Comparative Biochemistry and Physiology, Part A 268 (2022) 111199

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Influence of temperature during incubation on the mRNA levels of temperature sensitive ion channels in the brain of broiler chicken embryos

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ARTICLE INFO

Editor: Michael Hedrick

Keywords:
Transient receptor potential (TRP) channel
Acclimatisation
qPCR
Thermal programming
Poultry

ABSTRACT

Thermosensation is crucial for the survival of any organism. In animals, changes in brain temperature are detected via sensory neurons, their cell bodies are located in the trigeminal ganglia. Transient receptor potential (**TRP**) ion channels are the largest temperature sensing family. In mammals, 11 thermoTRPs are known, as in poultry, there are only three. This research further elucidates TRP mRNA expression in the brain of broiler embryo's. Three incubation treatments were conducted on 400 eggs each: the control (**C**) at 37.6 °C; **T1** deviating from C by providing a + 1 °C heat stimuli during embryonic day (**ED**) 15–20 for 8 h a day; and **T2**, imposing a + 2 °C heat stimuli. After each heat stimuli, 12 eggs per treatment were taken for blood sampling from the chorioallantoic membrane and brain harvesting. Incubation parameters such has residual yolk (free embryonic) weight, chick quality and hatch percentage were collected. After primer optimization, 22 target genes (13 TRPs and 9 non-TRPs) were measured on mRNA of the brain using a nanofluidic biochip (Fluidigm Corporation). Four target genes (ANO2, TRPV1, SCN5A, TRAAK) have a significant treatment effect - independent of ED. Another four (TRPM8, TRPA1, TRPM2, TRPC3) have a significant treatment effect visible on one or more ED. Heat sensitive channels were increased in T2 and to a lesser degree in T1, which could be part of an acclimatisation process resulting in later life heat tolerance by increased heat sensitivity. T2, however, resulted in a lower hatch weight, quality and hatchability. No hormonal differences were detected.

1. Introduction

Thermosensation is crucial for the survival of any organism as temperature (ambient and/or body) has implications on cellular and metabolic homeostasis, and behaviour (McKemy, 2013). At a cellular level, biological macromolecules such as proteins and nucleic acids can be degraded due to extreme high and low temperatures, leading to metabolic imbalances and accumulation of byproducts such as reactive oxygen species (**ROS**) (Hayes et al., 2021). Therefore, bacteria, plants, and animals all have mechanisms in place to detect and react to environmental temperature (Tattersall et al., 2012). In animals, changes in temperature are detected via sensory neurons, having temperature sensitive ion channels embedded in their membranes (Patapoutian et al., 2003). In many cases, the molecular entities responsible are conserved across species with the largest group being the transient receptor potential (**TRP**) ion channels (McKemy, 2013). In mammals, 11 TRP channels, all members of four subfamilies: TRP canonical (**TRPC**), vanilloid (**TRPV**), melastatin (**TRPM**), ankyrin (**TRPA**), show a temperature dependence, as seen by a steep but gradual increase in channel opening when subjected to activating changes in temperature (Voets, 2012). Interestingly, the TRP family members are all polymodal channels, being activated by different physical and chemical stimuli other than temperature (García-Ávila and Islas, 2019). In the 1990's, it was discovered that capsaicin, the compound responsible for the burning sensation when eating chili peppers, operates via TRPV1 (Caterina et al., 1997). Similarly, the cold sensation of mint, containing the compound

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https://doi.org/10.1016/j.cbpa.2022.111199
Received 28 January 2022; Received in revised form 17 March 2022; Accepted 18 March 2022
Available online 23 March 2022

menthol, activates TRPM8 (Peier et al., 2002). Despite thermal activation not being an all-or-nothing event, a yardstick for quantitative comparison is the thermal threshold, or activation temperature (Voets, 2012). Heat sensing TRPs are activated if ambient temperatures exceeds the threshold, respectively, drops below the threshold activate cold sensing TRPs. In mammals, these channels are embedded in the neural membrane of sensory neurons with their cell bodies located in the trigeminal ganglia (**TG**) located in the brain, respectively cell bodies are located in the dorsal root ganglia (**DRG**) which can be found in the spine (García-Ávila and Islas, 2019). Next to the TRP family, several non-TRP ion channels are possibly thermosensitive in warmblooded animals and are well reviewed by Korogod and Demianenko (2017) and Vriens et al. (2014). In this study, the focus was placed on the following non-TRP ion channel: ANO1 and 2, abbreviated as ANO1 and ANO2 (Cho et al., 2012; Vriens et al., 2014); K^+ channels TREK1 (KCNK2),[1] TREK2 (KCNK10),[2] and TRAAK (KCNK4)[3] (Kang et al., 2005; Maingret et al., 2000); STIM1-ORAI1[4] (Xiao et al., 2011); and HCN1[5] (Nakamura et al., 2013).

Avian TRPs have been scarcely studied, especially in their link with temperature sensitivity. In vitro studies cloning chicken genes revealed TRPM8 to be cold sensitive (activated with decreasing temperature, activation temperature being 29.5 °C) (Myers et al., 2009). In the same way, both TRPA1 as TRPV1 are shown to be heat sensitive (activated with increasing temperature, activation temperature around 39.4 °C, respectively 45 °C) (Jordt and Julius, 2002; Saito and Shingai, 2006). A thorough literature search done by the authors has not revealed studies on other temperature sensitive TRPs in avian species.

Nevertheless, studying biological development in avian embryos (either chicken or duck) does have a special advantage as there are no direct influences of the mother during the gestation period, making the embryo a standalone study object. And more importantly, both chicken and ducks are thermally programmable during the embryonic phase. Improving later life heat tolerance can be done by providing heat stimuli during incubation, respectively, improving later life cold tolerance can be done by providing cold stimuli. This aspect, provides additional rationale for specifically studying the thermoregulatory system in avian embryos. In fact, several studies proved successful at this thermal programming as an improved thermal tolerance was achieved by using incubation temperatures of 39–39.5 °C, with the period in which these manipulations were performed ranging from embryonic day (**ED**) 11 to ED 20, ED 13 to ED 17, ED 16 to ED 18, ED 7 to ED 16. The duration of the manipulation ranged from 3 h/ED to 6 h/ED to 12 h/ED (Collin et al., 2005; Iqbal et al., 1990; Moraes et al., 2004; Piestun et al., 2008; Yahav et al., 2004). A study by Yalçin et al. (2008) used an amplitude of 38.5 °C, 6 h/ED, ED10–18, and found an improved heat tolerance in later life. Piestun et al. (2008) found improvements for a treatment of 39.5 °C, 12 h/ED, ED7–16. Collin et al. (2005) on the other hand, did not find those improvements using the same treatment. This study tested the effect of two similar, although not identical, treatments: T1 (38.5 °C, 8 h/ED, ED15–20) and T2 (39.5 °C, 8 h/ED, ED15–20), as we still don't fully understand the underlying mechanism of the thermoregulatory response and its control. This motivates the authors to start improving the understanding of thermosensitivity. All in all, the influence of elevated incubation temperatures and its effect on mRNA expression levels of potential thermosensitive ion channels in poultry has been scarcely investigated, leading to three objectives. Firstly, based on the literature in mammals and birds, we aim to investigate which TRPs and non-TRPs are expressed in the brain during embryonic development. Secondly, how does the expression changes with time? Thirdly, we aim to determine which of these genes are thermosensitive. Therefore, samples were taken after the daily heat challenge when the embryos were back at control temperature, adapting to the normal temperature.

2. Material and methods

2.1. Incubation treatments

Incubation Period. A total of 1400 eggs of 39-week-old Ross 308 layers were stored for 3 days in a climate controlled room at a relative humidity of 60% and 18 °C (Walk-in stability chamber, Weiss Technic, Liedekerke, Belgium). Using individual egg weights, three equivalent groups ($n = 400$) were created. Each group received a different incubation treatment (see Table 1): [1] In treatment 1, temperature was kept constant at 37.6 °C in the control group (**C**) until ED10, after which a mild decrease was conducted to provide a comfort temperature for chicks during hatching, which is an industry recommended target (incubation expert team, Petersime N.V.) Specifically, the temperature dropped to 37.3 °C at ED10 (240 h (**h**) of incubation) until ED14 (336 h of incubation), where it was decreased further to 37.1 °C until hatch. [2] In the first experimental treatment (**T1**), the temperature was increased by 1 °C to 38.6 °C from ED15 to ED20 for 8 h/day. In incubation hours, the peaks were given from 365 h – 372 h on ED15, from 389 h – 396 h on ED16, from 413 h – 420 h on ED17, from 437 h – 444 h on ED18, from 461 h – 468 h on ED19, and finally from 485 h – 492 h on ED20. In between these heat stimuli, the temperature of the incubator was allowed to drop to the control temperature of 37.6 °C. After the last peak on ED20, the temperature dropped to 37.1 °C following the profile of the control group C. [3] In the second experimental treatment (**T2**) the temperature was increased 2 °C to 39.6 °C from ED15 to ED20 for 8 h per day, and was dropped to that of the control group thereafter. The heat stimuli were given on the same incubation hours specified in T1. The temperature profile used in T1 and T2, were designed from a large literature study, in an attempt to find a treatment that improves later life heat tolerance, that has a reasonable performance decrease and has a minimal amount of treatment days / minimal duration of the heat stimuli itself. This "minimal treatment", if effective, would mean less hours of stress to the embryos and less energy costs due to the increased temperatures. Three identical incubators (SmartPro-Combi™ 41, Pas-Reform, The Netherlands) controlled by X-streamer™ (Petersime N.V., Belgium) which operated in an identical manner, were each assigned one of the three treatments,. Machine and eggshell temperature (Ovoscan™, Petersime N.V., Zulte, Belgium) were monitored closely (accuracy +/− 0.5 °C) to ensure the theoretical temperature profiles were achieved in practise.

Table 1
Theoretical temperature profiles used in the control, treatment +1 °C (T1) and treatment +2 °C (T2). If certain temperatures are not indicated, it is identical to 37.6 °C (control temperature).

Treatment	Time	Heat stimuli	Theoretical Temperature (°C)	Duration
Control (C)	ED0–21	No	37.6	24 h
Treatment +1 (T1)	ED15–20	Yes	38.6	8 h
Treatment +2 (T2)	ED15–20	Yes	39.6	8 h

[1] Abbreviation: KCNK2: Potassium two pore domain channel subfamily K member 2 (alias: TREK1)

[2] Abbreviation: KCNK10: Potassium two pore domain channel subfamily K member 10 (alias: TRAAK)

[3] Abbreviation: KCNK4: Potassium two pore domain channel subfamily K member 4 (alias: TREK2)

[4] Abbreviation: STIM1-ORAI1: Stromal interaction molecule 1 – Calcium release activated calcium modulator 1

[5] Abbreviation: HCN1: Hyperpolarization activated cyclic nucleotide gated potassium channel 1

2.2. Data collection

2.2.1. Incubation parameters

On ED 14 until ED 18, and at the biological stages of internal pipping (**IP**), external pipping (**EP**), and hatch (**H**), 12 eggs per treatment were collected for blood sampling and tissue harvesting. The biological stages IP, EP and H coincided with ED19, 20 and 21 respectively. Laboratory analysis was performed on eggs collected on ED 14, ED 15, ED 17, IP and H; all collected within 7 h of the heat treatment. A precise timeline can be found in the appendix A (A1).

2.2.2. Blood sampling

Blood was drawn from the chorioallantoic membrane using a 1 ml syringe and 27 G needle. At hatch, blood was sampled after decapitation of the chick. Lithium Heparin coated tubes (MICROVETTE 5005 L LI HEP/PK100, Sarstedt Inc., USA) were used for assembling the blood, which was centrifuged for 10 min at 2000*g*, at 6 °C for plasma collection. All samples were kept on ice and stored at −20 °C.

2.2.3. Embryo and yolk weight

After blood sampling, the eggs were opened and embryos and their yolk were carefully separated and weighed. Excess fluids were removed by absorbent paper. Relative embryo and yolk weight were calculated (Willemsen et al., 2011).

2.2.4. Brain harvesting

Embryos were decapitated immediately for whole-brain harvesting. Samples were snap-frozen in liquid nitrogen and stored at −80 °C.

2.2.5. Performance parameters

After 510 h of incubation, all chicks were feather sexed and weighed. Hatch of fertile (**HOF**) was determined as the amount of hatched chicks per fertile incubated eggs. Hatch of treatment (**HOT**) was determined as the amount of hatched chicks per incubated eggs excluding eggs that died before the treatment began (stages: infertile, early death (death <48 h), blood ring (death ED 2.5–4), and black eye (ED5–12). The quality of all day-old broilers was determined using the Tona score method, in which eight parameters are scored independently (Tona et al., 2003). The different parameters evaluated were activity, feathers and appearance, yolk sac retraction, eyes, paws, navel, remaining membranes, and remaining yolk sac. The maximum scores given per parameter are 6, 10, 12, 16, 16, 12, 12, and 16 respectively, resulting in a maximum score of 100.

2.3. Laboratory analysis

2.3.1. Plasma hormones

Corticosterone and 3,5,3′-triiodothyronine (**T3**) levels were determined by radioimmunoassay (Bio-Connect Diagnostics B.V., The Netherlands; DIAscource Immuno Assays SA., Belgium).

2.3.2. Primer design and validation

Intron spanning primers were designed using Primer-BLAST (NCBI) or gathered from previous literature. PCR products were below 327 bp and validated with agarose gel electrophoresis confirming one single product, and by the melting curves during real-time PCR. Primer efficiencies were evaluated and an overview can be found in Appendix A (A2). Primers diluted to 100 μl were suspended in DNA buffer to form a PrimerMix which was stored at −20 °C to be used in the high throughput qPCR (Fluidigm Corporation). A complete overview of used primers is shown in Appendix A (A2).

2.3.3. mRNA extraction

Tissues were crushed in liquid nitrogen and mRNA was extracted using the ReliaPrepTM mRNA Cell and Tissue Miniprep System (Promega Corporation) following the manufacturer's instructions. The mRNA quality and concentration were determined by Nanodrop (Thermofisher Scientific Nanodrop 2000).

2.3.4. Quantitative PCR

Extracted mRNA was converted to cDNA by reverse transcription of 60 ng total mRNA using a Reverse Transcription Master Mix (Fluidigm Corporation) according to the manufacturer's instructions. In short, cDNA was pre-amplified using PreAmp MasterMix and the previously pooled PrimerMix in an appropriate thermal cycle. What followed was an exonuclease step (Thermo Fischer Scientific) using the appropriate thermal cycle. A 48 × 48 dynamic array integrated fluidic circuits (**IFC**, Fluidigm Corporation) was primed and loaded with the appropriate primers and prepared pre-amplified samples, all according to the manufacturer's instructions. A total of four arrays were used on which cDNA samples were randomised per treatment and embryonic day. A general pool was created by mixing 1 μl of each sample on each embryonic day. On each array, the same pooled pre-amplified cDNA samples were used in a 3-fold dilution for primer efficiency and standard curve set-up. Non-template controls were included to check for contaminations and nonspecific amplifications. High throughput quantitative PCR was conducted using the BioMark HD Real-Time PCR system using the following scheme: 60 s at 95 °C, thirty cycles with denaturing for 5 s at 96 °C, and annealing/elongation for 20 s at 60 °C. Quantification cycles (**Cq**) were collected by Fluidigm real-time PCR analysis 4.7.1. software. Relative mRNA concentrations were calculated using the dilution curve of the pooled sample of that particular gene on that particular plate (Applied Biosystems, 2004). Average primer efficiencies of the four plates are shown in Appendix A (A2). Reference genes' expression stability according to the treatment and embryonic day was calculated using NormFinder software (Andersen et al., 2004). The combination of reference genes EF1A1,[6] YWHAZ,[7] and GAPDH[8] proved most stable over the different treatments, thus being identical regardless of the incubation profile. Its geometric mean was used for normalisation to account for volume differences. However, this geometric mean was not considered stable enough over embryonic days, as statistical analysis found this variable to have a clear age effect.

2.4. Statistical analysis

First, a general description of used statistical techniques is given, followed by a more detailed description of the performance and genetic measures.

In general, all statistical analysis and data manipulation were performed in R version 4.0.0 (R Core Team, 2019). The 'emmeans' package (Lenth, 2020) was used for post-hoc analysis when relevant using the Kenward-Roger method for degrees of freedom estimation and the Tukey adjustment for multiple comparisons. When relevant, model residuals were tested for normality using the Shapiro-Wilk test and for variance homogeneity using Levene's test. When applicable, a Box-Cox data transformation was applied to normalize data following the formula: $x = \frac{X^{\lambda}-1}{\lambda}$ if $\lambda \neq 0$ using the 'MASS' package (Venables and Ripley, 2002). All hierarchical models were reduced by backward step-wise elimination using a 5% significance level.

2.4.1. Performance parameters

Relative yolk free embryo weight (**RYFEW**) and relative yolk weight (**RYW**) were analysed by linear regression model (after reduction) including treatment, embryonic day, and egg weight (before incubation) as main effects and the interaction of treatment and embryonic day. Weight at hatch (**D0**) was analysed by linear regression model (after

[6] Abbreviation: EF1A1: Elongation factor 1 alpha 1

[7] Abbreviation: YWHAZ: Tyrosine 3-monooxygenase/Tryptophan 5-monooxygenase activation protein zeta

[8] Abbreviation: GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

reduction) including treatment, sex, and egg weight (before incubation) as main effects. Corticosterone and triiodothyronine levels were analysed by linear regression model (after reduction) including treatment, embryonic day as main effects, and the interaction of treatment and embryonic day. Tona scores were analysed for treatment effects using the non-parametric Kruskall-Wallis tests. Dunn's test was used for post-hoc comparison between levels of treatment with Benjamin-Hochberg adjustment. Finally, the number of hatched males to females per treatment was analysed using a chi-squared test.

2.4.2. Quantitative PCR

Normalised relative mRNA values were analysed for each gene separately by linear regression model including treatment, embryonic day as main effects and if applicable, the interaction of treatment and embryonic day. Even though, no conclusions can be made on the effect of embryonic age on the expression level, it still explains a significant amount of the variation and is therefore included in the model. An extra variable 'plate' was included in the regression model when either the pooled cDNA assay, its 3-fold, or its 9-fold dilution had a coefficient of variation value between plates higher than 5% calculated over all four arrays. Analysis was conducted on data between ED15 to hatch, during which the treatment took place. If the proper conditions of normality and variance homogeneity were met, ANOVA followed by multiple comparison with Tukey correction was used. If the conditions were not met, the non-parametric Kruskall-Wallis tests combined with a Dunn's test was used for post-hoc comparison between levels of treatment with Benjamin-Hochberg adjustment. An overview is provided in Table 4.

2.5. Ethics

This study was approved by the Ethical Committee for Animal Experiments of the Catholic University of Leuven (approval number P138/2019, date of approval 07/01/2020).

3. Results

3.1. Temperature

Average monitored temperatures and their standard deviation for both machine and eggshell temperatures for each incubator(Appendix A, A3). No abnormalities were detected, guaranteeing the theoretical temperature profiles in practice.

3.2. Incubation performance

The levels of RYFEW, RYW, corticosterone, and T3 levels were all significantly affected by embryonic day (data not shown) but were not affected by treatment (*p*-values and absolute values is shown in Tables 2 and 3). Hatch of fertile for C, T1, and T2 was 93%, 92%, and 92%, respectively ($\chi^2 = 0.007$). Seven infertile eggs were found for C, one for T1, and five for T2. Hatch of treatment were 93%, 95%, and 94% ($\chi^2 = 0.021$, $P > 0.05$). Additionally, no significant differences were found between the number of hatched males (**M**) versus females (**F**) (C: 97 M/103F, T1: 98 M/93F, T2: 106 M/93F) between the treatments ($\chi^2 = 0.59$; $P > 0.05$).

3.3. Hatch

The total Tona scores for C, T1, and T2 were 97, 97, and 95, respectively, were significantly affected by treatment, due to the strong effect of treatment on the subcategories residual yolk sac and membrane, navel, and appearance (see Table *2*2). No difference exists between C and T1, but both are significantly higher than T2. Additionally, hatch weight was affected by treatment with C > T1 > T2 and by sex with males having a higher weight than females.

Table 2
Overview of incubation parameters (Residual yolk and yolk free embryo weight (g); hormonal concentrations (ng/ml) during development (corticosterone and triiodothyronine) and hatch performance (Hatch weight (g), hatch percentage (%) and Tona score). When treatment is significant, the measured differences resulting from the multiple comparisons are shown. When embryonic day or sex are significant, the measured differences are not shown. Non-significant output is marked by 'n.s.'. Significant output is marked by '***'; '**';'*' if the *p*-value is smaller than 0.001, 0.01, or 0.05 respectively. Number of ED analysed consists of ED15, ED17, internal pipping and hatching. Number of samples analysed per treatment on a specific day is 12.

	ED	Treatment	Treatment x ED
Residual Yolk free embryo weight (g)	***	n.s.	/
Residual Yolk weight	***	n.s.	/
Corticosterone	***	n.s.	/
Triiodothyronine ((T3) (ng/ml)	**	n.s.	/

	Sex	Treatment	Sex	Treatment
Hatch weight	n.s.	***	*	C > T1 > T2
Tona Score (Score out of 100)	n.s.	***		Total Tona score and subcategories: C = T1 > T2
a) Residual Yolk Sac	n.s.	*		
b) Residual Membrane	n.s.	**		
c) Navel	n.s.	**		
d) Down and appearance	n.s.	**		

Hatch percentage	Treatment
a) Hatch of Fertile	n.s.
b) Hatch of Treatment	n.s.

Table 3
Overview of incubation parameters (Residual yolk free embryo weight (g); Residual yolk weight (g); hormonal concentrations (ng/ml) during development (corticosterone and triiodothyronine)) and hatch performance (Hatch weight (g)) between treamtents. Parametric analysis (for RYFEW and RYW) is shown by the mean over the different embryronic days ± sem, non-parametric analysis (Corticosterone, Triiodothyronine, Hatch weight) by the median and the percentile confidence intervals (calculated for the group means by bootstrap). Number of ED analysed consists of ED15, ED17, internal pipping and hatching. Number of samples analysed per treatment on a specific day is 12. No data is shown for the age effect.

Parameter	C	T1	T2
RYEW (g)	46.1 ± 1.2	46.8 ± 1.4	45.9 ± 3.0
RYW (g)	21.6 ± 0.6	22.1 ± 1.2	22.0 ± 1.2
Tona score	97 ± 0.4	97 ± 0.3	95 ± 0.6
Corticosterone (ng/ml)	14.3, [12.8–16.5]	14.1, [10.3–16.2]	13.5, [11.5–15.3]
Triiodothyronine (ng/ml)	2.1, [0.9–2.5]	1.8, [1.5–2.0]	1.4, [0.3–1.8]
Hatch Weight Male (g)	46.0, [44.3–46.6]	44.6, [43.3–45.2]	42.3, [41.4–43.4]
Hatch Weight Female (g)	44.2, [43.3–45.6]	43.5, [42.7–44.2]	41.5, [40.4–43.4]

3.4. Quantitative PCR

Despite all targeted genes being measurable at ED15, two out of 22 genes (TRPM5 and TRPV4) did not generate a successful standard curve excluding these from further analysis. As indicated in Table 4, twelve target genes were affected by treatment either as a main effect or as an interaction effect of treatment with the embryonic day. From this, eight target genes remained affected after multiple comparisons between treatments. Four target genes (ANO2 ($p < 0.001$), TRPV1 ($p < 0.05$), SCN5A[9] ($p < 0.001$), and TRAAK ($p < 0.001$)) have a significant

[9] Abbreviation: SCN5A: Sodium channel voltage-gated, Type V, Alpha subunit (alias Nav1.5)

Table 4
Statistical output of successful target genes. Used statistical techniques are marked by parametric (ANOVA + Tukey correction for multiple comparisons) or non-parametric (Kruskall-Wallis + Dunn's test with Benjamni Hochberg correction for multiple comparisons). If data was transformed by Box-Cox transformation, the used lambda factor is shown. Models not including a certain effect are marked by a "/". Non-significant output is marked by 'n.s.'. Significant output is marked by '***'; '**';'*' if the p-value is smaller than 0.001, 0.01, or 0.05 respectively. Number of ED analysed consists of ED15, ED17, internal pipping and hatching. Number of samples analysed per treatment on a specific ED is 12.

Gene	Statistical Technique	Lambda transformation factor	ED	Treatment	Treatment x ED	Plate
ANO1	non-parametric	/	/	/	***	/
ANO2	parametric	/	***	***	n.s.	/
BDNF	non-parametric	/	***	n.s.	/	/
HCN1	non-parametric	/	***	n.s.	/	Yes
SCN5A	parametric	/	***	***	n.s.	/
STIM1	non-parametric	/	***	n.s.	/	/
TRAAK	parametric	-0,1,010,101	***	**	n.s.	/
TREK1	non-parametric	/	n.s.	**	/	/
TREK2	non-parametric	/	n.s.	***	/	/
TRPA1	parametric	/	/	/	***	Yes
TRPC1	parametric	/	***	n.s.	n.s.	Yes
TRPC3	non-parametric	/	/	/	*	Yes
TRPC5	non-parametric	/	**	n.s.	/	Yes
TRPM2	non-parametric	/			*	/
TRPM3	parametric	0.4646465	***	n.s.	n.s.	/
TRPM8	non-parametric	/	/	/	**	Yes
TRPV1	parametric	0,4,242,424	***	*	n.s.	Yes
TRPV2	non-parametric	/	***	n.s.	/	Yes
TRPV3	non-parametric	/	***	n.s.	/	Yes
TRPV6	non-parametric	/	***	n.s.	***	Yes

treatment effect - independent of ED. The fold expressions per treatment are shown in Fig. 1. Another four target genes (TRPM8 ($p < 0.01$), TRPA1 ($p < 0.001$), TRPM2 ($p < 0.05$), TRPC3 ($p < 0.05$)) have a significant treatment effect visible on one or more embryonic days. The fold expressions per treatment are shown in Fig. 2. Both TRPM8 and TRPA1 have a treatment effect on ED15, with no differences between T1 and T2, but both being higher than C. All four showed an effect at IP with T2 significantly higher than C. For TRPA1 and TRPM2 there is a significant higher mRNA level for T2 compared to T1. For TRPM8 and TRPC3, no difference between T1 and T2 is seen.

4. Discussion

The present study examined the effect of elevated incubation temperatures, either +1 °C or + 2 °C (T1 and T2), on the mRNA level of 22 target genes in the brain of broiler embryos from ED15 until hatch. Eight target genes ANO2, SCN5A, TRPV1, TRAAK, TRPM8, TRPA1, TRPC3, TRPM2 were affected by treatment either as main effect, meaning that it is independent of the embryonic day (Fig. 1: Fold expressions for target genes significantly influenced by treatment as main effect (ANO2: Anoctamin 2; TRPV1: Transient Receptor Potential subfamily V member 1; SCN5A: Sodium Voltage-Gated Channel Alpha Subunit 5; TRAAK: TWIK-Related Arachidonic Acid-Stimulated Potassium Channel). Differences between treatments ($n = 12$) are indicated by lower case letters which differ if a significant difference is present.Fig. 1), or the effect occurred within one or more embryonic day(s) (See Fig. 2.). As all the tested genes were selected from literature due to their thermoregulatory potential, our results may indicate that these eight genes play a functional role in the thermoregulatory development of broilers and the acclimatisation effect induced by the thermal programming.

4.1. Treatment effects on fold expressions of target genes

Treatment effects of ANO2, SCN5A, TRPV1, and TRAAK were independent of the embryonic day. As the effect of treatment could potentially be confounded with the effect of the incubator itself, the differences on ED 14, before temperature treatment started, were checked (Appendix A (A4)). ANO2 and SCN5A did not have a significant difference between incubators on ED14, reinforcing the detected temperature effects. TRPV1 and TRAAK did not show any difference between C and T2 (no T1 data available) on ED14. What follows is a short discussion of each of these genes.

4.1.1. ANO2

Anoctamin 2 (ANO2) seems to be a poorly studied ion channel in both mammals and avian species, as no studies were evident in the literature. ANO1 on the other hand is far more studied in mammals, especially in studies regarding temperature sensitivity. Research by Cho et al. (2012), has shown via an in vitro study (cloning mouse ANO1) that ANO1 is a noxious heat sensor with Cl^- currents increasing strongly with temperatures above 44 °C. ANO2 was activated by similar temperatures. As a tenfold lower transcription level was measured for ANO2 under the same temperature change, it was concluded that ANO1 is largely responsible for this noxious heat sensitivity. In our study, the lack of a treatment effect on ANO1, opposite to the main effect found for ANO2 seems to suggest ANO2 to be a relevant factor of thermal programming in avian species, and not ANO1.

4.1.2. SCN5A

The temperature sensitivity of voltage-gated sodium channels such as SCN5A in mammals is still under debate with several studies not finding any effect of temperature while others finding strong effects (Egri and Ruben, 2012). For poultry, SCN5A was found to be differentially expressed in the breast tissue between control broiler embryos, incubated at 37.8 °C and heat incubated embryos (39.5 °C, 12 h, ED7–16) (Loyau et al., 2016). Our results match these findings of increased expression for T2 (39.6 °C, 8 h, ED15–20) compared to C (37.6 °C) in the brain.

4.1.3. TRPV1

Research by Julius et al. identified TRPV1 as a heat sensitive ion channel in mammals (Caterina et al., 1997). A molecular understanding was gained by an in vitro study of cloned chicken TRPV1 (cTRPV1) showing the TRPV1 channel to open when temperature exceeds 45 °C, measuring noxious heat (Jordt and Julius, 2002). In our study, mRNA levels were increased in T2 compared to T1, not C. It seems that T2, the profile with the highest temperature peaks, stimulates the amplification of more noxious heat sensing channels, possibly as part of an acclimatisation process induced by the thermal programming.

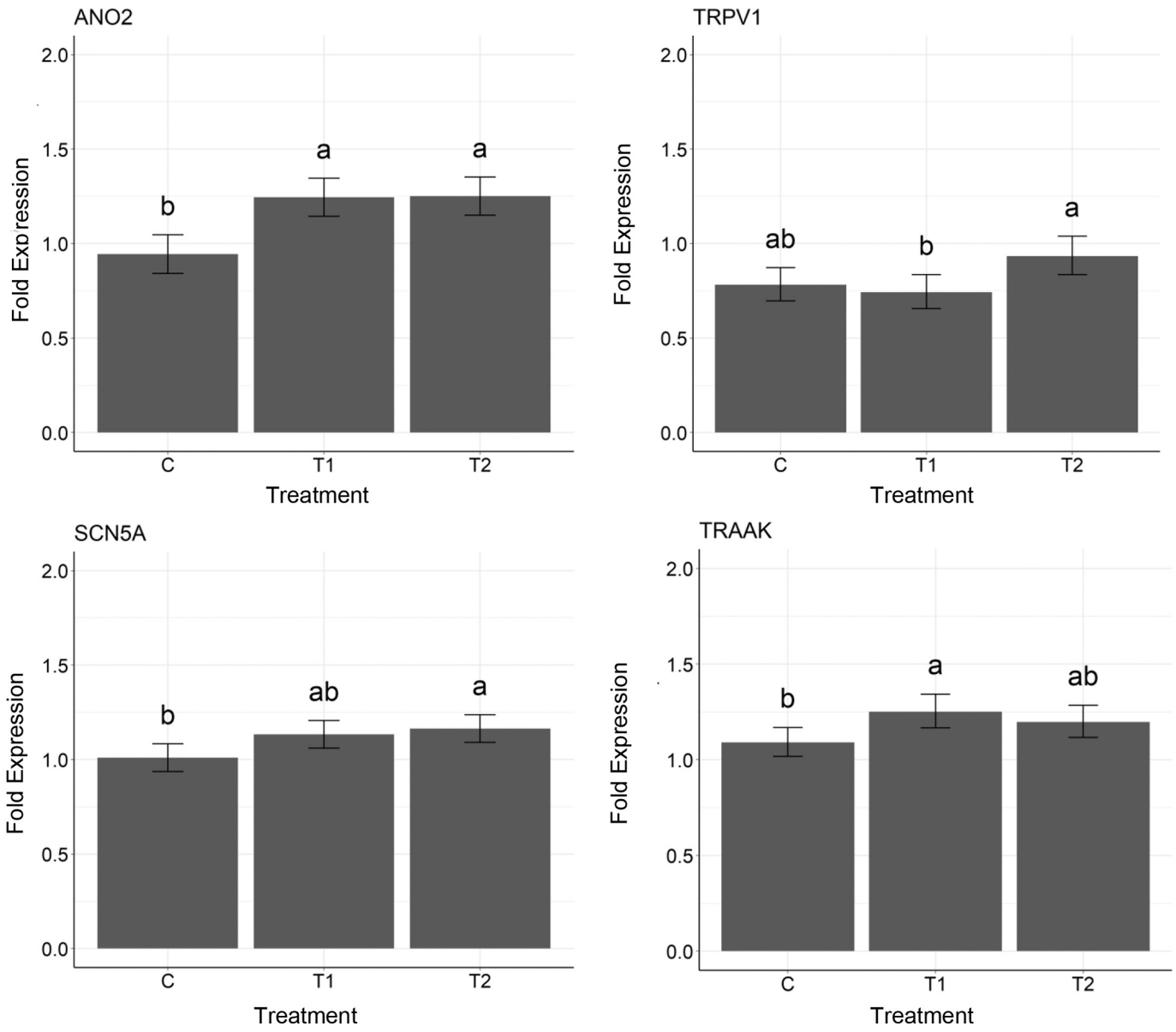

Fig. 1. Fold expressions for target genes significantly influenced by treatment as main effect (ANO2: Anoctamin 2; TRPV1: Transient Receptor Potential subfamily V member 1; SCN5A: Sodium Voltage-Gated Channel Alpha Subunit 5; TRAAK: TWIK-Related Arachidonic Acid-Stimulated Potassium Channel). Differences between treatments (n = 12) are indicated by lower case letters which differ if a significant difference is present.

4.1.4. TRAAK

The TWIK-related potassium channels consist of three members: TREK1, TREK2, and TRAAK. All three members are shown to be active near warm temperatures. Kang et al. (2005) showed this in an in vitro study in which the genes were cloned genes from the rat's genetic database, transfected on a DRG neurone culture. All channels exhibited a low activity at room temperature (24 °C), but are all active at 37 °C (Kang et al., 2005). Next to DRG neurons, all three are shown to be expressed in rat trigeminal ganglion cells where they are co-located with TRPV1, 2, and TRPM8 (Yamamoto et al., 2009). For avian species, no research could be found on this ion channel. The lack of a treatment effect for TREK1 and TREK2 and the main treatment effect for TRAAK from ED15 until hatch, may be marking the importance of this ion channel in thermoregulation in avian species.

Four target genes TRPM8, TRPA1, TRPC3, TRPM2 were affected by the interaction of treatment and embryonic day, meaning the treatment effect is only visible on certain embryonic day(s). Again, no incubator effects were found on ED14 (Appendix A (A4)). What follows is a short discussion of each of these genes.

4.1.5. TRPM8

TRPM8 was the second TRP in history to be designated as a thermoTRP activated by cold (Peier et al., 2002). It is found in a wide range of metazoans and is shown to be under strong evolutionary pressure. Its activation range depends on the body temperature of the animal. The TRPM8 activation temperature for chickens is around 30 °C (meaning the ion channel is opened if the temperature is below this threshold), 24 °C for rats, and 14 °C for frogs; consistent with the elevated body temperature of chickens over rats and frogs (Myers et al., 2009). Despite TRPM8 being a known cold sensor, chicken's cold sensitivity seems to be unique. Researched showed chicken DRG neurons to still be cold sensitive, even though they were menthol-insensitive, thus excluding TRPM8 as a candidate for this response. Other potential cold sensing

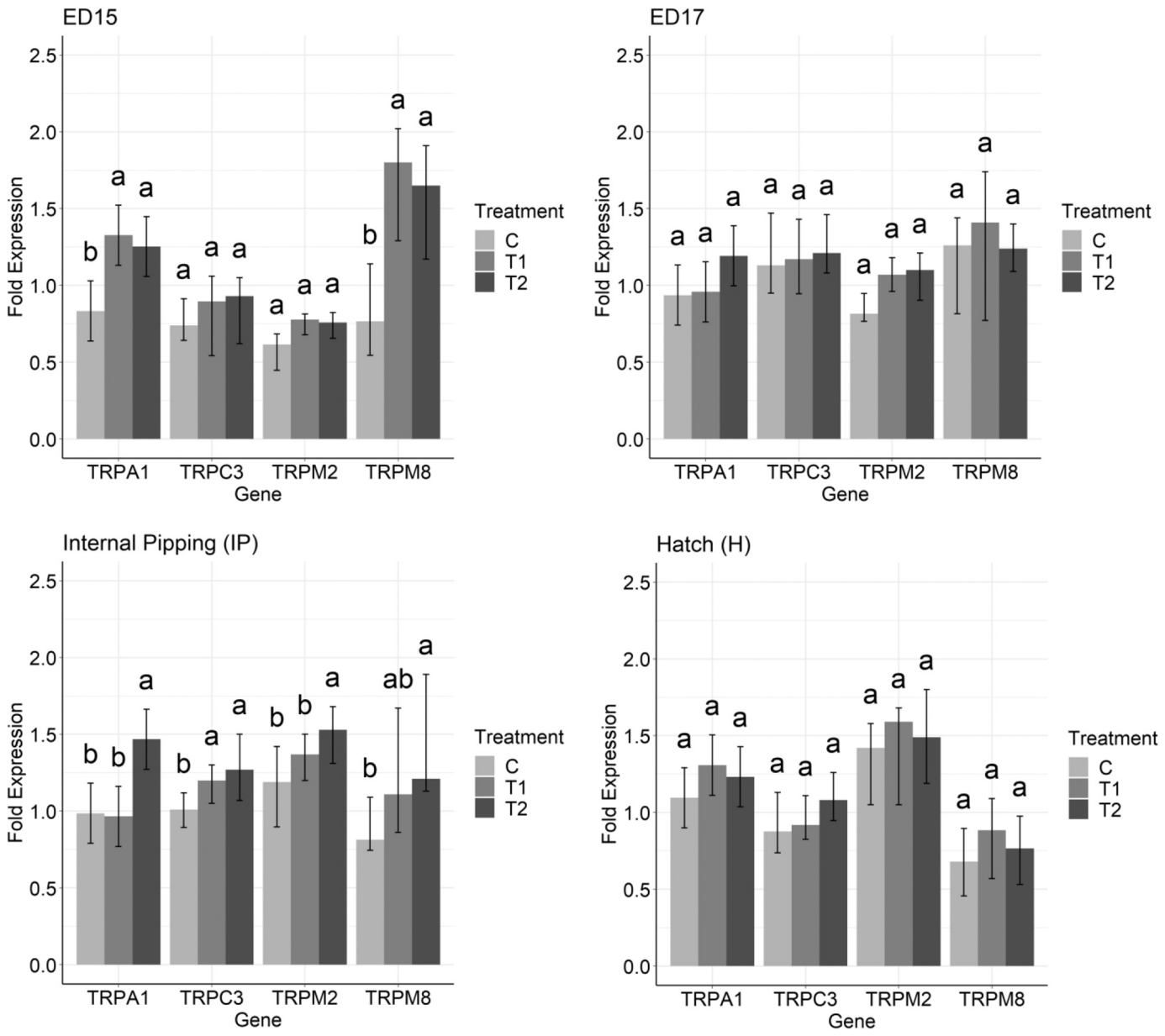

Fig. 2. Fold expressions for target genes significantly influenced by an interaction effect between treatment and age (TRPM8: Transient Receptor Potential subfamily M member 8; TRPA1: Transient Receptor Potential subfamily A member 1; TRPM2: Transient Receptor Potential subfamily M member 2; TRPC3: Transient Receptor Potential subfamily C member 3). Differences between treatments ($n = 12$) on a specific embryonic day are indicated by the lower case letters which differ if a significant difference is present.

candidates such as TRPC5, K2P[10] channels, or voltage-gated sodium channels were also proven not to be responsible, leaving this cold sensing channel a mystery (Yamamoto et al., 2016). The upregulation on ED15 for both T1 and T2 compared to C could be seen as an immediate, but unprecise or uncoordinated reaction to the treatment. A similar effect was seen in TRPA1, further elaboration on this theory is provided below.

4.1.6. TRPA1

Chicken TRPA1, being a heat sensor, is activated by temperatures exceeding 41–42 °C as shown by an in vitro study (Saito et al., 2014). In mice, TRPA1 has been reported as a cold sensitive neuron in both TG and DRG (Yamamoto et al., 2016). In this study, a significant elevation for T2 -compared to C- on ED15 and IP, numerically on ED17, seems to follow the same patterns as seen in ANO2 and SCN5A, which are also heat sensors, suggesting a potential acclimatisation process induced by T2. For T1, an on-off elevation is seen on ED15 -increased value compared to C-, never reoccurring in following embryonic days. It seems like the effect of T1 does not seem to be intense enough to create a longterm change as in T2.

4.1.7. ED15

On ED15, both TRPM8 (cold sensing) and TRPA1 (heat sensing) showed increased mRNA values. Considering their opposite functioning, heat versus cold sensing (provided that TRPM8 is not heat sensing in poultry), but both of them are reacting to the same heat stimulus, could be described as an immediate, but inappropriate, unprecise or uncoordinated response. Research by Nichelmann and Tzschentke, 2003, showed a similar reaction in the blood flow in the chorioallantoic

[10] Abbreviation: K2P: Tandem pore domain potassium channels

membrane of chicken. In that study, embryos were subjected to a daily heat stimulus. The first day, both vasoconstriction as vasodilation occurred. The response is thus immediate, but not appropriate, as one would only expect a dilatation. After repeating the heat stimulus the following days, similar to our experiments, blood flow adapted in all individuals, showing vasodilation as a response to the heat stimulus at hatch. This phenomenon was therefore described as going from an immediate, but uncoordinated (unprecise) reaction, to a coordinated, precise reactions (Nichelmann and Tzschentke, 2003). Our study showed both TRPA1 and TRPM8 to react on ED15 (immediate but unprecise), but only TRPA1 and other heat sensing channels kept their increased expression level until IP and/or hatch. Another example can be seen in the respiration rate of chicken embryos, showing general respiration responses before IP, but clear panting reactions after IP as a reaction to increased incubation temperatures (Nichelmann and Tzschentke, 2002).

4.1.8. TRPC3 and TRPM2

Both TRPC3 and TRPM2 were affected by the temperature treatment at the stage of internal pipping, but none of these have been investigated with regards to thermosensitivity in poultry. Research on bovine aortic endothelial cells showed TRPC3 to be activated by oxidative stress and function as a ROS-sensitive channel (Yao and Garland, 2005). Similarly, TRPM2 can be activated by H_2O_2 leading to cell death in rat cortical neural cells (Takahashi et al., 2011). Furthermore, TRPC3 is upregulated under acute heat stress conditions in oysters' adductor muscle, mantle and gill where it plays a functional role in energy regulation (Fu et al., 2021). These findings could imply that both T1 and T2 induced heat stress at the IP stage, with the potential creation of reactive oxidative species activating these channels. Alternatively, TRPC3 can be activated by BDNF[11] (Li et al., 1999), however, because of the steady BNDF expression between treatments, this activation does not seem plausible in our study.

4.1.9. Treatment effect at IP for TRPM8, TRPC3 and TRPM2

The reason for sampling at IP, is to enable a comparison at a same biological age, i.e. equal developmental stage. It should be kept in mind that, as the temperature influenced the time of hatch, first mainly samples were taken from C, followed by T1 and then T2. Therefore, effects found at IP can also be due to the chronological time effect (e.g. C samples collected mostly earlier on ED 19 than T2), although no treatment effects at IP, EP or hatch (biological ages) were found for RYFEW nor RYW.

4.2. Acclimatisation

Acclimatisation is a process induced by the environment, consisting of phenotypic, non-genetic changes, typically induced by sudden environmental changes such as the heat imposed during the incubation phase. The process takes several days to weeks and consist out of two stages; acute or short term and chronic or long term. The chronic phase is driven by continued exposure, in this study by repeating the heat stimulus multiple days in a row. As a result, there are altered receptor populations which change tissue sensitivity resulting in a new physiological state (Collier et al., 2019). The same principles seem applicable in this research. The first day of treatment, on ED15, an acute (immediate) uncoordinated (unprecise) responses is seen as both heat sensing and cold sensing TRPs expression levels were increased both for T1 as T2 compared to C. This is followed by a chronic phase where heat sensing TRPs keep increased mRNA levels until hatch. This chronic phase, however, is more visible in T2 compared to T1, as three heat sensing channels (ANO2, SCN5A and TRPA1) showed longterm increased expression whereas for T1 only 2 heat sensing channels showed such pattern (ANO2 and TRAAK). These longterm changed levels of TRPs could be changing the neuronal sensitive in the brain resulting in an acclimatized animal. In this study, the acquisition of heat tolerance is not measured, however, similar (but not identical) treatments have been shown successful in previous studies. Our findings indicate an acclimatisation caused by T2, by increasing the level of heat sensitive ion channels. An epigenetic mechanism seems to be behind it (Nichelmann and Tzschentke, 2002).

4.3. Target genes not affected by treatment

Remarkably, from the 12 channels not affected by temperature several are shown to be highly thermosensitive in mammals (Cho et al., 2012; Vriens et al., 2011). Differences between thermosensitive channels between mammals and poultry have been discovered before: TRPA1, warm sensitive in broilers but cold sensitive in mammals (Saito et al., 2014) or the loss of TRPM4 in poultry (Saito and Shingai, 2006). Besides species, another explanation could be originating from the studied tissue: most research conducted focused on expression levels at the level of DRG neurons located in the spinal column, not in the brain. Even ganglion specific regulation of TRP channel expression levels between DRG and TG neurons has been shown at the level of mRNA (Vandewauw et al., 2013).

4.3.1. Unknown effects for TRPM5 and TRPV4

Two target genes, TRPM5 and TRPV4, failed to produce a standard curve due to air bubble blockage in the microfluidic chip. No normalised relative mRNA values could be calculated and so no further results are shown. However, as all primers were validated in a pilot study, and as the reactions were successful in the target samples, this is the first study to confirm the existence of TRPM5 and TRPV4 in the brain of broiler embryos, expressed from ED14 onwards. TRPM5 has been measured before in the gastrointestinal tract of chicken, where it functions as a downstream signalling protein of taste receptor genes (Cheled-Shoval et al., 2015). TRPV4 is expressed in the mitochondrial region in avian spermatozoa where it acts as an important factor determining sperm motility (Majhi et al., 2020).

4.4. Performance

Although no treatment effect on RYFEW and RYW was observed during embryonic development, a lower hatch weight of T2 chicks compared to T1, in turn, lower than C was shown. Additionally, lowered hatchability (both HOF and HOT), and quality (mainly around the navel) suggest an impaired development for T2. These results are in line with Piestun et al. (2008), who tested a similar T2 treatment. The quality decrease could have an important practical implication as this could lead to longterm growth impairment caused by increased infection risks.

5. Conclusion

Thermosensation is crucial for any organism's survival. Transient receptor potential channels have been widely researched in mammals as they perform several functions like heat and/or cold sensing, embedded in the membranes of primary neurons. Surprisingly, research in avian species is very limited regardless of the well proven use of avian embryonic models in history for the study of several biological systems. Additionally, avian embryos can be thermally manipulated during incubation by subjecting the eggs to certain fluctuating heat elevated temperatures resulting in more heat tolerance at later life. A phenomenon assumed to be caused by epigenetic adaptations in the brain. We investigated the effect of two different fluctuating heat elevated treatments (T1 and T2) on mRNA levels of TRP and non-TRP ion channels in the brain. This study either confirmed or demonstrated for the first time, the existence of 22 target genes in the brain of broilers between ED14 until hatch. All 22 target genes showed expression at ED14, making

[11] Abbreviation: BDNF: Brain-derived neurotrophic factor

thermal programs at ED14 or later justifiable as temperature inputs could thus be detected by the embryos. Eight target genes were significantly different between the temperature treatments, four of them having a consistently elevated fold expression of T2 above C, similarly two for T1 above C. In the end, four genes (ANO2, SCN5A, TRPA1, and TRAAK) seem to play a functional role in the acclimatisation phenomenon, induced by the thermal programming.

CRediT authorship contribution statement

Sara Maria Daniel Verlinden: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Tomas Norton:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Mona Lilian Vestbjerg Larsen:** Formal analysis, Investigation, Supervision, Writing – review & editing. **Martine Schroyen:** Data curation, Formal analysis, Investigation, Methodology. **Ali Youssef:** Conceptualization, Supervision, Writing – review & editing. **Nadia Everaert:** Investigation, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was supported VLAIO (Vlaams agentschap innoveren en ondernemen, number HBC.2017.1001). This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

We gratefully acknowledge the infrastructure used by the group of Physiology, led by prof. J. Buyse at the department of Biosystems, KU Leuven for the use of incubators. We also thank Petersime N.V. for supplying the eggs used in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2022.111199.

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