דו"ח מסכם לתוכנית מחקר מספר 356-0613-12
שנת מחקר שלישית מתוך שלוש שנים

שינוי בבקרה של RNA קצרים (MicroRNA) על ביטויים של ההורמונים בהיפותלמוס, תביא לשיפור עמידות לתנאי סביבה.

Changes in MicroRNA regulation of the hypothalamic releasing hormones will lead to improved thermotolerance.

מוגש לקרן המדען הראשי במשרד החקלאות

ע"י

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תקציר

1. **הצגת הבעיה:** לאור התחממות כדור הארץ וההשפעות הברורות שיש לטמפרטורה קיצונית על רווחת עופות המשק, יש לפתח זני עופות עמידים לחום. ממחקרים שערכנו בשנים האחרונות זיהינו שכאשר חושפים אפרוחים בני שלושה ימים לטמפרטורה קיצונית אך מבוקרת, אפרוחים אילו עמידים יותר בבגרותם לשינויי אקלים קיצוניים מאשר אפרוחים שלא נחשפו לטמפרטורה גבוהה. במקביל זיהינו שעמידות זאת נובעת משינוי בביטוי גנים בהיפותלמוס הקדמי.
2. **מטרות המחקר**: אימות הנחת העבודה שהפעלה או השתקה ארוכת טווח של גנים הקשורים למבנה ההיפותלמוס ולהורמוני השחרור, בשלבים הראשונים של ההתפתחות שלאחר הבקיעה, תלויה בשינויים אפיגנטיים בכלל וב RNA קצרים בפרט ומביאה לשיפור העמידות של פטמים לחום לאורך חייהם.
3. **שיטות העבודה:** בחינת השפעת התניית חום ואתגור חום על הבקרה האפיגנטית בהיפותלמוס תוך התרכזות בבקרה על ביטוי גנים שקשורים למבנה ההיפותלמוס הקדמי, ולהורמונים המשחררים נבחנה ע"י מדידת טמפרטורת הגוף של האפרוחים, בחינת שינוי

במבנה המטילציה על ה DNA, ועל שינויי בביטוי microRNA בהיפותלמוס. בחינת ביטוי הגנים וה miR נעשתה בעזרת Real time PCR, בחינת המטילציה על ה DNA נבחנה ב ChiP, ובדיקת השפעתם של ההורמונים המשחררים וה miR על עמידות לחום נבחנה ע"י הזרקתם למוח האפרוח.

4. **תוצאות עיקריות:** כתוצאה מהתניית חום באפרוחי פטמים ביום השלישי לחייהם, מצאנו שינויים בביטוי ההורמונים המשחררים בהיפותלמוס, בבקרה אפיגנטית על ביטוי הורמונים אילו כולל RNA הקצרים ועל השפעתם של microRNAs על בקת טמפרטורה בכלל ועל גנים שקשורים לתוספת של תאים חדשים להיפותלמוס הקדמי. שינויי בקרה אילו בשלבים הראשונים של ההתפתחות שלאחר הבקיעה הביאו לשיפור בעמידות פטמים לחום.
5. **מסקנות והמלצות לגבי יישום התוצאות:** ישנה השפעה ארוכת טווח להתניית חום על בקרה אפיגנטית בהיפוטלמוס. יש לשים דגש על חלבונים שקשורים לבקרה אפיגנטית בפיתוח סלקציה של קווים עתידיים.

מעריכים מומלצים לבדיקת הדוח המדעי

1. פרופ' שלמה יהב
2. ד"ר איתמר ברש
3. ד"ר שלי דוריאן

הממצאים בדו"ח זה הינם תוצאות ניסויים.

הממצאים בשלב זה יכולים להיות מיושמים בפיתוח זנים חדשים של פטמים עמידים לעקות חום.

חתימת החוקר _____

Introduction

The temperature control system, like other sensory systems, develops during a critical period, which is characterized by high sensitivity to environmental influences and increased levels of synaptic plasticity (Hensch 2004). The critical period of the thermoregulatory system in chicks is between the third and the fifth day after hatching (Yahav and McMurtry 2010). Exposure to stressful events, among them heat stress, is differently perceived by organisms depending on the stringency of the stress. Whereas severe stress can be detrimental and result in a vulnerable response, mild stress can be beneficial and eventually lead to resilience (Franklin et al., 2012).

The key CNS site integrating the neuroendocrine adjustments to stress including thermal conditioning is the hypothalamic paraventricular nucleus (PVN) which produces hypothalamic releasing hormones such as corticotropin-releasing hormone (CRH) (Debonne et al. 2008; Kageyama and Suda, 2009), thyrotropin-releasing hormone (TRH) (Nilni, 2010) and chicken homologue of mammalian argininvasopressin peptide (AVP), arginin-vasotocin (AVT) (Grossmann et al., 1995). These releasing factors control stress behaviors through several mechanisms. One of them is the hypothalamic-pituitary-adrenal (HPA) axis, which is activated by CRH and AVT (McNabb, 2007). The end-hormones of the HPA axis are glucocorticoids, mineralocorticoids and catecholamines.

In this proposed project we will demonstrate that the hypothalamic releasing hormones are long-term regulated by heat, meaning their set-point for expression is altered differentially by different levels of heat stress. Furthermore they are regulated by epigenetic mechanisms including CpG methylation at their promoters and microRNAs (miRs). The epigenetic regulation of the releasing hormones determines their expression levels and hence their ability to response to heat-related stress.

Hypothesis, and Objectives (similar to the proposal)

The hypothesis to be tested in the proposed research is that thermal exposure during chick postnatal development changes microRNA expression and as a consequence alters the translation of the hypothalamic releasing hormones that finally results in long-lasting changes in thermal response set point.

Specifically I will check:

1. The level of miR-15a and/or let7b or miR-30a during heat conditioning in the third post-natal day, which is the critical period for hypothalamic development, and 10 days later to evaluate long-term effects.
2. If indeed the aforementioned miRs target the releasing hormones using luciferase reporter assay.
3. The effectiveness of intracranial microinjection of the aforementioned mimic– miRs and antimiR to penetrate the cells and the intracranial distribution of the drugs using real-time PCR and in situ hybridization.
4. The biochemical effects of intracranial injection of the aforementioned miRs on the level of the expression of CRH, TRH and AVT in the hypothalamus using the both real-time PCR and western blot analysis.
5. The phenotypic effect of mimic miR or antimiR "knock down" of the of the hypothalamic releasing hormones during both heat conditioning and heat challenge of previously conditioned chicks throughout the life span of the chick.

Results

The effect of heat conditioning on thermotolerance acquisition

Previous studies have shown that heat conditioning at 3 days post-hatching (during the critical period of thermal control establishment) of 36-37.5ºC for 24 hrs caused a higher resilience to heat later in life (Yahav and McMurtry, 2010). Further studies in our lab have shown that heat conditioning of 37.5±0.5ºC (day 3 post-hatch chicks) and heat challenge (35.5±0.5ºC on day 10 post-hatch) of the conditioned chicks caused an increase in *Bdnf* mRNA levels compared to non-conditioned age-matched chicks (Katz and Meiri, 2006; Yossifoff et al., 2008; Kisliouk and Meiri, 2009).

In order to fine-tune the suitable temperature for heat conditioning, two temperatures were tested: 36±0.5ºC and 37.5±0.5ºC (heat challenge in both was at 35.5±0.5ºC). Heat conditioning of 37.5±0.5ºC caused a significantly higher increase in body temperature than 36º±0.5C at 6 and 24 hrs during the conditioning (differences between 36±0.5°C and 37.5±0.5°C ~ 0.4°C; $P<0.01$ and $P<0.05$ for 6 and 24 hrs, respectively; Fig. 1A). During heat challenge, a week after the conditioning, the body temperatures of the 36±0.5ºC conditioned chicks were significantly lower than those of both 37.5±0.5ºC conditioned and non-conditioned chicks ($P<0.001$; Fig. 1B). While the

body temperature of chicks that were conditioned at 36±0.5ºC on the third day post-hatch was only modestly increased during heat challenge (by about 1°C), the body temperature of chicks that were conditioned at 37.5±0.5ºC were significantly raised by more than 2°C (42.19ºC, 42.17ºC and 41.8ºC at 2, 6 and 24 hrs respectively for the 36±0.5°C conditioned chicks; 43.33ºC, 42.65ºC and 42.67ºC for the 37.5±0.5°C conditioned chicks). Body temperatures of non-conditioned chicks were higher than those of 37.5±0.5ºC conditioned chicks only at 6 hrs during the conditioning (P<0.05; Fig. 1B). These results determined in favor of conditioning chicks at 36±0.5ºC as a better temperature for chicks' heat conditioning to achieve thermotolerance acquisition.

The expression of the mRNA of *Bdnf* in the PO/AH during heat conditioning of 3-day-old chicks, was increased (by 20%) in the 36±0.5ºC conditioned chicks 2 hrs during the conditioning (P<0.05) and the levels of the mRNA of *Bdnf* in 37.5±0.5ºC conditioned chicks were increased 2 and 6 hrs during the conditioning (by 24% and 32% respectively; P<0.01; Fig.1C). The difference in *Bdnf* mRNA expression between the two conditions at 6 hrs during the conditioning is correlated with the increase in body temperatures at the same time.

Evaluation of miR-15a and miR-30a-5p binding to *Bdnf* 3'-UTR

To test whether miRNAs are involved in controlling *Bdnf* expression in chick PO/AH, miRNAs that might contribute to *Bdnf* regulation were nominated computationally. *In silico* analysis of the gallus *Bdnf* 3'-UTR sequence using the prediction software program TargetScan that recognizes avian miRs identified the binding sites of numerous miRNAs. As a starting approach, two of them were selected: miR-15a and miR-30a-5p (Fig. 2A). These miRNAs were chosen because they are highly conserved among vertebrates and they have high affinity in their binding sites (Fig. 2B). Furthermore, previous studies confirmed that miR-15a and miR-30a-5p binds to the *Bdnf* 3'-UTR in vertebrates (Mellios et al., 2008; Friedman et al., 2009).

To examine whether miR-15a and/or miR-30a-5p binds to the 3'-UTR of *Bdnf*, luciferase reporters encoding either the *Bdnf* 3'-UTR or a mutant of *Bdnf* 3'-UTR at the miR-15a binding site or miR-30a-5p were generated. Relative luciferase activity was not changed with transfection of 10 pmol or 40 pmol of miR-30-5p (Data not shown). However, transfection of 40 pmol of miR-15a caused a significant decrease of 22% of the relative luciferase activity compared to the luciferase activity in a mutant for the miR-15a binding site on the *Bdnf* 3'-UTR (There was no inhibitory effect using 10 pmol of miR 15a; Fig. 2C).

MiR-15a expression during thermotolerance acquisition

In the next stage, I examined if the expression profile of miR-15a was affected by heat conditioning. Inversely with *Bdnf* expression, miR-15a levels during heat conditioning were decreased during the conditioning in both condition temperatures (36±0.5ºC and 37.5±0.5ºC; Fig. 3). While the expression of miR-15a was decreased at both 2 and 6 hrs at 37.5±0.5°C (by 50%, $P<0.01$ and 33%, $P<0.05$ respectively), it was decreased only after 2 hrs at 36±0.5°C and only by 25% ($P<0.05$; Fig. 3). Furthermore, conditioning at 37.5±0.5°C results in significantly lower miR-15a levels than conditioning at 36±0.5°C at 2 and 6 hrs during the conditioning (by 33% and 50% respectively, both $P<0.05$; Fig. 3) The levels of miR-15a returned to the levels of naïve untreated chicks at both conditioned temperatures after 24 hrs.

MiR-15a pharmacokinetics in the PO/AH

To explore whether intracranial injection of miR-15a is effective *in vivo*, mimic-miR-15a was intracranially injected into the third ventricle of 3-day-old chicks.

Its levels in the PO/AH were evaluated using real-time PCR in comparison to those of saline injected counterparts 2, 6 and 24 hrs after the injection. As depicted in Figure 4A, a 2.5 *μ*g dose of mimic-miR-15a results in a decrease of miR-15a after 2 hrs (by 40%, $P<0.05$) following by an increase as time went by. The maximum amount of miR-15a was observed after 24 hrs, at which time it was almost four times higher than the saline injected chicks at the same time ($P<0.05$; Fig. 4A).

The physiological effectiveness of miR-15a incorporation was evaluated by measuring the *Bdnf* mRNA levels 2, 6 and 24 hrs after the injection. In parallel to the decrease in miR-15a expression, intracerebralventricular microinjection of 2.5 *μ*g of mimic miR-15a results in higher levels of *Bdnf* when compared with its saline injected chicks counterparts at the same time (Fig. 4B). *Bdnf* mRNA levels were significantly higher by 65% at 2 hrs after the injection compared to saline injected chicks ($P<0.05$; Fig. 4B).

CyclinD, another possible target for miR-15a (3 potential seed sites) was also examined by its mRNA levels after mimic-miR-15a intracranial injection. There were no significant differences in the mRNA levels of CyclinD between the chicks 2, 6 and 24 hrs after miR-15a injection, compared with those in saline injected chicks 2 hrs after the injection. *Bdnf* mRNA levels were significantly higher 2 and 6 hrs after miR-15a injection

compared with CyclinD mRNA levels along the heat conditioning (P<0.01 and P<0.05 respectively; Fig. 4C).

The phenotypic effect of miR-15a injection was examined by measuring the injected chicks' basal body temperature. Intracranial injection of miR-15a resulted in a decrease in the chicks' basal body temperatures. As shown in Figure 4D, 2 hrs after miR-15a treatment the body temperature of miR-15a injected chicks declined to its lowest level, 40.38 ± 0.39 ºC (P<0.01), whereas that of saline injected counterparts was 41.65 ± 0.09 ºC. At 6 hrs after miR-15a injection, the body temperature had risen to 41.18 ± 0.12 ºC; at 24 hrs it remained at the same level - 41.19 ± 0.16 ºC (Fig. 4D). The body temperatures of saline injected chicks were 41.42 ± 0.12 ºC and 41.26 ± 0.13 ºC after 6 and 24 hrs of treatment, respectively (Fig. 4D).

Effect of miR-15a injection during heat conditioning

After determining the pharmacokinetic effect of miR-15a on body temperature and *Bdnf* levels, I examined the effect of intracranial administration of miR-15a during heat conditioning.

The effect of intracranial injection of miR-15a on body temperature of 3-day-old chicks during heat conditioning was opposite to the effect on chicks that were not exposed to heat. The administration of the miR caused a significant increase in body temperatures compared with those of saline injected or non-injected chicks throughout the treatment both in 36º±0.5C and in 37.5±0.5ºC conditioned chicks, 2, 6 and 24 hrs after the injection, during heat conditioning (Fig. 5A). The differences between body temperatures of 37.5±0.5ºC conditioned chicks remained significantly higher than 36±0.5ºC conditioned chicks at 6 and 24 hrs during the conditioning, similar to the responses without intracranial injection (P<0.01 and P<0.05 at 6 and 24 hrs respectively for the miR injected and P<0.01 in both durations for the saline injected chicks; Fig. 5A). Saline injection caused higher body temperatures compared with non-injected chicks only at 6 hrs during heat conditioning and after the injection both in 36±0.5ºC and in 37.5±0.5ºC conditioned chicks (P<0.001 and P<0.01 respectively; Fig. 5A).

Intracranial injection of miR-15a had an effect on the *Bdnf* mRNA levels of the 36±0.5ºC conditioned chicks. The injection had a transient effect as depicted by the induction of *Bdnf* in the saline injected chicks (at 2 hrs; 35% induction; P<0.01), whereas miR-15a had a longer and a more pronounced effect (by 60%; 2 and 6 hrs into conditioning; P<0.001; Fig. 5B). Moreover, miR-15a injection resulted in significantly

higher *Bdnf* levels than saline injection 6 hrs into conditioning (by 37%; $P<0.01$). After 24 hrs of heat conditioning, miR-15a injection had no effect on *Bdnf* expression, its levels returned to the levels of *Bdnf* before intracranial injection and heat conditioning.

MiR-15a levels were affected by both stresses of injection and heat exposure in a biphasic manner. A short-term effect 2 hrs after the injection and heat exposure caused an increase in the expression profile of saline injected chicks (by 127%; $P<0.01$) and 50% higher miR-15a levels than the miR injected chicks ($P<0.05$; Fig. 5C). While after 24 hrs of conditioning miR-15a was induced only in the miR injected group (by 157%; $P<0.05$) and its expression was twice than the saline injected chicks ($P<0.05$; Fig. 5C).

The injection of miR-15a to the chicks' PO/AH before heat conditioning seemed to have different effect in different conditioned temperatures. *Bdnf* levels were significantly higher when chicks were exposed to the lower temperature of 36±0.5ºC compared with 37.5±0.5ºC ($P<0.05$ at 2, 6 and 24 hrs during the conditioning; Fig. 5D). As shown in Fig. 5B, miR-15a injected chicks who conditioned at 36±0.5ºC have shown an increase in *Bdnf* mRNA levels by up to 60%. However, miR-15a injected chicks who conditioned at 37.5±0.5ºC have shown a modest significant increase (of 28%) in *Bdnf* mRNA levels only after 6 hrs of conditioning and intracranial injection ($P<0.05$), while 24 hrs during the conditioning and after the treatment, *Bdnf* mRNA levels decreased by 19% ($P<0.05$; Fig. 5D).

MiR-15a levels were higher in the 36±0.5ºC conditioned chicks than in the 37.5±0.5ºC conditioned chicks (Fig. 5E). 2 hrs during the conditioning, the levels of the 37.5±0.5ºC conditioned chicks significantly decreased ($P<0.05$) and were three times lower than those of the 36±0.5ºC conditioned chicks ($P<0.001$). Expression of miR-15a was increased at both 36±0.5ºC and 37.5±0.5ºC conditioned chicks 24 hrs during the conditioning and after miR-15a injection (by 85% and 157% respectively; $P<0.05$; Fig. 5E).

Long-term effect of miR-15a and heat conditioning

In order to evaluate the long-term phenotypic effect of miR-15a injection during the critical period for thermotolerance acquisition, the effect of miR-15a injection was evaluated a week after the injection – before heat challenge and 2 and 6 hrs during heat challenge. Three groups were tested: chicks that were conditioned on day 3 at 36±0.5ºC or 37.5±0.5ºC, and age matched non-conditioned chicks. All three groups were heat challenged to 35.5±0.5ºC and have shown high temperatures during the heat exposure as expected.

Conditioning in both temperatures was effective and the body temperatures of saline injected chicks on day 3 caused attenuation of the increase in body temperature when they were re-exposed to heat on day 10, compared with chicks that were exposed to heat at the first time 6 hrs after the beginning of heat challenge (43.56ºC for the non-conditioned chicks, compared with 42.90ºC and 42.93ºC for the 36±0.5ºC conditioned and 37.5±0.5ºC conditioned chicks respectively; $P<0.001$; Fig. 6A).

MiR-15a injection caused a long-term effect. A week after the injection the basal body temperatures of chicks that were conditioned at 36±0.5ºC on day 3 were higher than those of age matched saline injected chicks (41.59ºC and 41.36 respectively, $P<0.05$). Conditioning at 37.5±0.5ºC did not have long-term effect on the basal body temperatures of the conditioned chicks a week after conditioning (Fig. 6A).

While the injection of miR-15a to 3-day-old chicks that were conditioned at 36±0.5°C did not impair the long-term thermotolerance acquisition, the additive effect of both miR-15a and 37.5±0.5°C conditioning resulted in a weakened thermotolerance effect. Their body temperatures were higher than of saline injected chicks that were conditioned at 37.5±0.5°C both after 2 and 6 hrs into heat challenge. (The body temperature of the miR-injected chicks was 43.41°C compared with 43.06ºC in the saline injected chicks 2 hrs after the beginning of heat challenge and 43.85°C compared with 42.93°C after 6 hrs; $P<0.01$; Fig. 6A).

Furthermore, injection of miR-15a almost abolished the long-term thermotolerance effect of the 37.5±0.5°C conditioned chicks. Compared to non-conditioned chicks there was only a 0.3°C difference in body temperature 2 hrs during heat re-exposure ($P<0.05$; Fig. 6A).

It should be noted that injection of miR-15a had a different effect, depended on the conditioning temperature. Conditioning at 36±0.5°C induced thermotolerance and the body temperature of the chicks was lower than the temperature of the chicks that were injected with miR-15a but were not exposed to heat on day 3 of their lives (42.76°C and 42.74°C, 2 and 6 hrs respectively during heat exposure for the 36±0.5°C conditioned chicks compared with 43.73°C and 43.79°C, 2 and 6 hrs respectively during heat exposure in the non-conditioned chicks; $P<0.001$). In contrast, intracranial injection of miR-15a followed by 37.5±0.5°C conditioning resulted in impaired thermotolerance acquisition. The body temperature of these chicks during heat re-exposure was much higher than the body temperature of the 36±0.5°C conditioned chicks and similar to the non-conditioned chicks

(43.41°C and 43.85°C, 2 and 6 hrs respectively during hear challenge in chicks that conditioned at 37.5±0.5°C; *P*<0.01; Fig. 6A).

The biochemical long-term effect of miR-15a intracranial injection was tested by measuring *Bdnf* mRNA levels and miR-15a levels one week after the injection and heat conditioning.

Before heat exposure of the 10-day-old chicks, as a consequence of the conditioning on day 3 the levels of *Bdnf* in chicks that were conditioned at 36±0.5ºC were the highest in both saline and miR-15a treated chicks. The levels of *Bdnf* mRNA was significantly lower by 44% in the miR-15a injected chicks compared with the saline injected chicks, (*P*<0.01; Fig. 6B). There was no long-term effect of either saline or miR-15a in chicks that were conditioned at 37.5±0.5ºC on the basal *Bdnf* mRNA levels, as with the non-conditioned chicks. The levels of the mRNA of *Bdnf* in the PO/AH of chicks that were conditioned at 37.5±0.5°C or non-conditioned chicks were lower by two folds than those in chicks that were conditioned at 36±0.5°C (*P*<0.001; Fig. 6B).

Basal miR-15a levels were also tested on 10-day-old chicks before heat exposure in order to evaluate the long-term consequences of heat conditioning on day 3. The effect of conditioning was opposite between conditioning at 36±0.5°C and 37.5±0.5°C. While there was a decrease in miR-15a expression in chicks that were conditioned at 36±0.5°C and intracranially injected with miR-15a, the levels of miR-15a in chicks that were conditioned at 37.5±0.5°C were significantly elevated (three times than the 36±0.5°C conditioned chicks; *P*<0.01; Fig. 6C). Nonetheless, as with *Bdnf* levels, chicks that were conditioned at 36±0.5ºC showed significant difference in the levels of miR-15a between saline and miR-15a treated chicks. MiR-15a injection on day 3 caused a long-term attenuation by 50% in the miR-15a levels a week later compared with saline injection (*P*<0.001). Furthermore, miR-15a levels in chicks that were conditioned on day 3 at 37.5±0.5ºC (both saline and miR injected chicks) were higher than in non-conditioned chicks by 50% one week after the injection (*P*<0.05; Fig. 6C).

Bdnf and miR-15a levels were examined during heat exposure of 10-day-old chicks in order to evaluate the long-term effect of intracranial injection of miR-15a a week earlier. Since the baseline of both *Bdnf* and miR-15a levels was affected by conditioning at 36±0.5°C, all the results are presented as differences from the levels of the group without heat treatment on day 10.

Saline injection had no long-term effect on *Bdnf* expression. Injection of miR-15a on day 3 and conditioning at 37.5±0.5ºC caused a transient attenuation in the expression

levels of *Bdnf* 2 hrs into heat challenge a week later (by 30%, compared with naive non-conditioned chicks; $P<0.01$). The levels of *Bdnf* returned to its levels before heat challenge after 6 hrs. MiR-15a injected chicks without heat exposure disrupted the thermoregulation system and caused an elevation of 25% 2 hrs into heat exposure ($P<0.01$). There was no long-term effect to miR injection with the mild conditioning of 36±0.5ºC (Fig. 6D).

MiR-15a levels were transiently induced by 90% 2 hrs within heat exposure in saline injected non-conditioned chicks, while there was no long-term effect in the expression levels of miR-15a in challenged previously conditioned chicks ($P<0.001$; Fig. 6E). In the miR-15a injected chicks there were more pronounced long-term effects of miR expression in both non-conditioned and chicks that were previously conditioned at 36±0.5ºC which lasted at both 2 and 6 hrs into heat challenge (increased by up to 95%). The levels of the 37.5±0.5ºC conditioned chicks did not change during heat exposure and were significantly lower than the aforementioned groups ($P<0.05$; Fig. 6E). Furthermore, miR-15a levels of chicks that were injected with miR-15a and conditioned at 36±0.5ºC were higher at both 2 and 6 hrs during heat challenge then their saline injected counterparts ($P<0.05$). Non-conditioned and miR-15a injected chicks' levels were higher than their saline counterparts only at 6 hrs during heat challenge ($P<0.001$; Fig. 6E).

Different intensities of heat conditioning on day 3 posthatch result in either a heat-resilient or vulnerable 1 week later

To study the mechanism underlying the development of thermotolerance in the PVN, I studied the difference between heat resilient and vulnerable responses. Chicks were exposed to heat stress at moderate (36°C) or harsh (40°C) temperatures for 24 h at the age of three days; during the critical period of temperature-control development. We measured the differences in body temperature along their exposure to the heat treatments (Fig. 7A). It is noticeable that the body temperature of chicks exposed to 40°C rose very quickly and sharply, while chicks exposed to 36°C demonstrated a much lower elevation in body temperature; from 10 min of heat treatment onward, the body temperature of chicks exposed to the very harsh 40°C was significantly higher than that of chicks exposed to 36°C (Fig 7A, $F(1,243)=93.67$, for the overall difference between heat treatments, and $F(4,243)=5.05$ for the heat treatment X time interaction, both $p<0.01$). The HPA axis is a highly adaptive neuroendocrine system which is strongly implicated in stress-resilience and vulnerability. Therefore, in order to determine whether it is activated differently in chicks exposed to the different ambient temperatures, the levels of Corticosterone (CORT)

were measured in the plasma (Fig. 7B). Similar to the differences in body temperature, cort levels in chicks that were exposed to 40°C was significantly higher than those of chicks exposed to 36°C 2 h and 6 h after initiating the heat stress (n = 10 in each group, $F(1,35)=29.2$, for the overall difference between heat treatments $p<0.01$).

Heat Stress attenuates neurogenesis

To evaluate the role of heat stress intensity on hypothalamic new cell generation, we injected 3-day-old chicks with BrdU prior to exposing them to the different ambient temperatures as discussed above. Exposure to 40°C for 24 h resulted in a 25% decrease in the total number of BrdU+ cells compared to control chicks 1 week after the treatment (age 10 days posthatch; control chicks represented 1440±90 BrdU+ cells per 100,000 counted cells; 17-20 chicks in each treatment group; $p<0.05$; Fig. 2A). This heat effect was only exerted on non-neuronal precursors (BrdU+/DCX- cells). While control chicks represented 680±40 BrdU+/DCX- cells per 100000 counted cells, the chicks exposed to 40°C had 500±50 BrdU+/DCX- cells per 100000 analyzed cells ($p<0.05$; Fig. 2C) The number of BrdU+/DCX+ cells in chicks exposed to 40°C did not change (Fig. 2B). Interestingly, exposure to mild heat stress (36°C) for 24 h did not affect the number of new cells in the Ant Hyp 7 days after the treatment, regardless of whether they were neuronal precursors (BrdU+/DCX+) or non-neuronal cells (BrdU+/DCX-; Fig. 2A–C) (kisliouk et al., 2014).

To evaluate the long-term effect of the heat stress inflicted on 3-day-old chicks by the different heat treatments, both conditioned chick groups (36 and 40°C), as well as a group of chicks which had not been heat-stressed, were exposed 1 week after conditioning to moderate heat challenge of 36°C for 24 h (Fig. 8A). Chicks that had been previously exposed to extreme 40°C were vulnerable to the heat stress, i.e. their body temperature rose significantly higher than that in chicks that had been exposed to heat for the first time in their lives; on the other hand, chicks that had been previously exposed to moderate heat (36°C) were resilient to heat stress, i.e. their body temperature increased to a lesser degree than those in the 40°C-conditioned chicks and their nonconditioned counterparts (Fig. 8B, $F(2,159)=84.74$, for the overall difference between heat treatments, $F(8,159)=9.51$ for the heat treatment X time interaction, both $p<0.01$).

In order to verify whether the changes in new cell generation had a long term effect, chicks were injected with BrdU. 12 h after the injection the chicks were exposed to moderate heat challenge of 36°C for 24 h (Fig. 9A). The percentage of BrdU-labeled cells incorporated into the anterior hypothalamus was quantified by FACS a week after the heat challenge. Effect of heat challenge on newborn cell number in the Ant Hyp was found to depend on the level of stress inflicted during the initial conditioning on day 3. As demonstrated in Fig. 4B–D, the lowest number of newborn cells, of both neural (BrdU+/DCX+) and non-neural (BrdU+/DCX-) origin, was observed in chicks conditioned on day 3 at 40°C. The number of neuronal precursors (BrdU+/DCX+) in chicks that had been exposed to heat for the first time on day 10 was much higher (25-fold) than that in heat-challenged 40°C-conditioned chicks ($p<0.05$), but was similar to that of naïve chicks (Fig. 9C). The effect of heat challenge on the percentage of non-neural (BrdU+/DCX-) cells in the Ant Hyp of nonconditioned chicks 7 days after the treatment was reflected by an almost 75% decrease in the number of newly generated cells compared to naïve chicks ($p<0.05$; Fig. 4D).

Interestingly, the same thermal challenge on day 10 of 36°C-conditioned chicks did not affect the number of newborn cells of either neural or non-neural origin in the Ant Hyp. These results are consistent with the idea that neurogenesis may be part of a resilience repertoire in which severe stress can be detrimental, but mild stress may be beneficial for neuronal network remodeling (Franklin et al., 2012).

Intracranial injection of miR-138 increases the number of newly generated cells in the hypothalamus.

We have previously demonstrated a decrease in miR-138 levels in the PO/AH during heat exposure (Kisliouk et al., 2011). It was therefore of interest to examine whether miR-138 is able to regulate the different hypothalamic cell populations in the postnatal critical period of thermal-control establishment. To this end, miR-138 mimic, miR-138 hairpin inhibitor (Inh-miR-138) or saline were intracranially injected into the 3V of 3-day-old chicks 12 h after BrdU injection (Fig. 10A). The effect of exogenous miR-138 on new cell generation was evaluated in structures adjacent to both LV and Ant Hyp on day 4 post-treatment by FACS analysis (age 7 days posthatch). As demonstrated in Fig. 10B, miR-138 injection resulted in an almost 3.5- and 2.5-fold increase in the number of BrdU-labeled cells in the structures adjacent to the LV ($P<0.001$) and Ant Hyp ($p<0.015$), respectively, whereas Inh-

miR-138 tended to reduce the number of new cells in both areas (saline injected chicks represented 1100±200 and 2100±600 BrdU+ cells per 100000 counted cells in the structures adjacent to the LV and Ant Hyp, respectively; each group included 8–12 chicks, of which each two brains were pooled together during tissue dispersion). Interestingly, a stimulatory effect of miR-138 on new cell generation was observed for both neural (BrdU+/DCX+) and non-neural (BrdU+/DCX-) cell types: a threefold increase in the percentage of the BrdU+/DCX+ population was found in both examined brain areas (saline injected chicks represented 600±100 and 1400±500 BrdU+/DCX+ cells per 100000 counted cells in the structures adjacent to the LV and Ant Hyp, respectively; Fig. 10C), and 3.2-fold and 1.5-fold increases in BrdU+/DCX- cell numbers were determined in the structures adjacent to the LV and Ant Hyp, respectively (saline injected chicks represented 500±100 and 700±150 BrdU+/DCX- cells per 100000 counted cells in the structures adjacent to the LV and Ant Hyp, respectively; Fig. 10D). A decreasing trend in the population number of both neuronal precursors (BrdU+/DCX+) and non-neural cell types (BrdU+/DCX-) was detected after the treatment with Inh-miR-138 (Fig. 10C, D). Note that injections of miR-138 and Inh-miR-138 did not significantly influence the total number of DCX+ cells and in both cases, the percentage of DCX+ cells was similar to that in saline-treated cells (data not shown).

miR-138 can modulate hypothalamic neurogenesis by direct targeting of Reln

We have recently demonstrated that miR-138 binds directly to the 3'-UTR of Reln mRNA, and moreover, exogenous miR-138 has been found to significantly abolish both Reln mRNA and Reln protein levels in primary hypothalamic cell culture (Kisliouk and Meiri, 2013). We therefore examined the effect of intracranial injection of miR-138 mimic on *Reln* mRNA expression in the anterior hypothalmus of 3-day-old chicks. The time course of *Reln* inhibition was determined by measuring its mRNA levels in the Ant Hyp between 2 and 24 h after the injection and comparing them with those in chicks injected with non-targeting miRNA, mimic-negative control (miR-neg). As depicted in Fig. 5E, 20% inhibition of *Reln* mRNA was observed 6 h after miR-138 mimic injection compared to miR-neg treatment at the same time point. To check the possibility that the effect of miR-138 on hypothalamic neurogenesis is mediated by direct inhibition of Reln, Reln-antisense "knockdown" was performed by intracranial injection of Reln-antisense into 3-day-old chicks 12 h after BrdU injection (Fig. 11A). The effect of intracranial injection of Reln-antisense on the amount of newborn cells in the Ant Hyp was consistent with the findings

from the miR-138 mimic injection. As demonstrated in Fig. 11F, in contrast to Reln-sense injection, Reln-antisense treatment resulted in an almost twofold increase in the number of BrdU-labeled cells in the Ant Hyp 4 days after the injection (age 7 days posthatch; Reln-sense injected chicks represented 1300±170 BrdU+ cells per 100000 counted cells; each group included 5 chicks; $p<0.05$) . The stimulatory effect of Reln-antisense treatment on newborn cell number was observed for both neural (BrdU+/DCX+) and non-neural (BrdU+/DCX-) cell types, with a 2.2-fold increase in the percentage of BrdU+/DCX+ cells and a 1.8-fold increase in BrdU+/DCX- cells relative to the Reln-sense-injected counterparts (Reln-sense injected chicks counted 490±20 BrdU+/DCX+ cells and 810±110 BrdU+/DCX- per 100000 analyzed cells; $p<0.05$; Fig. 11F). These data further support the hypothesis that miR-138-induced hypothalamic neurogenesis is at least partially mediated by the blocking of Reln expression.

Evaluating the epigenetic effect of heat conditioning and challenge on the expression levels of TRH

Since TRH plays an essential role in regulation of homeostasis we checks its expression levels during heat conditioning in the PVN of 3-day-old chicks and found a significant increase in its expression starting 10 min. of after the beginning of the heat exposure (increase of 13.7±7.56) and lasting up to 2 h (increase of 3.1±0.99) (data not shown).

As a result of the heat conditioning the expression levels of TRH was altered and a week after heat conditioning it was higher by 2±0.66 (Fig 12 A, n=10 in each group, $p<0.05$) than in 3-day old chicks. Heat conditioning at day 3 caused a decrease in the level of TRH to the expression levels in day 3 (1.1±0.18). In addition it was found that during heat challenge the expression pattern of TRH in the two groups i.e. heat conditioned (12 B) and age matched naïve 10 days old chicks (12 C) is different. While in conditioned chicks TRH stays constant (or even rises although not significantly) in non-conditioned chicks there is a clear reduction in the expression of TRH.

Since there is a long-term change in the expression pattern of TRH we assumed it was caused by alterations in epigenetic marks. We evaluated 5 CpG sites 4 on the promotor which were all changed as a result of conditioning (data not shown) and one in the intron at an HSF1 binding sit which is demonstrated in Fig 13. to prove the concept of alteration in DNA methylation as a result of heat conditioning. N5 is located 1115 bp downstream from the ATG. At this site we identified a clear opposite correlation between

the expression levels of TRH and the methylation levels. At the times where there is an increase in the expression of TRH there is a decrease in the methylation levels and vice versa (Fig. 13).

Since this site is a binding site for the transcription factor HSF2 we checked its expression pattern at day 10 as a result of heat conditioning at day 3 and found that it is affected by the conditioning. With age, between day 3 and day 10, there is a decrease of 38%±0.06 in the basic expression of HSF2 (n=10 in each group, $p<0.01$, Fig 14A). Heat conditioning causes an additional decrease of 25%±0.07. In addition the expression pattern of HSF2 is different during heat challenge between 10-day-old previously conditioned and non-conditioned chicks (14B, C). While in non-conditioned chicks there is a decrease in the expression levels of HSF2 during heat challenge, (10 and 30 min into challenge a decrease of 0.08±0.84 and 0.09±0.8 respectively, n=10 in each group, $p<0.01$) and no effect during longer exposures to heat. In contrast in previously conditioned chicks there is a significant increase in the expression levels of HSF2 after 6 h of heat challenge (6 and 24 h into challenge 0.21±1.6-ı 0.17±2.13 respectively).

Since in the literature it is not clear if HSF2 activates TRH alone or in combination with HSF1 we proved using immunostaining that they are co-localized in the same cells and are induced together as a result of heat conditioning (Fig 15).

Evaluating the role of CRH, both as a target and as a modulator of the stress vulnerability and resilience response

CRH plays an essential role in determining the stress reaction of the HPA axis. Therefore, it has an important potential role in determining the vulnerability and resilience reaction to heat stress during secondary encounters with a stressor. In order to verify its role, we monitored the level of *Crh* expression in the PVN of chicks exposed to either 36 or 40°C on the 3rd day of their lives, during the critical period of temperature-control development (Fig. 16). Whereas *Crh* expression did not increase during heat exposure, and even declined with time, in the PVN of chicks exposed to 36°C (reduction of 50% at 24 h), that of chicks exposed to 40°C rose rapidly: after 10 min, *Crh* expression increased by 50%, 2 h into the treatment it doubled, and then remained high, even at 6 h of heat exposure. That is to say, from 10 min of heat exposure onward, the *Crh* level in the PVN of chicks exposed to the very harsh 40°C was significantly higher than that of chicks exposed to 36°C ($n = 7–16$, $F(1,112)=27.2$, for the overall difference between heat treatments $p<0.01$).

To study the involvement of CRH in the long-term stress effects, its mRNA expression was measured in the PVN during the heat challenge performed 1 week after the initial exposure to heat stresses, conferring either resilience or vulnerability according to changes in body temperature. The expression level of *Crh* mRNA in chicks that had been previously exposed to 36°C (resilient) was significantly reduced compared to stressed nonconditioned chicks at 6 h into the challenge (reduction of 60%, n=30-40 in each group), whereas *Crh* mRNA expression in chicks that had been conditioned at 40°C (vulnerable) was significantly higher than its expression in nonconditioned chicks at 2 and 6 h into the challenge (elevation of 50 and 90% respectively, $F(2,517)=13.6$, for the difference between heat treatments, $F(8,517)=4.54$ for the heat treatment X time interaction, both $p<0.01$. Fig. 17).

These results further indicate a strong connection between different intensities of heat conditioning on day 3 and a heat-resilient or vulnerable memory 1 week later. Furthermore they demonstrate a potentially direct connection with CRH.

Therefore, in the next stage, we studied the direct effect of CRH on the acquisition of heat resilience or vulnerability. We first measured the effect of intracranial injection of either specific *Crh*-antisense or CRH into the third ventricle without stress, i.e. at the chicks' optimal environmental temperature (30°C). Both treatments were effective: the plasma levels of cort in chicks injected with CRH was three times higher 2 h after injection than those in naïve chicks and almost five times higher than those of chicks injected with saline ($F(2,59)=9.67$, for the difference between injected groups $p<0.01$, Fig. 18A). The levels of *Crh* in the hypothalamus in chicks injected with specific *Crh*-antisense were 50% lower 30 min after injection than those in saline injected chicks ($F(1,50)=3.65$, for the overall difference between injected groups, and $F(2,50)=2.97$ for the injection treatments X time interaction, both $p<0.05$, Fig. 18B). The inhibition of *Crh* mRNA still remained high after 2 h. There was no justification in measuring the CRH mRNA after a microinjection of the CRH protein. The effect of CRH injection on the body temperature was opposite to that of *Crh*-antisense treatment. While CRH injection resulted in significant elevation of body temperature, *Crh*-antisense caused a reduction in body temperature (Fig. 18C). Two hours after injection of CRH, chick body temperature was more than half a degree higher than that in chicks injected with saline, an effect which remained 6 h postinjection. In contrast, the body temperature of the chicks injected with *Crh*-antisense was reduced almost by 0.5ºC 30 min and 2 h after injection (n=17 in each group, $F(2,177)=18.69$, for the overall

difference between injected groups, and F(6,177)=6.72 for the injected treatment X time interaction, both p<0.01, Fig. 16C).

Since intracranial injection of CRH or *Crh*-antisense caused an alteration in the body temperature and cort level of 3-day-old chicks, it was pertinent to study whether this mimicked stress would have a long-term effect. To this end, 3-day-old chicks were injected with CRH, *Crh*-antisense or saline, but were not subjected to heat-conditioning. These chicks were then subjected to a mild environmental temperature challenge (36°C) 1 week after the injection (Fig. 19A). By measuring the body temperature, CRH-injected chicks exhibited a vulnerable response while *Crh*-antisense exhibited resilience. Although all groups experienced the same environmental temperature, the body temperature of chicks that were injected 1 week earlier with CRH was significantly higher than that of chicks injected with saline (Fig. 19B). In comparison with CRH injected chicks, *Crh*-antisense treated chicks had significantly lower body temperature (n = 17 in all groups, F(2,186)=27.56, for the overall difference between previously injected groups, and F(6,186)=4.21 for the previously injected groups X time interaction, both p<0.01).

To determine whether the difference in the body-temperature response to heat challenge 1 week after CRH injection is dependent on differential activation of the HPA axis, the levels of both *Crh* mRNA and cort were measured 1 week postinjection of either CRH or *Crh*-antisense during exposure to mild heat (36°C). As depicted in Fig. 19C, throughout the challenge, plasma cort level was higher in chicks that had been injected a week earlier with CRH and lower in chicks injected with *Crh*-antisense in comparison with their saline-injected counterparts. The cort level in chicks that were injected with *Crh*-antisense did not change during heat exposure, and was significantly lower than that in both CRH and saline injected counterparts, indicating stress resilience (F(2,180)=5.5, for the overall difference between previously injected groups, p<0.01, Fig. 19C). In response to heat challenge, *Crh* expression in the PVN was affected similarly to the cort almost in all groups. As shown in Fig. 19D, the level of *Crh* mRNA in chicks previously injected with CRH was the highest at 6 h into heat challenge, resembling vulnerable response, while its expression level in *Crh*-antisense treated chicks was the lowest throughout the challenge, indicating resilience (F(2,172)=5.05, for the overall difference between previously injected groups, p<0.01, Fig. 19D, Cramer et al. 2015).

Determining whether an epigenetic regulation of the CRH gene is part of the mechanism underlying stress vulnerability and resilience.

Since we found that the heat stress adaptive processes include long-term differential expression of the hormones and receptors of the HPA axis between resilience and vulnerability states, it is pertinent to assume that in accordance, there will be a difference in distribution of epigenetic marks which govern the expression levels of these proteins. This stress set-point "Early programming" might include alterations in patterns of DNA methylation and microRNA within different regions of the CRH gene (Lin and Dent, 2006).

CpG Methylation

An epigenetic mechanism influencing transcription is the methylation of CpG dinucleotides - CpG (Bernstein et al., 2007; Davey et al., 1997; Miller and Sweatt, 2007; Tsankova et al., 2004). The promoter region alongside the first intron of the CRH gene, contain both CpG stand-alone and CpG islands (regions rich with CpG repetition). These CpG repetitions are targets of dynamic methylation changes which occur throughout lifespan. DNA methylation can be induced by environmental alterations. In particular, early heat conditioning may alter DNA methylation patterns (Yossifoff et al., 2008). In order to assess whether DNA methylation has a role on determining the expression level of CRH, we sequenced two bisulfit treated DNA segments of the CRH gene; 1) A promoter region. 2) Section from the first intron. Both regions contain multiple CpG sites (Fig 20A). By comparing the DNA methylation pattern of 10 day old chicks previously conditioned at 36°C on the promoter area and at the first intron region, it is highly noticeable that the intron region exhibits a very rich methylation pattern (hypermethylation), in contrast to that of the promoter which displays very low percentages of methylation (hypomethylation). Furthermore, by comparing these chicks before the moderate heat challenge at day 10, and 6 h into heat challenging, their methylation pattern differs significantly displaying an elevation in methylation percentage along time (Chi square analysis performed, $P<0.05$ for each methylation site with a significant difference Fig 20B).

Discussion

The expression levels of the releasing hormones in the frontal hypothalamus are affected by heat conditioning at day 3 post hatch. This effect lasts beyond the immediate effect, and a week after conditioning, when heat challenged is applied, both the body temperature and the expression levels of the releasing hormones and genes that are involved in plasticity of the nucleuses where the releasing hormones are expressed, is different than

that of aged matched non-conditioned chicks. This long-term alteration in gene expression is regulated by alterations in epigenetic marks including changes in the pattern of DNA methylation that affect transcription factor binding and alteration in microRNA expression. Here we showed that indeed the DNA methylation of CpG sites at both TRH and CRH are altered during heat conditioning. This change in pattern lasts and should be taken into consideration when planning to improve the breeding of chicks to withstand heat sepals.

In contrast to CpG methylation which affected directly the expression of the releasing hormones, microRNA that we checked affected the resilience of chicks to heat but they seem to affect genes that are involved in neuronal plasticity in the hypothalamic nucleus in chich the releasing hormones resides i.e. the frontal hypothalamus. We demonstrated effects of Mir-15a on BDNF which is a neurotrophic factor and miR-138 on relin which is involved in neurogenesis.

In order to fully understand the role of microRNAs in heat resilience and vaulnerability more work should be done. Nevertheless their role in heat conditioning has been established and it is crucial for inducing heat resilience.

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Appendix

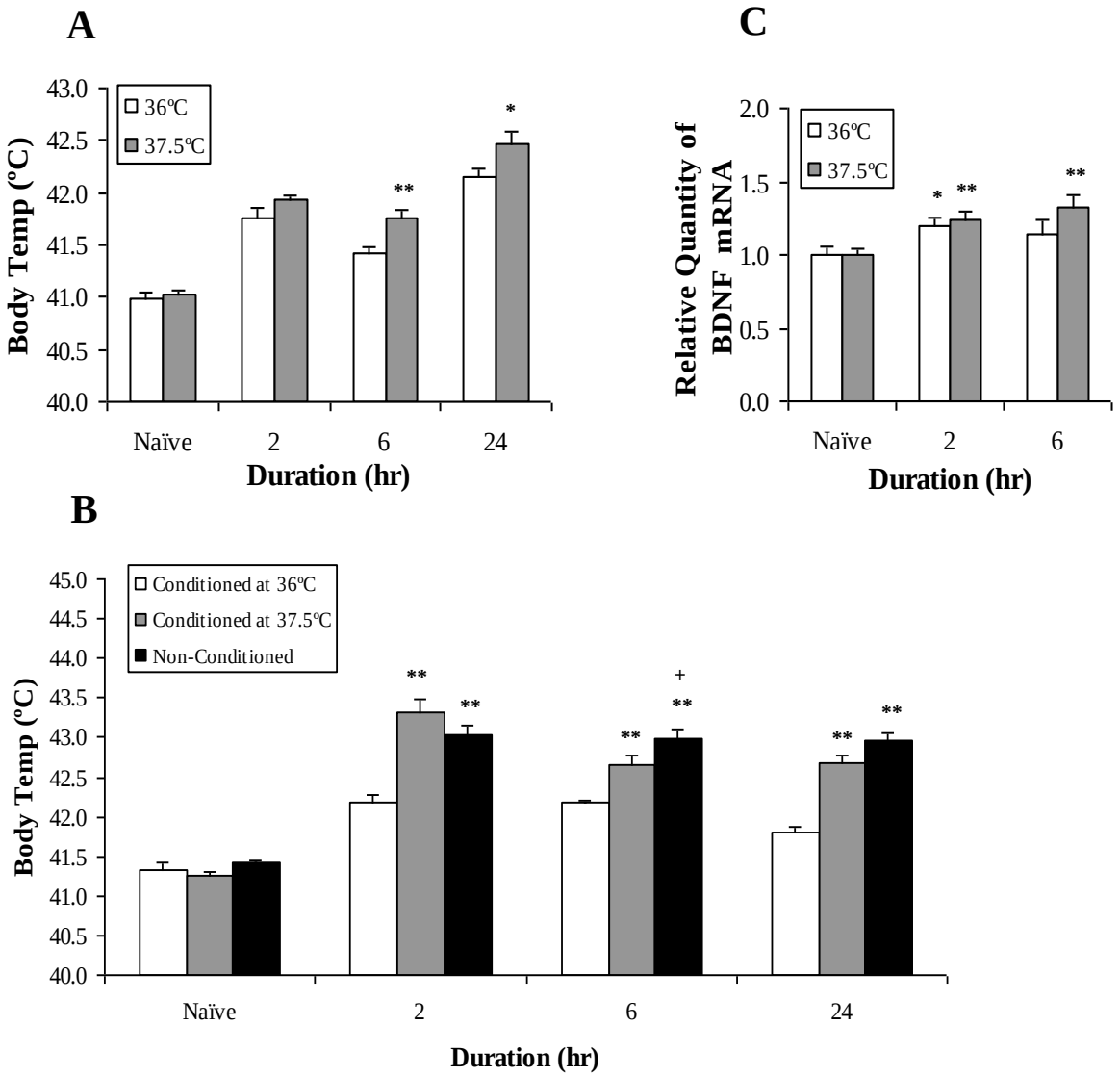
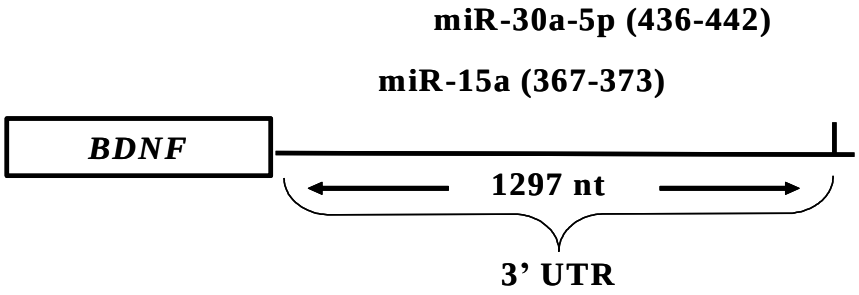


Figure 1 – Conditioning of chicks at 36ºC as a better temperature of conditioning for thermotolerance acquisition. A. Comparison of chicks' body temperatures during heat conditioning (day 3 post-hatch) between 36±0.5ºC and 37.5±0.5ºC of heat exposure. B. Comparison of chicks' body temperature one week after the conditioning (heat challenge) between chicks conditioned at 36±0.5ºC, at 37.5±0.5ºC and non-conditioned chicks (heat stress). Asterisks indicate differences between 36±0.5ºC conditioned to 37.5±0.5ºC conditioned and non-conditioned chick's body temperatures; + indicates differences between 37.5±0.5ºC conditioned to non-conditioned chicks (Student's *t*-test). Each bar represents the mean ± SEM of 10-30 chicks. C. Comparison of chicks' *Bdnf* mRNA levels during heat conditioning between 36±0.5ºC and 37.5±0.5ºC of heat exposure. Asterisks indicate significant differences between 36º±0.5C conditioned and 37.5±0.5ºC conditioned chicks to naïve chicks (Student's *t*-test). Each bar represents the mean ± SEM of 9-20 chicks.

A



B

>gga-miR-15a
UAGCAGCACAUAAUGGUUUGU

>gga-miR-30a-5p
UGUAAACAUCCUCGACUGGAAG

Position 367-373 of BDNF 3' UTR:

5' ...AAGAUUCAAAUUGCAUGCUGCU
3' UGUUUGGUAAUACACGACGA

Position 436-442 of BDNF 3' UTR

5' ...GAACCAAAACAUUCCGUUUACAU...
3' GAAGGUCAGCUCCUACAAAUGU

C

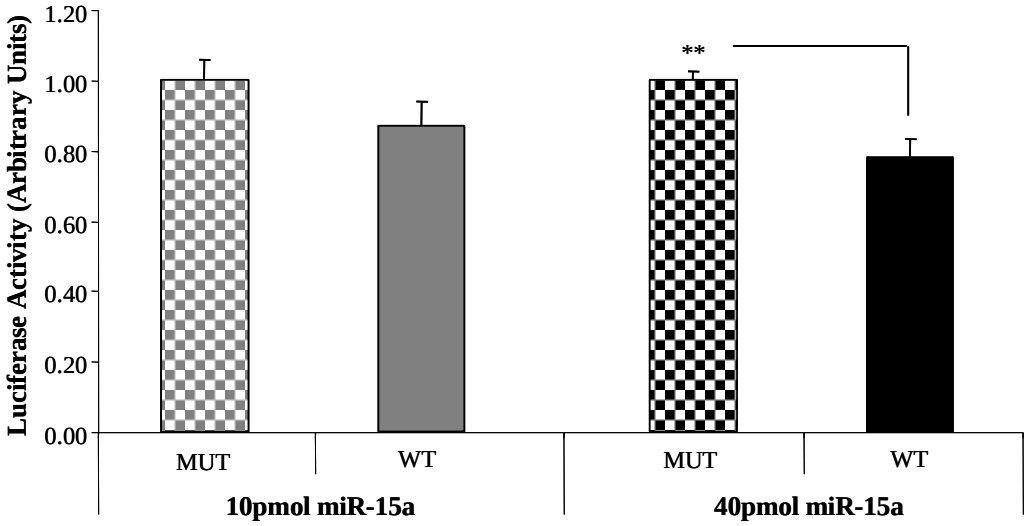


Figure 2 - *Bdnf* mRNA is a target for miR-15a. A. Schematic representation of miR-15a and miR-30a-5p potential binding sites along 1297 nucleotides in *Bdnf* 3'-UTR adjusted to the coding region. Numbers in parenthesis indicate miRNAs-complementary positions in the 3'-UTR downstream of the last protein codon. B. Complementarities between the putative *Bdnf* 3'-UTR and miR-15a (left) and miR-30a-5p (right). C. Luciferase activity in HEK 293T cells cotransfected with psiCHECK-2 vector containing wild-type (WT) or mutated (MUT) *Bdnf* 3'-UTR and miR-15a. Each bar represents the mean ± SEM of 9-12 independent repeats. The Renilla luciferase (RL) reporter signal was normalized to the firefly luciferase (FL) signal used as an internal control. Asterisks indicate differences between wild-type and mutated *Bdnf* 3'-UTR (Student's *t*-test).

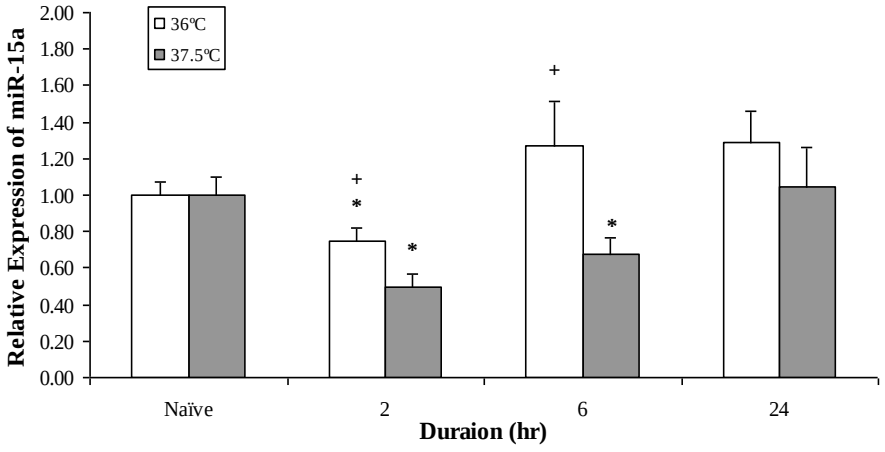


Figure 3 – Evaluation of miR-15a expression during heat conditioning. Comparison of chicks' miR-15a levels during heat conditioning between 36±0.5ºC and 37.5±0.5ºC of heat exposure. Asterisks indicate differences between 36±0.5ºC conditioned and 37.5±0.5ºC conditioned chicks to naïve chicks; + indicate differences between 36±0.5ºC conditioned and 37.5±0.5ºC conditioned chicks (Student's *t*-test). Each bar represents the mean ± SEM of 6-10 chicks.

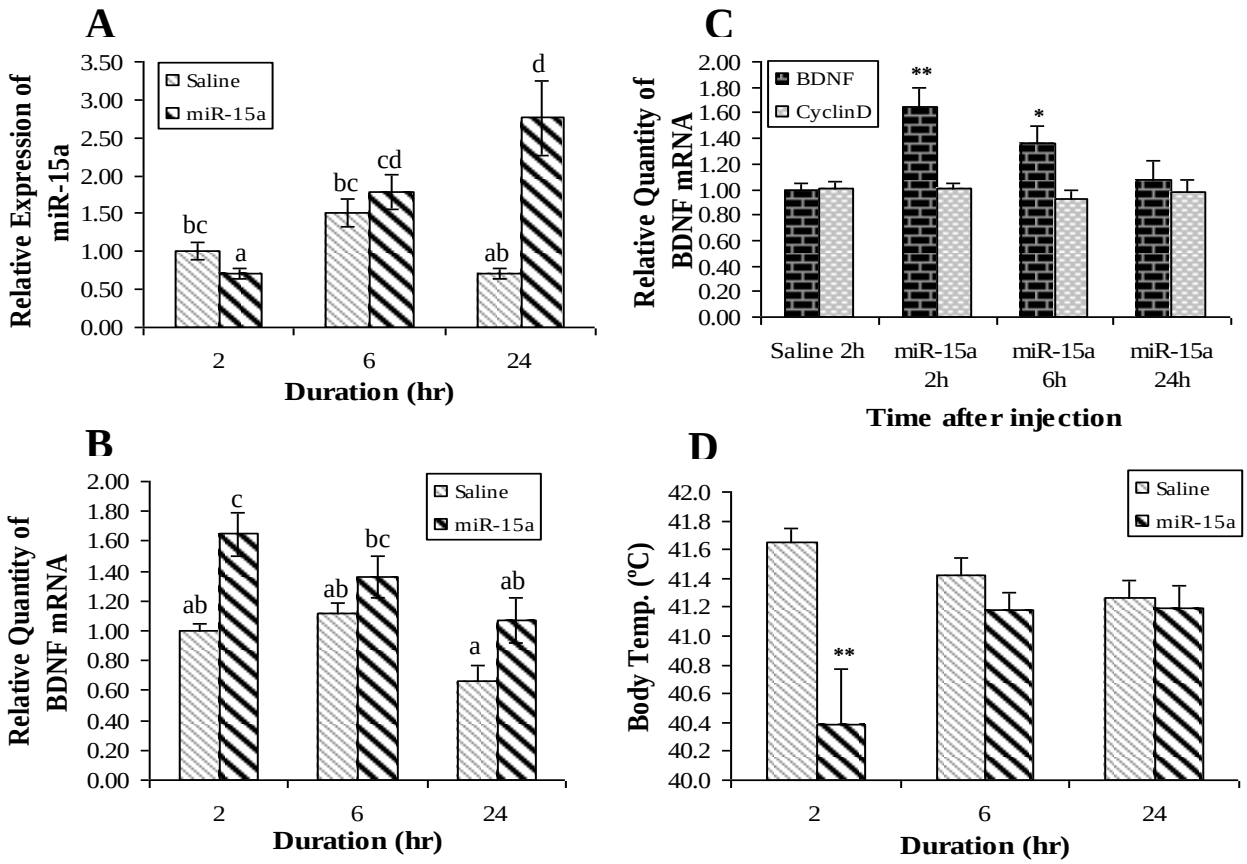


Figure 4 – Pharmacokinetic evaluation after miR-15a injection to the PO/AH of 3-day-old chicks. A. Time course of miR-15a incorporation into the PO/AH. Chicks were killed 2, 6 or 24 hrs after injection of miR-15a-mimic. Chicks injected with saline, 2 hrs after injection, were used as controls in all presented experiments. Each bar represents the mean ± SEM of 7-9 chicks at the same time point; Different letters indicate significant differences ($P<0.05$; Tukey-Kramer HSD). B. Time course of *Bdnf* mRNA levels in the PO/AH. Chicks were killed 2, 6 or 24 hrs after injection of miR-15a mimic. Each bar represents the mean ± SEM of 9-10 chicks at same time point; Different letters indicate significant differences ($P<0.05$; Tukey-Kramer HSD). C. Effect of miR-15a injection into the PO/AH on BDNF and CyclinD mRNA. Chicks were killed 2, 6 or 24 hrs after injection of miR-15a mimic. Each bar represents the mean ± SEM of 9-10 chicks at same time point; Asterisks indicate significant differences between BDNF and CyclinD at the same time point (Student's *t*-test). D. Alteration in body temperature of 3-day-old chicks after miR-15a injection. Chicks' body temperature was measured 2, 6 or 24 hrs after saline or miR-15a injection. Each bar represents the mean ± SEM of 10 chicks at same time point; Asterisks indicate significant differences between saline and miR-15a counterparts at the same time point (Student's *t*-test).

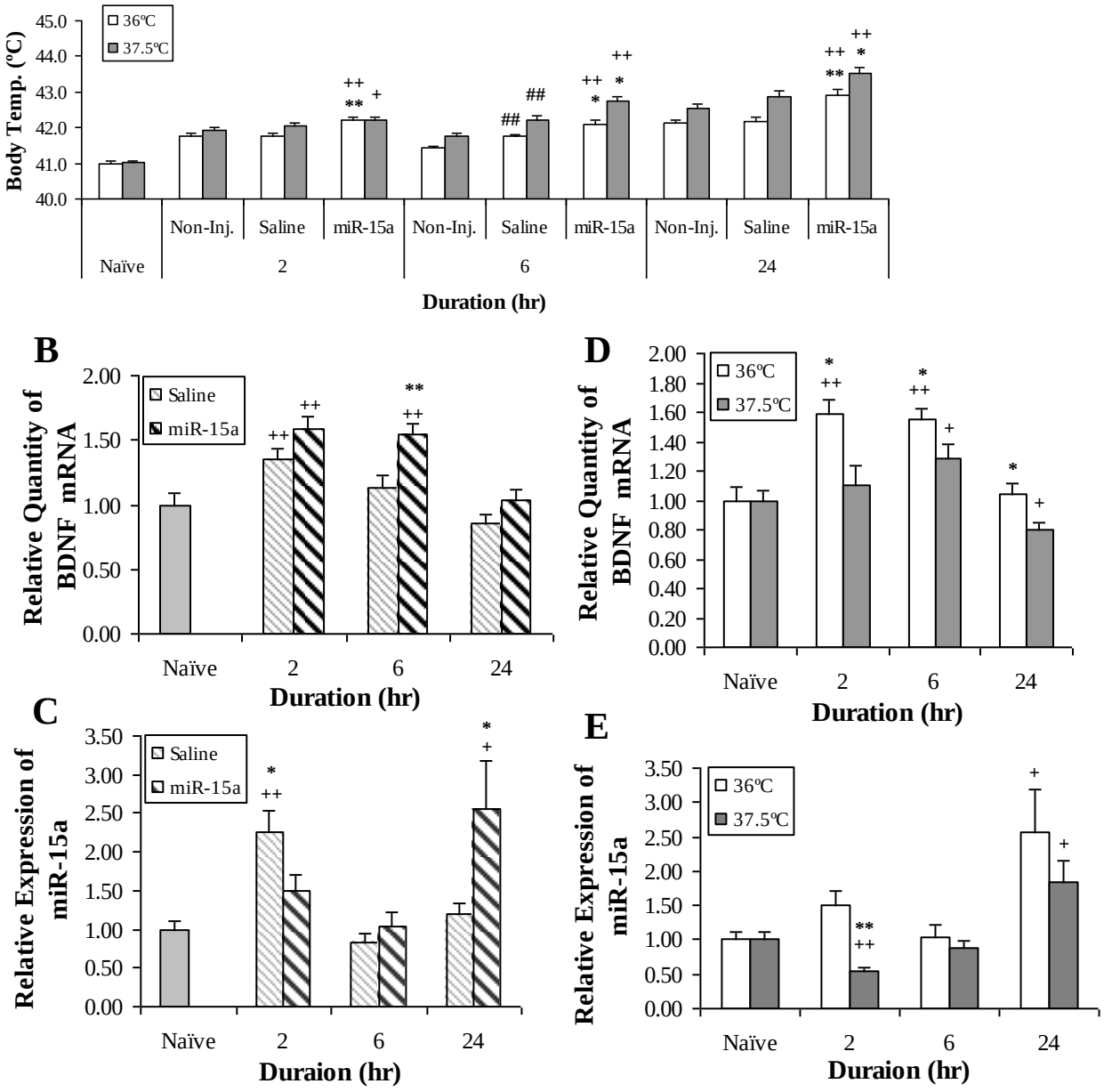


Figure 5 – Effect of miR-15a injection into the PO/AH during heat conditioning. A. Comparison of chicks' body temperatures during heat conditioning (day 3 post-hatch) between non-injected, saline-injected and miR-15a-injected chicks at 36±0.5ºC and 37.5±0.5ºC of heat exposure. Asterisks indicate differences between miR-15a-injected and saline-injected chicks at the same time and in the same heat exposure; + indicate differences between miR-15a-injected chicks and non-injected chicks at the same time and in the same heat exposure; # indicate differences between saline-injected and non-injected chicks at the same time and in the same heat exposure (Student's *t*-test). Each bar represents the mean ± SEM of 9-10 chicks. B. Comparison of chicks' *Bdnf* mRNA levels during heat conditioning of 36±0.5ºC (day 3 post-hatch) between saline-injected and miR-15a-injected chicks at the same time. C. Comparison of chicks' miR-15a levels during heat conditioning of 36±0.5ºC (day 3 post-hatch) between saline-injected and miR-15a-injected chicks at the same time. Asterisks indicate differences between saline-injected and miR-15a-injected chicks; + indicate differences between saline or miR-15a injected chicks and naïve chicks (Student's *t*-test). Each bar represents the mean ± SEM of 8-10 chicks. D. Comparison of chicks' *Bdnf* mRNA levels who injected with miR-15a between conditioning of 36±0.5ºC to conditioning at 37.5±0.5ºC at the same time. E. Comparison of chicks' miR-15a levels who injected with miR-15a between conditioning of 36±0.5ºC and conditioning at 37.5±0.5ºC at the same time. Asterisks indicate differences between conditioning of 36±0.5ºC chicks and conditioning at 37.5±0.5ºC chicks; + indicate differences between conditioning of 36±0.5ºC chicks or conditioning at 37.5±0.5ºC chicks and naïve chicks (Student's *t*-test). Each bar represents the mean ± SEM of 8-10 chicks.

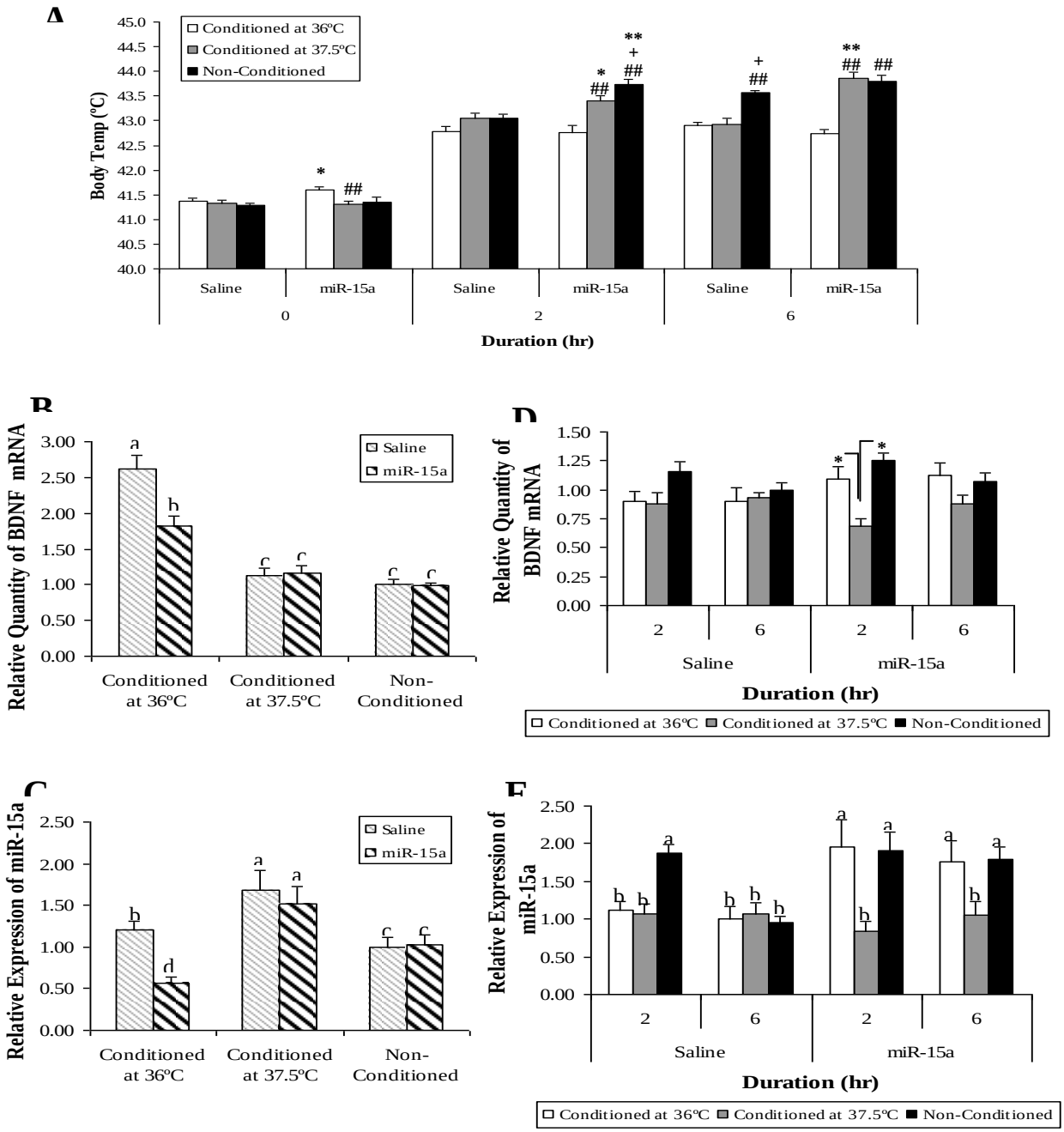


Figure 6 – The long-term effect of miR-15a injection into the PO/AH. A. Comparison of chicks' body temperatures during heat challenge of 35.5±0.5ºC (day 10 post-hatch) between saline-injected and miR-15a-injected chicks that were conditioned at 36±0.5ºC, conditioned at 37.5±0.5ºC and non-conditioned chicks (heat stress). Asterisks indicate differences between miR-15a-injected to saline-injected chicks at the same time; + indicate differences between non-conditioned chicks to 37.5±0.5ºC conditioned chicks at the same time; # indicate differences between non-conditioned and/or 37.5±0.5ºC conditioned chicks to 36±0.5ºC conditioned chicks at the same time (Student's *t*-test). Each bar represents the mean ± SEM of 8-12 chicks. B. Comparison of chicks' basal *Bdnf* mRNA levels before heat challenge (day 10 post-hatch) between saline-injected and miR-15a-injected chicks that were conditioned at 36±0.5ºC, conditioned at 37.5±0.5ºC and non-conditioned chicks (heat stress). Different letters indicate significant differences ($P<0.05$; Student's *t*-test). Each bar represents the mean ± SEM of 8-19 chicks. C. Comparison of chicks' basal miR-15a levels before heat challenge (day 10 post-hatch) between saline-injected and miR-15a-injected chicks that were conditioned at 36±0.5ºC, conditioned at 37.5±0.5ºC and non-conditioned chicks (heat stress). Different letters indicate significant differences ($P<0.05$; Student's *t*-test). Each bar represents the mean ± SEM of 7-10 chicks. D. Comparison of chicks' *Bdnf* levels during heat challenge of 35.5±0.5ºC (day 10 post-hatch) between saline-injected and miR-15a-injected chicks that were conditioned at 36±0.5ºC, conditioned at 37.5±0.5ºC and non-conditioned chicks (heat stress). Asterisks indicate significant differences ($P<0.05$; Student's *t*-test). Each bar represents the mean ± SEM of 8-17 chicks. E. Comparison of chicks' miR-15a levels during heat challenge of 35.5±0.5ºC (day 10 post-hatch) between saline-injected and miR-15a-injected chicks that were conditioned at 36±0.5ºC, conditioned at 37.5±0.5ºC and non-conditioned chicks (heat stress). Different letters indicate significant differences ($P<0.05$; Student's *t*-test). Each bar represents the mean ± SEM of 7-10 chicks.

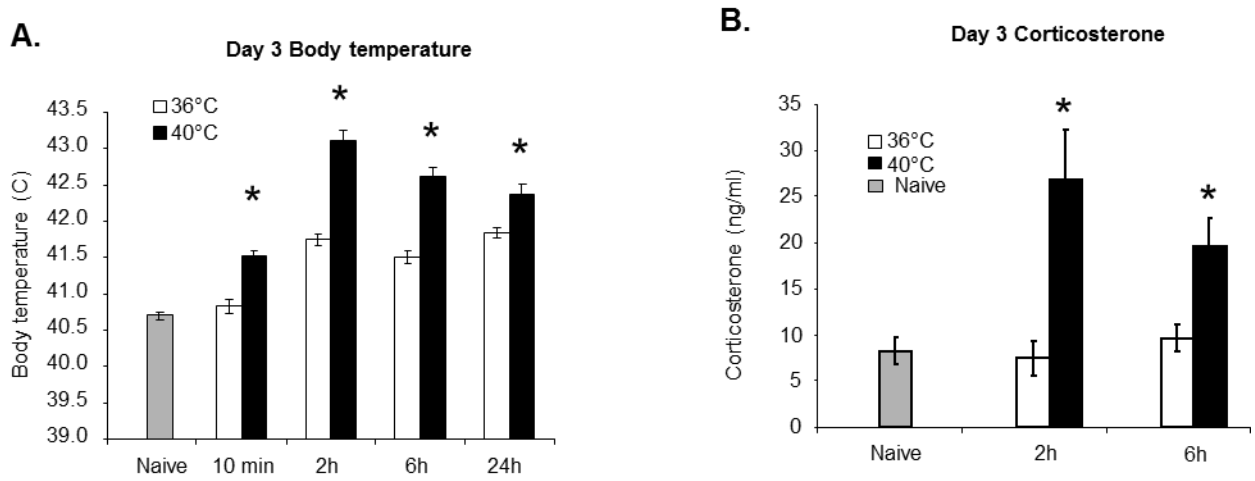


Figure 7. Heat-stress response is dependent on ambient temperature in 3-day-old chicks. Chicks were exposed for 24 h to moderate (36 ± 0.5°C) or extreme (40 ± 0.5°C) ambient temperatures. At various times during heat exposure, cloaca temperature was measured, blood was sampled, and chicks were sacrificed and their PVN surgically removed. All presented results are average ± SEM. (A) Body temperature. (B) Plasma CORT levels were evaluated using a CORT-specific RIA kit. * indicates significant difference between heat treatments at the same time point # indicates significant difference from naïve.

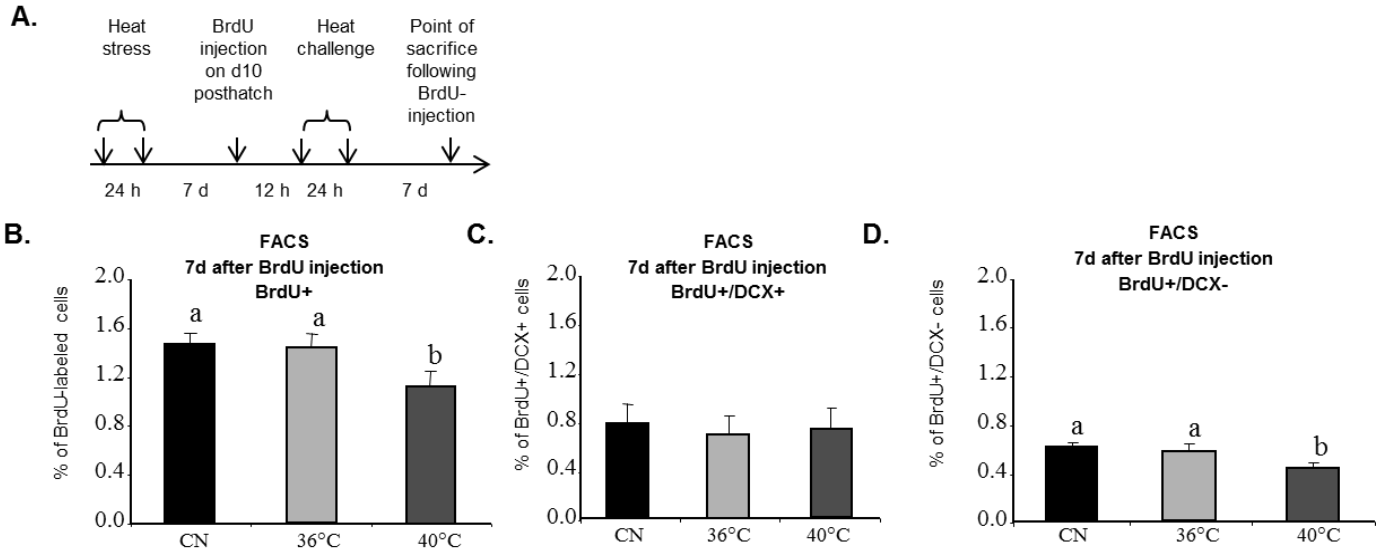


Figure 8. Effect of different intensities of heat stress on the quantity of newly generated cells in the Ant Hyp 7 days after heat exposure by FACS analysis. (A) Schematic representation of the treatment protocol. BrdU was injected on day 3 posthatch 12 h prior exposure to heat (36 °C or 40°C) for 24h. The hypothalamuses of control (CN) and heat-exposed (36 °C and 40 °C) chicks were dissected 7 days after BrdU injection, dissociated, fixed and double-immunostained with anti-BrdU and anti-DCX antibodies. (B) The percentage of BrdU-labeled cells (BrdU+) out of the total counted cells in the Ant Hyp. Each bar represents the mean ± SEM of 17–20 individual chicks. (C) The percentage of neural precursors, BrdU and DCX double labeled cells (BrdU+/DCX+) out of total counted cells in the Ant Hyp. Each column represents the mean ± SEM of the same subjects represented in (B). (D) The percentage of non-neural precursors, BrdU-positive but DCX-negative cells (BrdU+/DCX-), out of total counted cells in the Ant Hyp. Each column represents the mean ± SEM of the same subjects represented in (B) and (C). Different letters indicate significant differences ($P < 0.05$) among the different treatments.

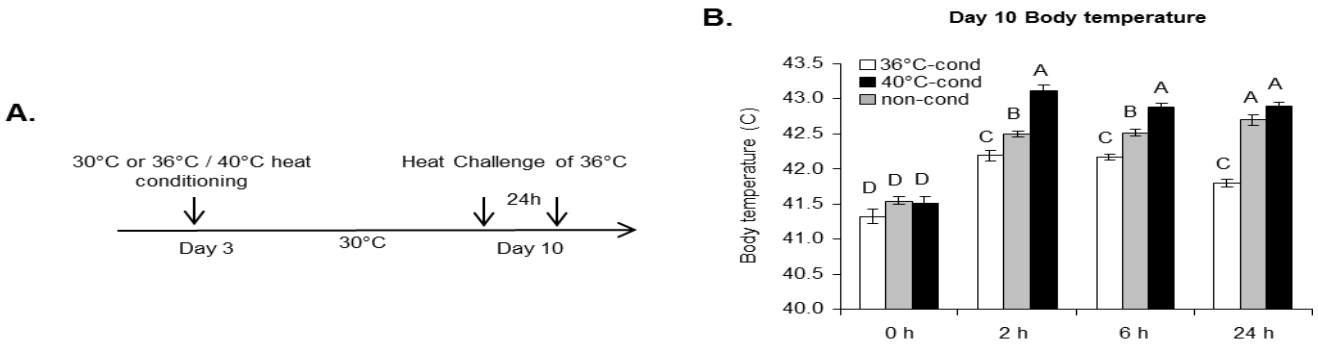


Fig. 9. Exposure to different intensities of heat-stress on day 3 resulted in either a heat-resilient or vulnerable response 1 week later.(A) Chicks that were exposed to moderate (36 ± 0.5°C; 36°C-cond) or extreme (40 ± 0.5°C; 40°C-cond) ambient temperature on day 3 posthatch were re-exposed to the moderately high ambient temperature of 36 ± 0.5°C a week later. Control chicks (non-cond) were exposed to 36°C on day 10 for the first time in their lives. (B) Body temperature of chicks exposed to the different heat treatments. Cloaca temperature was measured just before sacrifice. Presented results here and in the rest of the figure are average ± SEM.

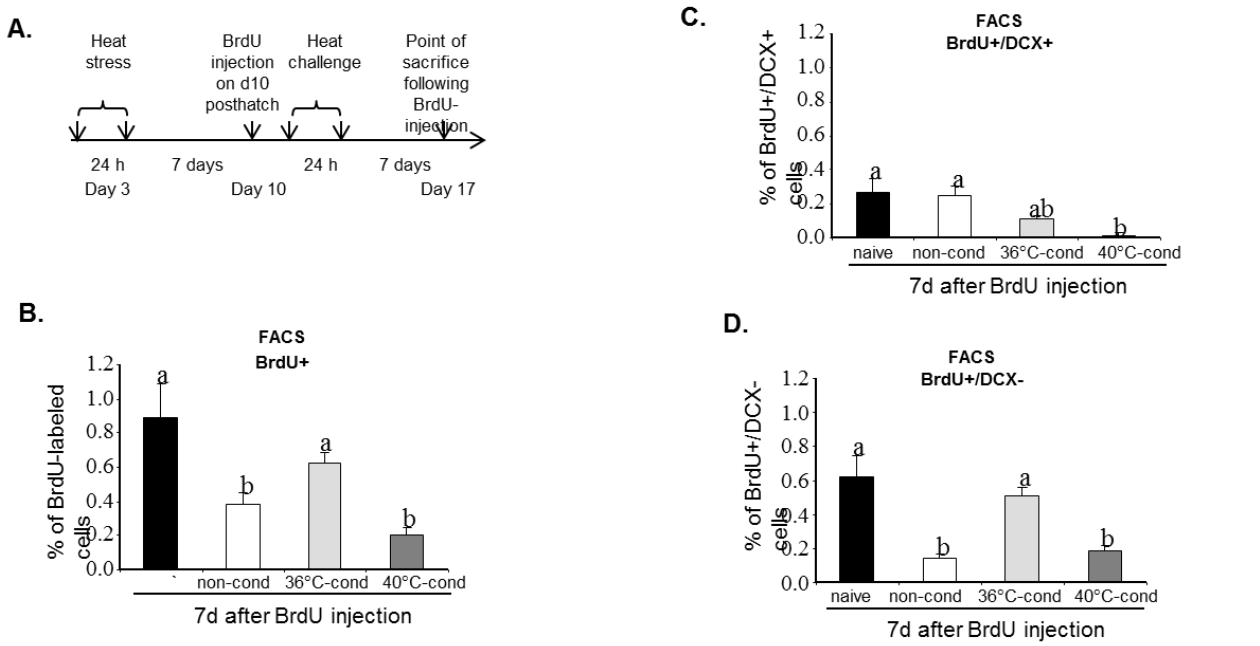


Fig. 10. Effect of heat challenge of previously conditioned chicks on the quantity of newborn cells in the Ant Hyp. (A) Schematic representation of the treatment protocol. Chicks were conditioned at mild (36 °C) or high (40 °C) ambient temperature for 24 h on day 3 posthatch. A week after conditioning, the chicks were injected with BrdU and 12 h after subjected to heat challenge (36 °C) for 24 h. The hypothalamuses of naïve, nonconditioned (non-cond) and both groups of conditioned chicks (36 °C-cond and 40 °C-cond) were dissected 7 days after BrdU injection (n = 9–10 per treatment group), dissociated, fixed and double-immunostained with anti-BrdU and anti-DCX antibodies. (B) The percentage of BrdU-labeled cells (BrdU+) out of the total counted cells in the Ant Hyp. Each bar represents the mean ± SEM of 9–10 individual chicks. (C) The percentage of neural precursors, BrdU and DCX double labeled cells (BrdU+/DCX+), out of total counted cells in the Ant Hyp. Each column represents the mean ± SEM of the same subjects represented in (B). (D) The percentage of non-neural precursors, BrdU-positive but DCX-negative cells (BrdU+/DCX-), out of total counted cells in the Ant Hyp. Each column represents the mean ± SEM of the same subjects represented in (B) and (C). Different letters indicate significant differences ($P < 0.05$) among the different treatments.

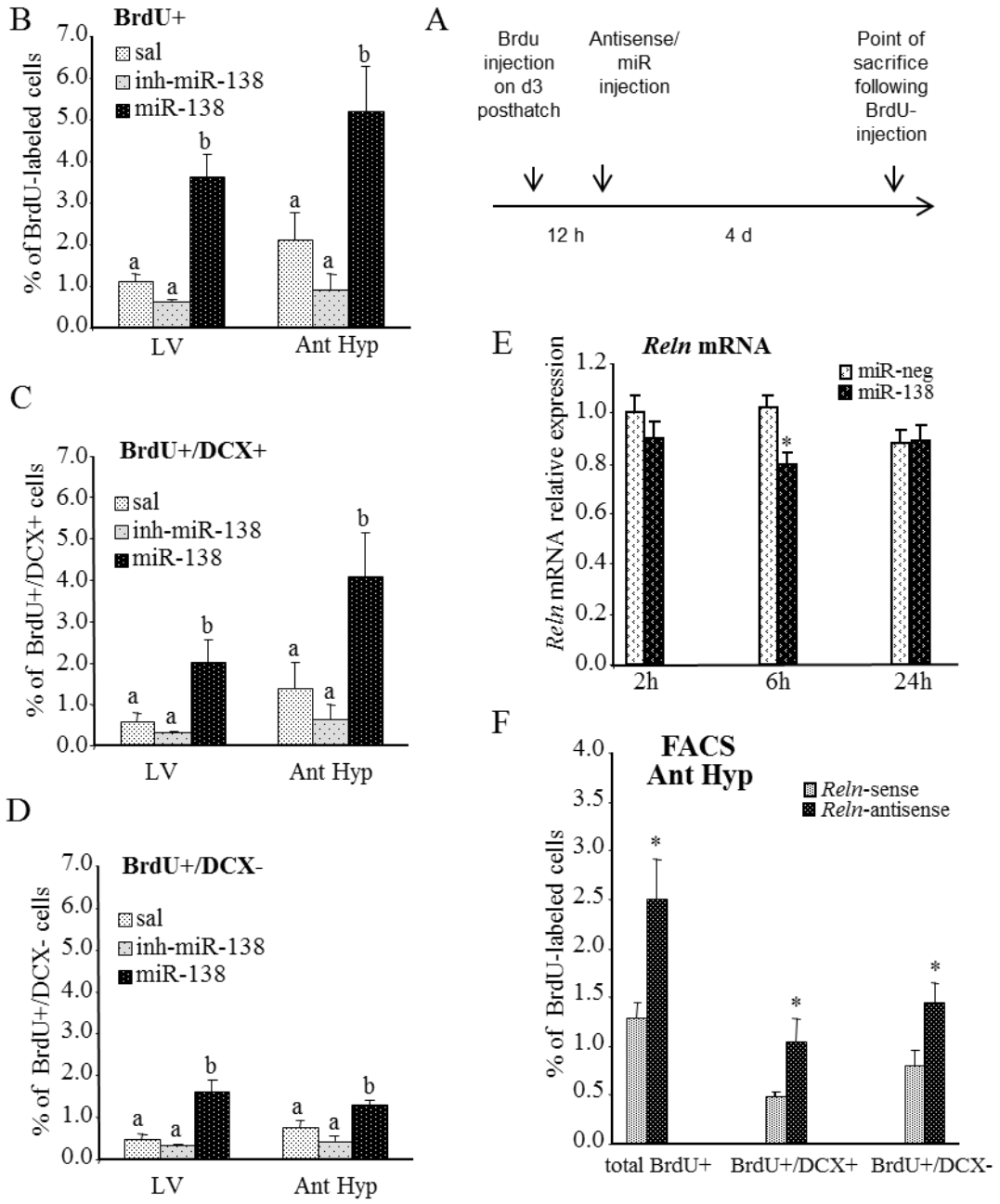


Fig. 11. Effect of miR-138 on new cell generation. (A) Time chart of the BrdU injection experiment. BrdU was injected on day 3 posthatch. Hsa-miR-138 mimic (miR-138), hsa-miR-138 hairpin inhibitor (Inh-miR-138), saline (Sal), Reln-antisense or Reln-sense DNA were intracranially injected 12 h following BrdU treatment. Four days after BrdU injection, both LV-adjacent areas and the Ant Hyp were dissected, dissociated and double-immunostained with anti-BrdU and anti-DCX antibodies and analyzed by FACS. (B–D) Effect of miR-138 injection on the number of newborn cells in the structures adjacent to LV and Ant Hyp. (B) The percentage of BrdU-labeled cells (BrdU+) out of total counted cells. Each bar represents the mean ± SEM of 8–12 chicks, where each two brains were pooled together during tissue dispersion. Different letters indicate significant differences ($P < 0.001$ for LV and $P < 0.015$ for Ant Hyp, respectively) among the different treatments. (C) The percentage of neural precursors (BrdU+/DCX+) out of total counted cells. Each column represents the mean ± SEM of the same subjects represented in (B) Different letters indicate significant differences ($P < 0.001$ for LV and $P < 0.015$ for Ant Hyp, respectively) among the different treatments.

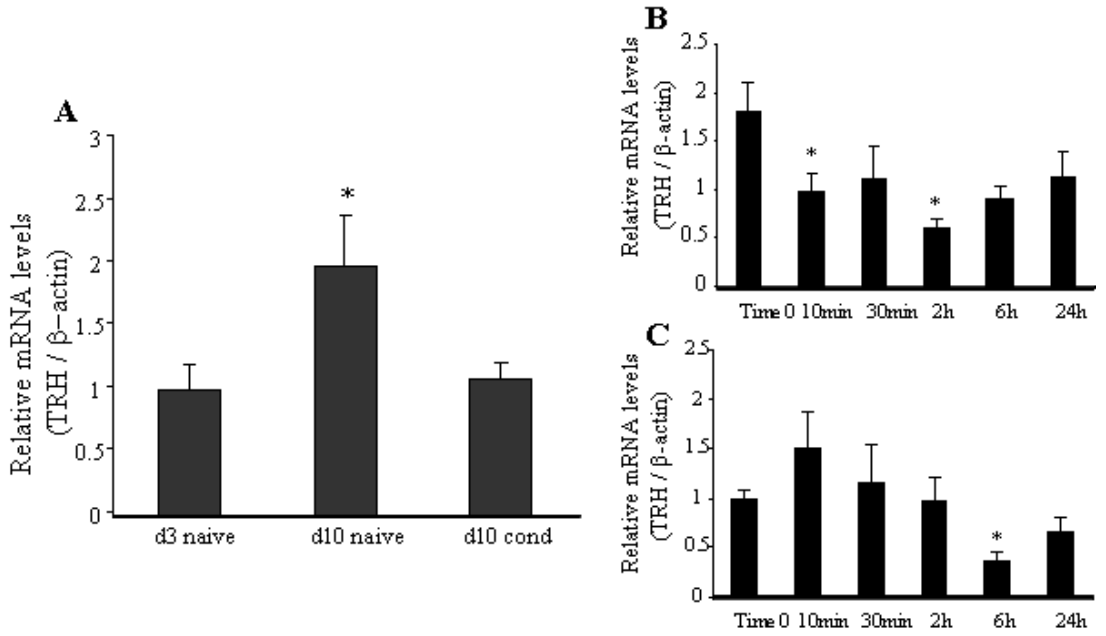


Figure 12. TRH expression is dependent on ambient temperature in 3-day-old chicks. Chicks were exposed for 24 h to extreme (40 ± 0.5°C) ambient temperatures. At various times during heat exposure, chicks were sacrificed and their PVN surgically removed TRH mRNA was evaluated using real time PCR. A. A comparison between day 10 naïve and day 10 previously conditioned chicks. B. The expression levels of TRH in at day 10 in non conditioned chicks. C. The expression levels of TRH at day 10 in previously conditioned chicks. All presented results are average ± SEM.

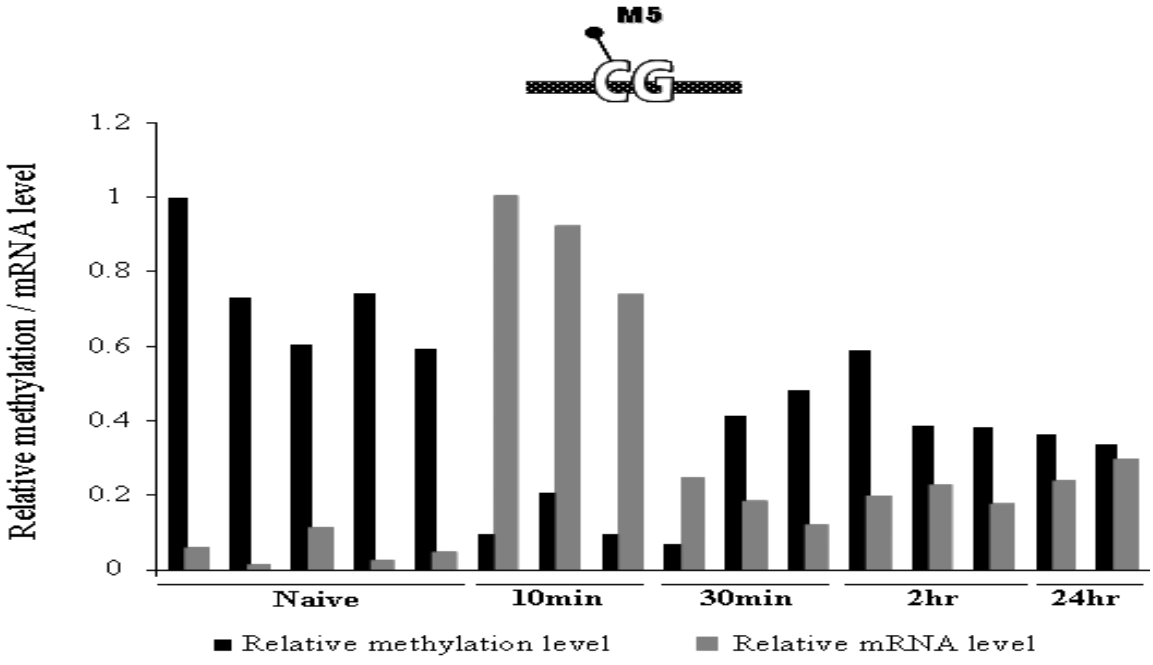


Figure 13. A comparison between the expression level of TRH and the methylation levels on site M5 at the first intron. Chicks were sacrificed in a time course during conditioning and the frontal hypothalamus dissected. The DNA and RNA were extracted methylation was determined after bisulfit reaction of the DNA and RNA was determined using real-time PCR.

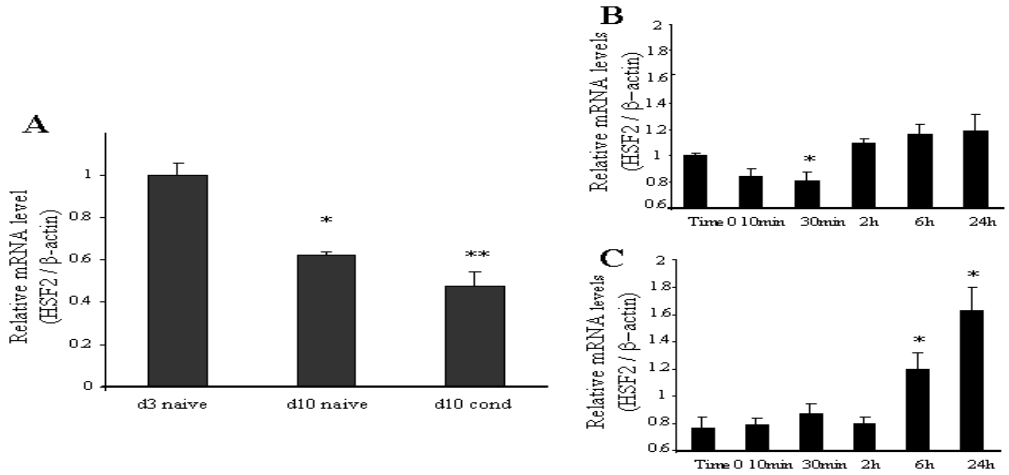


Figure 14. HSF2 expression is dependent on ambient temperature in 3-day-old chicks. Chicks were exposed for 24 h to extreme (40 ± 0.5°C) ambient temperatures. At various times during heat exposure, chicks were sacrificed and their PVN surgically removed HSF2 mRNA was evaluated using real time PCR. A. A comparison between day 10 naïve and day 10 previously conditioned chicks. B. The expression levels of HSF2 in at day 10 in non conditioned chicks. C. The expression levels of HSF2 at day 10 in previously conditioned chicks. All presented results are average ± SEM.

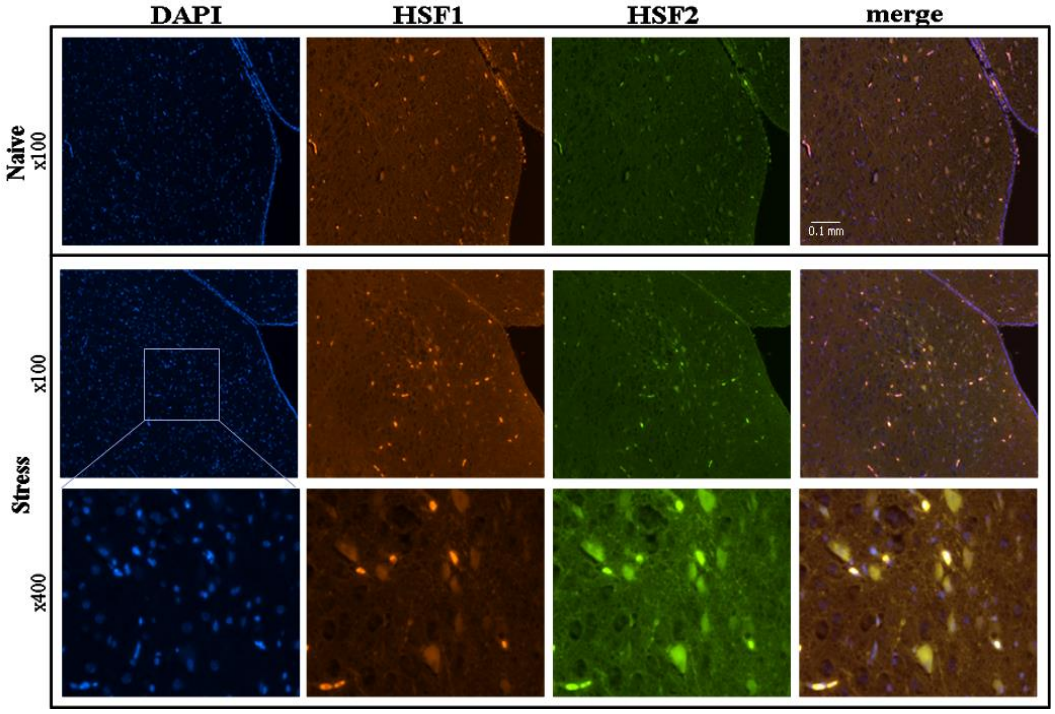


Figure 15 Colocalization of the transcription factors HSF1 & HSF2. Immunohistochemical staining in sagital brain slices of the frontal hypothalamus before the exposure to heat (upper row) and 30 min after the beginning of heat exposure (Stress lower 2 panels). The left lane is stained with DAPI to stain nuclei. In the next lane a staining with a specific polyclonal AB to HSF1 and a secondary AB stained with CY3. The 3rd lane is staind with aspecific monoclonal AB to HSF2 and the last lane is a merge of all 3 lanes.

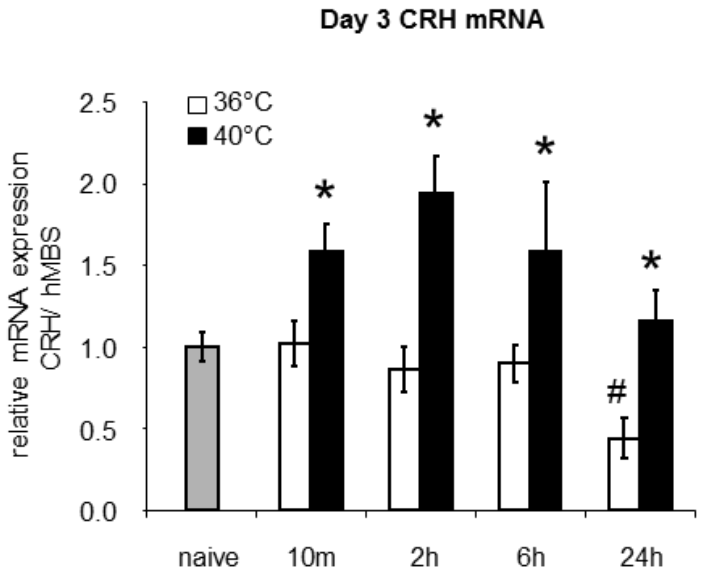


Figure 16. CRH expression is dependent on ambient temperature in 3-day-old chicks. Chicks were exposed for 24 h to moderate (36 ± 0.5°C) or extreme (40 ± 0.5°C) ambient temperatures. At various times during heat exposure, chicks were sacrificed and their PVN surgically removed CRH mRNA was evaluated using real time PCR. All presented results are average ± SEM.

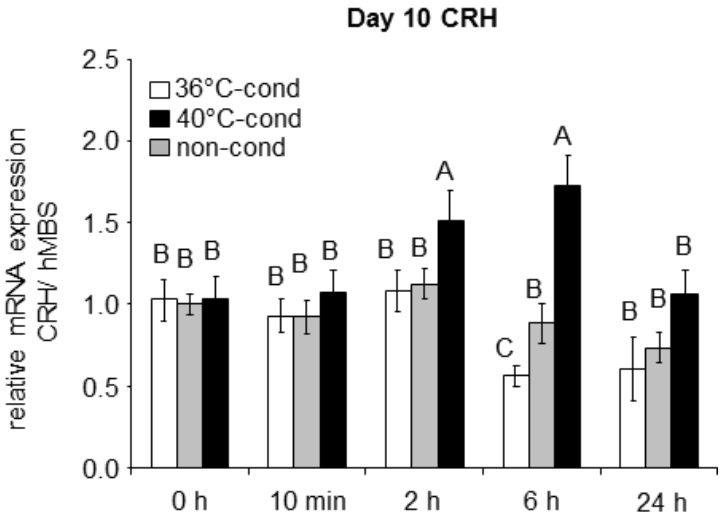


Figure 17. Exposure to different intensities of heat-stress on day 3 affects CRH expression 1 week later. Chicks that were exposed to moderate (36 ± 0.5°C; 36°C-cond) or extreme (40 ± 0.5°C; 40°C-cond) ambient temperature on day 3 posthatch were re-exposed to the moderately high ambient temperature of 36 ± 0.5°C a week later. Control chicks (non-cond) were exposed to 36°C on day 10 for the first time in their lives. CRH was evaluated using real time PCR. Presented results are average ± SEM.

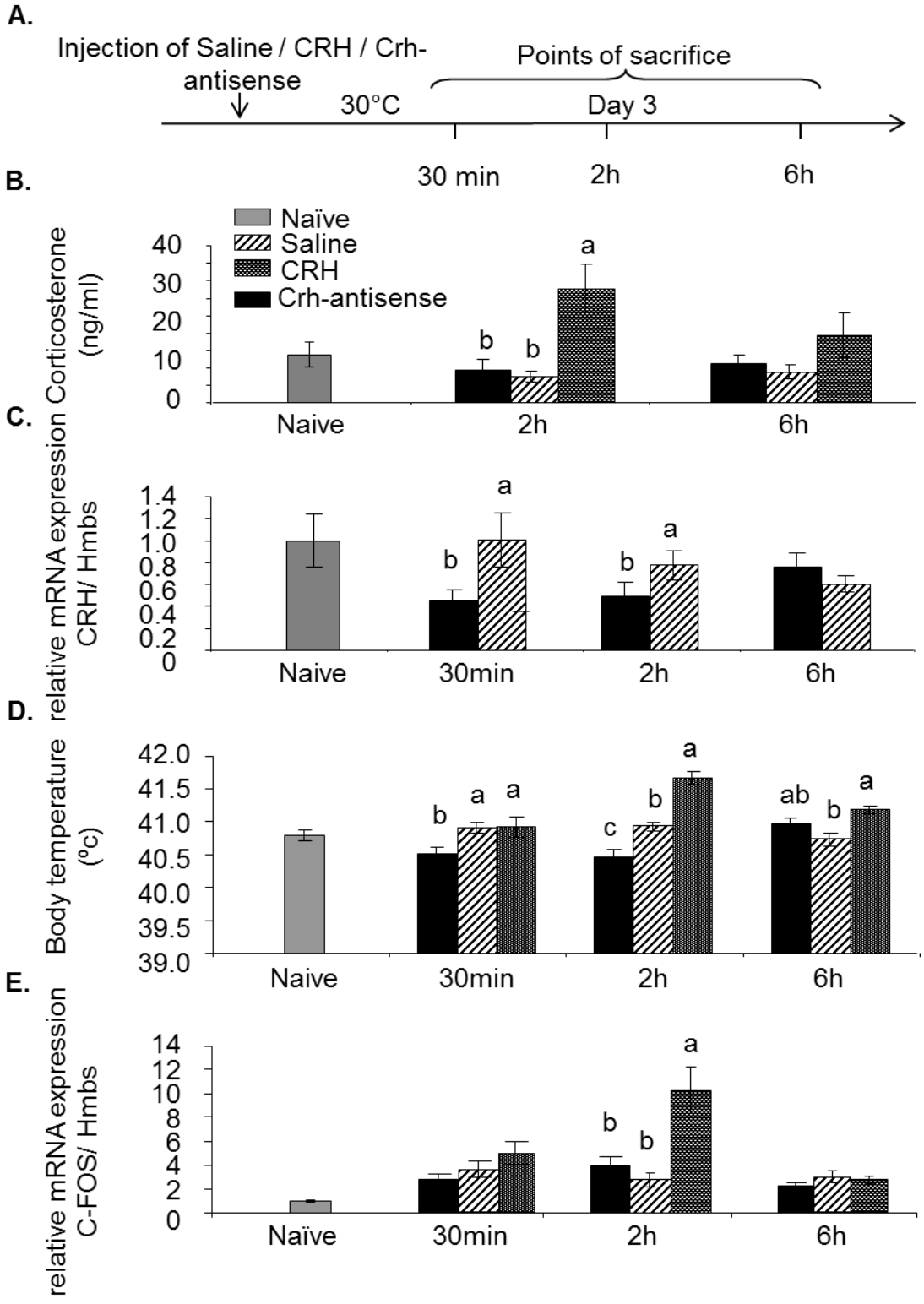


Figure 18. Intracranial injection of CRH elevates body temperature, plasma CORT, and *c-Fos* expression. (A) CRH or *Crh*-antisense or saline were intracranially injected into the third ventricle of 3-day-old chicks. Cloaca temperature was measured, blood was sampled, chicks were sacrificed and their PVN surgically removed. (B) Plasma CORT level in injected chicks was evaluated using a CORT-specific RIA kit. (C) *Crh* mRNA expression level in the PVN was evaluated using real time PCR. (D) Body temperature of chicks. (E) c-Fos mRNA expression level in the PVN was measured using real-time PCR. Different letters indicate significant differences at each time point.

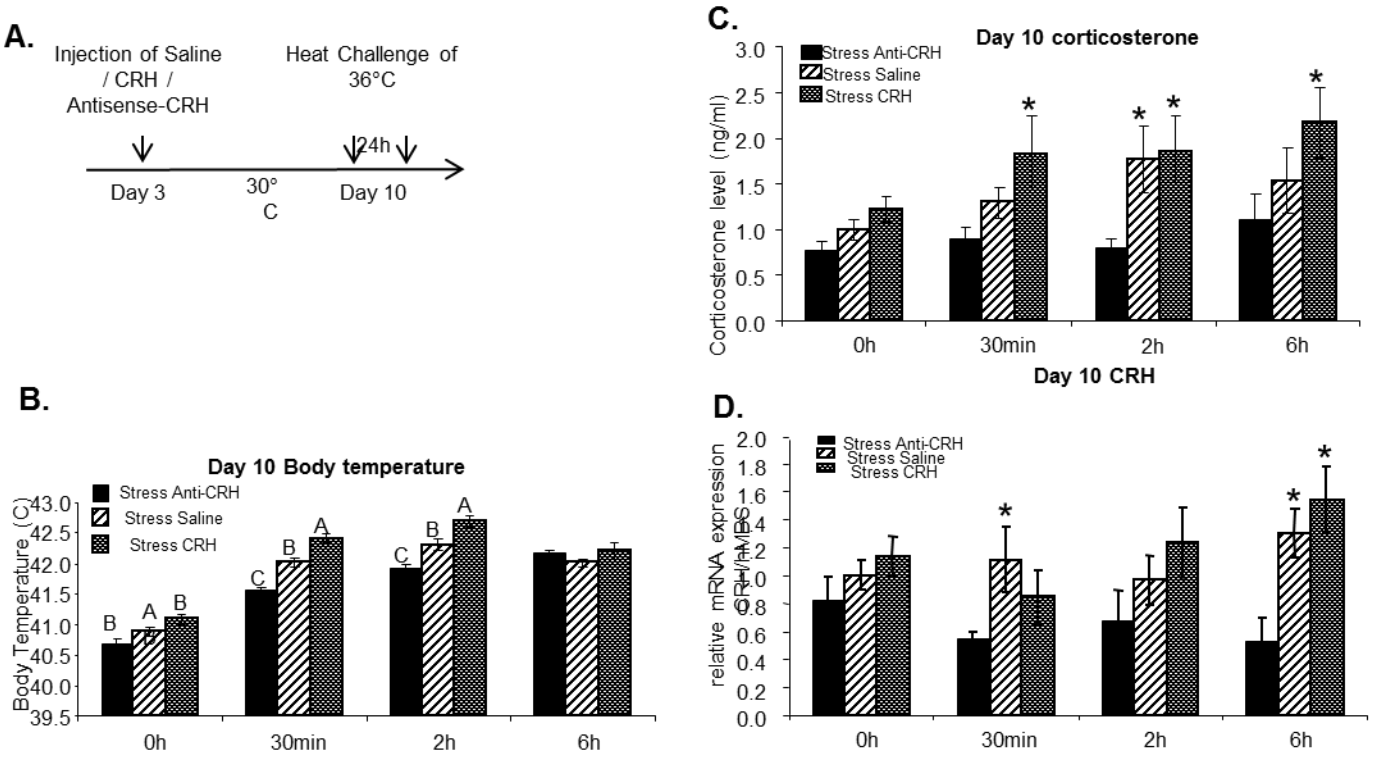


Fig. 19. Intracranial injection of CRH during the critical period of thermal-control establishment produces a long-term effect on body-temperature response, cort secretion CRH mRNA expression. (A) CRH or saline or CRH-antisense were intracranially injected into the third ventricle of 3-day-old chicks. Control chicks were injected with a similar volume of 0.9% NaCl. A week later, the chicks were heat-challenged at 36 ± 0.5°C for 6 h. The cloaca temperature was measured and blood samples were collected at 30 min, 2 and 6 h postinjection. (B) Body temperature of chicks exposed to heat challenge. (C) Plasma cort levels of chicks injected with CRH or saline was evaluated using a cort-specific RIA kit. (D) CRH mRNA expression level in the PVN was evaluated using real time PCR

* indicates significant difference between CRH-injected and saline-injected chicks at the same time point ($P < 0.05$).

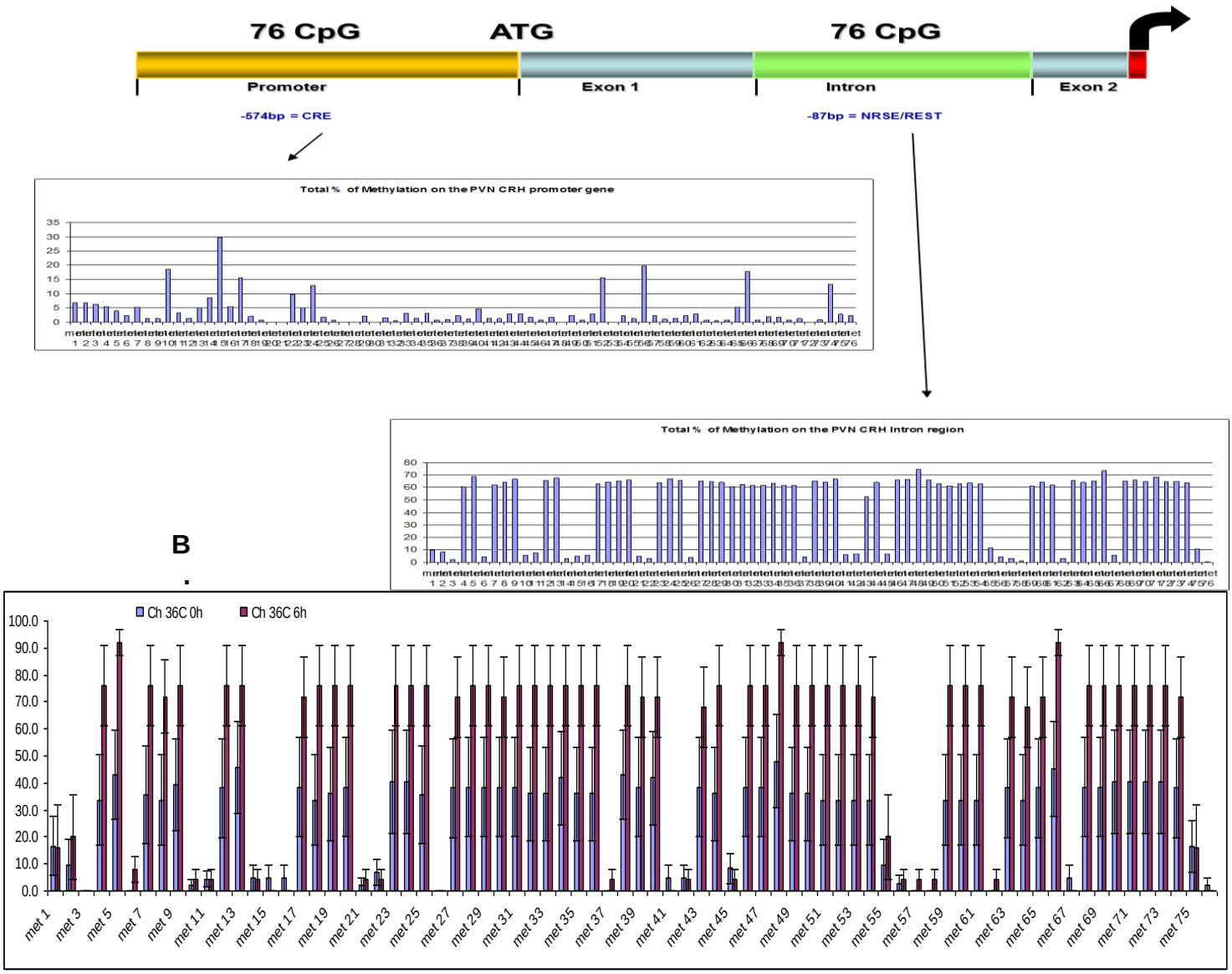


Fig. 20. (A). Illustration of the CRH gene, indicating the amount of CpG sites on the promoter and the first intron, and exhibiting the differences in methylation percentages in each region. (B). CpG methylation percentage on 10 day old previously mild heat conditioned chicks, before heat challenge (0 h) and 6 h into heat challenging. * indicatin a significance difference between 0 h and 6 h. Chi square analysis performed, ($P<0.05$ for all significant methylation sites).

<u>סיכום עם שאלות מנחות</u>

נא לענות על <u>כל</u> השאלות, <u>בקצרה ולעניין</u>, ב 3 עד 4 שורות <u>מכסימום</u> לכל שאלה (לא תובא בחשבון חריגה מגבולות המסגרת המודפסת).

שיתוף הפעולה שלך יסייע לתהליך ההערכה של תוצאות המחקר.

הערה: נא לציין הפנייה לדו"ח אם נכללו בו נקודות נוספות לאלה שבסיכום.

1. מטרות המחקר לתקופת הדו"ח תוך התייחסות לתוכנית העבודה.
מטרת המחקר הייתה לבדוק האם יש שינוי ביטויו של ההורמונים המשחררים כתוצאה מהתניות חום ובאתגור חום שבוע לאחר מכן. לבדוק האם הבקרה על ביטויים של גנים אילו היא אפיגנטית כולל בקרה של microRNA. לבדוק השפעה של עקת חום על נוירוגנזה בהיפותלמוס ובהשפעת microRNA.
2. עיקרי הניסויים והתוצאות שהושגו בתקופה אליה מתייחס הדו"ח.
הניסויים והממצאים העיקריים היו 1. חשיפת פטמים לעקות חום הביאו לשינוי בריכוזי ההורמונים המשחררים בזמן התניית החום ובזמן אתגור חום שבוע מאוחר יותר. 2. השינויים בביטוי ההורמונים המשחררים תלויים בשינויים אפיגנטיים בעיקר שינויים במטילציה על ה DNA אבל כנראה לא שינויים ב microRNA. 3. להתניית חום ואתגור ישנה השפעה על נוירוגנזה בהיפותלמוס שאושרה ע"י הזרקה של מירים למוח האפרוח – ההזרקה אפקטיבית ומשפיעה על טמפרטורת הגוף 3 ל microRNA השפעה על האפקטיביות של התניית החום.
3. המסקנות המדעיות וההשלכות לגבי יישום המחקר והמשכו. האם הושגו מטרות המחקר בתקופת הדו"ח.
המחקר התקדם היטב ולפי התוכנית. מהממצאים של המחקר ניתן ליראות שאכן ההורמונים המשחררים בהיפוטלמוס מושפעים מהתניית חום בתקופה הקריטית דרך מנגנונים אפיגנטיים. בנוסף ברור של microRNA תפקיד בבקרת העמידות לחום אם כי כנראה דרך נירוגנזה ובקרת גדילה ולא ישירות כבקרה על ביטוי ההורמונים המשחררים.
4. הבעיות שנותרו לפתרון ו/או השינויים שחלו במהלך העבודה (טכנולוגיים, שיווקיים ואחרים); התייחסות המשך המחקר לגביהן, האם יושגו מטרות המחקר בתקופה שנותרה לביצוע תוכנית המחקר.
הפרויקט התקדם לפי התוכנית והורחבו למעבר למירים שהוצאו למירים נוספים ובהמשך לגנים נוספים שקשורים למבנה הגרעין בהיפותלמוס שאחראי על ההורמונים המשחררים.
5. האם הוחל כבר בהפצת הידע שנוצר בתקופת הדו"ח - <u>יש לפרט</u>: פרסומים – כמקובל בביבליוגרפיה, פטנטים - יש לציין מס' פטנט, הרצאות וימי עיון - יש לפרט מקום ותאריך.
<u>מאמרים</u> Kisliouk, T., Cramer, T. and **Meiri, N.** (2014) heat stress attenuates new cell generation in the hypothalamus: a role for mir-138. <u>Neuroscience</u> 277: 624-663

Cramer, T., Kisliouk, T., Yeshurun, S., and **Meiri N.** (2015) The balance between stress resilience and vulnerability is regulated by corticotropin-releasing hormone during the critical postnatal period for sensory development. Developmental Neurobiology (In Press).

<u>תקצירים לכינוסים</u>

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פרסום הדו"ח: אני ממליץ לפרסם את הדו"ח: (סמן אחת מהאופציות)
➢ ללא הגבלה (בספריות ובאינטרנט)
ניתן לפרסם את הדו"ח בספריות למרות שהפרק על מיר a15 והפרק על TRH לא פורסמו