Cutaneous TRPV4 Channels Activate Warmth-Defense Responses in Young and Adult Birds

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Transient receptor potential vanilloid 4 (TRPV4) channels are sensitive to warm ambient temperatures (Tₐ), triggering heat loss responses in adult rats in a Tₐ range of ~26–30°C. In birds, however, the thermoregulatory role of TRPV4 has never been shown. Here, we hypothesized that stimulation of TRPV4 induces thermolytic responses for body temperature (Tₜₜ) maintenance in birds, and that this function is already present in early life, when the Tₐ range for TRPV4 activation does not represent a warm condition for these animals. We first demonstrated the presence of TRPV4 in the dorsal and ventral skin of chickens (Gallus gallus domesticus) by immunohistochemistry. Then, we evaluated the effects of the TRPV4 agonist, RN1747, and the TRPV4 antagonists, HC067047 and GSK2193874, on Tₜₜ and thermoeffectors at different Tₐs in 5-day-old chicks and 60-day-old adult chickens. For the chicks, RN1747 transiently reduced Tₜₜ both in thermoneutrality (31°C) and in a cold Tₐ for this phase (26°C), which relied on huddling behavior inhibition. The TRPV4 antagonists alone did not affect Tₜₜ or thermoeffectors but blocked the Tₜₜ decrease and huddling inhibition promoted by RN1747. For the adults, TRPV4 antagonism increased Tₜₜ when animals were exposed to 28°C (suprathermoneutral condition for adults), but not to 19°C. In contrast, RN1747 decreased Tₜₜ by reducing metabolic rate and activating thermal tachypnea at 19°C, a Tₐ below the activation range of TRPV4. Our results indicate that peripheral TRPV4 receptors are functional in early life, but may be inhibited at that time when the range of activation (~26–30°C) represents cold Tₐ for chicks, and become physiologically relevant for Tₜₜ maintenance when the activation Tₐ range for TRPV4 becomes suprathermoneutral for adult chickens.

Keywords: peripheral thermoreceptor, chicken, thermoregulation, Metabolism, body temperature, thermolysis, huddling.
1 INTRODUCTION

The maintenance of body temperature (Tb) in an endotherm exposed to ambient temperature (Ta) variation depends on the activity of neural circuits, which detect Ta in the peripheral thermoreceptors, integrate the thermal information in the brain, and recruit appropriate thermoeffectors for heat gain or heat loss (Bicego et al., 2007; Morrison, 2016). The thermosensitive transient receptor potential channels, called thermo-TRPs, emerged two decades ago as putative molecules involved in thermosensitivity (Patapoutian et al., 2003; Wang and Siemens, 2015). They are located on the cell membrane of a variety of cell types, such as neurons and epithelial cells, and have six transmembrane domains. In vitro studies of thermo-TRPs in mammals have shown that TRPV1, TRPV2, and TRPM3 are activated by noxious heat, TRPA1 is activated by noxious cold, TRPM8 and TRPC5 are activated by innocuous cold, and TRPM2, TRPM4, TRPM5, TRPV3, and TRPV4 are activated by moderate heat (Guler et al., 2002; Clapham, 2003; Patapoutian et al., 2003). Functional evidence (in vivo) of a thermoregulatory role for thermo-TRPs, however, is available for only a few channels, including TRPM8, TRPV3 and TRPV4, in rodents (Peier et al., 2002; Almeida et al., 2012; Vizin et al., 2015; Wang and Siemens, 2015; Scarpellini et al., 2019). In vitro, TRPV4 channels are specifically stimulated at temperatures of 27–34°C (Guler et al., 2002; Watanabe et al., 2002; Kauer and Gibson, 2009). At least in rats they are expressed peripherally in the keratinocytes (Guler et al., 2002). In vivo, we have shown that peripheral TRPV4 receptors activate warmth-defense responses when adult rats are exposed to a range of T a between ~26 and 30°C (Vizin et al., 2015). Besides that, hypothalamic TRPV4 channels, which seems to be modulated by neurochemical signaling instead of changes in temperature, are also important for thermoregulatory responses to warm conditions in adult rats (Vizin et al., 2015; Scarpellini et al., 2019).

TRP channels are well preserved throughout evolution, and a phylogenetic analysis in birds, a vertebrate group that seems to have evolved endothermy following a different pathway from mammals (Legendre and Davesne, 2020), indicates that they have a copy of each gene coding for TRPs: V1, V2, V3, V4, V6, M2, M5, M8, and A1 (Saito and Shinagai, 2006). Some studies in vitro show that similarly to mammals, chicken TRPM8 is activated in mild cold and by menthol (Chuang et al., 2004; Myers et al., 2009), whereas chicken TRPA1 is activated by noxious heat, contrary to what is found in mammals (Saito et al., 2014). Additionally, chicken TRPV1 also senses noxious heat (Jordt and Julius, 2002) and seems to be involved in the T a drop during endotoxemic shock in chicks (Nikami et al., 2008). TRPV4 channels were described in the rictus associated with Herbst corpuscle mechanoreceptors in pigeons (Cabo et al., 2013). However, as far as we know there is no study showing the presence of any TRP channels in the skin or exploring their role in avian thermoregulation (in vivo).

In mammals and birds, along with development and growth, the maturation of thermogenesis and increase in insulation make the thermal comfort and thermoneutral zone shift overtime from warmer to colder T a (Alberts, 1978; Nichelmann and Tzschentke, 2002; Mortolla and Labbe, 2005). As a consequence, changes in thermoregulation are observed during the animal’s development, as thermosensitivity changes with aging, i.e., a T a that is considered neutral in early life, is normally supraneutral for adults. In this way, precocial birds are excellent models for studying the development of thermogenesis since endothermy is already present as soon as they hatch and the shift in thermosensitivity can be observed by activation of heat loss/heat conservation thermoeffectors in early life (Mathiu et al., 1991; Khandoker et al., 2004; Mortola and Maskrey, 2011; Toro-Velasquez et al., 2014; Cristina-Silva et al., 2021; Amaral-Silva et al., 2022).

In the present study, we tested the hypothesis that TRPV4 stimulation induces thermolytic responses for T b maintenance in birds at warm T a,s, and that this function is already present in early life, when the TRPV4 activation T a range does not represent a suprathermoneutral condition. First, immunohistochemistry was used to detect TRPV4 channels in the dorsal and ventral skin of 60-day-old adult chickens. To assess the role of TRPV4 channels in thermoregulation in vivo, we treated 5-day-old chicks and 60-day-old chickens with a selective agonist (RN1747) and antagonists (HC067047 and GSK 2193874) of TRPV4 (Vincent et al., 2009; Everaerts et al., 2010). The effects of the TRPV4 agonist and antagonists were evaluated on T a and the thermoeffectors, such as oxygen consumption (index of thermogenesis), pulmonary ventilation (index of thermal tactypnoea in heat) and huddling behavior (heat conservation mechanism in chicks) in different T a,s.

2 MATERIALS AND METHODS

2.1 Animals

2.1.1 Five-Day-Old Chicks

Hatchlings from Gallus gallus domesticus, of the Carijo lineage (Plymouth rock), were locally supplied (Globoaves, Itirapina, SP, Brazil) and raised at the Department of Animal Morphology and Physiology (FCAV-UNESP). The chicks were kept in climatic chambers (Premium Ecologica, Belo Horizonte, MG, Brazil) at 31–32°C (age-appropriate) with a light:dark cycle of 14:10 h (lights on at 6 a.m.) (Amaral-Silva et al., 2020, Amaral-Silva et al., 2021; Cristina-Silva, 2021). The experiments were conducted in 5-day-old chicks (60–70 g), a phase when thermogenesis is considered to be fully established (Nichelmann and Tzschentke, 2002; Tazawa et al., 2004; Amaral-Silva et al., 2020).

2.1.2 Sixty-Day-Old Chicks

A separate group of Carijo hatchlings (Globoaves, Itirapina, SP, Brazil) were housed in climatic chambers of the FCAV-UNESP-Jaboticabal poultry sector, and raised at an appropriate T a for each stage of development (first week, 31–32°C; second week, 25–28°C; third week, 20–22°C; forth through eighth weeks, 19–20°C; De Oliveira et al., 2006). A light:dark cycle of 14:10 h was kept the entire time. The experiments were then conducted when the birds achieved 55–65 days (2.5–3.5 kg), an age when birds were considered adults for this lineage.

All animals were provided standard food and water ad libitum. The experiments were carried out between 8:00 a.m. and 5:00
p.m. to avoid any influence of the daily $T_b$ cycle. All procedures were conducted according to the guidelines of the National Animal Experimentation Control Council (CONCEA-Brazil) and with the approval of the local Animal Care and Use Committee (CEUA-FCAV-UNESP-Jaboticabal).

### 2.2 Immunohistochemistry of the Dorsal and Ventral Skin

After the end of the experiments, 60-day-old adult chickens were deeply anesthetized and skin from the dorsal and ventral regions of the body was sampled, allocated in embedding cassettes and immediately immersed in 4% paraformaldehyde solution (PFA, Sigma-Aldrich Brazil Ltda. Sao Paulo, SP, Brazil) for 2 h. In sequence, the cassettes containing the skin were immersed in 70% alcohol solution for at least 2 days. The skin samples were then embedded in paraffin and the blocks were sliced in 40-µm transversal sections using a microtome (Leica RM2255, Wetzlar, HE, Germany).

For immunohistochemistry, the sections were deparaffinized and incubated for 30 min in an antigen retrieval solution (Dako, Glostrup, Denmark) at 70°C. Subsequently, the slices were washed in 1.5% hydrogen peroxide for 30 min, and were then incubated in 3% horse serum (Sigma-Aldrich Brazil Ltda. Sao Paulo, SP, Brazil) for 1 h at room temperature to prevent non-specific binding. The slices were incubated at 25°C for 24 h with a rabbit anti-TRPV4 primary antibody (Abcam 39260; dilution 1:200; Cambridge, United Kingdom), followed by a 4-h incubation in darkness with a fluorescent anti-rabbit secondary antibody (Alexa Fluor 488; dilution 1:500; Thermo Fisher Scientific Inc. Waltham, MA, United States). Finally, the sections were placed on gelatin-coated slides. After drying, they were covered by coverslips glued with a specific fluorescence preservative (ProLong Gold Antifade Reagent, Invitrogen, Carlsbad, CA, EUA) and observed under a microscope (Axio Imager Z2; Carl Zeiss do Brasil Ltda. Sao Paulo, Brazil) at a wavelength of 488 nm. A negative control was prepared following the same steps, except for the absence of the primary antibody.

### 2.3 Body Temperature ($T_b$)

Chicks, free to walk around the brooder (Protocols 1 and 2, described below), had their $T_b$ measured by a colonic sensor (Yellow spring Instrument, Co., Ohio, United States). The sensor was inserted 3 cm through the cloaca into the colon and was connected to a tele-thermometer (45TUC, Yellow spring Instrument Co., Ohio, United States). To avoid the influence of stress on $T_b$ due to manipulation on the day of the experiment, the chicks were previously trained to the procedure from day 2 to day 4 (3 measurements/day, 1/hour). Chicks in the respirometry chamber (Protocol 4, described below) had their $T_b$ monitored by telemetry using a temperature-sensitive tag (Biomark HPR Plus Reader, Boise, ID, United States). The Tb of adult animals (Protocols 5 and 6 described below) were recorded in real time by telemetry using a Biotherm reader (Biomark HPR Plus Reader, Boise, ID, United States) and uploaded to a computer (BioTerm software). Both sensors (colonic and abdominal) were calibrated against a certified thermometer (0.1°C precision). The $T_b$s measured by the colonic and abdominal sensors were highly correlated ($R^2 = 0.9782$).

The $T_b$ of adult animals (Protocols 5 and 6 described below) was monitored by temperature dataloggers (SubCue, Calgary, AB, Canada) implanted in the coelomic cavity via median laparotomy. To this end, animals were anesthetized using isoflurane (5% for induction using a face mask followed by intubation and maintenance with 1% in pure O$_2$). A small incision in the skin and muscle layers at the ventral midline, just caudal to the breast muscle, allowed for the insertion of the sensor into the coelomic cavity. Subsequently, the abdominal muscles and skin were sutured in layers, the isoflurane flow was closed, and we waited until animal itself removed the anesthesia tube from the trachea by reflex. The chickens were prophylactically treated with antibiotic (enrofloxacin, intramuscular; 10 mg kg$^{-1}$; Bayer SA, Sao Paulo, SP, Brazil) and with analgesic anti-inflammatory (flunixin meglumine, intramuscular; 2.5 mg kg$^{-1}$; MSD Saude Animal, Sao Paulo, SP, Brazil) agents. The surgery was performed at least 2 days before the experiment. At the end of the experiments, chickens were deeply anesthetized with 2,2,2-tribromoethanol (250 mg kg$^{-1}$; Sigma-Aldrich Brasil Ltda. Sao Paulo, SP, Brazil) to remove the sensors. The data stored in the sensors was uploaded to the computer (Subcue software, Calgary, AB, Canada) and calibrated according to the manufacturer’s recommendations.

### 2.4 Oxygen Consumption

Oxygen consumption (VO$_2$) was measured using an open-flow respirometry system. One animal at a time was placed inside a respirometer (3 L for chicks and 40 L for adult chickens) and continuous gas flow (room air) was maintained through the chamber at 1 L min$^{-1}$ for chicks and 5 L min$^{-1}$ for adult chickens using a mass flow system with flowmeters (MSF and FK-100; Sable Systems, Las Vegas, NV, United States). The temperature within the respirometer was set according to the protocol described below. A subsample of the outflow air was pulled (180 mL min$^{-1}$; SS4; Sable Systems, Las Vegas, NV, United States), passed through a water vapor pressure analyzer (RH300; Sable Systems, Las Vegas, NV, United States), dried (Drierite, with indicator, eight mesh, Sigma-Aldrich Brazil Ltda. Sao Paulo, SP, Brazil), and finally pulled into an O$_2$ analyzer (PA-10; Sable Systems, Las Vegas, NV, United States). Water vapor...
pressure (WVP; kPa) and barometric pressure (kPa) were later used to correct the flow. The analyzer and flowmeters were connected in line with an analog-to-digital converter (PowerLab; ADInstruments, Sydney, NSW, Australia), and data were recorded using LabChart (ADInstruments, Sydney, NSW, Australia). The analyzers were calibrated before each experiment using nitrogen as zero and dry ambient air as 20.95% oxygen. As CO₂ was neither analyzed nor scrubbed, experiment using nitrogen as zero and dry ambient air as (PowerLab; ADInstruments, Sydney, NSW, Australia), and connected in line with an analog-to-digital converter (standard conditions of temperature, pressure and dry air).

Quotient (considered to be 0.85). Data are shown in STPD fractional concentration of oxygen, and RQ is the respiratory quotient (considered to be 0.85). Data are shown in STPD (standard conditions of temperature, pressure and dry air).

2.5 Pulmonary Ventilation

Pulmonary ventilation (Ve) was measured concurrently with V̇E02 using a FD141 pressure transducer-based spirometer (ADInstruments, Sydney, NSW, Australia) connected to the respirometer. Breathing frequency (f) was calculated by counting the pressure peaks per time. Tidal volume (VT) was calculated using the following formula (Drorbaugh and Fenn, 1955): VT = A(Vcal / Pcal) [Tb (P₂ - PCH₂O)] - [Tb (P₂ - PB₂)], where V̇E is the tidal volume, A is the wave amplitude, Vcal is the calibration volume, Pcal is the calibration pressure, Tb is the body temperature, PB is barometric pressure, PCH₂O is the water vapor pressure in the chamber, and PB₂ is the water vapor pressure of the air inside the animal’s body. The system was calibrated for volume by comparison with the pressure produced by known volumes of air injected into the system with a syringe. Finally, pulmonary ventilation was calculated as Ve = f × VT.

2.6 Drugs

The selective TRPV4 antagonist, HC-067047 [2-Methyl-1-[3-(4-morpholinyl) propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1H-pyrrole-3-carboxamide] (Eveeraets et al., 2010; Shibasaki et al., 2015), was purchased from Tocris Bioscience (Bristol, United Kingdom). It was dissolved in 10% propylene glycol; 3.75 ml kg⁻¹ was chosen based on the results of the Protocol 1. The drugs were applied intramuscularly (i.m.) to its presumed site of action (skin receptors) before being subjected to the thermal stimulus (26°C for chicks; 28 and 19°C for adults), as previously described in rats (Almeida et al., 2012; Vizin et al., 2015). The temperatures of 31 and 26°C were chosen for Protocols 1–4 because 31°C is thermoneutral for five- to eight-day-old chicks (Coleone et al., 2009; Amaral-Silva et al., 2021, 2022; Cristina-Silva et al., 2021) and 26°C is in the range of TRPV4 activation in vivo, but is considered to be cold for chicks (Vizin et al., 2015; Amaral-Silva et al., 2021, 2022; Cristina-Silva et al., 2021). Different animals were used for each protocol, and they were deeply anesthetized and killed immediately after the end of the experiments.

2.7 Protocols

Protocols 1–4 were performed in 5-day-old Carijo chicks, and Protocols five and six were performed in 60-day-old adult Carijo chickens. To investigate the effects of TRPV4 activation on thermoeffectors, we combined pharmacological blockage of TRPV4 (using specific antagonists) with both chemical (topical application of RN1747 in chicks and adults) and thermal (exposure of adults to 28°C) activation of the channel. The animals were kept at 30–31°C for at least 60 min in order to promote skin vasodilation and deliver the circulating antagonist (i.m. injected) to its presumed site of action (skin receptors) before being subjected to the thermal stimulus (26°C for chicks; 28 and 19°C for adults), as previously described in rats (Almeida et al., 2012; Vizin et al., 2015).

2.7.1 Protocol 1: Effect of Chemical Activation and Inhibition of the TRPV4 Channels on T₂ of 5-day-Old Chicks at 31°C and 26°C

Chicks received: 1) topical application of the TRPV4 agonist, RN1747 (0.2, 0.5 or 1.0 mg ml⁻¹), or its vehicle (100% propylene glycol; 3.75 ml kg⁻¹) on the dorsal skin; or 2) intramuscular (i.m.) injection of the TRPV4 agonist, HC067047 (10, 50 or 100 μg kg⁻¹), or its vehicle (90% saline +10% ethanol; 1 ml kg⁻¹). Colonic temperature was measured just before and hourly up to 240 min after each treatment.

2.7.2 Protocol 2: Effect of the TRPV4 Antagonist on T₂ of 5-Day-Old Chicks at 31°C and 26°C Following Chemical Activation of TRPV4

Chicks were injected with 50 μg kg⁻¹ (i.m.) of HC067047 or its vehicle (1 ml kg⁻¹). One hour later, they were topically applied with RN1747 (0.5 mg ml⁻¹) or its vehicle (3.75 ml kg⁻¹). This generated four treatment combinations: 1) vehicle of HC067047 + vehicle of RN1747 (sham); 2) HC067047 + vehicle of RN1747 (agonist effect only); 3) vehicle of HC067047 + RN1747 (agonist effect only); 4) HC067047 + RN1747 (inhibition of TRPV4 chemical activation). Colonic temperature was measured before the first treatment and hourly for five consecutive hours in all groups. The antagonist and agonist doses were chosen based on the results of the Protocol 1. The
experiment was conducted: 1) at a neutral $T_a$ of 31°C during the whole period of $T_b$ measurements; and 2) started at 31°C, which was maintained for 1 h after the antagonist injection (to facilitate access of the circulating drug to the skin, as explained above), and at the moment of the agonist topical application, the $T_a$ was reduced to 26°C.

2.7.3 Protocol 3: Effects of the TRPV4 Antagonist on Huddling Behavior of 5-Day-Old Chicks Exposed to 26°C Following Chemical TRPV4 Activation

One day before the experiments, chicks were separated into groups of five individuals and kept in climatic chambers at 31°C. On the following day, chicks were injected with HC067047 (50 μg kg$^{-1}$) or vehicle (90% saline +10% ethanol; 1 ml kg$^{-1}$) and maintained at 31°C for 60 min. Then, they were topically applied with RN1747 (0.5 mg ml$^{-1}$) or its vehicle (100% propylene glycol; 3.75 ml kg$^{-1}$) and exposed to 26°C for the manifestation of cold-induced huddling behavior. The treatment combinations presented in Protocol two were repeated in this protocol (vehicle of HC067047 + vehicle of RN1747; HC067047 + vehicle of RN1747; vehicle of HC067047 + RN1747; and HC067047 + RN1747). Chicks were monitored using a webcam (LifeCam HD-3000, Microsoft, Redmond, WA, EUA) fixed about 1 m above the experimental chambers and programmed to take pictures with a 2-min interval from the time of pretreatment up to about 270 min after the topical treatment. The number of single chicks (ungrouped animals) was counted, and the area occupied by the five chicks in the group was measured using ImageJ (FIJI) to infer huddling behavior for heat conservation (Dantionio et al., 2015).

2.7.4 Protocol 4: Effects of the TRPV4 Antagonist on the Effect of Chemical TRPV4 Activation on $T_b$, Oxygen Consumption ($\text{VO}_2$) and Ventilation ($\text{VE}$) of 5-Day-Old Chicks at 31°C

The selective TRPV4 antagonist, GSK2193874, was used in this protocol (Cheung et al., 2017; Scarpellini et al., 2019) to confirm the TRPV4 inhibition effect on the $T_b$ of chicks and to verify the metabolic thermoeffectors affected. Resembling Protocols 2 and 3, chicks were pretreated with 50 μg kg$^{-1}$ (i.m.) of the TRPV4 antagonist, GSK2193874, or its vehicle (1% DMSO; 1 ml kg$^{-1}$), and after 60 min, were topically applied with 0.5 mg ml$^{-1}$ of the TRPV4 agonist, RN1747, or its vehicle (100% propylene glycol; 3.75 ml kg$^{-1}$). This also generated four treatment combinations: 1) vehicle of GSK2193874 + vehicle of RN1747 (sham); 2) GSK2193874 + vehicle of RN1747 (agonist effect only); 3) vehicle of GSK2193874 + RN1747 (agonist effect only); 4) GSK2193874 + RN1747 (inhibition of TRPV4 chemical activation). The chicks in this protocol were allocated for 30 min in the respirometer for habituation. After that, oxygen fraction was recorded continuously from 20 min before the pretreatment until 240 min after the topical treatment. Ventilation was recorded during 2 min every 20 min, when the respirometer was closed for baseline recordings (FiO$_2$). $\text{VO}_2$, $\text{VE}$, and $f$ were determined for each 20-min interval. $T_b$ was recorded every 5 min. $T_a$ in the respirometer was monitored in real time by thermopar sensors (ADInstruments, Sydney, NSW, Australia) and controlled inside a climatic chamber (BOD, FANEM, Sao Paulo, SP, Brazil).

2.7.5 Protocol 5: Effects of Chemical Inhibition of TRPV4 on $T_b$, $\text{VO}_2$ and $\text{VE}$ in Adult Chickens Exposed to 28°C and 19°C

Sixty-day-old chickens, previously implanted with a $T_b$ sensor, were exposed to 30°C for 50 min before the experiments (to facilitate the access of the circulating drug to the skin, as explained above), $T_b$ was measured during this phase. After that, this protocol was conducted in two different ways: 1) animals were injected with 100 or 500 μg kg$^{-1}$ of the TRPV4 antagonist, GSK2193874, or its vehicle (1% DMSO; 1 ml kg$^{-1}$) and transferred to a respirometer at 28°C (warm for this age) for recordings of $T_b$, $\text{VO}_2$, $\text{VE}$, and $f$ for 180 min; or 2) animals were injected with 500 μg kg$^{-1}$ of the TRPV4 antagonist, GSK2193874, or its vehicle (1% DMSO; 1 ml kg$^{-1}$) and transferred to a respirometer at 19°C (mild cold for this age) for recordings of $T_b$, $\text{VO}_2$, $\text{VE}$, and $f$ for 180 min. The respirometer was placed inside a climatic chamber (BOD, FANEM, Sao Paulo, SP, Brazil) continuously programmed to meet $T_a$s set according to the experimental protocol considering the internal temperature of the respirometer measured by thermopar sensors (ADInstruments, Sydney, NSW, Australia).

2.7.6 Protocol 6: Effects of Chemical Activation of TRPV4 on $T_b$, $\text{VO}_2$ and $\text{VE}$ of Adult Chickens Exposed to 19°C

After the initial warming phase at 30°C (see Protocol 5), 60-day-old chickens were topically applied with 0.5 mg ml$^{-1}$ of the TRPV4 agonist, RN1747, or its vehicle (100% propylene glycol; 3.75 ml kg$^{-1}$) equally distributed on the ventral and dorsal skin. They were then transferred to a respirometer at 19°C for recordings of $T_b$, $\text{VO}_2$, $\text{VE}$, and $f$ for 180 min. Similar to Protocol 5, the respirometer was placed inside a climatic chamber (BOD, FANEM, Sao Paulo, SP, Brazil) and its internal temperature was precisely monitored in real time by thermopar sensors (ADInstruments, Sydney, NSW, Australia).

2.8 Data Processing and Analysis

All data are presented as mean ± standard error. The effects on the peak of delta $T_b$ (calculated by subtracting the peak value of the treatments from the respective controls) of the animals that received different doses of the TRPV4 agonist and the antagonist were compared using one-way ANOVA. The effects of the TRPV4 agonist and antagonists on the variables evaluated in chicks ($T_b$, $\text{VO}_2$, $\text{VE}$, $\text{VE}$, and $f$, number of single chicks, and area occupied by a group) and adult chickens ($T_b$, $\text{VE}$, $\text{O}_2$, $\text{VE}$, $\text{VE}$, and $f$) were tested using repeated measures two-way ANOVA with treatment and time as factors. The differences among means were evaluated by the Sidak post-hoc test. Significant differences were declared at $p < 0.05$.

3 RESULTS

3.1 Immunohistochemistry for TRPV4 in the Dorsal and Ventral Skin of Adult Chickens

Demonstration of the presence of TRPV4 channels (green immunostaining) in the ventral and dorsal skin of chickens is
shown in fluorescence photomicrographs (Figure 1A,B, respectively). TRPV4 was expressed in the layers of the dermis and subcutis both in the ventral and dorsal sites. A negative control is presented in Figure 1C to confirm the absence of unspecific staining without the primary antibody, anti-TRPV4.

### 3.2 Thermoregulatory Responses to Chemical Activation and Inhibition of TRPV4 Channels in 5-Day-Old Chicks

**Protocol 1**—The topical application of the selective agonist for TRPV4, RN1747, significantly reduced the peak of delta $T_b$ (at 120 min for all) in 5-day-old chicks when applied in concentrations of 0.5 and 1.0 mg ml$^{-1}$, but not when applied at 0.2 mg ml$^{-1}$ (Table 1). On the other hand, the selective TRPV4 antagonist, HC067047, alone did not affect the chicks’ $T_b$ in the three doses applied, 10, 50 and 100 μg kg$^{-1}$ (peak at 120 min; Table 1).

**Protocol 2**—RN1747 (0.5 mg ml$^{-1}$) caused a decrease in $T_b$ from 120 to 180 min following application (Figure 2A; 180–240 min; treatment effect: $p < 0.0001$, $F_{(3, 36)} = 10.42$) when the chicks were in the thermoneutral condition. A more accentuated $T_b$ decrease from 60 to 240 min following application of RN1747 (Figure 2B; 120–300 min; treatment effect: $p < 0.001$, $F_{(3, 71)} = 16.79$) was observed when chicks were exposed to cold (26°C—activation range of TRPV4). The antagonist injection

### Table 1

Peak values of delta body temperature ($T_b$) of 5-day-old chicks under thermoneutral condition ($T_a = 31^\circ$C) that received either topical administration (3.75 ml kg$^{-1}$) of the TRPV4 agonist RN1747 (0.2, 0.5 and 1 mg ml$^{-1}$) on the dorsal skin, or intramuscular (i.m.; 1 ml kg$^{-1}$) injection of the TRPV4 antagonist HC067047 (10, 50 or 100 μg kg$^{-1}$), compared to the respective vehicles (topical: propylene glycol; i.m.: 90% saline +10% ethanol).

| Treatment | Peak ± SEM (delta $^\circ$C) | Peak diff * (delta $^\circ$C) | p value |
|-----------|-------------------------------|-------------------------------|---------|
| Vehicle (100% propylene glycol, topical) | $-0.05 ± 0.05$ | | 0.999 |
| RN1747, 0.2 mg ml$^{-1}$ topical | $-0.05 ± 0.05$ | | 0.9255 |
| RN1747, 0.5 mg ml$^{-1}$ topical | $-0.30 ± 0.07$ | $-0.25$ | 0.0022 |
| RN1747, 1.0 mg ml$^{-1}$ topical | $-0.45 ± 0.07$ | $-0.40$ | 0.0022 |
| Vehicle (90% saline +10% ethanol, i.m.) | $0.30 ± 0.1$ | | 0.7503 |
| HC067047, 10 μg kg$^{-1}$ i.m. | $0.15 ± 0.08$ | $-0.15$ | 0.9829 |
| HC067047, 50 μg kg$^{-1}$ i.m. | $0.40 ± 0.05$ | 0.10 | 0.9038 |
| HC067047, 100 μg kg$^{-1}$ i.m. | $0.40 ± 0.07$ | 0.10 | 0.9038 |

Values are expressed as mean ± SEM. Number of animals in each group is shown in parentheses.

*Peak differences were calculated subtracting the peak value of the treatments (RN1747 and HC067047) from the respective controls (100% propylene glycol and 90% saline +10% ethanol) 2 hours after the applications at 31°C. **Significant p value for peak differences (one-way ANOVA; Sidak post-hoc test).
alone had no effect on T\textsubscript{b}, which followed the same pattern as the control animals (vehicle i.m. + vehicle topical). However, the HC067047 effectiveness in inhibiting TRPV4 was demonstrated by the inhibition of the T\textsubscript{b} decrease caused by the agonist, RN1747, at both thermoneutral and cold conditions (Figures 2A,B).

**Protocol 3**—The effect of the agonist and antagonist drugs on T\textsubscript{b} was accompanied by the behavioral thermoregulation. Exposure to cold (26°C) induced huddling behavior in the 5-day-old chicks, which was observed by the reduction of the number of single chicks (Figure 2C; p < 0.001, F(3, 20) = 47.65) and the area occupied by the group of five chicks (Figure 2C; p < 0.001, F(3, 20) = 59.08). Topical application of RN1747 inhibited the huddling behavior in response to cold, as it did not reduce the number of single chicks (Figure 2C; 170–220 min; p < 0.001, F(3, 20) = 11.39) or the area occupied by the group of five chicks (Figure 2D; 160–250 min; p < 0.001, F(3, 20) = 50.21). Injection of HC067047 alone did not affect the chicks’ behavior, but its injection prevented the inhibition of huddling caused by the agonist, RN1747; thus, the chicks huddled in response to cold, similarly to the controls. The effects of cold and pharmacological activation/inhibition of TRPV4 in 5-day-old chicks’ behavior is exemplified in images in Figure 2D. It is possible to observe that the only group showing no huddling is the one that received the “vehicle + RN1747” treatment.

**Protocol 4**—Under thermoneutrality, the selective TRPV4 antagonist, GSK2193874, had a similar effect on T\textsubscript{b} compared to the antagonist, HC067047. By itself, it did not affect T\textsubscript{b}, but it...
prevented the $T_b$ decrease caused by RN1747 (Figure 3A; 120–140 min; 200–280 min; treatment effect: $p < 0.01$, $F_{(3,21)} = 5.40$), and the chicks’ $T_b$ was comparable to that of the controls. Despite the $T_b$ changes, no difference in $V_O_2$ (Figure 3B; treatment effect: $p = 0.66$, $F_{(3,21)} = 0.53$), $V_E$ (Figure 3C; treatment effect: $p = 0.53$, $F_{(3,21)} = 0.76$), $V_T$ (Figure 3D; treatment effect: $p = 0.69$, $F_{(3,21)} = 0.48$), or $f$ (Figure 3E; treatment effect: $p = 0.58$, $F_{(3,21)} = 0.66$) was observed among the treatments.

### 3.3 Thermoregulatory Responses to Thermal and Chemical Stimulation and Inhibition of TRPV4 Channels in 60-Day-Old Adult Chickens

**Protocol 5**—Injection of 500 μg kg$^{-1}$ (i.m.) of GSK 2193874 in adult chickens resulted in a higher $T_b$ compared to the vehicle group at 28°C (Figure 4A left panel; 130–260 and 230 min; treatment effect: $p = 0.01$, $F_{(2, 24)} = 5.13$). A lower dose of the antagonist (100 μg kg$^{-1}$) did not affect $T_b$ significantly. No difference in $V_O_2$ (Figure 4B, left; treatment effect: $p = 0.81$, $F_{(2, 24)} = 0.21$), $V_E$ (Figure 4C, left; treatment effect: $p = 0.28$, $F_{(2, 24)} = 1.14$), $V_T$ (Figure 4D, left; treatment effect: $p = 0.96$, $F_{(2, 24)} = 0.04$) or $f$ (Figure 4E, left; treatment effect: $p = 0.26$, $F_{(2, 24)} = 1.44$) was observed among the treatments in chickens at 28°C.

Different from the exposure to 28°C, chickens at 19°C and injected with the higher dose of GSK 2193874 (500 μg kg$^{-1}$) did not differ in $T_b$ from the vehicle group (Figure 4A, right panel; treatment effect: $p = 0.90$, $F_{(1,9)} = 0.02$). In this condition, the antagonist also did not affect the thermoeffectors $V_O_2$ (Figure 4B right; treatment effect: $p = 0.82$, $F_{(1,8)} = 0.05$), $V_E$ (Figure 4C, right; treatment effect: $p = 0.98$, $F_{(1,8)} < 0.01$), $V_T$ (Figure 4D, right; treatment effect: $p = 0.60$, $F_{(1,8)} = 0.30$) or $f$ (Figure 4E, right; treatment effect: $p = 0.33$, $F_{(1,8)} = 1.05$).

**Protocol 6**—Topical application of the TRPV4 agonist, RN1747, in 60-day-old chickens at 19°C resulted in a significant drop in $T_b$ when compared to the vehicle group (Figure 5A; 130–250 min; treatment effect: $p = 0.03$, $F_{(1,9)} = 7.03$). This $T_b$ decrease in response to the agonist was preceded by a reduction in $V_O_2$ (Figure 5B; 110–130 min; treatment effect: $p = 0.03$, $F_{(1,9)} = 6.54$). No difference in $V_E$ was observed between groups (Figure 5C; treatment effect: $p = 0.70$, $F_{(1,9)} < 0.01$). However, chickens treated with RN1747 had a lower $V_T$ (treatment effect: $p = 0.03$, $F_{(1,9)} = 6.43$) and a higher $f$ (treatment effect: $p = 0.03$, $F_{(1,9)} = 6.20$) than chickens injected with vehicle (Figures 5D,E), indicating activation of respiratory heat loss.

### 4 DISCUSSION

Our results show the first evidence of the presence of TRPV4 channels in the skin of the dorsal and ventral surfaces of the body,
FIGURE 4 | Effect of intramuscular injection (i.m.; 1 ml kg$^{-1}$) of the TRPV4 antagonist, GSK2193874 (100 and 500 μg kg$^{-1}$), or vehicle (1% DMSO) on body temperature ($T_b$; A), oxygen consumption ($\dot{V}O_2$; B), ventilation ($\dot{V}E$; C), tidal volume ($V_T$; D) and breathing frequency ($f$; E) of 60-day-old chickens under warm condition ($T_a$ = 28°C; graphs in the left). Effect of the i.m. injection of GSK2193874 (500 μg kg$^{-1}$) or 1% DMSO (1 ml kg$^{-1}$) on the same respective variables in 60-day-old chicken under mild cold condition ($T_a$ = 19°C; graphs on the right). $T_{bi}$, initial body temperature. The arrow indicates the moment of injection. Number of animals in each group is shown in parentheses. All the animals were pre-exposed to a hot condition (30°C) for inducing cutaneous vasodilation and facilitate the distribution of drugs throughout the peripheral sites of receptors (Almeida et al., 2012). * $p < 0.05$, significant difference from the vehicle group at the same time point (two-way ANOVA; Sidak post-hoc test).
and their role in the activation of warmth-defense responses in birds. These thermosensors are functional in early life, despite having no physiological meaning for thermoregulation in chicks, which sense its temperature activation range (~26–30°C) as cold to neutral (Figure 6).

Immunohistochemistry revealed the presence of TRPV4 channels spread throughout the dermis of chickens. At least in humans, these channels are highly diffuse in skin, including vascular endothelial cells, eccrine sweat gland secretory cells, and keratinocytes (Kida et al., 2012; Fusi et al., 2014; Olivan-Viguera et al., 2018). In rats, evidence exists for the presence of TRPV4 in keratinocytes (Guler et al., 2002; Wang and Siemens, 2015). In this case, it is suggested that some humoral pathways promote communication between the keratinocytes in the epidermis and the nerve endings in the dermis, which is essential for the thermal information to be sent to the brain (Wang and Siemens, 2015). In chickens, TRPV4 may reside in primary afferent sensory neurons and/or other cell types that are connected to nerve endings, which make possible the transmission of afferent signals for thermoeffector activation.

Chemical stimulation of cutaneous TRPV4 (topical application of a selective agonist) resulted in a decrease of Tb in 5-day-old chicks when allocated in both thermoneutral (31°C) and cold (26°C) conditions. The hypothermia observed during TRPV4 stimulation occurred together with the inhibition of huddling in response to cold (26°C), a behavioral heat conservation mechanism well known in birds, including chicken chicks (Kleiber and Winchester, 1933; McKechnie and Lovegrove, 2001; Gilbert et al., 2010; Amaral-Silva et al., 2022). Both the Tb decrease and the prevention of huddling behavior were inhibited by pretreatment with the TRPV4 antagonist (HC067047) at a dose that did not affect behavior when injected alone, highlighting the specificity of TRPV4 in this response. Interestingly, a similar pattern is observed in adult rats, i.e., the selection of colder Ts upon chemical activation of cutaneous TRPV4, and the inhibition of cold-seeking behavior by HC067047 following exposure to 28–31°C (Vizin et al., 2015).

Despite the pharmacological evidence that 5-day-old chicks have functional TRPV4 channels, and that HC06747 blunted the agonistic effect, the inhibition of TRPV4 channels alone at 31°C or 26°C did not increase Tb, as observed in adult rats (Vizin et al., 2015; Scarpellini et al., 2019). This is probably because the TRPV4 thermosensors are not thermally activated at this age by either of the Ts used. Even though the chicks were exposed to Ts in the range that this thermoreceptor is shown to sense in vivo (Vizin et al., 2015), 31 and 26°C do not represent a warm T for the 5-day-old chicks. In fact, a T of 26°C triggers activation of thermogenesis instead of thermolysis in the first week of life in chicks (Amaral-Silva et al., 2021; Cristina-Silva et al., 2021;
Amaral-Silva et al., (2022). Thus, these animals are not expressing any heat loss response, which would be inhibited by HC06747 in the case where TRPV4 was active, and $T_b$ would be consequentially increased by the lack of this response. We speculate that, at this age, TRPV4 channels may be chemically inhibited or present a very high activation threshold, becoming physiologically relevant only for older animals.

Later in life, when 28°C represents a supraneutral $T_s$ for adult chickens, TRPV4 seems to be active and responsive, preventing hyperthermia in this condition. In support of this, when the adult chickens were exposed to mild cold (19°C), which does not activate TRPV4, the inhibition of these channels, using the same dose of antagonist as that which reduced $T_b$ at 28°C, did not affect $T_b$. The hyperthermic response to the TRPV4 antagonist at 28°C did not involve thermogenesis, considering that GSK2193874 did not change $O_2$ consumption. Besides that, the thermal tachypnea (high $f$ and low $V_T$, known to provide respiratory heat loss; Hammel and Pierce, 1968; Richards, 1970; Mortola and Maskrey, 2011) was also not affected by the inhibition of TRPV4. In this case, if GSK2193874 only inhibited cutaneous vasodilation, an impairment of heat dissipation through the body-ambient thermal gradient (~41.1–28°C) would be enough to cause the observed $T_b$ increase (Richards, 1970; Wolfenson et al., 1981). This suggests that TRPV4 might maintain regular $T_b$ in chickens exposed to mild suprathermoneutral $T_s$ by peripheral vasomodulation, which may be enough for thermoregulation in the range of $T_s$ sensed by TRPV4. Although it was not measured here, there is evidence that cutaneous vasodilation is activated by chemical stimulation of TRPV4 in adult rats (Vizin et al., 2015) and humans (Fuji et al., 2019). In the latter, however, that response is partially, but not completely, inhibited by local microinfusion of HC067047 or GSK2193874 (Fuji et al., 2021). This issue remains to be confirmed in birds. Another matter for future investigation is the possibility that TRPV4 channels are present in the brain of chickens. If this was the case, an influence of GSK2193874 inhibiting brain TRPV4 channels would be speculated. At least in rats, TRPV4 channels located in the hypothalamus region seem to play a role in thermolytic responses together with those channels present in the skin (Vizin et al., 2015; Scarpellini et al., 2019). Regarding chickens, although one cannot rule out a possible central action of GSK2193874, its efficiency in inhibiting cutaneous TRPV4 channels was confirmed by blockade of the hypothermia induced by topical application of RN1747 in chicks (Figure 3).

When TRPV4 was chemically stimulated at 19°C, adult chickens decreased $T_b$, similarly to what was observed in chicks. This response in the adults relied on the activation of tachypnea, providing evaporative heat loss (Wolfenson et al., 1981; Arad and Marder, 1982), and also on metabolic rate inhibition, reducing heat gain (Amaral-Silva et al., 2021; Amaral-Silva et al., 2022). In this case, the pharmacological effect of the TRPV4 agonist may be more potent to stimulate the specific skin sensors for activating those thermoeffectors than stimulation by the warm condition at 28°C. A possibility exists that such a $T_s$ is inside the common temperature range of activation of other temperature-sensitive receptors, for example TRPV3 (Luo and Hu, 2014; Wang and Siemens, 2015; Ishikawa et al., 2019), overlapping the influences of different thermoreceptors.

5 CONCLUSION

Our data indicate the presence of TRPV4 channels in the chicken’s dermis, which present a thermolytic role for $T_b$ maintenance. Chicken TRPV4 thermosensors are functional in early life, when the range of TRPV4 activation (~26–31°C) actually represents a cold to thermoneutral ambient condition. In this phase, these channels may remain suppressed, but become physiologically relevant only later in life, when the $T_s$ they sense represent a warm condition for adult chickens and thermolysis is evoked to maintain $T_b$. If there is a specific thermoeffector activated by TRPV4 channels in warm conditions, or if they induce small modulations of different thermoeffectors for maintaining $T_b$, are still pending questions for birds.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.
ETHICS STATEMENT

The animal study was reviewed and approved by all procedures were conducted according to the guidelines of the National Animal Experimentation Control Council (CONCEA-Brazil) and with the approval of the local Animal Care and Use Committee (CEUA-FCAV-UNESP-Jaboticabal).

AUTHOR CONTRIBUTIONS

Conception and designed experiments: CC-S, LA-S, and KB. Conducted experiments: CC-S, LA-S, KS, WS, MF, and GC. Analyzed and interpreted data: CC-S, LA-S, MA, LG, GS, and KB. Wrote the manuscript: CC-S, LA-S, MA, LG, GS, and KB. Edited and revised the manuscript: CC-S, LA-S, MA, WS, MF, LG, GS, and KB.

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