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Original article

Effect of neonatal or adult heat acclimation on plasma fT3 level, testicular thyroid receptors expression in male rats and testicular steroidogenesis *in vitro* in response to triiodothyronine treatment

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Abstract

This study aimed to evaluate the effect of heat acclimation of neonatal and adult rats on their testes response to *in vitro* treatment with triiodothyronine (T3). Four groups of rats were housed from birth as: 1) control (CR) at 20°C for 90 days, 2) neonatal heat-acclimated (NHA) at 34°C for 90 days, 3) adult heat-acclimated (AHA) at 20°C for 45 days followed by 45 days at 34°C and 4) de-acclimated (DA) at 34°C for 45 days followed by 45 days at 20°C. Blood plasma and both testes were harvested from 90-day old rats. Testicular slices were then submitted to *in vitro* treatment with T3 (100 ng/ml) for 8 h. Plasma fT3 level was lower in AHA, NHA and DA groups than in CR group. Basal thyroid hormone receptor α1 (*Thra1*) expression was higher in testes of NHA and DA and β1 receptor (*Thrb1*) in DA rats *vs.* other groups. In the *in vitro* experiment, T3: 1) decreased *Thra1* expression in all groups and *Thrb1* in DA group, 2) increased *Star* expression in CR, NHA and DA groups, and *Hsd17b3* expression in NHA group, 3) decreased the expression of *Cyp11a1* in NHA and DA groups, and *Cyp19a1* in all the groups, 4) did not affect the activity of steroidogenic enzymes and steroid secretion (A4, T, E2) in all the groups. These results indicate, that heat acclimation of rats, depending on their age, mainly affects the testicular expression of steroidogenic enzymes in response to short-lasting treatment with T3.

Key words: thyroid hormone, heat acclimation, thyroid receptors, steroidogenesis

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Introduction

In the course of heat acclimation, substantial adaptations to these environmental conditions have been found in the male organism (Horowitz 2010). One of the criteria for heat-acclimated state is metabolic rate reduction (Horowitz 2010), mediated by the thyroid gland activity (Seebacher 2009). Heat acclimation diminishes thyroxin (T4) and triiodothyronine (T3) secretion from the thyroid gland. These effects were found in both adult and perinatal heat-acclimated rats (Arieli and Chinet 1986, Maloyan and Horowitz 2002, White et al. 2005, Durst et al. 2010, Horowitz 2010) as well as in pigs subjected to tropical ambient temperature (Christon 1988).

Thyroid hormones among others are involved in the regulation of reproductive processes (Wagner et al. 2008). Thus, disturbance of T4 and T3 secretion might alter them unfavorably. In males with decreased plasma thyroid hormone concentration, adverse changes in the morphology of the reproductive tract, suppressed activity of Leydig and Sertoli cells, and in consequence hypospermatogenesis have been demonstrated (Ariyaratne et al. 2000, Oner et al. 2006, Wagner et al. 2008, Auharek et al. 2010, Tousson et al. 2012). In the testes, T3 may act through genomic or non-genomic mechanisms (Bassett et al. 2003, Zamoner et al. 2011). The expression of nuclear thyroid hormone receptors (THR) in testes diminishes during the development of males from neonatal up to pubertal stage (Buzzard et al. 2000, Jannini et al. 2000), leading to their lower expression in adult rats. However, the *in vitro* T3 treatment of senescent rat testes improves their potential to produce testosterone (T) (Kim et al. 2002). It may indicate that even low THR expression is sufficient to mediate the T3 action in testes of adult males.

There are two types of thyroid receptors (THR) in the testes, namely THR1A/THR2A and THR1B (Buzzard et al. 2000, Jannini et al. 2000, Rao et al. 2003, Wagner et al. 2008). The expression of THRA1 and THRA2 mRNA and proteins found in the adult rat and men testes suggestes that mainly these subtypes of THRs mediate the action of thyroid hormones in males gonads (Buzzard et al. 2000, Jannini et al. 2000, Canale et al. 2001). Nevertheless, the expression of *Thrb1* gene was subsequently revealed in the testes of adult male rats (Rao et al. 2003). It was also suggested that THRB1 is responsible for sustaining T production in transgenic mice with Sertoli and Leydig cells bearing the conditional dominant-negative *Thra1* allele (Fumel et al. 2015).

The reproductive processes (Hansen 2009, Durairajanyagam et al. 2015) as well as thyroid function in mammals are affected by ambient temperature, particularly by heat (Durst et al. 2010, Kuba et al. 2015). Interestingly, severity of heat-induced changes in these processes (Kurowicka et al. 2015a,b) and the expression of THRs in testes (Buzzard et al. 2000) are dependent on the age of animals. The age-related differences in the testicular steroidogenesis and sperm production were noted between neonatal and adult heat-acclimated rats (Kurowicka et al. 2015a,b). Collectively, it seems that T3 may mediate the adaptive changes of reproductive processes in male rats, housed at high ambient temperature. Thus, we hypothesize that neonatal heat acclimation may differently affect the testicular response to T3 compared to adult heat acclimation in rats. To clarify this hypothesis, we determined: 1) the plasma free T3 (fT3) concentration in neonatal and adult heat-acclimated rats, 2) the testicular *Thra1* and *Thrb1* expression, 3) the expression and activity of steroidogenic enzymes, 4) the accumulation of androstenedione (A4), testosterone (T), and estradiol-17β (E2) secretion *in vitro* by the testicular slices in response to short-lasting treatment with T3.

Material and Methods

Experimental animals

Twenty male Wistar rats were randomly divided to four groups: 1) control males (CR, n=5) – born and housed until the 90th day of life at an ambient temperature of 20±1°C; 2) adult heat-acclimated males (AHA, n=5) – born at 20±1°C and after reaching puberty (45th day) housed at 34±1°C for the next 45 days; 3) neonatal heat-acclimated males (NHA, n=5) – born and housed at 34±1°C for 90 days, and 4) de-acclimated males (DA, n=5) – housed at 34±1°C from birth to the 45th day, and then at 20±1°C for the next 45 days. Both, the humidity and the light:dark cycle (12:12 hours) were controlled. The rats were provided with free access to tap water and chow pellets (Labofeed H; Feeds and Concentrates Production Plant, Kcynia, Poland). The local Ethics Committee for Animal Experimentation of the University of Warmia and Mazury in Olsztyn approved all the experiments.

Tissue collection and *in vitro* incubation of testicular slices

The rats were sacrificed at the 90th postnatal day by cervical dislocation and then blood samples and whole testes were collected. The plasma was isolated and stored at -20°C for future analysis of fT3 and

Table1. Primers used for qPCR estimation of thyroid hormone receptors mRNAs.

Gene	NCBI Accession No.	Primer sequence	Product length (b)
Thra1	NM_00101796	F: GTTTGAGCACTACGTCAACCACC R: CACTCGACTTTCATGTGGAGGAA	132
Thrb1	NM_012672	F: GTGCTGGATGACAGCAAGAGG R: CTGGCTTATGCCCAATGGATT	103
Actb	NM_031144	F:ACCATGTACCCAGGCATTGC R:GCCAGGATAGAGCCACCAATC	131

steroid hormones. Testes were aseptically cut into equal slices (2.1 mm thick and 250±10 mg of weight/slice), which were used for control (two) and treatment with T3 (two). In the *in vitro* experiment, the testicular slices were incubated separately in a six-well plate (BD Labware, Europe) in 3 ml of F-12 medium (pH=7.4; AppliChem, Germany) supplemented with 1% BSA (AppliChem, Germany). Two slices were used from each rat for each treatment group. After a one-hour pre-incubation in a shaking water bath at 34ºC under an atmosphere of 95% O_2 + 5% CO_2, the medium was changed, and the incubation was continued for the next 8 hours in medium alone (basal) or in medium containing T3 (100 ng/ml, Sigma-Aldrich, Germany). Following incubation, the media and tissues were collected and frozen at -20ºC (media) and at -80ºC (tissues) for hormone and gene expression analysis, respectively.

Isolation of total RNA and quantitative Real-time PCR

For each treatment group, half of one testicular slice (~125 mg) per animal was homogenized, and total RNA was isolated using Trizol (Life Technologies, USA). The quantity and quality of the isolated RNA was determined by Nano Drop (Thermo Scientific, Wilmington, USA) and by gel electrophoresis. A constant amount of RNA (1 µg) was reverse transcribed using a Qiagen kit (Qiagen Corp., USA), according to manufacturer's instructions, and the cDNA was stored at -20ºC until qPCR analysis. The expression of following genes was analyzed: *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3 Cyp19a1, Thra1* and *Thrb1*. As a reference gene, *Actb* was used. The primers were previously published (Kurowicka et al. 2015a), except these for *Thra1* and *Thrb1*, which are presented in Table1. The qPCR was performed using a SYBR Green Mix (Applied Biosystems, USA). The reaction parameters were: 95ºC for 10 min, then 40 cycles at 95ºC for 15 s, followed by 60ºC (for *Thra1, Thrb1, Star, Cyp19a1* and *Actb*) or 61ºC (for *Cyp11a1, Hsd3b1, Cyp17a1* and *Hsd17b3*) for 30 s and 72ºC for 5 min. The dissociation curve parameters were: 65ºC for 1 s, followed by 95ºC for 5 s. Serial dilutions of the appropriate cDNA reaction products were used as standard curves for DNA quantification. The expression levels of all genes studied were normalized to *Actb.* All reaction products were separated and their specificity was verified by sequencing.

Western blot for STAR protein

The half of testicular slice/animal (~125 mg) was used for STAR protein determination by Western blot as described previously (Kurowicka et al. 2015a).

Isolation of mitochondrial and microsomal protein fractions from testicular slices and determination of steroidogenic enzymes activity

The second series of testicular slices (two/rat) was homogenized in Tris-HCl-sucrose (pH=7.4) buffer (McVay et al. 2004), supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Germany). The mitochondrial and microsomal fractions were obtained *via* sequential centrifugation of the homogenates (Kurowicka et al. 2015a). These fractions were re-suspended in the same buffer and stored at -80ºC for further analysis. The steroidogenic enzymes activities were determined based on the conversion of tritiated substrates into their appropriate products as described previously (Kurowicka et al. 2015a).

Determination of free triiodothyronine in blood plasma and steroid hormones in media

Plasma concentration of fT3 was measured in one run, using commercially available fT3 RIA kit (Isotopes, Hungary), according to the manufacturer's instructions. The assay sensitivity was 0.3 nmol/l and intra-assay variation coefficient was 5%.

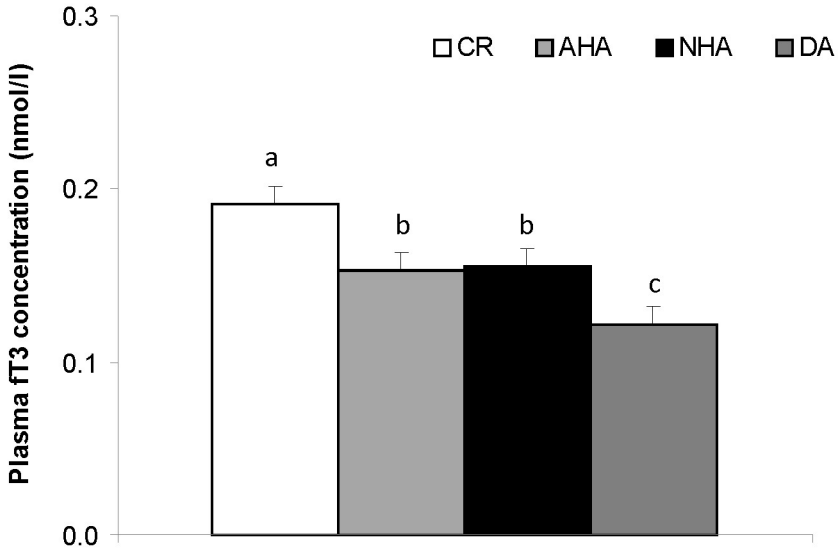

Fig. 1. Free triiodothyronine (fT3) plasma concentration in control (CR), adult heat-acclimated (AHA) neonatal heat-acclimated (NHA), and de-acclimated (DA) rats (n = 5/group). a, b, c – different letters indicate statistically significant differences between the groups.

Concentrations of A4, T and E2 in media were measured by RIA (Ciereszko et al. 1998, Szafranska et al. 2002). The intra- and inter-assay coefficients of variance were as follows: for A4 – 2% and 13.3%, respectively; for T – 2.1% and 5.1%, respectively; and for E2 – 1.4% and 10.6%, respectively. The assay sensitivity for A4 and T was 2 pg/tube and for E2 – 1 pg/tube.

Statistical analysis

Data were expressed as the mean ±SEM for plasma fT3 concentration and *Thra1* and *Thrb1* expression in the testicular tissue. The expression and activity of steroidogenic enzymes in testicular slices and steroids *in vitro* secretion by this tissue following treatment with T3 (100 ng/ml) were presented as fold changes in relation to the corresponding basal levels, since in these cases the basal values have been already published (Kurowicka et al. 2015a). Significant differences between the means were determined *via* one-way analysis of variance (ANOVA) (Statistica, StatSoft, Tulsa, USA) followed by LSD post-hoc test. The significant effect of the T3 treatment was determined *via* Student's-t test. Significant differences between the means were determined based on $p<0.05$.

Results

Plasma fT3 concentration in neonatal and adult heat-acclimated rats

Plasma fT3 concentration was lower in all heat-acclimated groups (AHA, NHA, DA) compared to that found in CR group (Fig. 1) and in DA group than that observed in AHA and NHA groups.

Basal and T3-influenced expression of *Thra1* and *Thrb1* in testes of neonatal and adult heat-acclimated rats

Basal *Thra1* expression was higher in testes of NHA and DA rats (Fig. 2A) and *Thrb1* in DA rats (Fig 2B) compared to that found in CR rats. Treatment of testicular slices with T3 decreased *Thra1* expression in CR, AHA, NHA and DA rats, and *Thrb1* in the DA rats *vs.* the corresponding basal expression (Fig. 2A, 2B).

Effect of T3 treatment on steroidogenic enzymes gene expression and enzymatic activity in testes of neonatal and adult heat-acclimated rats

The expression of studied genes in testes changed in response to T3 treatment. Up-regulation of *Star* was found in CR, NHA and DA rats, and *Hsd17b3* in NHA rats, but down-regulation of *Cyp11a1* occurred

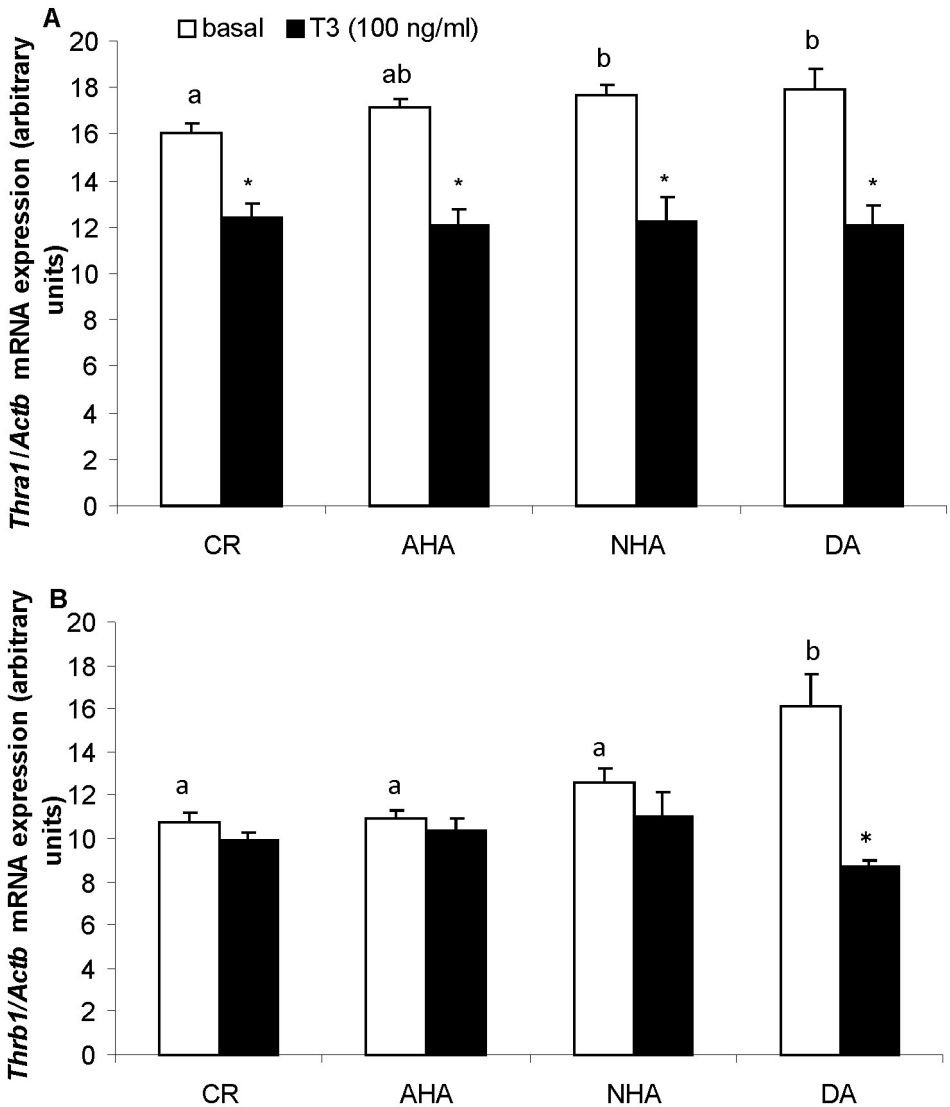

Fig. 2. Basal and T3 (100 ng/ml) stimulated levels of intra-testicular *Thra1* (A) and *Thrb1* (B) mRNA expression in the testicular slices from control (CR), adult heat-acclimated (AHA), neonatal heat-acclimated (NHA) and de-acclimated (DA) rats (n = 5/group). a, b – different letters indicate a statistically significant differences between the groups; * – asterisks indicate a statistically significant difference between the basal and T3-induced mRNA expression.

in NHA and DA rats, and *Cyp19a1* in all the experimental groups (Fig. 3). T3-induced expression of *Cyp11a1* was lower in NHA and DA groups compared to that found in CR and AHA groups, whereas that of *Hsd17b3* and *Cyp19a1* was higher in NHA rats than in the rest of the studied groups. There were no changes in STAR protein expression and steroidogenic enzymes activity in testes treated with T3 *in vitro* in comparison to basal values (data not shown).

Effect of T3 treatment of testicular slices on A4, T and E2 levels in incubation media

There were no significant effects of T3 treatment on A4, T and E2 concentrations in media following the 8-h incubation of testicular slices in all the studied groups, although the E2 secretion in response to T3 in NHA and DA rats was higher than that in CR and AHA animals (Fig. 4).

Discussion

The present study has revealed differences in fT3 plasma concentration in rats exposed to various regimes of heat acclimation. It was also demonstrated the expression of two types of *Thr* (*Thra1* and *Thrb1*) in testes of these animals as well as some *in vitro* effects of T3 on their expression. The testicular tissue treatment *in vitro* with T3 also affected the expression of *Star*, *Cyp11a1*, *Hsd17b1* and *Cyp19a1* genes in rats

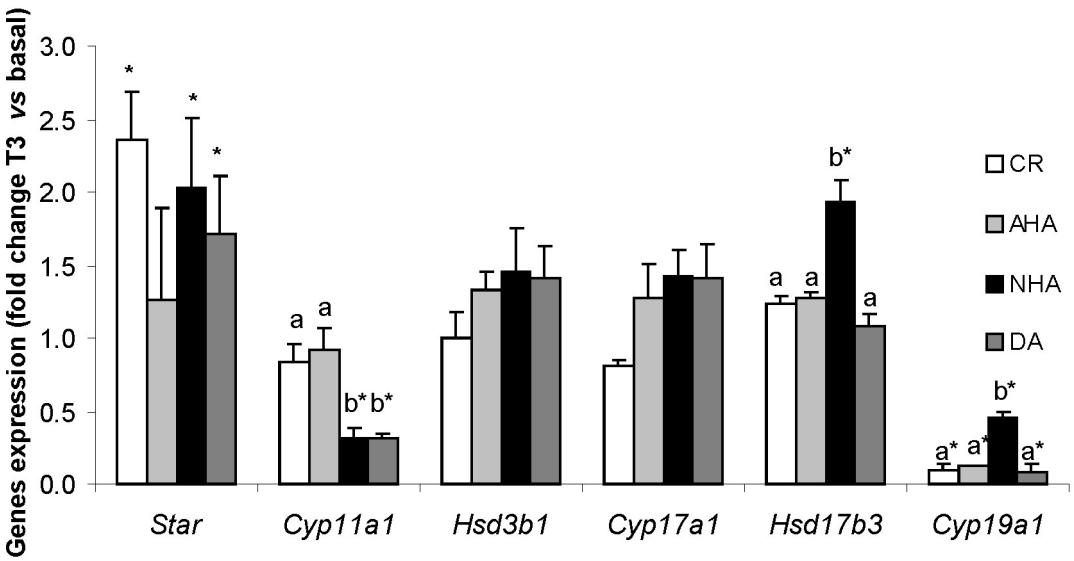

Fig. 3. Changes in the levels of intra-testicular mRNA expression of steroidogenic pathway enzymes in testicular slices from control (CR), adult heat-acclimated (AHA), neonatal heat-acclimated (NHA) and de-acclimated (DA) rats (n = 5/group) in response to T3 (100 ng/ml); * – asterisks indicate a statistically significant difference between the basal and T3-stimulated levels; a, b - different superscripts indicate significant differences in T3-induced mRNA expression between the experimental groups.

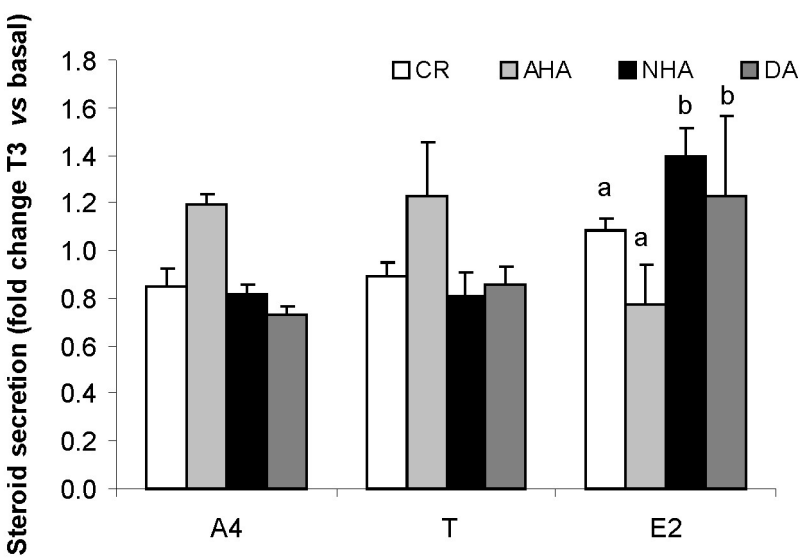

Fig. 4. Changes in the *in vitro* secreted A4, T and E2 from rat testicular slices stimulated by T3 (100 ng/ml). Rat groups: control (CR), adult heat-acclimated (AHA), neonatal heat-acclimated (NHA) and de-acclimated (DA) rats (n = 5/group); a, b - different superscripts indicate significant differences in T3-induced steroid secretion between the experimental groups.

submitted to different heat acclimation schedule without influence on the activity of steroid enzymes and steroid secretion.

In previous studies, the heat acclimation was accompanied by decreased plasma thyroid hormones concentration (Arieli and Chinet 1990, Maloyan and Horowitz 2002, Seebacher 2009), which was also observed in all the groups of heat-acclimated rats in this study. The lowest plasma fT3 concentration was found in de-acclimated rats. Unexpectedly, in this group, the decrease in fT3 concentration was deepen in spite of long-lasting exposure of rats to room temperature after their neonatal heat acclimation. This observation may indicate a disturbed return of the thyroid gland to normal function after postnatal heat exposure of rats.

The action of T3 on testicular function depends on the presence of its receptors as well as it might reciprocally affect their expression in testes. In this study, we have confirmed the expression of two types of *Thr* (*Thra1* and *Thrb1*) mRNAs in testes of studied rats. The expression of THRA1 and THRA2 mRNA and proteins was found in adult rats (Buzzard et al.

2000, Jannini et al. 2000, Canale et al. 2001). In turn, Rao et al. (2003) have also revealed the expression of *Thrb1* gene in testes of adult rats. In addition, we found up-regulated testicular expression of *Thra1* in NHA and DA rats and *Thrb1* in DA rats compared to the control animals. These data imply that neonatal heat acclimation increases testicular sensitivity to T3. In response to treatment with T3, the down-regulation of the testicular *Thra1* expression to a similar level in all the studied groups and *Thrb1* in DA group was observed. This observation is in agreement with results of earlier study (Rao et al. 2003). Therefore, it seems that up-regulation of *Thr* (especially *Thra1*) in neonatally heat-acclimated rats, at least partially, may result from decreased T3 plasma concentration. On the other hand increased testicular thyroid receptor expression in NHA and DA rats may explain the lack of changes in the gonado-somatic index in these rats (Kurowicka et al. 2015a), in contrast to the enlargement of the testes in neonatally hypothyroid animals detected by others (Ariyaratne et al. 2000, Holsberger et al. 2005, Oner et al. 2006, Wagner et al. 2008, Auharek and Franca 2010, Tousson et al. 2012).

In hypothyroid animals, altered reproductive tract morphology, spermatogenesis and Leydig cell functions were shown (Ariyaratne et al. 2000, Holsberger et al. 2005, Oner et al. 2006, Wagner et al. 2008, Auharek and de Franca 2010, Tousson et al. 2012). We have also reported severe histological changes in the testes and abnormal sperm production mainly in adult heat-acclimated males (Kurowicka et al. 2015b).

The results of our present and previous studies (Kurowicka et al. 2015b) indicate differentiated impact of heat acclimation on testicular function despite similar plasma fT3 concentrations found in AHA and NHA rats. Therefore, in further *in vitro* experiments, we aimed to delineate T3 effects on the testicular steroidogenesis at the molecular level in heat-acclimated rats. In response to T3, testicular expression of *Star* and *Cyp19a1* was up- and down-regulated, respectively in the most of the studied groups (except *Star* expression in AHA rats). Similar effects of T3 on the testicular *Star* and *Cyp19a1* expression were observed by others (Manna et al. 1999, 2001, Pezzi et al. 2001, Catalano et al. 2003). It is also worth to note that inhibition of *Cyp19a1* expression by T3 was weaker in NHA compared to that found in the other groups. Moreover, we revealed down-regulation of *Cyp11a1* and up-regulation of *Hsd17b3* by T3 in the testes of neonatal heat-acclimated and/or de-acclimated rats in contrast to those determined in CR and AHA groups. Such effects of T3 in heat-acclimated rats have not been reported yet. In earlier studies, *Cyp11a1* expression was rather up- than down-regulated by T3 in adult rats (Manna et al. 2001, Park et al. 2014). Interestingly, in NHA and DA rats, basal and prolactin stimulated *Cyp11a1* testicular expression was also different compared to that found in CR and AHA rats (Kurowicka et al. 2015a).Thus, the regulation of *Cyp11a1* expression in testes of NHA rats requires further experimentation.

In the present study, significant effects of T3 were observed neither on the activity of steroidogenic enzymes: CYP11A1, HSD3B1, CYP17A1, HSD17B3 and CYP19A1 nor on steroid secretion by the testicular tissue of the studied rats. Admittedly, in this study we used a short incubation period of testicular slices (8 h) in the presence of T3 in order to observe early, extra nuclear effects of T3 action on steroidogenesis in heat-acclimated rats (Wagner et al. 2008, Zamoner et al. 2011). However, we were not able to determine any extra nuclear effects of T3 in testes of the studied rats. It cannot be excluded that the effect of T3 on testicular steroidogenesis in heat-acclimated rats would occur using extended tissue incubation with the treatment (Manna et al. 1999, 2001, Pezzi et al. 2001, Catalano et al. 2003, Park et al. 2014). This notion is supported by observed differences in E2 secretion in response to T3 between the neonatally heat-acclimated (NHA and DA) and the other studied groups (CR and AHA).

In conclusion, this study has documented decreased fT3 plasma concentration in neonatal and adult rats submitted to heat acclimation, which in the neonatally heat-acclimated rats was accompanied by increased testicular expression of *Thra1*and/or *Thrb1*. Short-lasting treatment of the testicular tissue with T3 altered *Cyp11a1* and *Hsd17b3* expression only in the neonatally heat-acclimated rats without substantial changes in the activity of steroidogenic enzymes and steroids secretion. This indicates that testes of neonatally heat-acclimated rats are more susceptible to T3 action than testes of adult heat-acclimated rats.

Acknowledgments

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