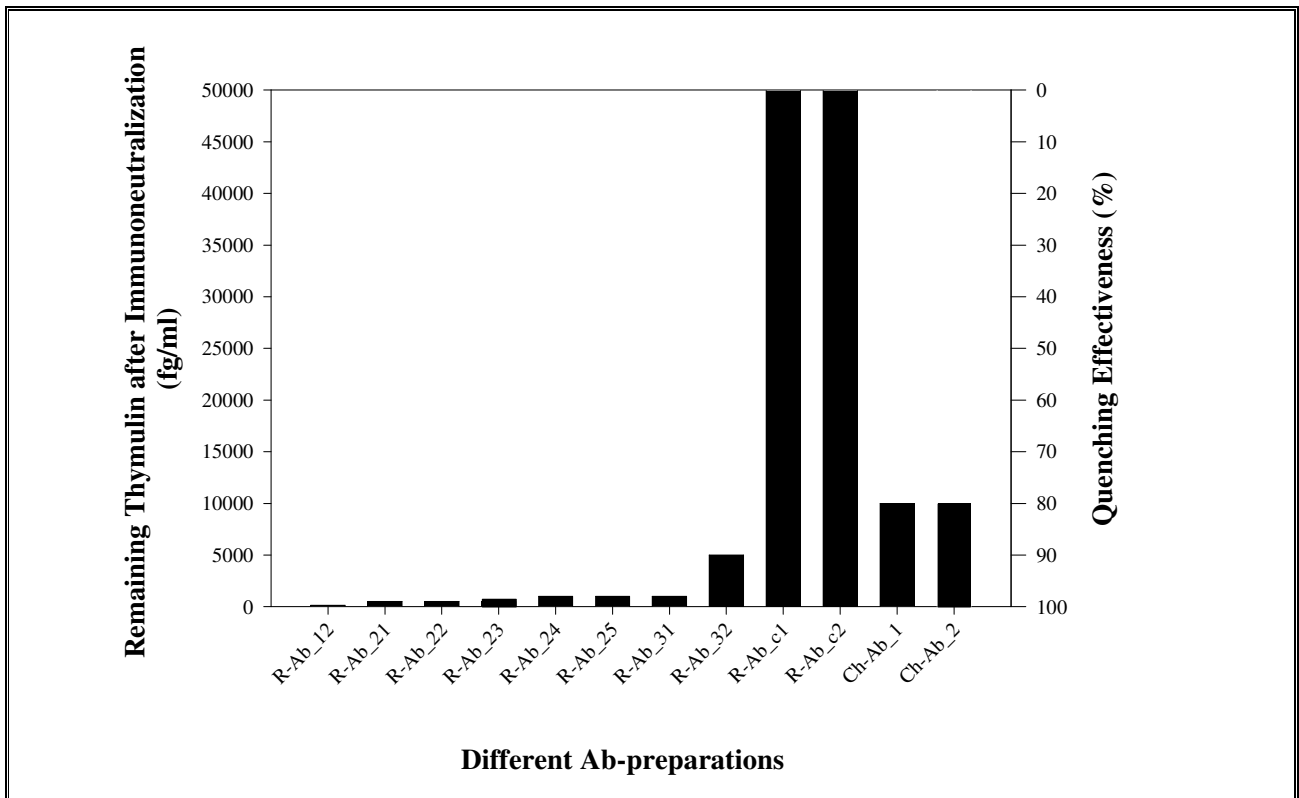


Figure 18: *In Vitro*-Immunoneutralization of Thymulin Using Different Immunoglobulin-Preparations. - 100µl of each immunoglobulin preparation or serum was pre-incubated with 100µl FTS-Zn²⁺-standard (100000fg/ml) during 1h at 37°C. The solution was filtered through an Amicon membrane (cut-off: 30000 Da) and serial dilutions from 1/25 to 1/100000 were made. Remaining Thymulin in the filtrate was measured by the rosette bioassay. The immunoneutralization effectiveness is expressed as % of the initial concentration (50000fg/ml). Remaining thymulin concentration is shown on the left Y-axis. - Data plotted with SigmaPlot 4.0 -

4.2 Immunohistochemistry

Immunohistochemistry was performed on different TEC-cell-lines using both mammalian and avian antibodies. In all cell lines used (rabbit-, mouse-, and human fetal thymic epithelial cells) strong unspecific reactions were encountered. No clear conclusions could be drawn. Data not shown.

5 In Vivo Immunoneutralization of the Thymic Hormone Thymulin With Anti-Thymulin Specific Mammalian Antibodies During Early Life – its Impact on Pituitary: Hormone Levels and Pituitary Morphology

5.1 Assessment of the Antibody's Biological Activity In Vivo: Preliminary Studies on its Effectiveness and Half Life

5.1.1 Long-Term Immunoneutralization Studies

These data are published elsewhere and therefore not shown here. Please refer to the original publication:

Goya RG, Reggiani PC, Vesenbeckh SM, Pleau JM, Sosa YE, Console GM, Schade R, Henklein P, Dardenne M. Thymulin gene therapy prevents the reduction in circulation gonadotropins induced by thymulin deficiency in mice. *Am J Physiol Endocrinol Metab.* 2007 Mar 27; [Epub ahead of print] PMID:17389714

5.1.2 Short-Term Immunoneutralization Studies

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5.2 Quantification of Pituitary Hormones by Means of RIA

5.2.1 Effect of Long-Term Immunoneutralization on Pituitary Hormone Serum Levels

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5.3 Histological Processing and Analysis

5.3.1 Gonads

Histological assessment of gonads did not reveal major differences between NRS- and anti-FTS-treated mice. Additionally, this analysis suggested that neither NRS- nor anti-FTS-injected mice of either sex had attained puberty at the time of sacrifice (32 days of age). Data are shown in figure 23a for testes and 23b for ovaries.

a)

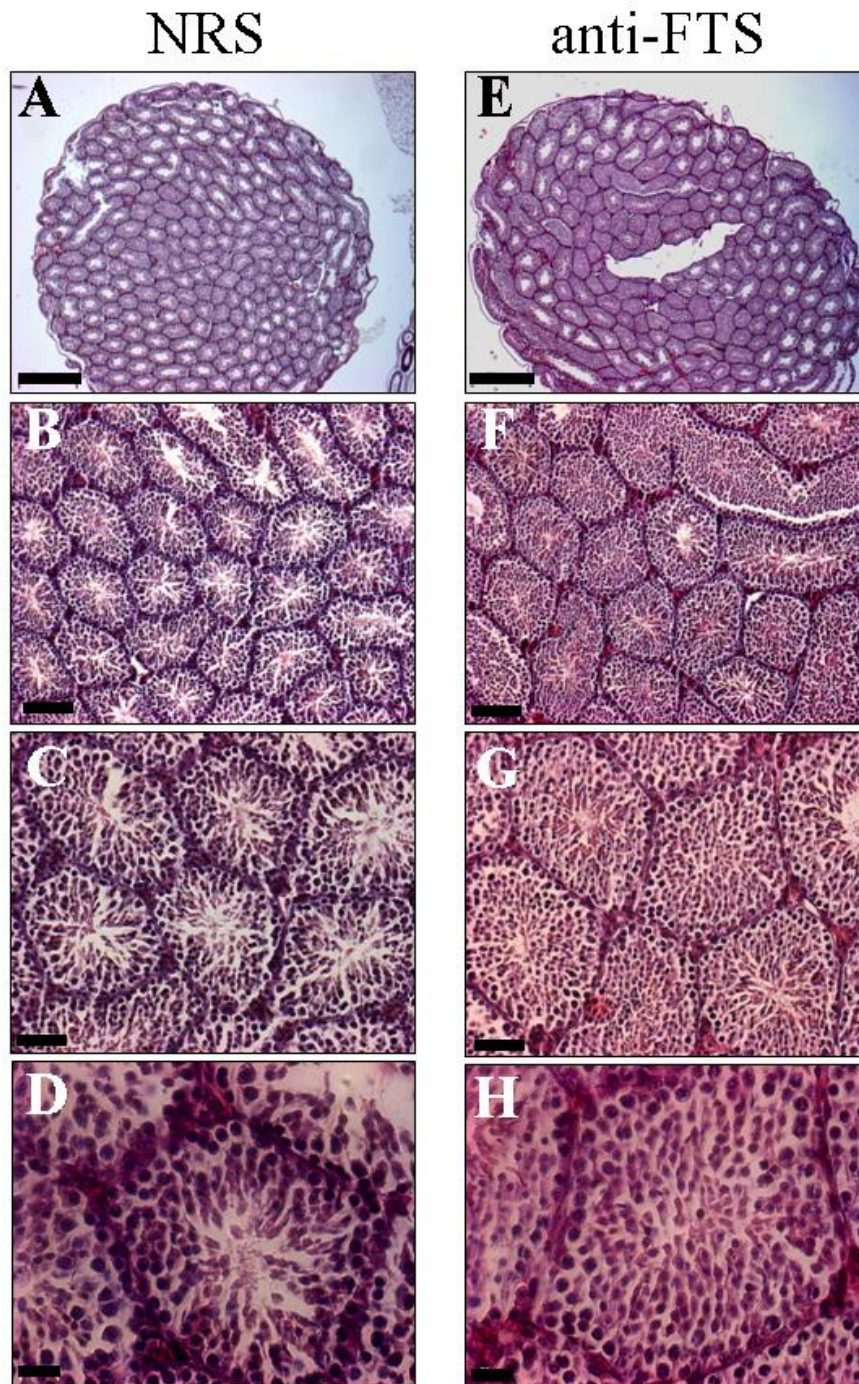


Figure 23a: Impact of Long-Term FTS Quenching During Early Life on Gonads' Morphology. – Testes were embedded in paraffin. Microtome-sections (6 μ m) were prepared and stained with hematoxylin-eosin (HE). The NRS-treated control group (A-D) was compared with an anti-FTS-treated experimental group (E-H), the magnification being 25x (A+E), 100x (B+F), 200x (C+G) and 400x (D+H). The crossbars represent different scales: 500 μ m (A+E), 100 μ m (B+F), 50 μ m (C+G) and 20 μ m (D+H).

- Leica-Light Microscope DM LB2, PixelINK 1394 Megapixel Fire Wire Camera #PL-A662, AxioVision 3.1 -

b)

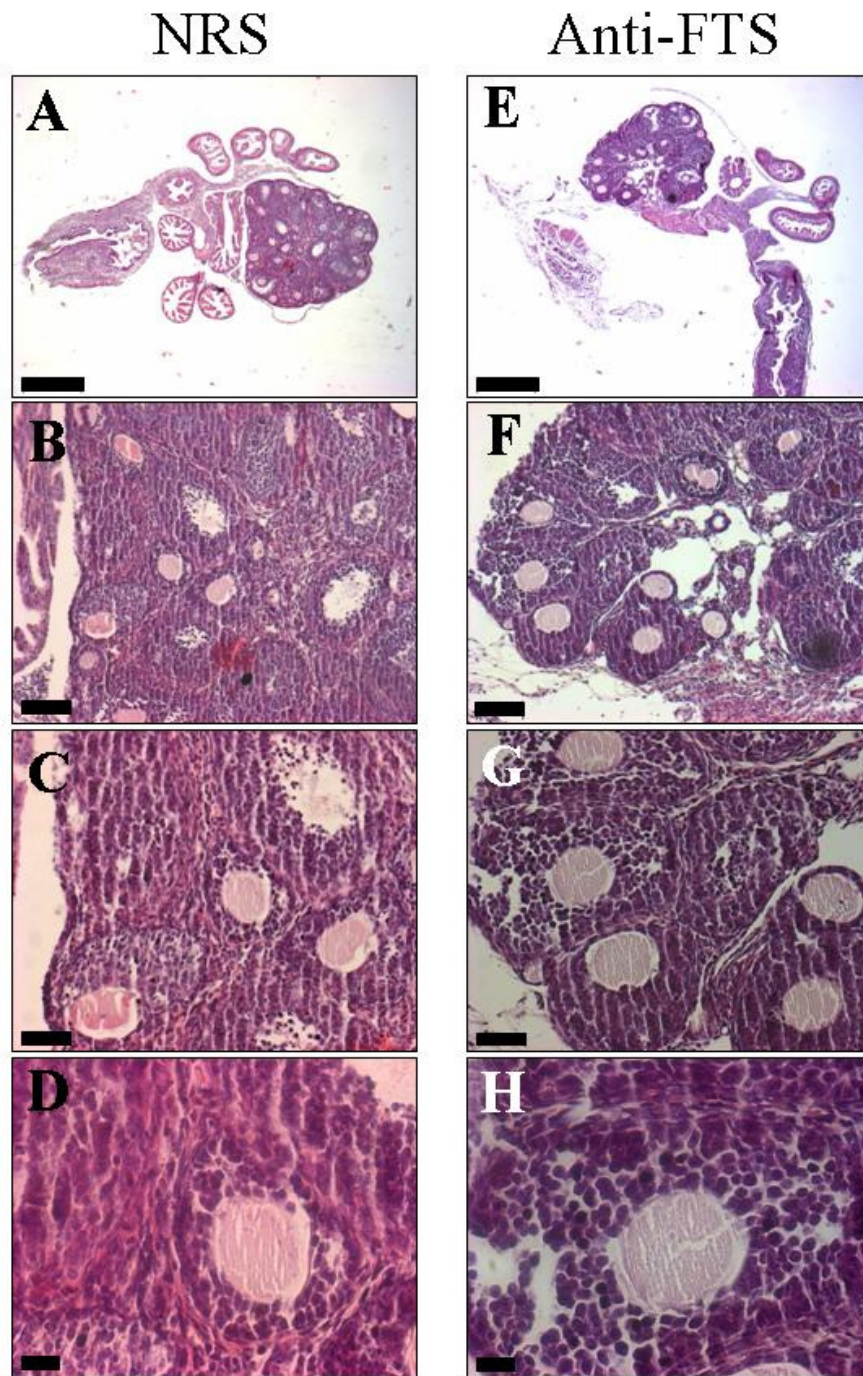


Figure 23b: Impact of Long-Term FTS Quenching During Early Life on Gonads' Morphology. – Ovaries were embedded in paraffin. Microtome-sections (6 μ m) were prepared and stained with hematoxylin-eosin (HE). The NRS-treated control group (A-D) was compared with an anti-FTS-treated experimental group (E-H), the magnification being 25x (A+E), 100x (B+F), 200x (C+G) and 400x (D+H). The crossbars represent different scales: 500 μ m (A+E), 100 μ m (B+F), 50 μ m (C+G) and 20 μ m (D+H).

- Leica-Light Microscope DM LB2, PixeLINK 1394 Megapixel Fire Wire Camera #PL-A662, AxioVision 3.1 -

5.3.2 Pituitary Gland

These data are published elsewhere and therefore not shown here. Please refer to the original publication:

Camihort G, Luna G, Vesenbeckh S, Ferese C, Dardenne M, Goya R, Console G. Morphometric assessment of the impact of serum thymulin immunoneutralization on pituitary cell populations in peripubertal mice. Cells Tissues Organs. 2006;184(1):23-30. PMID:17190977

5.4 Quantification of Thymocytes

At 32 days of age, following long-term-immunoneutralization, the total number of thymocytes was found to be significantly higher in the female anti-FTS subgroup compared to the NRS control subgroup. In males, a slight tendency towards an elevated number of thymocytes in the FTS subgroup was observed with $p>0.05$ (see figure 26a/c). However, due to the small and differing numbers of observed animals ($n = 5-13$) those results have to be discussed carefully.

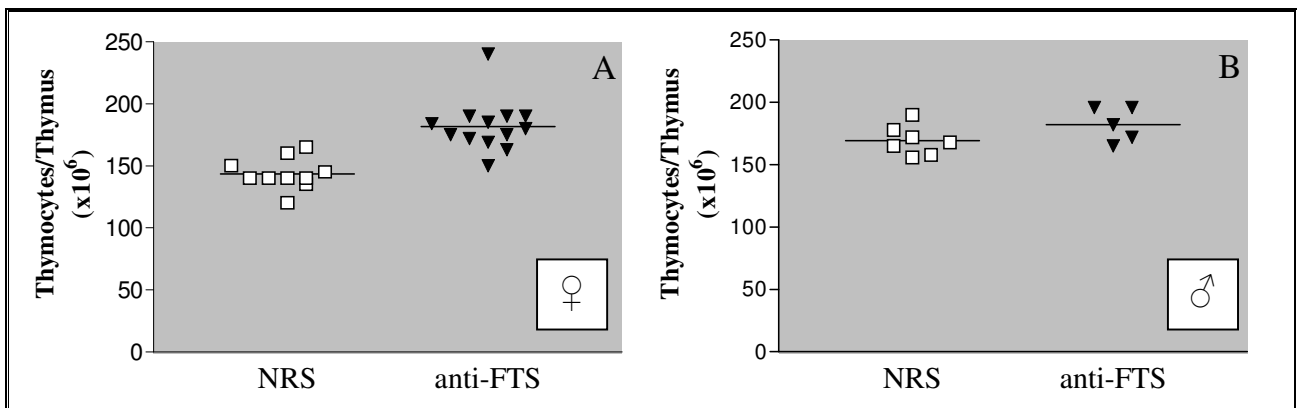


Figure 26 a: Impact of L-T Serum-Thymulin Quenching on the Total Number of Thymocytes at Peripuberty in Mice. – Animals were injected with normal rabbit serum (\square NRS-subgroup, control) or anti-FTS serum (\blacktriangledown FTS-subgroup) during their first month of life, afterwards sacrificed, and their thymocytes counted after thymus-dissection. Results are shown separately for females (A) and males (B). N-values were between 5 and 13. Means are indicated by horizontal lines. $p<0.01$ in (A) and $p>0.05$ in (B).

5.5 Animal Weights

In the main experiment, comparisons of regular body weight recordings in the control-subgroups of NRS (long-term quenching) and anti-KLH (short-term quenching) and the experimental subgroup of anti-FTS animals showed a significantly slower body weight gain in the anti-FTS subgroups treated by long-term quenching (see table 3 and figure 26b). Short-term treated animals remained unaffected in this regard.

Table 3: Effect of L-T/S-T Serum Thymulin-Immunoneutralization on Body Weight in C57BL/6 Mice. - Mice were submitted to either long-term (L-T; 32 days) or short-term (S-T; 7 days) thymulin immunoneutralization beginning at postnatal day 1 or 2. Control mice were i.p. injected with either normal rabbit serum (L-T quenching) or anti-KLH serum (S-T quenching). In the S-T study, thymulin immunoneutralization was terminated by i.p. injection of synthetic thymulin at postnatal days 8 and 9. Body weight (BW) was recorded at indicated ages, expressed in g. The rightmost column indicates the significance of BW differences between control and anti-FTS treated mice for the corresponding treatment. The total number of mice weighed (males and females considered together) in each group is indicated in parentheses. *NS* = non-specific

Treatment	Age (days)	Control (NRS or anti-KLH)	anti-FTS	p-value
L-T quenching	32	14.22 ± 0.36 (17)	12.08 ± 0.51 (18)	p < 0.05
S-T quenching	45	18.43 ± 0.46 (46)	19.19 ± 0.36 (41)	NS

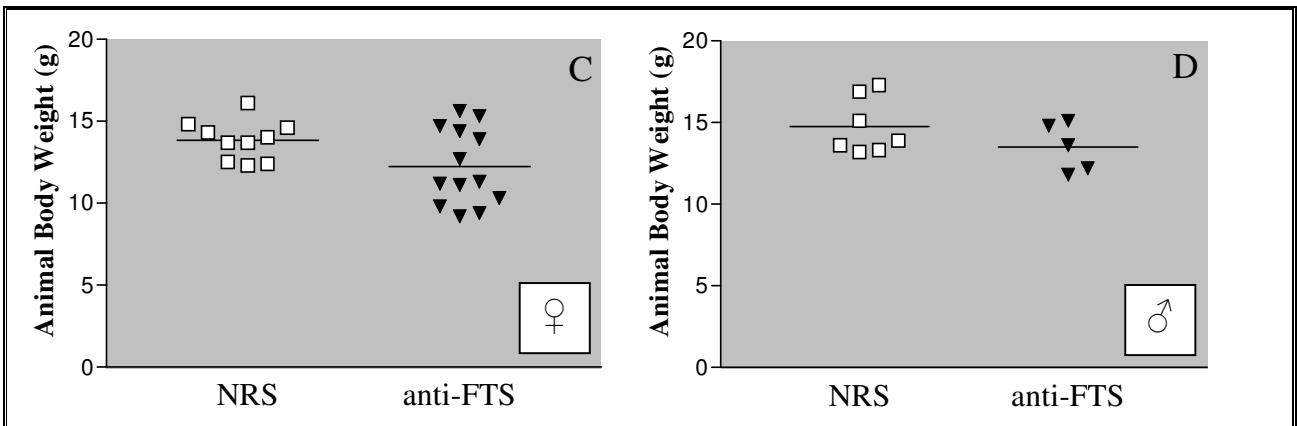


Figure 26 b: Impact of L-T Serum-Thymulin Quenching on the Body Weight at Peripuberty in Mice. – Animals were injected with normal rabbit serum (□ NRS-subgroup, control) or anti-thymulin serum (▼ FTS-subgroup) during their first month of life (32 days), afterwards sacrificed and their body weight recorded. Results are shown separately for females (C) and males (D). N-values were between 5 and 13. Means are indicated by horizontal lines. p>0.05 in (C) and (D).

5.6 Ratio of Thymocytes and Body Weight

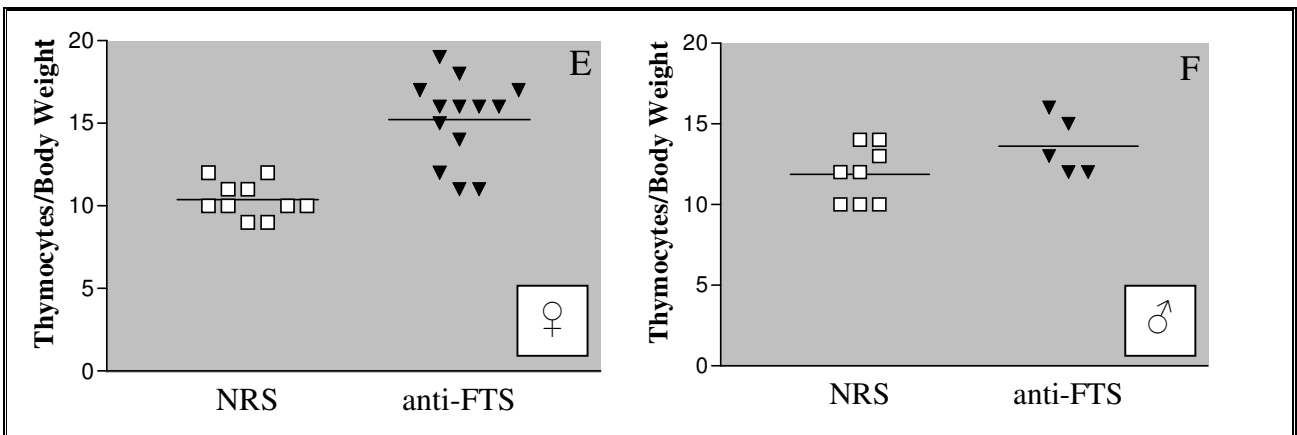


Figure 26 c: Impact of L-T Serum-Thymulin Quenching on Total Number of Thymocytes per gram Body Weight at Peripuberty in Mice. – Animals were injected with normal rabbit serum (□ NRS-subgroup, control) or anti-thymulin serum (▼ FTS-subgroup) during their first month of life, afterwards sacrificed. Data were deduced from experimental results from figure 26a/b. Results are shown separately for females (C) and males (D). N-values were between 5 and 13. Means are indicated by horizontal lines. p<0.01 in (E) and p>0.05 in (F).

5.7 Impact of Thymulin Quenching on the External Appearance of the Animal

The animals' external appearance was observed throughout the experiment, from the first day of life until day 32 (45) of life when all animals were killed. All animals were found to behave normally and no signs of increased susceptibility for illnesses were detected. No signs of 'wasting' were detected. Vaginal opening had not occurred in the female subjects when checked on day 32.