CHAPTER III: Animals, Materials and Methods

1 Animal Keeping

1.1 General Facts

All animals were kept in air-conditioned laboratories with an artificial light-dark regime of 12:12 h and were supplied with food and water ad libitum. An institutional ethics committee approved the animal experiments reported here. Official Permission for antibody-generation in hens and rabbits was given by authorities (#H0069/03).

1.2 Chickens

Laying hens (Lohmann white and brown, 22-24 weeks old) were obtained from commercial breeders (Legehennen Vermehrungsbetrieb, Bestensee, Germany). The hens were kept in cages (EBECO, Castrop-Rauxel, Germany).

1.3 Rabbits

Rabbits (HSDCPB:NZW, 2500-3000g) were obtained from Harlan Winkelmann, Borchen, Germany, and kept in commercial cages (Ehret, Emmedingen, Germany).

1.4 Mice

Male and female C57BL/6J mice were obtained from the 'Centre d'Elevage des Animaux de Laboratoire', Orleans, La Source, 45, France and kept in commercial cages (Charles River, L'Arbresle, France), age ranging 5-6 weeks, weight 20-25g. Newborn mice were obtained in our animal facilities.

2 Antibody Generation

2.1 Antigen Synthesis

The antigen was provided by Dr. Peter Henklein from the Biochemical Institute of Charité. It was chemically synthesized (Pyr-AKSQGGSN-COOH) and coupled to keyhole limpet haemocyanin (KLH) as previously described (Sambrook 1989).

2.2 Preparation of the Antigen for Immunization

For immunization, a mixture of FTS/KLH-antigen solution and Freund's Complete/Incomplete Adjuvants (FCA/FIA, Sigma) at a ratio of 3:2 in a total volume of 600-800 µl was used. The peptide (5mg FTS) was previously incubated with 5mg $ZnCl_2$.

2.3 Immunization

2.3.1 Chickens

Hens were immunized by injection in the pectoral muscle using FCA, the first two boosters were given every four weeks, followed by boosters every two months using FIA and injecting into the same muscle. Eggs were collected 10 days after boostering and antibodies extracted non-invasively as described below.

2.3.2 Rabbits

Rabbits were immunized by injecting the above solution (using FCA) near the nodi lymphatici poplitea (s.c.), the boosters (using FIA) were given i.c. in the shoulder region. Time lapse between immunization and the first booster was 4 to 6 weeks. The antibody-containing serum was obtained 7 to 10 days after boostering by taking blood from the ear vessel and separating the cellular components.

3 Antibody Purification

3.1 PEG-Precipitation

For IgY extraction from egg-yolk, the PEG-method was used: first, the egg was cracked open carefully and the yolk was separated from the egg white by rolling it on filterpaper, letting the paper soak up the traces of the clear part. Then, the yolk was transferred into a 50 ml polypropylene conical tube (Falcon® Blue Max™) and total yolk volume was recorded. PBS (phosphate-buffered saline, in all presented experiments commercial PBS 10x from Roche Diagnostics Corporation, Germany was used) was added, at twice the volume of the yolk, and the mix was stirred moderately for about 10 min at RT on a Stuart roller mixer (Sigma SRT2). PEG 6000 (Polyethylene glycol 6000, #0158.2, Carl Roth GmbH, Karlsruhe, Germany) at 3.5% was added and the tube was stirred again for about 10 min. The tubes were centrifuged at 11000 rpm for 10 min at 4°C (5000-10000g). The pellet was discarded and the supernatant was filtered through filter paper into a measuring

cylinder. The volume of the filtrate was measured and transferred to a new Falcon tube. This time, 8.5% of PEG was added and the tube was stirred again, and centrifuged as described above. The supernatant was discarded and the sediment dissolved in PBS (same amount of PBS as the original yolk volume). Then, 12% of PEG was added, the tube stirred, centrifuged, the supernatant discarded and the sediment dissolved in PBS (this time half the initial yolk-volume).

Antibody extract was dialyzed against PBS for at least 24 h at 4°C using dry membranes (Visking type 27/32, #1780.1ff, Roth; cut off=14 kDa). The membranes were pretreated with 5mM EDTA-solution by repeated cooking (5min each) and washed with distilled water followed by sterilization (10min, 120-125°C). Protein concentration was estimated by spectrophotometric analysis (Shimadzu UV-160A) at 280nm and aliquots were stored at –20°C.

3.2 Ammonium Sulfate-Precipitation

For IgG purification from rabbit serum, the Ammoniumsulfate method (AS) with saturated ammonium sulfate solution was applied to the sera.

First, a saturated ammonium sulfate solution was prepared by adding 69.7g solid ammonium sulfate to 100ml of PBS. It was stored at 4°C. IgG-containing serum samples were placed in 50ml Falcon-tubes. To each sample 66.7% of the previously prepared saturated (AS)-solution was slowly added, e.g. 10ml were added to 15ml of serum sample. After each addition performed step by step (starting with 500µl, then 1000µl each) the mixture was stirred at 4°C. After the last addition the solution was left at 4°C and was stirred overnight. The tube was centrifuged for 20 min at 4°C and 10000g. The supernatant was discarded and the sediment dissolved in 1ml PBS. Antibody-extract was dialyzed and photometric absorption measured as described for IgY-extraction. Aliquots were stored at -20°C.

3.3 Affinity Chromatography

As a ligand, chemically synthesized FTS (see 2.1) was used. It was coupled to NHS-activated Sepharose columns (HiTrap™ 4B, 1ml, Amersham Biosciences, Germany) according to the following protocol:

Ligand coupling: The gel is supplied in 100% isopropanol to prevent deactivation of the NHS-groups prior to coupling. In the first step isopropanol was removed by washing off the column with 1mM ice-cold HCl. All injections onto the column were performed with 3x2ml, slowly (½ drop/sec) to avoid irreversible compression of the column. Immediately, 1ml of ligand solution

(10mg FTS dissolved in 1ml standard coupling buffer) was injected. The column was left to stand for 15-20min at RT. Then, any excess active NHS-groups were deactivated by injecting buffer A (containing 0.5M ethanolamine) and non-specifically bound ligands were washed out by using buffer B (containing 0.1M acetate). This step was repeated (buffer A-B-A), the column left for incubation as described above, and the process repeated again (buffer B-A-B) before a pH-neutral PBS-buffer was added (protocol according to the provider's instructions).

Purification: For purification, ÄKTA^{TM}-prime (Amersham Pharmacia Biotech) was used. The column was washed with 3ml start buffer (PBS), 3ml elution buffer (glycine/HCl 0.1M, pH 2.5) and equilibrated with 10ml PBS. The hyperserum was added slowly, the column washed and eluted according to standard instructions provided by Amersham Biosciences.

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

4.1 Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on isolated IgY and IgG. All samples (20µl each) were treated for 3 min at 100°C in SDS-loading buffer (Roti-Load 1, #K929.1, Carl Roth GmbH Germany), and were further subjected to SDS-PAGE on 12% polyacrylamide gel slabs (Mini-PROTEAN 3 Cell, #165-3301, BIO-RAD, USA). The gels were stained with Coomassie Brilliant Blue (Roti-Blue, #A152.1, Carl Roth GmbH, Germany). Electrophoretic transfer was performed on a Mini Trans-Blot Cell (BIO-RAD, USA) using a Tris-glycine continuous buffer system (0.025 M Tris, 0.192 M glycine, 20% methanol, pH8.3). The gels were electroblotted for 1h at 100V.

4.2 Dot Blot

The antibodies were tested by Dot Blot as previously described (Sasse, Lengwinat et al. 2000). Briefly, synthetic peptides FTS and FTS previously incubated with $ZnCl_2$ were dotted in concentrations of 100ng/µl, 10ng/µl and 1ng/µl onto a nitrocellulose membrane (Millipore, Immobilon P®, pore size 0.45 µm) that had been pretreated with a 30-sec methanol, 60-sec aqua dest. and 5-min TBS-T (TBS+0.05% Tween) baths followed by 30-min incubation at 37°C. Dots on this membrane were dried for 30 min at 37°C. Antibody-antigen reactions were made visible according to conventional immunohistochemical procedure. Non-specific binding was blocked by using 10% skimmed milk (#232100 Difco, Becton Dickinson) in TBS-T for 1 h at room temperature

with constant shaking. Primary antibodies (different IgG and IgY, each 1:50, 1:500, 1:1000) and secondary antibody (with IgG-primary antibody: Sigma #A-6154, anti-rabbit IgG from rabbit; with IgY-primary antibody: Sigma #A-9046, anti-chicken IgG from rabbit; both POD-labeled, used 1:500) were diluted in 10% skimmed milk solution and incubated for 1 h and 45 min, respectively. After blocking and every antibody incubation step, the membranes were washed several times in TBS-T. The membrane was stained for 20-30 min in DAB solution (Sigma Fast 3.3-Diaminobenzidine, # D-4418). The reaction was stopped by adding distilled water.

4.3 ELISA

A series of studies were performed to optimize an ELISA for thymulin. Previously published protocols did not work under present conditions, or they revealed weak sensitivity. Changes in parameters like incubation conditions and background blocking conditions led to reproducible and valid results. After introducing a significantly better amplification system, the kit ABC, we established the following final protocol:

The synthetic peptide as target antigen was coated onto 96 well ELISA microtiter plates (Nunc Immuno Plates MaxiSorp, #456540, flat bottom) using 100ng FTS/well in ELISA coat buffer (carbonate buffer, pH=9.5) incubated for 24 h at RT. The coating was not washed out, but tipped over. Non-specific binding was blocked with 3% casein (skimmed milk, Régilait®) in carbonate buffer for 2 h at 37°C. After washing with PBS containing 0.05% Tween 20, primary antibody dilutions in PBS + 4.1%NaCl + 3%casein + 0.1%Tween 20 were added and incubated overnight at 4°C. Plates were washed after each of the following incubation steps:

The biotin labeled secondary donkey-anti-rabbit antibody provided with the kit ABC (Vectastain Elite, #PK-6200 Vector Laboratories) was used in a dilution of 1/500 in PBS, incubated for 45 min at 37°C, followed by the reagent A+B at the same dilution for 30 min at 37°C. Reagent A+B contains biotinylated peroxidase and avidin and requires preincubation (at least 30 min) to form large complexes. Those complexes generate a large increase in signal owing to the increase in bound enzyme molecules. When IgY was used as primary antibody an intermediate anti-chicken IgG from rabbit was additionally used (Sigma #C-2288, used 1/200, 1h at 37°C). The Avidin-Biotin Complex (ABC)-system has already been described for the use in ELISA by John R. Crowther (Crowther 1995). Now, ABTS substrate (Sigma #A-1888) in citric acid plus hydrogen peroxide were added and left to stand for 15 min at RT, after which the reaction was stopped with NaN_3. The

optical density was measured at 405nm with a Tecan Spectra Classic using the software easyWin curvefitting. Data were analyzed using Microsoft Excel, SigmaPlot and GraphPadPrism.

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

5.1 In Vitro Quenching

5.1.1 Competitive ELISA to Detect Remaining FTS

All the generated mammalian antibodies to be checked were used in appropriately set concentrations (2xOD50). In a low binding microtiter plate (NUNC) 60 µl of the FTS-containing standard or sample were incubated overnight at 4°C with 60 µl of the respective primary antiserum. Then, 100µl of the mix was transferred to a high binding microtiter plate previously coated with 50ng FTS/well and blocked as described above (4.3). After 24 h incubation at 4°C, the liquid was discarded and the plate was washed 3 times with wash buffer (4.3). All further steps were performed as described above (4.3)

column 1	non specific binding: achieved by using buffer alone
column 2	B0, maximum binding: achieved by using antibody alone
column 3-12	Decreasing standard FTS-concentrations: e.g. 500pg/well-1pg/well

5.1.2 Sheep Cell Rosette Assay (SCRA)

5.1.2.1 Principles

Rosette-forming cells (RFC) of adult thymectomized mouse spleen were found to be less sensitive to azathioprine than those of normal mouse spleen. Rosette formation with normal splenocytes is inhibited by 1 µg/ml whereas 70µg/ml is required to inhibit rosettes using splenocytes from thymectomized animals. This reduction in sensitivity to azathioprine is reversed by the addition of thymic extracts or serum from normal mice. In the early '70s, these findings led Dardenne and Bach to establish the first and so far only existent reproducible and semiquantitative bioassay for thymic hormones, especially thymulin (Dardenne 1975).

5.1.2.2 Thymectomy and Preparation of Spleen Cells

Thymectomy was performed on male and female C57BL/6J mice by suction with a Pasteur pipette after anaesthesia with 0.008-0.01ml Avertine/g bodyweight (Avertine is a mixture of 10g of 2,2,2-tribromethanol: Aldrich #T4,840-2, and 10ml of 2-methyl-2-butanol: Aldrich #15,246-3; it is

used as a 2.5% dilution in saline buffer). The skin was cut, presternal fascia were dissected and the superior part of the sternum was opened to give better access to the thymus. With a pipette connected to a vacuum system the thymus was suctioned with a mechanical vacuum pump. The wound was sutured afterwards. Animals were used at the age of 6-10 weeks, 1 to 4 weeks after thymectomy.

Mice were bled from the retro-orbital plexus as completely as possible, in order to diminish contamination with red blood cells that would falsify rosette formation. All further manipulations were performed at 4°C, the tubes and medium being placed on ice water. The whole spleen was placed at the bottom of a Potter glass to teflon homogenizer in 3-4 ml RPMI (Rooswell Park Memorial Institut) medium (RPMI-1640 with L-Glutamine, Gibco) where it was homogenized by moving the pestle three times, pressing slightly on the bottom of the tube. Cells were then filtered on tergal and centrifuged for 10 min, 1200rpm, at 4°C. The pellet was carefully re-suspended in 1 ml of RPMI medium/spleen. After adding 10µl of Trypan Blue, lymphocyte count was performed in Neubauer chambers. The values found for the spleen are generally in the order of 80 to 120 million nucleated cells, with quite significant variations according to mice. Then, the number of nucleated cells was set at 40 million/ml by adding the appropriate amount of RPMI medium.

5.1.2.3 Test Samples and Azathioprine

Serial test sample dilutions in RPMI medium were made from 1/4 up to 1/128, or, if high thymulin concentrations were suspected, up to 1/1000. If serum samples were used, serum had to be ultra-filtered (10 min at 3000rpm and 4°C) on Amicon membranes (30kDa cutoff, YMT micropartition system, PMS-I Amicon Corporation, Denver, CO, USA), in order to eliminate all components with a molecular weight higher than 30000, such as inhibiting proteins. 50µl-samples of every dilution were prepared and placed on ice water until use.

Azathioprine had been previously prepared at a concentration of 20µg/ml in RPMI medium. Finally, 50 µl of Azathioprine and 50 µl of the lymphocyte suspension were added to each test sample dilution. Control tubes were prepared by replacing the test sample or Azathioprine by a similar volume of RPMI medium. Tubes were closed, slightly mixed and incubation was performed in a 37°C water bath for 75 to 90 min. Later, the tubes were placed again on ice water.

5.1.2.4 Rosette Formation and Reading

200µl of sheep red blood cells (Eurobio, 7 avenue de Scandinavie, 91 Les Ullis) containing 1% formol were diluted in 15ml of RPMI medium leading to a final cell-concentration of 200 million/ml. Again, 50 µl of this suspension was added to the tubes. The cellular preparation thus obtained was immediately centrifuged for 5 min at 1200 rpm (150g) and 4°C. Now, re-suspension was made on a rotating apparatus (APELAP, 29 rue des Ecoles, 92 Bagneux) for three min at 10 rpm, with the apparatus being inclined between 0° and 30° from the vertical axis.

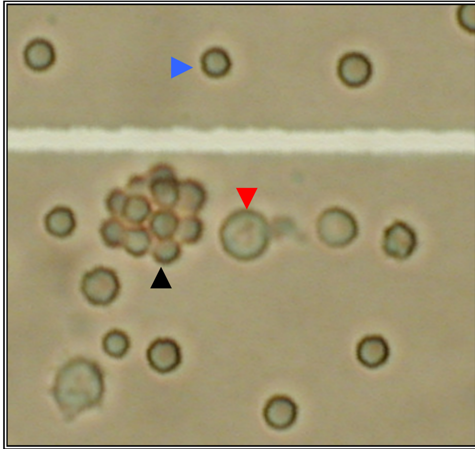

Figure 9: Rosette Formation in Neubauer Chamber. – Adherence of at least four sheep red blood cells to a splenocyte (lymphocyte circulating in the spleen) is considered necessary to define a true rosette. A splenocyte completely surrounded by erythrocytes is called morula (▲). Single erythrocytes (▶) and splenocytes (▼) shown in neighborhood.

After re-suspension the contents of each tube were quickly distributed into the two chambers of NEUBAUER cells. For each tube, the content of the two chambers was read and compared. Rosettes are defined as the agglutination of at least four red cells by a nucleated cell. The maximum dilution of the sample still giving rosette inhibition of more than 50 per cent is considered as the active dilution.

5.1.2.5 SCRA to Detect Remaining FTS

100µl of the antibody preparation was incubated with 100µl of FTS in a concentration of 10ng/ml for 60 min at 37°C. Then, antibody-FTS-complexes were filtered out with Amicon-membranes (see above). Different dilutions of the resulting preparations were made (1/1, 1/5, 1/25, 1/125, 1/500, 1/1000, 1/5000, 1/10000, 1/50000, 1/100000) and remaining FTS was measured by SCRA (see above).

5.2 Immunohistochemistry

TEC lines of rat, mouse and human origin were used. Cells were seeded on sterile coverslips in 6-well plates. Two or three days later, when the cultures reached 70-80% confluency, the medium was discarded and the cells quickly washed with PBS. Then, cells were fixed for 15-20 min in 1ml PBS-10% formol, washed twice with PBS for 5 min and impermeabilized with 1 ml PBS-0.3% Triton X-100. Cells were then carefully washed two times with PBS for 5 min. Blockade of endogenous peroxidase was achieved by incubating the cells with normal horse serum 1:100

(provided with the ABC kit) for 30 min at room temperature. The blocking serum was then removed by aspiration and the excess liquid was blotted on absorbent paper. Then, the cultures were incubated for 30 min with a rabbit anti-FTS serum. Afterwards, the primary antiserum was removed and the cells washed twice with PBS for 5 min and subsequently incubated with a biotinylated anti-rabbit IgG antibody (a component of the ABC kit) for 30 min at room temperature. The cells were washed twice with PBS for 5 min and incubated with the ABC reagent (kit ABC) during 30 min at room temperature. The cells were washed twice with PBS for 5 min and incubated for 5-10 min with the DAB substrate (DAB Substrate kit from Vector), checking color development by visual inspection under the microscope. When the appropriate level of color was achieved, cells were washed three to four times with distilled water, the excess moisture blotted off with absorbent paper and the coverslips mounted on microscope slides with Fluoromount (Electron Microscopy Sciences Inc., Hartfield, Pennsylvania) mounting medium. The slides were left at 37º C for 48 h, then cleaned with a water-moistened cotton swab and stored until microscopic observation and photographic documentation.

6 *In Vivo* Immunoneutralization of the Thymic Hormone Thymulin during Early Life – Its Impact on Pituitary: Hormone Levels and Pituitary Morphology

6.1 Immunoneutralization of Thymulin with anti-Thymulin Specific Mammalian Antibodies

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

In initial experiments designed to assess the immunoneutralizing effectiveness and duration of the anti-FTS serum used, adult- (4 months) and baby- (15 days) mice were i.p. injected once with 0.8ml anti-FTS hyperserum/animal and 0.08ml/animal respectively. At 2-3 day intervals, subgroups of 3-4 mice were bled in order to measure serum thymulin profiles. The experiment was continued for 27 days in adult mice, and for 10 days in baby mice.

6.1.2 Long-Term Immunoneutralization Studies

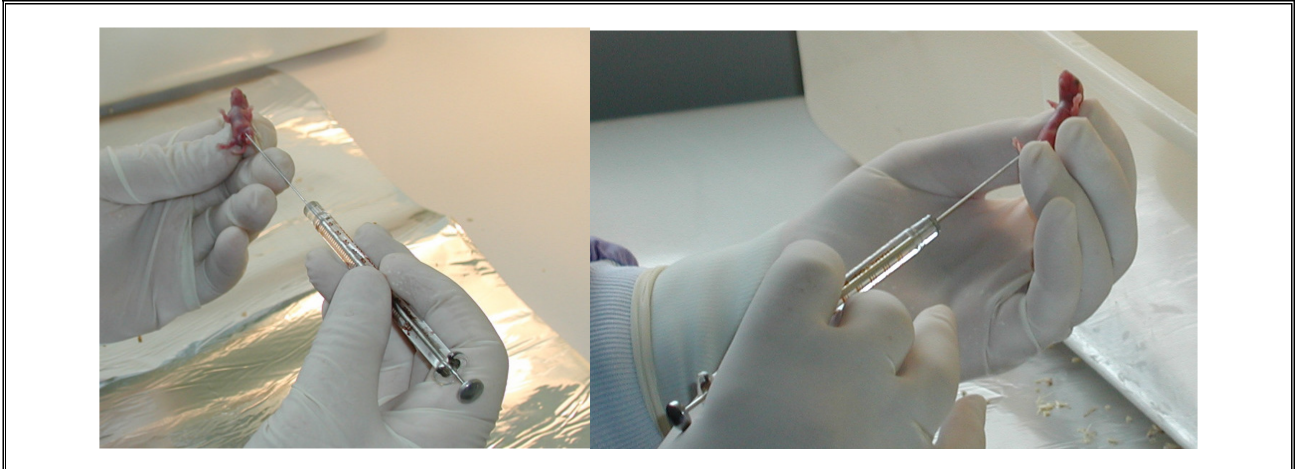

Figure 10: Procedure of i.p.-Injection of Antisera into Newborn C57BL/6J-Mice. – For animal manipulations gloves were worn. At the time of injection animals were removed from their cages one by one, injected, and immediately brought back to their mothers. Antisera-application was performed by slow i.p.-injection using a 0.1ml-Hamilton syringe. - *CNRS, Hôpital Necker, Paris 2003* -

Newborn mice were divided into two subgroups initially consisting of 40 pups each. Animals from the experimental subgroup were i.p. injected at the age of one and two days with 20µl rabbit anti-FTS serum each day. Beginning seven days later, the animals were subjected to a schedule of weekly i.p. injections of antiserum at a dose of 8µl/g bodyweight until the end of the experiment at day 31-32 (long-term immunoneutralization, L-T). As the control group, the second subgroup received i.p. injections of normal rabbit serum (NRS) in the same volumes as indicated above, at the same ages. During the study, body weights (BW) were recorded at regular intervals, approximately every three days.

Figure 11: Dissection of Thymus from Adult Rat. – The Animal was killed by decapitation. The Thymus (*) is easily accessed by opening of the thoracic cage. After photographic documentation the organs were removed for further processing.
Instituto de Investigación Bioquimica de La Plata (INIBIOLP), Argentina 2003.

6.1.3 Short-Term Immunoneutralization Studies

In a second experiment a subgroup of experimental animals was subjected to i.p. injections of 20µl of rabbit anti-FTS serum at postnatal days 1 and 2 which was followed by two i.p. injections of synthetic thymulin (60pg/g BW) at

postnatal days 8 and 9 (short-term immunoneutralization, S-T). Thymulin ($FTS\text{-}Zn^{2+}$) was prepared 30 min before injection by mixing equimolecular (10nM) volumes of synthetic FTS and $ZnCl_2$, both prepared in PBS. Control mice were subjected to the same treatment except that they received a rabbit anti-KLH serum instead of anti-FTS. Those mice were sacrificed at 45 days of age for hormone measurement.

6.1.4 Parameters Studied

At the age of 32 days (S-T: 45 days) all the animals were sacrificed by decapitation in the late morning, after having recorded the animals' weight and outer appearance. Trunk-blood was collected and the thymus, pituitaries and gonads were dissected (for dissection procedures compare figure 11). The thymus was weighed, homogenized and thymocytes were immediately counted. Serum was obtained and frozen at –20°C until ready for hormone assays. Pituitaries and gonads were fixed in Bouin's fluid and formol, respectively.

6.2 RIA

Serum PRL-, GH-, LH-, FSH- and TSH-levels were measured by a specific radioimmunoassay (RIA) using the mouse materials provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance CA, USA. Iodination grade mouse hormones were radiolabeled by the Iodo-Gen^R method (Fraker and Speck 1978). The iodinated hormone was purified on PD-10 Sephadex® G-25 M columns (Pharmacia, Uppsala, Sweden) equilibrated with 0.01M phosphosaline, pH 7.6. A 1/10 goat anti-rabbit IgG serum in 0.05M phosphate buffer, 1% normal rabbit serum and 8% polyethyleneglycol, was used to separate bound from free hormone. Serum PRL-, GH-, LH-, FSH- and TSH-levels were expressed in terms of rPRL RP-2, rGH RP-2, rLH RP-2, rFSH RP-2 and rTSH RP-2, respectively.

6.3 Histological Studies

6.3.1 Gonads

Testes, epididymides and ovaries were removed, fixed in 4 % formaldehyde at RT for 48 h, dehydrated and embedded in paraffin, and sectioned at 6µm thickness. Sections were stained with hematoxylin and eosin following the classical protocols described at www.mta-labor.info (Hallmann 1966). The reproducibility of all of the morphological data was verified by similar findings in at

least three different animals. Photographs were taken using a digital camera and Leica microscope Axio Vision 3.1.

6.3.2 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

7 Statistical Analysis

Data were expressed as mean ± SEM, unless otherwise indicated. Histometrical data were analyzed by the least significant difference test (95% significance level) for multiple comparisons (Zar 1974), and serum data were assessed by ANOVA followed by Duncan's multiple range test, when appropriate. Prism 3.0 and SigmaPlot 4.01 were used for graphs.