Metabolic trade-offs favor regulated hypothermia and inhibit fever in immune-challenged chicks

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Summary statement: Thermal response to an immune challenge is tied to the metabolic balance in birds. Endotoxin-injected chicks preserve energy favoring regulated hypothermia and eliminating fever in case of competing metabolic demands.

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ABSTRACT

The febrile response to resist a pathogen is energetically expensive while regulated hypothermia seems to preserve energy for vital functions. We hypothesized here that immune challenged birds under metabolic trade-offs (reduced energy supply / increased energy demand) favor a regulated hypothermic response at the expense of fever. To test this hypothesis, we compared 5-days old broiler chicks exposed to fasting, cold (25^{o}C), and fasting combined with

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cold to a control group fed at thermoneutral condition (30°C). The chicks were injected with saline or with a high dose of endotoxin known to induce a biphasic thermal response composed of body temperature (Tb) drop followed by fever. Then Tb, oxygen consumption (metabolic rate), peripheral vasomotion (cutaneous heat exchange), breathing frequency (respiratory heat exchange), and huddling behavior (heat conservation indicator) were analyzed. Irrespective of metabolic trade-offs, chicks presented a transient regulated hypothermia in the first hour, which relied on a suppressed metabolic rate for all groups, increased breathing frequency for chicks fed/fasted at 30°C, and peripheral vasodilation in fed/fasted chicks at 25°C. Fever was observed only in chicks kept at thermoneutrality and was supported by peripheral vasoconstriction and huddling behavior. Fed and fasted chicks at 25°C completely eliminated fever despite the ability to increase metabolic rate for thermogenesis in the phase correspondent to fever when it was pharmacologically induced by 2.4-Dinitrophenol. Our data suggest that increased competing demands affect chicks' response to an immune challenge favoring regulated hypothermia to preserve energy while the high costs of fever to resist a pathogen are avoided.

K**eywords:** birds, endotoxin, cold, fasting, fever, regulated hypothermia

INTRODUCTION

Challenging conditions that animals face daily in a natural environment, such as changes in ambient temperature, food scarcity, different seasons, extreme weather events, predators, amongst others, may require either extra energy to meet a higher maintenance cost or an alternative physiological adjustment to cope with insufficient energy supply/stores. For example, endotherms can increase metabolic rate to maintain core body temperature (Tb) and activity in the cold, or alternatively decrease Tb (torpor) to save energy during cold season or food shortage (Bicego et al., 2007; Hohtola, 2012; McKechnie, 2008; Ruf and Geiser, 2015). Independent on the condition the animal is facing, concurrent pathogenic infections are always possible; hence, adequate energy management to fight an infection while in a hostile environment might be vital. For birds, the potential trade-off between maintenance in an adverse condition and resist a pathogen infection is particularly significant because they already present an intrinsic energetic

costly lifestyle, with the highest Tb and metabolic rate amongst vertebrates (Hohtola, 2012; Legendre and Davesne, 2020).

The thermoregulatory component of the immune response to a pathogen can be a key cost on birds' energetic budget when the response launched is fever (Marais et al., 2011). Fever is characterized as an elevated Tb actively established and defended by heat producing and/or conserving mechanisms, which consist in an important adjuvant to immune function protecting the host against pathogen infections (Blatteis, 2003; Gray et al., 2013; IUPS Thermal Physiology Commission, 2001; Lochmiller and Deerenberg, 2000). On the downside, the high costs of the febrile response represent a metabolic challenge that may exceed its' benefits under circumstances of reduced physiological fitness (Garami et al., 2018).

We recently showed that birds, in addition to mammals, can switch the thermal defense strategy from fever to a regulated form of hypothermia (often called anapyrexia) in response to a severe immune challenge (Amaral-Silva et al., 2021). Such regulated hypothermia stands for the opposite thermal response of fever, that is, a result of thermolytic effectors to actively defend a lower Tb (Bicego et al., 2007; Garami et al., 2018; Romanovsky et al., 1996). Thus, while fever is considered a thermal response that provides resistance to pathogen infection at a high cost, regulated hypothermia seems to be activated for saving energy to defend vital systems "tolerating" the pathogenic presence, a response that is also considered beneficial to the host (Amaral-Silva et al., 2021; Corrigan et al., 2014; Ganeshan et al., 2019; Liu et al., 2012; Steiner and Romanovsky, 2019).

Because the energy budget for the thermal response to fight pathogens and for maintenance is the same, the fever-regulated hypothermia switch seems to be related to the energy expended on competing demands and triggered only when the metabolic costs of the pro-inflammatory response exceed the available resources (Ganeshan et al., 2019). For example, rodents only display regulated hypothermia during systemic inflammation when concurrently challenged with cold or fasting (Almeida et al., 2006; Corrigan et al., 2014; Ganeshan et al., 2019; Krall et al., 2010). In contrast, broiler chicks can launch a regulated hypothermic response when challenged with high doses of endotoxin (lipopolysaccharide, LPS), even when in a thermoneutral condition (Amaral-Silva et al., 2020; Amaral-Silva et al., 2021; Dantonio et al., 2016). Likewise, several species of small passerines are reported to present only Tb decrease after an immune stimulus, even though none of the studies addressed the possible regulated

nature of such response (Coon et al., 2011; Cornelius et al., 2017; King and Swanson, 2013; Owen-Ashley et al., 2006; Sköld-Chiriac et al., 2015). Yet, passerines may be already specially challenged on the energy budged because their small size entails a large relative surface area for passive heat loss, additionally to their limited fasting capacity or tolerance to anorexia (Hohtola, 2012). On the other hand, adult chickens, which present larger energy storage seem to need much higher doses of LPS to challenge their energy resources resulting in decreased Tb (Leshchinsky and Klasing, 2001). Another evidence of the metabolic status influencing the thermal response to an immune challenge in birds is that layer chicks display a persistent hypometabolic response to LPS when extra energy needs to be directed to maintenance because of embryonic exposure to a pollutant (dioxin) (Amaral-Silva et al., 2020). Nevertheless, the concept of switching from fever to regulated hypothermia was not considered yet as a possible thermal strategy to manage an immune challenge when birds are exposed to metabolic trade-offs.

Here we hypothesized that competing demands caused by fasting (reduced energy supply), cold (increased energy demand), or a combination of both fasting and cold favor regulated hypothermia at the expense of fever in birds challenged with LPS. By using a high dose of LPS we could analyze the effect of the environmental trade-offs in both regulated hypothermia and fever in the same individual, as it responds to such immune challenge with a biphasic thermal response. This treatment model is commonly used to simulate severe inflammation. Within the first hour after LPS injection its circulating concentration is high and hypothermia occurs, overtime, LPS circulating concentration decreases and a mild inflammation triggers fever ~4-5hs after LPS injection (Amaral-Silva et al., 2020; Dantonio et al., 2016; Liu et al., 2012; Romanovsky et al., 1996). To characterize the hypothermic and febrile responses to LPS in the environmentally challenged chicks (*Gallus gallus*), we analyzed Tb, O_2 consumption (thermogenesis index), cutaneous temperature (vasoconstriction/vasodilation index), breathing frequency (respiratory heat loss), and huddling (thermoregulatory behavior) as thermoeffectors. An extra protocol was made to assure thermogenic capacity during fever elimination by measuring Tb and the autonomic thermoeffectors in immune challenged chicks exposed to 2,4-dinitrophenol, a mitochondrial uncoupler that increases metabolic rate.

MATERIAL AND METHODS

Animals and housing

Hatchlings and fertile eggs from *Gallus gallus domesticus*, lineage Cobb 500 were supplied by a local commercial hatchery (Pluma, Descalvado-SP, Brazil). Fertile eggs were incubated at 37.5^{o}C with 60% of relative humidity and were rotated every 2hs in an automatic incubator (Premium Ecologica, Belo Horizonte-MG, Brazil). On day 19 of embryonic development, eggs were transferred to a hatcher (Premium Ecologica, Belo Horizonte-MG, Brazil) kept at 37.5^{o}C and 70% humidity. Few eggs were incubated daily in order to have only a couple of hatchlings every day for respirometry experiments. Small batches of hatchlings were purchased multiple times for supplying all other procedures. All hatchlings were housed in temperature-controlled brooders (Premium Ecologica, Belo Horizonte-MG, Brazil) at 33^{o}C, a temperature that was progressively decreased to 30^{o}C until the 5th post-hatching day. The brooders were placed in a room with a 14h:10h light-dark cycle and the chicks were supplied with water *ad libitum* and standard food according to protocols detailed below. All experiments were performed in 5-day-old chicks (80 ± 10 g with no sex distinction) when they are known to present thermogenesis fully established and the ability to display regulated hypothermia and fever in response to endotoxin (Amaral-Silva et al., 2020; Amaral-Silva et al., 2021; Dantonio et al., 2016; Tazawa et al., 2004; Tzschentke and Nichelmann, 1999).

All procedures were previously approved by the local Animal Care Committee (CEUA-FCAV, protocol number 5140/17) in agreement with the guidelines of the National Council of Control in Animal Experimentation (CONCEA-Brazil).

Body temperature measurements

Three days before the experiment, chicks were anesthetized with isoflurane (3% for induction, 1% for maintenance in 100% of O_2) and a temperature sensor, biotag (BioTherm13, ~13 mm, 134.2 kHz FDX-B, Biomark, Boise-ID USA), was inserted into the celomatic cavity via an implanter syringe (AnimalTAG, São Carlos-SP, Brazil). Muscle and skin layers were then closed by surgical glue (Dermabond® Topical Skin Adhesive, Johnson & Johnson, São Paulo-SP, Brazil) and chicks received antibiotic (enrofloxacin, 10 mg/kg, intramuscular) and anti-inflammatory (flunixin meglumine, 2.5 mg/kg, subcutaneous) drugs to avoid infection and pain.

On the day of the experiment, Tb was measured by telemetry using a reader antenna, and data were recorded (Biomark HPR Plus™ Biomark, Boise-ID, USA).

Metabolic and ventilatory measurements

Oxygen consumption ($\dot{V}O_2$) was measured to indirectly access metabolic rate as previously described (Amaral-Silva et al., 2021). The $\dot{V}O_2$ of each chick inside a 3 L chamber allocated in a temperature-controlled room was measured via flow-through respirometry. Ambient air was pulled (MFS, Sable Systems, Las Vegas, NV, USA) at 1000 mL min^{-1} rate through the respirometer, into a water vapor pressure (WVP) analyzer (RH300; Sable Systems, Las Vegas, NV, USA). The outflow was then subsampled (160 mL min^{-1}; SS4, Sable Systems, Las Vegas, NV, USA), and sequentially pulled through a drying column (Drierite, Sigma Aldrich, St. Louis, MO, USA), into a calibrated O_2 analyzer (PA-10; Sable Systems, Las Vegas, NV, USA). All equipment was connected to an analogic-digital converter and signals recorded using PowerLab (LabChart; ADInstruments, Dunedin, Otago, New Zeland). The recordings were composed of 18 min of outflow analysis followed by 2 min of inflow analysis for baseline. $\dot{V}O_2$ was calculated using the following equation: $\dot{V}O_2 = [FRe\ (FiO_2 - FeO_2)] / [1 - FiO_2\ (1 - RER)]$, where FRe is the excurrent flow rate (outflow), FiO_2 is the incurrent fractional concentration of oxygen, FeO_2 is the excurrent fractional concentration of oxygen, and RER is the respiratory exchange ratio (considered 0.85; Koteja, 1996). All values were compared at standard temperature and pressure, dry (STPD).

Breathing frequency (f) was concurrently measured with $\dot{V}O_2$ using the barometric method. During $\dot{V}O_2$ baseline the chamber was closed and f measured using a pressure transducer (ADInstruments, Dunedin, Otago, New Zeland) connected to the experimental chamber. The pressure signal was then converted by an analog-digital converter (Powerlab, ADInstruments, Dunedin, Otago, New Zeland), and recorded inline using LabChart (ADInstruments, Dunedin, Otago, New Zeland). Breathing frequency was determined by counting the peaks of pressure waves.

Skin Temperature - Heat loss index

An infrared camera (FLIR E40, Wilsonville, OR, USA) connected in line with a computer using Flir Tools software (FLIR, Wilsonville, OR, USA), was positioned below a bottomless custom-made chamber built as described previously (Amaral-Silva et al., 2021) where chicks were accommodated. Infrared images were then used to measure skin temperature (Ts) from the inferior surface of the feet, a thermal window for birds (Amaral-Silva et al., 2021; Cristina-Silva et al., 2021; Hillman et al., 1982; McCafferty, 2013). Similarly, ambient temperature (Ta) was measured from thermal images of a black tape (emissivity 0.95) attached to the chamber's bottom close to the chick´s feet (Amaral-Silva et al., 2021; Tattersall, 2016).

The thermal images were analyzed using ThermaCam (FLIR, Wilsonville-OR, USA), and Ta, Ts, and Tb were used to calculate the heat loss index (HLI) as: HLI = (Ts − Ta) / (Tb − Ta) (Romanovsky et al., 2002). The HLI results in a range from 0 to 1 where 0 indicates maximum vasoconstriction and 1 maximum vasodilation.

Behavioral thermoregulation

Huddling behavior was analyzed as a thermoeffector for heat conservation, which is commonly observed in chicks during cold and fever challenges (Dantonio et al., 2016; Gilbert et al., 2010). One day before the experiment, a webcam (LifeCam Hd-300-Microsoft, Redmond, WA USA) was positioned above the brooders, and chicks were separated into groups of 5 individuals. On the next morning, the brooders were uncovered and the chicks were photographed using the time-lapse function of HandyAvi (AZcendant Mesa, AZ, USA). The total area occupied by a group of five chicks was calculated in the photos using ImageJ (FIJI). The average area that the chick occupy before injection was considered 100% and the changes in area were calculated relative to those initial values. A reduction of the area occupied by a group of chicks is indicative of huddling behavior.

Protocols

Chicks used in all the five protocols described below had systemic inflammation induced by intramuscular injection of 100 μg kg^{-1} of LPS (1 mL kg^{-1}; *E. coli*, O127:B8; Sigma, St. Louis-MO, USA) dissolved in pyrogen-free saline, or were injected with 1 mL kg^{-1} of pyrogen-free saline as a control. The chosen LPS dose applied intramuscularly was previously reported to

induce a biphasic thermal response in chicks (Amaral-Silva et al., 2020; Amaral-Silva et al., 2021; Dantonio et al., 2016). All experiments were conducted during the light phase between 7:00 am and 7:00 pm. The experiments were performed in four different conditions: 1) fed in thermoneutrality (30^{o}C; control for ambient conditions); 2) fasted in thermoneutrality; 3) fed in cold (25^{o}C) and; 4) fasted in cold. For fasting, food was taken from the brooder on the day preceding the experiments at the time lights went off (8:00 pm) since chicks naturally stop eating in the dark phase. On the following day, experiments started always at 7:30 am, standardizing 11:30h of food deprivation prior to the experiment (established after pilot experiments seeking fasting effect whilst keeping chicks' welfare). Chicks in the fed groups started eating at 6:00 am when the lights turned on in the chicks' facility, and food was offered *ad libitum* throughout the light phase. All ambient conditions were kept during the experiments for every chick, for example, chicks in the fasting groups were kept fasted during the whole experiment, having access to water only, while chicks in the fed groups had access to food and water during the analysis. Cold exposure (25^{o}C) started 90 min before and lasted the entire duration of the experiment. Since repeated injections of LPS are described to attenuate fever in mammals and birds (Bennett and Beeson, 1953; Branco et al., 2014; Dias et al., 2005; Gray et al., 2013), each chick was used for only one experiment, totalizing 441 chicks used for all experiments described in this study. At the end of each experimental protocol, chicks were killed using an isoflurane overdose followed by cervical dislocation to assure death.

I. Trade-offs influence on the LPS effect on body temperature: Chicks previously implanted with a temperature sensor were submitted to the different ambient conditions described above. Body temperature was recorded once by telemetry, the chicks were injected with LPS or saline and Tb was measured hourly for additional 6 hours after injection. Sixty-two chicks were used for this protocol

II. Trade-offs influence on the LPS effect on metabolic and respiratory rates: Chicks exposed to one of the four different environmental conditions were habituated inside the respirometry chamber for 30 min. After two concurrent measurements of $\dot{V}O_2$ and f, the respirometer was opened, the chick was injected with LPS or saline and immediately returned to

the chamber. $\dot{V}O_2$ and f were then measured for additional 240 min. A total of 49 chicks were used for this protocol.

Since relative humidity can interfere with heat loss, we analyzed WVP in the chamber during respirometry experiments (Fig S1). Similar WVP was observed in the chambers of chicks injected with saline and LPS regardless of the environmental challenge. Also, no difference in WVP was observed due to the feeding compared to the fasting protocol at 25°C or 30°C. As expected, the only factor affecting WVP was Ta, in which chicks at 30°C were in chambers with a slightly higher WVP (~3 kPa) than chicks at 25°C (~2.5 kPa) for most of the experimental period whether injected with saline or LPS. Since WVP is directly affected by temperature, we believe that the small difference in WVP (~0.5kPA) is intrinsic to Ta and may not affect any comparisons made in this study.

III. Trade-offs influence on the LPS effect on heat loss index: Chicks previously implanted with a temperature sensor were habituated in the chambers keeping the environmental conditions experimented for at least 40 min. After that, thermal images of the feet and body temperature were recorded every 15 minutes during the whole experiment. Two initial measurements preceded the injection of LPS or saline, which was followed by continuous Ts and Tb measurements for the next 240 minutes. This protocol was applied to 62 chicks.

IV. Trade-offs influence on the LPS effect on huddling behavior: Groups of 5 chicks kept at one of the different ambient conditions described above were photographed every minute for one hour. The chicks were rapidly taken from the brooder for injection of LPS or saline and returned to the brooder for an additional 240 min of image recording. Forty-eight groups with 5 chicks each were analyzed in this protocol totalizing 240 chicks. All 5 individuals in each group received the same treatment, LPS or saline.

V. Induction of mitochondrial uncoupling for testing thermogenic capacity during trade-off influence on the LPS effects: Only chicks challenged with fasting combined with cold were used in this protocol. 2,4-Dinitrophenol (DNP, Sigma, St. Louis, MO, USA), a drug known to enhance metabolic demand through mitochondrial uncoupling in many species, including birds, was used to pharmacologically increase metabolic rate of the chicks (Amaral-Silva et al., 2021;

Gleeson, 1986; Stier et al., 2014). DNP was administered 220 minutes after the LPS/saline injection because two reasons: i) chicks at a control environment (fed at 30°C) present fever at about 240 minutes (Fig2); and ii) DNP affects $\dot{V}O_2$ about 20 min after its injection in 5-day old chicks (Amaral-Silva et al., 2021).

For this protocol, 2 sets of experiments were performed. At the first, chicks previously implanted with a temperature sensor were placed in a respirometer and $\dot{V}O_2$, *f*, and Tb were concomitantly measured. An intramuscular injection of LPS or saline was carried after two initial measurements and the chicks returned to the respirometer for additional 220 minutes of analysis. The chicks were then intraperitoneally injected with 18 mg kg^{-1} of DNP dissolved in saline, resulting in 2 groups: Saline+DNP and LPS+DNP. After DNP injection, chicks were placed back in the respirometer for further 80 minutes of $\dot{V}O_2$, *f*, and Tb measurements (300 minutes after LPS/saline injection). A second group of chicks, also previously implanted with a temperature sensor, was acclimated for 40 min in the chambers for HLI measurements. After that, thermal images of the feet and body temperature were recorded with a 15 minutes interval. Two initial measurements preceded the injection of LPS or saline, and a second injection of 18 mg kg^{-1} of DNP was administered after 220 min of LPS/saline injection. Twenty-eight chicks were used for this protocol.

Statistical analysis

Data are shown as mean ± standard error (SEM). The number of animals used in each experiment (*n*) is indicated in the figure legends. The effects of the ambient conditions alone on Tb, $\dot{V}O_2$, HLI, *f*, and the area occupied by the chicks were analyzed using one-way ANOVA. Since $\dot{V}O_2$ changes allometrically with body mass, an analysis of covariance was performed to check whether the effect of the ambient conditions on $\dot{V}O_2$ was influenced by the body mass of the chicks in the different groups. The effect of different ambient conditions on the chicks' Tb, $\dot{V}O_2$, HLI, *f*, and behavioral responses to LPS or saline were analyzed using RM two-way ANOVA considering treatment x time as factors. For treatment, the ambient conditions were considered together with LPS/saline injections: "30°C-fed, saline"; "30°C-fed, LPS"; "30°C-fasted, saline"; "30°C-fasted, LPS"; "25°C-fed, saline"; "25°C-fed, LPS"; "25°C-fasted, saline"; "25°C-fasted, LPS". RM two-way ANOVA was also performed for DNP experiments considering the effects of the treatments ("Saline+DNP" and "LPS+DNP") x time. The

differences among the averages were evaluated by Tukey´s post hoc test. Significant differences were considered for $p<0.05$.

RESULTS

Effect of metabolic trade-offs on the chicks' body temperature and thermoeffectors

We first evaluated how the ambient conditions alone affected the chicks' Tb and thermoeffectors. Body temperature (Fig 1A) was not significantly affected by the environmental competing demands ($p=0.063$). Challenged chicks also present a similar $\dot{V}O_2$ (Fig 1B) to the chicks in the control condition (fed at 30°C), however, a slight increase in $\dot{V}O_2$ in fed chicks at 25°C and a slight decrease in $\dot{V}O_2$ in fasted chicks at 25°C resulted in difference between these two groups ($p=0.033$). Breathing frequency (Fig 1C) was slightly decreased by fasting in general and fasted chicks at 25°C or 30°C had lower f than fed chicks at 25°C ($p=0.002$ for both). The chicks' HLI indicated progressive peripheral vasoconstriction along with the increase of competing energetic demands (Fig 1D). Compared to fed chicks at 30°C (controls), fasted chicks at 30°C lowered HLI by 49% ($p=0.012$), fed chicks at 25°C decreased HLI by 72% ($p<0.001$), and fasted chicks exposed to 25°C presented the most dramatic HLI decrease, 92% ($p<0.001$). In the same way, chicks adopted a huddling behavior to cope with fasting and cold (Fig 1E). The area occupied by challenged chicks was 31%, 24%, 50% smaller for those fasted at 30°C, fed at 25°C, and fasted at 25°C compared to fed chicks at 30°C ($p<0.001$ for all). Fasted chicks at 25°C also occupied a smaller area compared to chicks fasted at 30°C ($p=0.008$) and fed at 25°C ($p<0.001$).

Effect of metabolic trade-offs on the thermal response to LPS

Next, we evaluated the Tb and thermoeffectors ($\dot{V}O_2$, f, HLI, and huddling behavior) launched in response to an immune challenge by chicks under reduced energy supply and/or increased energy demand. Fed chicks at 30°C presented a biphasic thermal response to LPS (Fig 2A) in which Tb decreased 60 min after injection compared to the onset (up to -0.6°C, $p=0.011$) and at 60 and 120 min compared to saline chicks ($p=0.005$ for both). The Tb drop was followed by an increase in Tb at 240 and 360 min compared to the initial values (up to 0.6°C, $p=0.019$ and 0.004) and from 240 to 360 min compared to saline ($p<0.001$ to 0.014). For fasted chicks at 30°C

(Fig 2B), LPS injection also caused a decrease in Tb at 60 min compared to the onset (-0.8^{o}C, p=0.001), and Tb was higher from 180 to 360 min compared to saline treatment (p<0.001 for all) but not higher than the initial values. This probably occur because fasting *per se* decreased Tb from 120 to 360 min compared to initial values (p=0.001 to 0.009) as observed in saline-injected chicks at 30^{o}C. Fed chicks at 25^{o}C (Fig 2C) decreased Tb in response to LPS at 60 and 180 min compared to the onset (up to -1.3^{o}C, p<0.001 to 0.008), and Tb was lower than the saline group from 60 to 180 min (p=0.002 to 0.045) with no subsequent fever, differing from fed chicks at 30^{o}C. Finally, fasted chicks at 25^{o}C (Fig 2D) also had only a sharp Tb drop in response to LPS from 60 to 180 min compared to initial values (up to -2^{o}C, p<0.001 for all) and to saline chicks at 60 and 120 min (p<0.001 and 0.006), with no fever response. Fasted 25^{o}C chicks injected with saline decreased Tb at 300 and 360 min compared to the onset (p<0.001 to 0.032). When saline chicks from different ambient conditions were compared, it made clear the fasting effect on Tb at both Tas since fasted chicks reduced Tb from 60 to 360 min whether at 30^{o}C (p<0.001 to 0.032) or 25^{o}C (p<0.001 to 0.012) compared to fed chicks at 30^{o}C (Fig 2E). Among LPS treated chicks, the fasted ones at 25^{o}C had a greater decrease of Tb (-1.5^{o}C) than control chicks (fed at 30^{o}C) at 60 and 120 min (p=0.008 and 0.016) (Fig 2F).

Oxygen consumption was decreased in the first hour after LPS injection in every ambient condition, which was followed by a subsequent increase of $\dot{V}O_2$ towards initial values. Fed chicks at 30^{o}C decreased $\dot{V}O_2$ 60 min after LPS injection compared to initial values (-27%, p=0.001) and at 40, 60 and 140 compared to saline ones (p=0.006, 0.002 and 0.022; Fig 3A). When chicks kept at 30^{o}C were fasted, they also decreased $\dot{V}O_2$ in response to LPS at 60 min compared to initial values (-26%, p<0.001), and to saline (p=0.027; Fig 3B). LPS-injected fed chicks at 25^{o}C had lower $\dot{V}O_2$ than saline-injected chicks at 40, 60, and 80 min after injection (p=0.007, 0.018, and 0.018, Fig 3C). Also, fasted chicks at 25^{o}C decreased $\dot{V}O_2$ at 40 min when injected with LPS compared to initial values (-26%, p<0.001) and at 40 and 60 min compared to the saline group (p<0.001 and 0.005 respectively; Fig 3D). There was no effect of different ambient conditions alone on chicks' $\dot{V}O_2$ whether injected with saline (p=0.586; Fig 3E) or LPS (p=0.352; Fig 3F).

Concurrently to the decrease in $\dot{V}O_2$, LPS-injected chicks had a higher *f* than the saline ones at 60 min when fed at 30°C (p=0.039; Fig4A), fasted at 30°C (p=0.018; Fig4B) and fed at 25°C (p=0.045; Fig 4C). Additionally, fed chicks at 30°C had higher *f* than saline ones at 100 min (p=0.041). Fasted chicks at 25°C did not show a significant difference in *f* when injected with LPS (p=0.142; Fig 4D). Competing environmental demands did not affect the *f* of chicks injected with saline (p=0.251; Fig 4E) or LPS (p=0.422; Fig 4F).

Peripheral vasomotion contributed differently to the thermoregulation of immune-challenged chicks depending on the ambient condition they were exposed to. For fed chicks at 30°C, LPS caused vasoconstriction on the feet (lower HLI) during most time between 90 and 240 min, compared to the saline ones (p=0.008 to 0.047) and at 180 and 210 min compared to the onset (p=0.038 and 0.041; Fig 5A and 5B). Fasted chicks injected with LPS at 30°C also presented lower HLI than saline ones from 135 to 175 min (p=0.003 to 0.025; Fig 5C and 5D). On the other hand, LPS-treated fed chicks at 25°C showed peripheral vasodilation (higher HLI) at 45 and 60 min compared to saline chicks (p<0.001 and 0.004; Fig 5E and 5F). Similarly, fasted chicks at 25°C increased HLI at 45 min (395%, p<0.001, Fig 5G and H) compared to the initial values, and at 15 and 45 min compared to saline ones (p=0.038 and <0.001). The ambient condition alone affected the HLI of chicks injected with saline (Fig 5I). Compared to fed chicks at 30°C, fasted animals at 30°C had lower HLI at 90 min (p=0.008) while both groups at 25°C presented a lower HLI from -15 to 240 min whether fed (p<0.00 to 0.032) or fasted (p<0.0001 to 0.003). Regarding LPS injected chicks, there was an initial difference of HLI among ambient conditions but from 90 min after LPS injection until the end of the experiment, all groups had similar HLI (Fig 5J). The HLI of 30°C fed chicks was higher compared to 30°C fasted chicks at 15 and 45 min (p=0.014 and 0.032), 25°C fed chicks from -30 to 45 min (p<0.001 to 0.041); and 25°C fasted chicks from -30 to 75 min (p<0.001 to 0.037).

Huddling behavior was used as a heat conservation mechanism in chicks exposed to all environmental conditions but the chicks separation for heat loss was observed only when they were fed in a neutral condition. Fed chicks at 30°C that were injected with LPS occupied a larger area compared to saline-injected chicks from 44 to 56 min (p=0.11 to 0.037), time that preceded the decrease in body temperature at 60 min (Fig 2A). In sequence, they started to huddle to increase body temperature, occupying a smaller area than the saline controls for the first time at 84 min, since then, kept huddled for 85% of the time until the end of the experiment (p<0.001 to

0.036; % calculated from the 1st area significantly reduced until the end of the experiment, at 240 min). Compared to the initial values, fed chicks at 30°C injected with LPS also decreased the occupied area during 35% of this same period (1st point of reduced area until the end of the experiment, p=0.007 to 0.032; Fig 6A). Fasted chicks at 30°C first huddled after 80 min of LPS injection, and from this moment until the end of the experiment, occupied a smaller area than the saline chicks for 46% of the time (p<0.001 to 0.039; Fig 6B). When fed chicks at 25°C were injected with LPS they occupied a smaller area than their controls for 55% of the time, from 56 min until the end of the experiment (p=0.001 to 0.042; Fig 6C), and decreased the area occupied compared to the initial values during 23% of this period (p=0.003 to 0.048; Fig 6C). Fasted chicks at 25°C treated with LPS occupied a smaller area than their saline pairs for 42% of the time starting at 44 min until the end of the experiment at 240 min (p<0.001 to 0.044, Fig 6D). The saline treatment *per se* did not induce huddling independent of the environmental condition (Fig 6E). In general, LPS seems to cause a similar response among the different conditions, except for the time 30°C fed chicks increased the area occupied for heat loss. On this occasion, the area occupied by fed chicks at 30°C was larger than the area occupied by fed chicks at 25°C from 40 to 76 min (p<0.001 to 0.027) and by fasted chicks at 25°C from 56 to 64 min (p<0.001 to 0.032).

Thermogenesis ability during fever elimination

To investigate if the chicks that eliminated the fever response to LPS were able to increase metabolic rate for thermogenesis, chicks at the most challenging ambient condition (fasted at 25°C) were treated with DNP, a mitochondrial uncoupler known to increase metabolic rate of birds (Amaral-Silva et al., 2021; Gleeson, 1986; Stier et al., 2014). This injection was carried 220 min after the saline/LPS injection, time that fever starts in fed chicks at 30°C (Fig 2). Regulated hypothermia was confirmed in this group before the DNP injection. $\dot{V}O_2$ decreased up to 28% after 60 and 80 min of LPS injection compared to the onset (p<0.01 and 0.004) and at 60 min compared to saline chicks (p=0.007, Fig 7A). Tb also decreased from 60 to 80 min compared to saline chicks (p=0.036 to 0.043) and from 60 to 90 and 120 min compared to the initial values (p=0.006 to 0.047, Fig 7C). Breathing frequency was unchanged by the LPS treatment (p=0.807, Fig 7B) as well as HLI, which did not significantly differ from the saline group despite a clear increase after LPS injection (Fig 7D). DNP was injected subsequently to

the measurement of $\dot{V}O_2$ and f at 220 min, thus the 220 min measurements were used as the references for DNP effects on $\dot{V}O_2$, f, and Tb. HLI was measured in different groups of chicks with a 15 min interval, thus 210 min was the last measurement before DNP injection and this time point was used as a reference for DNP in these groups.

Twenty min after the DNP injection (240 min of the experiment), chicks increased $\dot{V}O_2$ by 20% both for the group pre-injected with saline (p=0.041) and LPS (p=0.048) compared to the values before DNP injection (220 min). At the same time, f increased 27% for the saline+DNP group (p=0.679, non-significant) and 51% for the LPS+DNP group (p=0.009) compared to 220 min. Due to DNP injection, chicks also presented a dramatic increase in HLI. Saline chicks increased HLI from 240 to 285 min compared to values before DNP injection (210 min, p<0.001 to 0.002), and also compared to initial values (p<0.001 to 0.016), while chicks pre-treated with LPS increase HLI from 240 to 300 min compared to 210 min (p<0.001 to 0.009) and compared to the onset (p<0.001 for all). Body temperature was lower than the initial values at 240 and 255 min for the saline+DNP chicks (p=0.016 and 0.007) and from 240 to 300 min for LPS+DNP injected chicks (p<0.001 to 0.002). The LPS+DNP group also presented a decrease in Tb at 240 min (20 after DNP, p=0.044) compared to the last measurement before DNP injection at 220 min. The saline+DNP chicks seemed to recover faster from the DNP-induced Tb drop and had higher Tb than the LPS+DNP injected chicks from 280 to 300 min (p=0.013 to 0.033, Fig 7C).

DISCUSSION

Our data support the idea that chicks change their thermoregulatory strategy during systemic inflammation in case of environmental energy trade-offs. Using the model of biphasic thermal response to endotoxin (characterized by initial regulated hypothermia followed by fever), we observed that increased energy demand (cold) alone or together with reduced energy supply (fasting) favor regulated hypothermia and inhibit fever. Chicks challenged with cold and cold combined to fasting presented pronounced regulated hypothermia by suppressing thermogenesis and promoting thermolysis. Even though fasted chicks in cold are capable of increasing metabolic rate (demonstrated with DNP) in a phase correspondent to the fever

observed in the chicks at 30°C, no additional energy was relied for thermogenesis and the fever phase was completely eliminated in those groups at 25°C.

The changes in the ambient conditions applied in the present study represented a challenge for our chicks. Despite chicks in challenging ambient conditions had similar Tb and $\dot{V}O_2$ to the control group, they responded autonomically and behaviorally to fasting and cold. The small decrease in f of the fasted chicks in comparison to the fed ones (Fig. 1C) followed the same pattern of the slight decrease in $\dot{V}O_2$ during fasting (Fig. 1B). Thus, f, as a ventilation component, may decrease to match a lower oxygen supply requirement, not in order to reduce respiratory heat loss. On the other hand, a decrease in HLI shows that all the trade-offs applied triggered activation of vasoconstriction to impair peripheral heat exchange. This response occurs in a range where vasoconstriction was progressively increased from fasted chicks at 30°C (relatively mild vasoconstricted), then fed at 25°C, and finally fasted chicks at 25°C (completely vasoconstricted), reflecting a progression on the challenging intensity for the chicks. Peripheral vasoconstriction is indeed a broadly described mechanism used for heat conservation in birds not only when they are exposed to cold (Ederstrom and Brumleve, 1964; Johansen and Bech, 1983; Steen and Steen, 1965), but also in response to fasting, possibly saving energy in this condition (Winder et al., 2020). The huddling behavior was also an intense response to fasting, cold, and fasting and cold together, which was the group occupying the lowest area. A reduced surface area to the surrounding temperature conserves heat among the individuals in the group contributing to significant energy saving when exposed to cold (Gilbert et al., 2010; McKechnie and Lovegrove, 2001; Mortola, 2021; O'Connor, 1975). In the case of fasting, huddling behavior may also be triggered for energy saving, similar to what is observed in mice and rats, which seek warm temperatures when fasted (Craig et al., 2021; Sakurada et al., 2000; Yoda et al., 2000). For birds, huddling behavior triggered by fasting *per se* has not been shown previously, as far as we know, but it is considered vital for the emperor penguins' survival during approximately 4 months of fasting while incubating the eggs during winter (-28°C on average) (Le Maho et al., 1976).

Environmental challenges have been shown to interfere in some components of the acute phase response to an immune challenge in birds, suggesting that the energetic outcome invested for pathogen resistance depends on the metabolic status and reserves (Ashley and Wingfield, 2011; Evans et al., 2017). Our study integrates the ideas that the thermal component of an acute

phase response is related to the metabolic status of a bird and that a switch from fever to regulated hypothermia occurs when the bird faces a severe immune challenge. The competing environmental demands employed in this study generated a spectrum of metabolic trade-offs, in which the costs for maintenance seem to increase progressively in comparison to the energy resources/reserves available for the chick. In this spectrum, the fasting protocol alone used in the present study characterized a mild metabolic challenge, cold represented a bigger challenge, and cold combined with fasting was the most severe stimulus. Consequently, chicks progressively reduced the energy spent with fever and migrated from a thermal biphasic response to regulated hypothermia only, which was prioritized during severe trade-offs, as discussed below.

Metabolic trade-offs enhance hypothermia in chicks treated with LPS

Metabolic suppression was the primary thermoeffector for Tb drop in immune challenged chicks, which occurred similarly for chicks at all experimental conditions. Indeed, we have shown that chicks are able to display Tb and $\dot{V}O_2$ drop in response to high doses of LPS both in warm and cold conditions and that these responses are regulated, not resultant from metabolic failure (Amaral-Silva et al., 2021). Here, besides confirming the key role of $\dot{V}O_2$ drop during Tb decrease in response to LPS, we show that it occurs even for the most challenged chicks (fasted in cold). Thus, the results support the idea of metabolic suppression for reducing thermogenesis as a strategy to save energy and reduce Tb, even when heat loss is already facilitated by the cold environment. An increase in breathing frequency was also observed during the Tb drop in 30^{o}C fed and 30^{o}C fasted chicks injected with LPS compared to the saline group, indicating a possible activation of panting, a known mechanism for evaporative heat loss in many birds (Arad and Marder, 1982; Bícego and Mortola, 2017; McKechnie et al., 2016). Here, this mechanism is apparently evoked when needed to aid the cooling force, which seems to be unnecessary to decrease Tb in chicks at 25^{o}C. In the same way, only chicks fed at 30^{o}C occupied a larger area during the regulated hypothermia caused by LPS, a thermolitic behavior in case of heat stress (Alsam and Wathes, 1991) that also seem to be activated only when needed. Contrasting, the non-evaporative thermolytic effector peripheral vasodilation (McCafferty, 2013; Scott et al., 2008; Tattersall et al., 2009) was activated to support the decrease of Tb for chicks exposed to cold (fed or fasted) but not for chicks in thermoneutral condition. These responses corroborate our previous study in which a high thermal gradient between body and environment is required

to activate peripheral vasodilation during LPS-induced Tb drop (Amaral-Silva et al., 2021). At 30°C, chicks showed higher HLI than 25°C groups up to 75 min, a possible reason for no increase in HLI to assist regulated hypothermia in these groups. We further speculate that the higher ambient temperature might result in a different stimulus for the thermoreceptors resulting in alternative thermoeffectors recruited for heat loss during the LPS-induced regulated hypothermia. In any case, peripheral vasodilation seems an important mechanism for the LPS-induced heat loss since fasted chicks at 30°C displayed smaller decreases in Tb than chicks in cold, whether fed or fasted. The increased HLI in birds challenged with cold combined with fasting also corroborates the idea of a regulated origin of the Tb decrease to reduce energy expenditure during severe inflammation.

Different from adult rodents, which depend on a trade-off with cold or fasting to display regulated hypothermia in response to endotoxin (Ganeshan et al., 2019; Krall et al., 2010), our immune-challenged chicks decreased Tb in all ambient conditions they were exposed, even when fed at thermoneutrality. The chicks' ability to drop Tb in thermoneutral conditions may be then related to the higher basal metabolic rate and Tb of birds compared to mammals, as well as the early life costs for growth in chicks compared to adults, which imply that being a young bird might already represent a trade-off with fever costs (Clarke and Pörtner, 2010; Legendre and Davesne, 2020; Mortola and Maskrey, 2011; Tickle et al., 2018).

Metabolic trade-offs inhibit LPS-induced fever in chicks

The febrile response to LPS was supported by peripheral vasoconstriction in chicks exposed to 30°C, whether fed or fasted, observed by the clear reduction in HLI. At thermoneutrality, peripheral vasoconstriction seems to have a predominant role to increase Tb without activation of extra thermogenesis in chicks. This is indeed considered a low-cost mechanism that birds use for heat conservation (Cabanac and Aizawa, 2000; Tattersall et al., 2009). During cold exposure, the initial maximum vasoconstriction in fed and fasted chicks precluded a further decrease in HLI at the time metabolic rate returned to the pre-LPS injection values, and no fever was observed in these groups. Despite peripheral vasoconstriction was not an option available to increase Tb in these chicks, if fever was the elicited response, thermogenesis could be activated (Amaral-Silva et al., 2020), but it did not occur here. The absence of a further thermogenic activation, in this case, seems not to be caused by a metabolic

limitation since even the most challenged chicks (fasted at 25°C) were able to increase metabolic rate after DNP injection. This confirms that an increase in metabolic rate would be possible for 25°C-exposed chicks at least for a while during the phase correspondent to fever in controls, thus, the absence of fever seems to be a regulated event. In fact, the chicks that had $\dot{V}O_2$ increased by DNP displayed a hypothermic response, resultant of the activation of thermolytic responses such as tachypnea (Fig. 7B) and cutaneous vasodilation (Fig. 7D, E) facilitating the Tb drop in the cold. Therefore, the heat produced by DNP injection was not conserved but antagonized by strong activation of heat loss mechanisms, which reinforces the idea of a regulated inhibition of fever in case of competing energy demands. We believe that hypothermia was caused by the DNP treatment because maintain (saline group) or increase metabolic rate towards initial value (LPS group) for fasted chicks at cold may already be an expensive event. Once DNP generated a further increase in metabolic rate, hypothermia was activated as a defense mechanism, which is usually seen in birds suffering from unfavorable metabolic conditions caused by food shortage, drought, or short-day cycles (Geiser, 2010; Hiebert, 1990; Laurila et al., 2005; Ruf and Geiser, 2015). Our results also resemble a study in food-restricted rats treated with LPS doses 400x higher than the dose used to cause regulated hypothermia, which resulted in a second decrease in Tb after 180 min of the injection (Krall et al., 2010). For these rats, hypothermia seems also to occur due to the unfavorable metabolic condition caused by the high LPS dose combined with the environmental challenges.

When chicks have the opportunity to express behavioral thermoregulation, it became a significant mechanism for fever induction. This corroborates with our previous results showing the essential participation of huddling in fever since LPS-injected chicks in thermoneutrality may not increase Tb when alone, but do it when in a group (Amaral-Silva et al., 2020; Dantonio et al., 2016). Interestingly, in the present study, all groups of chicks reduced the occupied area after LPS exposure, regardless of a Tb increase for fever. Noticeable, however, was that the higher severity of environmental challenge resulted in earlier huddle. Chicks fed at 30°C started huddling 84 min after LPS, while fasted chicks at 30°C showed this response 76 min after LPS, chicks fed at 25°C started at 60 min, and chicks fasted at 25°C took only 48 min to start huddling behavior. In this case, it is possible that the energetic component of this response is playing a bigger hole than the instant thermoregulatory component. Return to euthermia after Tb reduction is considered an energetically expensive event for rodents after a LPS-induced Tb drop, and also

for birds after daily torpor (Ganeshan et al., 2019; Hiebert, 1990). Thus, for challenged groups that did not present fever, the earlier huddling may be launched to prevent extra energy expenditure to return $\dot{V}O_2$ and Tb to an euthermic state and not for fever production. Indeed, birds are known to launch pre-emptive heat loss conservation mechanisms before experiencing a shortfall in energy reserves (Winder et al., 2020). Thus, chicks experiencing a bigger energetic trade-off may have urged to use behavior to be able to return Tb to euthermy, which seems to be crucial for survival since mice that fail to recover from LPS-induced hypothermia present tissue dysfunction in multiple vital organs (Ganeshan et al., 2019). Alternatively, we acknowledge that behavior is a complex trait that reflects the sum of stimuli that an animal is receiving at the moment. In this way, we speculate that the huddling observed here may also be a component of sickness behavior and if this is the case it may be activated by a different mechanism than other thermoeffectors. For example, in rats, lethargy and loss of appetite occur during the LPS-induced hypothermia, which seems to be driven by a different pathway than thermoregulation considering that Tlr4 knockout mice still present such responses to LPS, while the decrease in Tb and metabolic rate are completely inhibited (Ganeshan et al., 2019).

Overall, the presence of regulated hypothermia and the elimination of fever observed in our chicks exposed to cold with or without food restriction corroborate findings in some immune-challenged passerines, which show only Tb drop that is sequentially recovered to euthermic levels with no fever (King and Swanson, 2013; Owen-Ashley et al., 2006; Sköld-Chiriac et al., 2015). Despite the high metabolic cost for maintenance and limited energy storage in passerines (Hohtola, 2012) can *per se* represent an energetic trade-off for the acute phase response, some studies had also shown the influence of different ambient conditions on the thermal response to an inflammatory stimulus, which adds support to our results. For example, zebra finches (*Taeniopygia guttata*) present hypothermia when LPS is injected during the light phase of the day when the birds are active and supposedly spending more energy, but show fever when injection occurs at night (resting phase) (Sköld-Chiriac et al., 2015). Additionally, black-capped chickadees (*Poecile atricapilus*) treated with LPS show a larger decrease in Tb when food is restricted every other day (Cornelius et al., 2017).

Conclusion and perspectives

Our data support that the metabolic status and the energy budget interfere in the thermal responses to an immune challenge in birds. Chicks that were not challenged with metabolic trade-offs present a biphasic thermal response characterized by initial regulated hypothermia followed by fever during systemic inflammation induced by high doses of LPS. In case of increased energetic demand combined or not with reduced energy supply, chicks launch pronounced regulated hypothermia and eliminate fever avoiding its high costs.

In natural environments, the immune challenge will most likely occur together with environmental challenges, which have been recognized to interfere in some components of the acute phase response such as sickness behavior, anorexia, and thermal response of immune challenged birds in wild and captivity (Bonneaud et al., 2003; Nord et al., 2020; Owen-Ashley and Wingfield, 2006; Owen-Ashley et al., 2006; Owen-Ashley et al., 2008; Ruhs et al., 2019; Sköld-Chiriac et al., 2015). Indeed even in a controlled condition, birds may face challenges to display the inflammatory response such as the accelerated growth in the modern poultry industry (Bennett et al., 2018), hygiene stress due to ammonia accumulation (Shah et al., 2020), and the stress caused by small space in layer chicken cages (Mashaly et al., 1984). In the present study, we showed that when the ambient factor represents an energetic trade-off to the fever costs, the bird may elicit an alternative thermal response to the immune challenge. In this way, our data provide valuable input to understand how integrated factors from the environment influence the bird's thermal response to an infection.

Besides that, both fever and regulated hypothermia seem to be conserved responses to an immune challenge among vertebrates (Amaral-Silva et al., 2021; Garami et al., 2018; Kluger et al., 1996; Merchant et al., 2008), thus, our data could contribute to understanding thermoregulation during inflammation in other species and phases of life. For example, thermal responses to endotoxins in adult mammals are affected by the environment (Ganeshan et al., 2019; Garami et al., 2018; Krall et al., 2010) but as far as we know, this effect throughout the development was not studied yet. In this way, our results shed light on the effect of metabolic trade-offs on the immune response in endotherms during early life, a phase in which growth requires high energy expenditure. Yet, our study can aid in understanding the thermal response to immune challenge in animals that Tb is more directly influenced by the environment since even ectotherms seem to regulate preferred Tb response to an immune stimulus based on its metabolic

status. For example, LPS challenged iguanas present behavioral fever when in prime energetic condition but select colder Tas when treated with the same LPS dose if energy reserves are not sufficient to sustain metabolism associated with the acute phase response (Deen and Hutchison, 2001). Also, snails (*Planorbarius corneuscan*) present a cold-seeking behavior in case of parasitic infection (Zbikowska and Cichy, 2012). This raises interesting questions on the evolutionary nature of regulated hypothermia as a thermal response to immune challenges, which shall be addressed in future studies.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

L.A.S. and K.C.B. conceived the study, K.C.B provided resources for the study. L.A.S and W.C.S. performed the experiments, L.A.S analyzed data, L.A.S., K.C.B, and L.H.G interpreted data, L.A.S., K.C.B, W.C.S. and L.H.G wrote the manuscript.

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DATA AVAILABILITY

Data supporting the present study are available from the corresponding author upon reasonable request.

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Figures

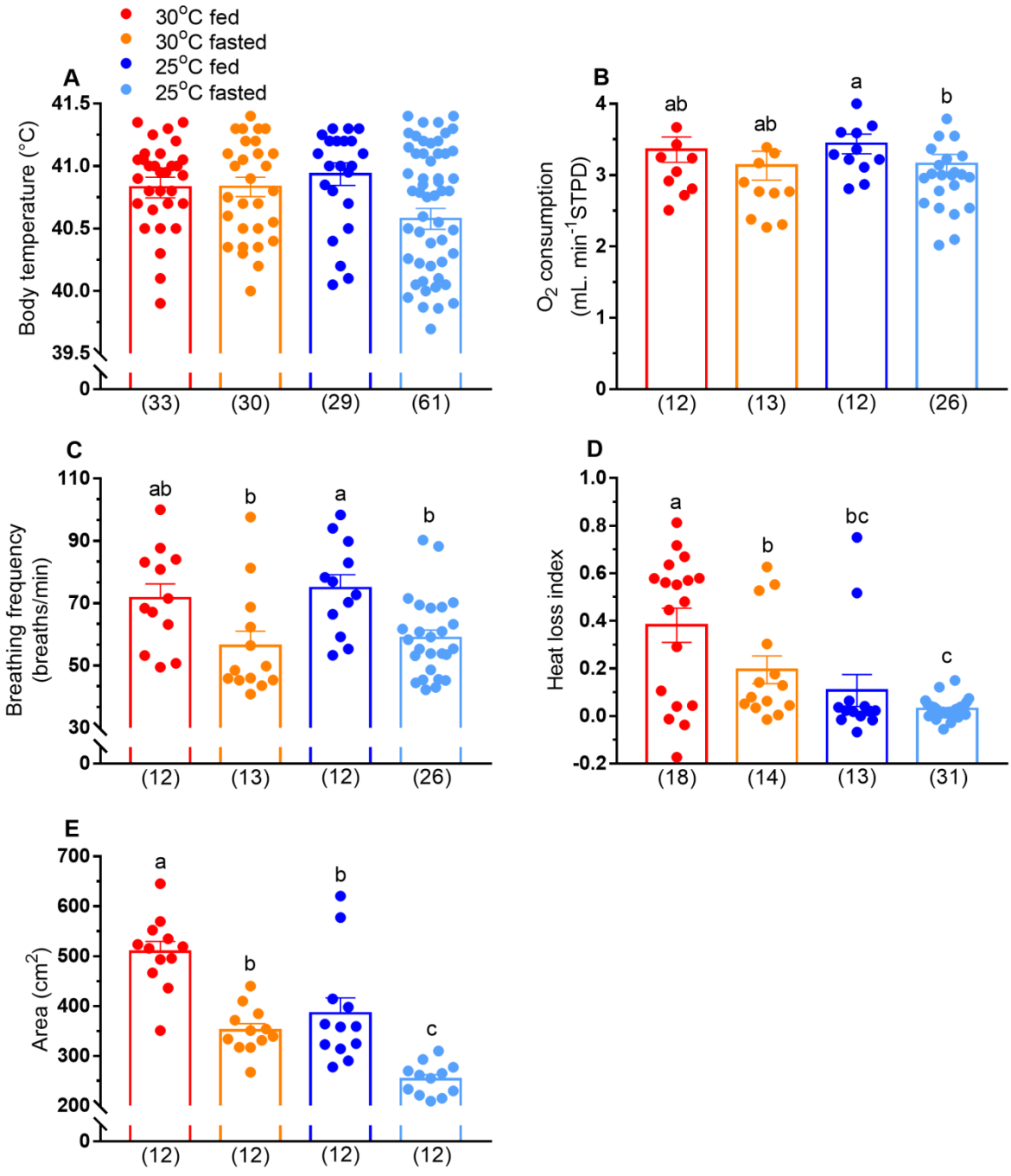

Figure 1. Body temperature (A), oxygen consumption (B), breathing frequency (C), heat loss index (D), and area occupied (E) by chicks exposed to competing environmental demands before saline or LPS injections. Data are means ± SEM. The number of subjects (A-D) or groups of 5 individuals (E) is shown in parenthesis. Different letters indicate significant differences among treatments ($p \leq 0.05$).

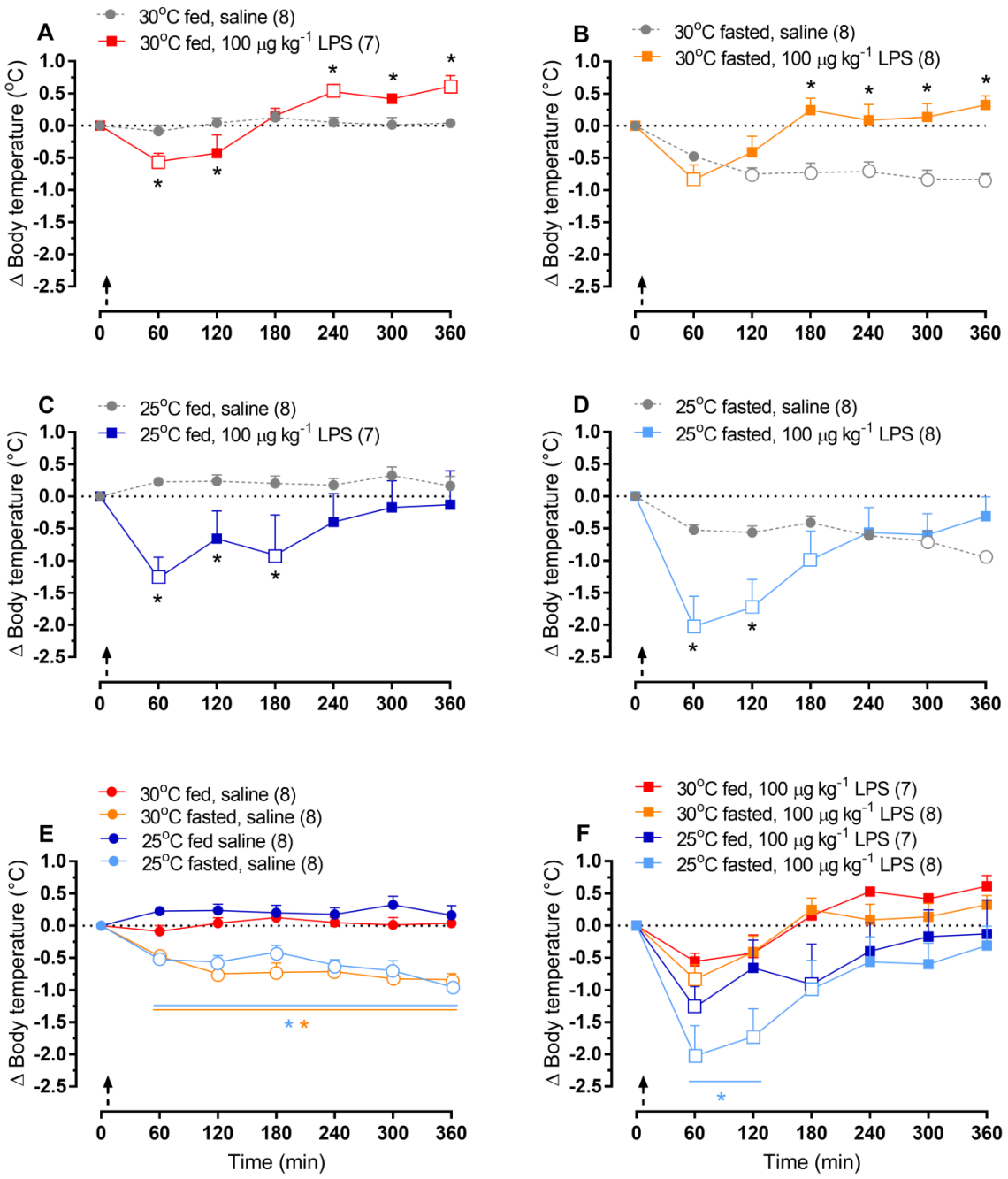

Figure 2. Ambient condition affects the chicks' thermal responses to an immune challenge. Lipopolysaccharide (LPS) effects on body temperature (Tb) of fed chicks at 30°C (A), fasted chicks at 30°C (B), fed chicks at 25°C (C), and fasted chicks at 25°C (D). Bottom panels show the effect of the ambient conditions on Tb of chicks respectively injected with saline (E) or LPS (F). Data are means ± SEM. Number of subjects is shown in parenthesis. Dashed arrows indicate

injection time. Open symbols represent statistical differences from initial values (0 min) within the same group. * represents difference from saline chicks (A to D). At the bottom panels * and * represent differences of the groups 30^{o}C fasted and 25^{o}C fasted, respectively, to the group 30^{o}C fed (control for ambient conditions). Delta temperature from pre-injection values.

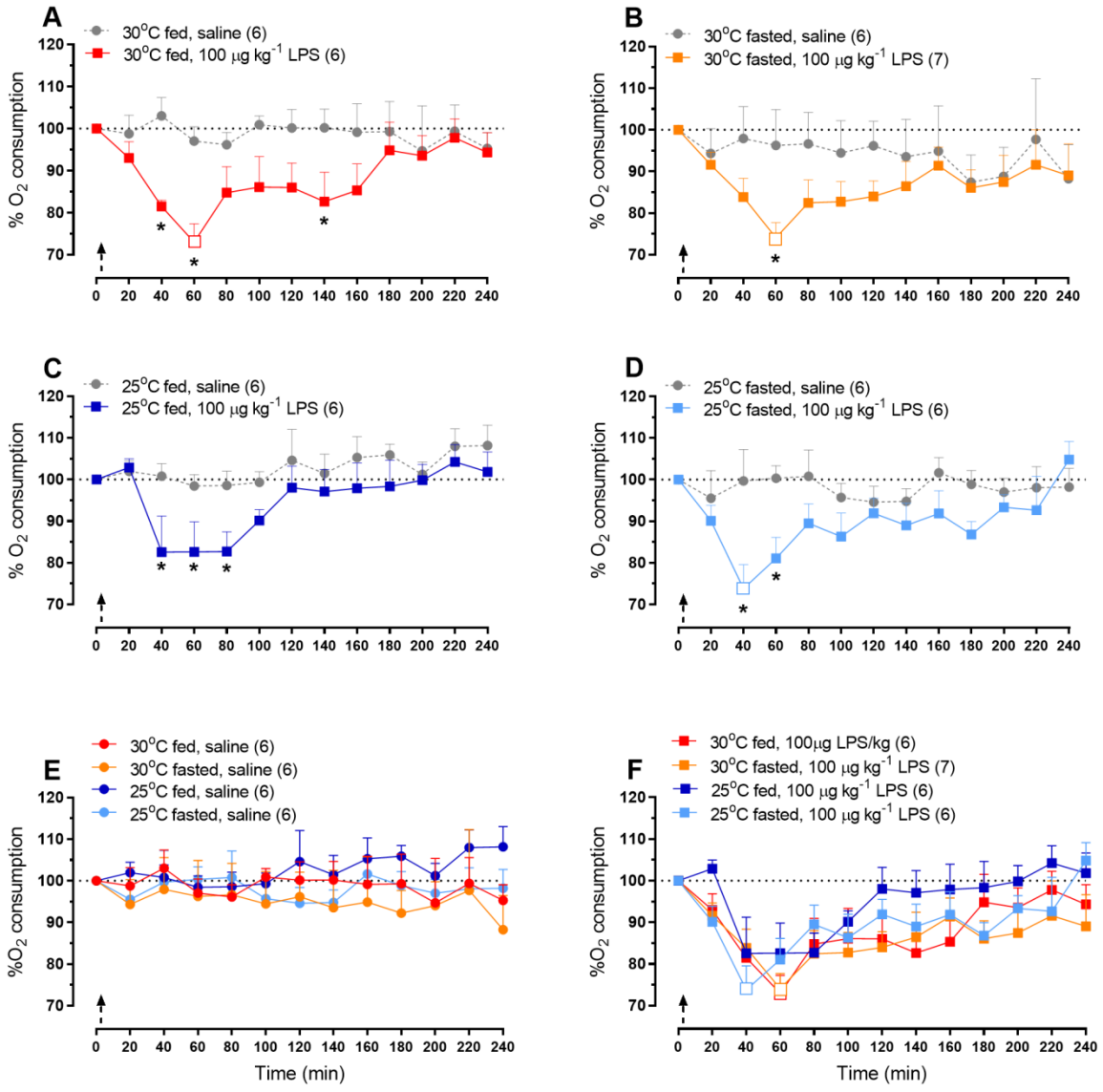

Figure 3. Changes in oxygen consumption (% from pre-injection values) in response to lipopolysaccharide (LPS) in chicks exposed to different ambient conditions. A) Fed chicks at 30°C. B) Fasted chicks at 30°C. C) Fed chicks at 25°C. D) Fasted chicks at 25°C. E and F) Effect of the ambient conditions in chicks treated with saline (E) or LPS (F). Data are means ± SEM. Number of subjects is shown in parenthesis. Dashed arrows indicate injection time. Open symbols represent statistical differences from initial values within the same group. * represents difference between treatments at the same time.

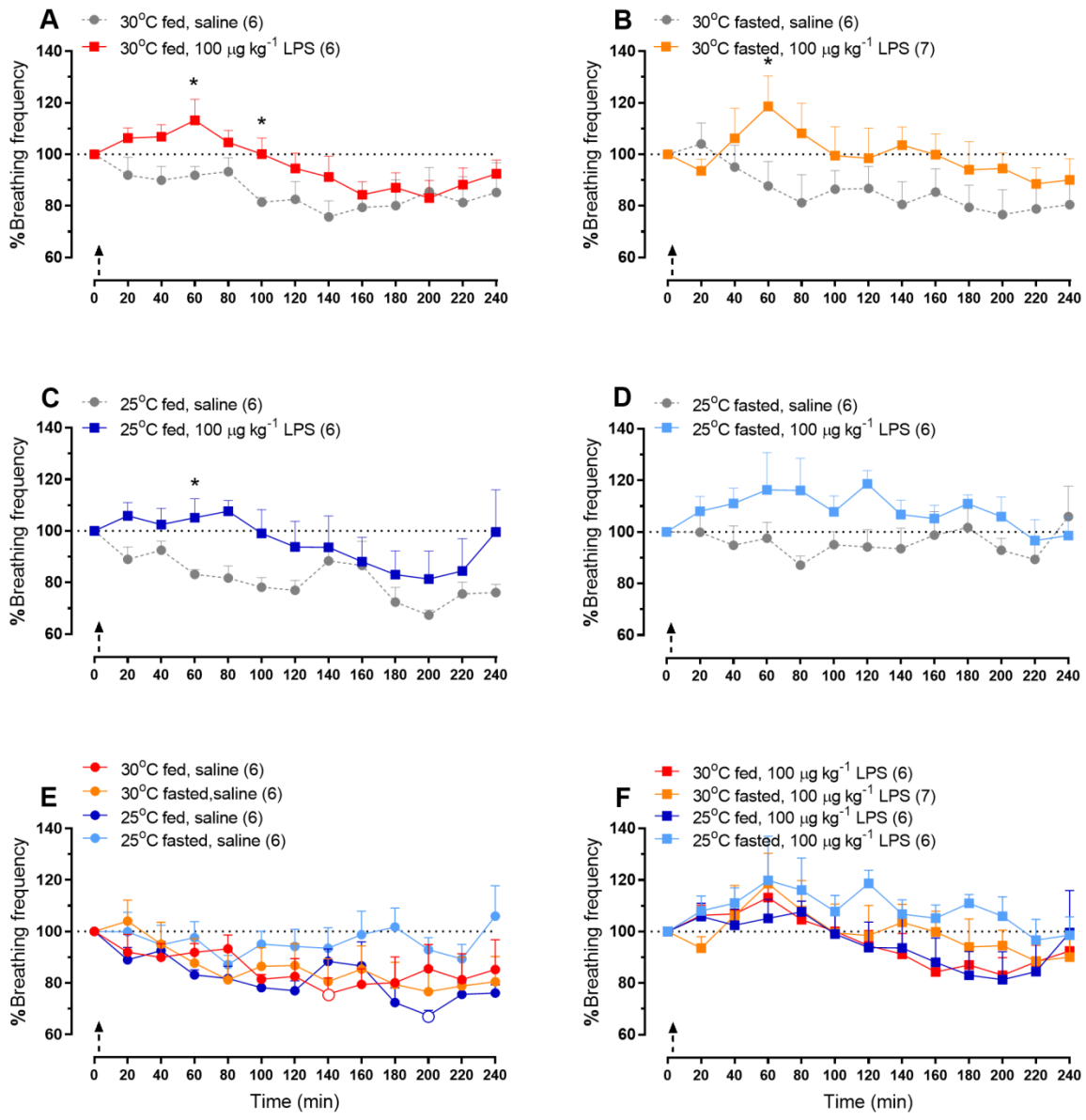

Figure 4. Changes in breathing frequency of lipopolysaccharide (LPS) challenged chicks exposed to different ambient conditions. Top and middle panels – LPS and saline effects in fed chicks at 30°C (A), fasted chicks at 30°C (B), fed chicks at 25°C (C), and fasted chicks at 25°C (D). Bottom panels - different ambient conditions effects in chicks treated with saline (E) or LPS (F). Data are means ± SEM. Number of subjects is shown in parenthesis. Dashed arrows indicate injection time. Open symbols represent statistical differences from initial values within the same treatment. * represents difference between treatments at the same time.

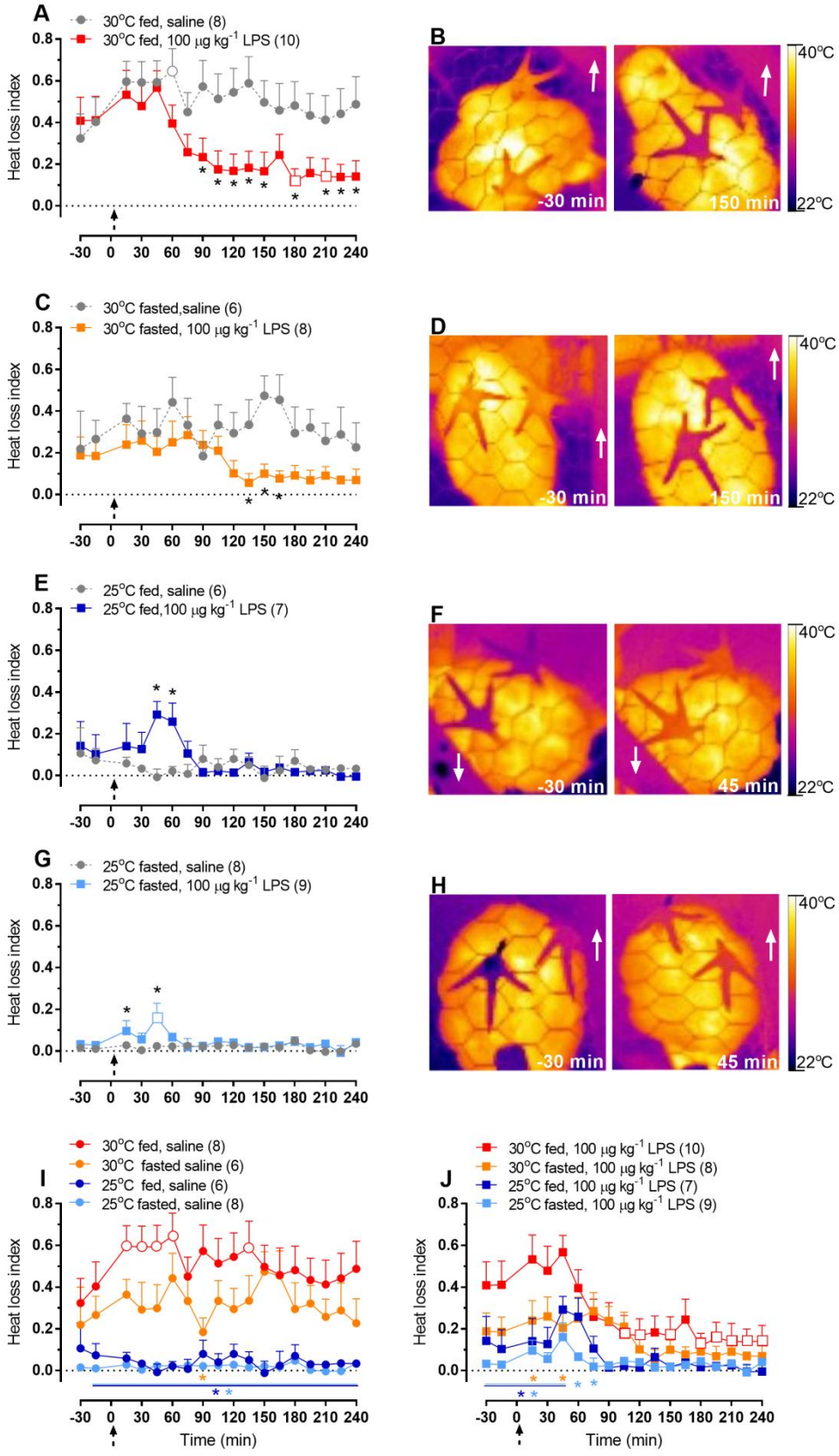

Figure 5. Heat loss index at different ambient conditions on immune challenged chicks. From A to H, left panels show the heat loss index of chicks treated with lipopolysaccharide (LPS) or saline while the right panels show the correspondent representative thermographic images from before (-30 min), and after 150 min (B and D), or 45 min (F and G) of LPS treatment. A and B) Fed chicks at 30^{o}C. C and D) Fasted chicks at 30^{o}C. E and F) Fed chicks at 25^{o}C. G and H) Fasted chicks at 25^{o}C. I and J) HLI from saline (I) and LPS (J) injected chicks compared by the different environmental demands. Data are means ± SEM. Number of subjects is shown in parenthesis. Dashed arrows indicate injection time. White arrows in the thermal images indicate a tape (ε= 0.95) used as a reference for ambient temperature measurement. Open symbols represent statistical differences from initial values (0 min) within the same group. * represents difference between treatments at the same time from A to G figures. For the bottom panels *, * and * respectively represent differences from the conditions 30^{o}C fasted, 25^{o}C fed, and 25^{o}C fasted to fed chicks at 30^{o}C (control for ambient conditions).

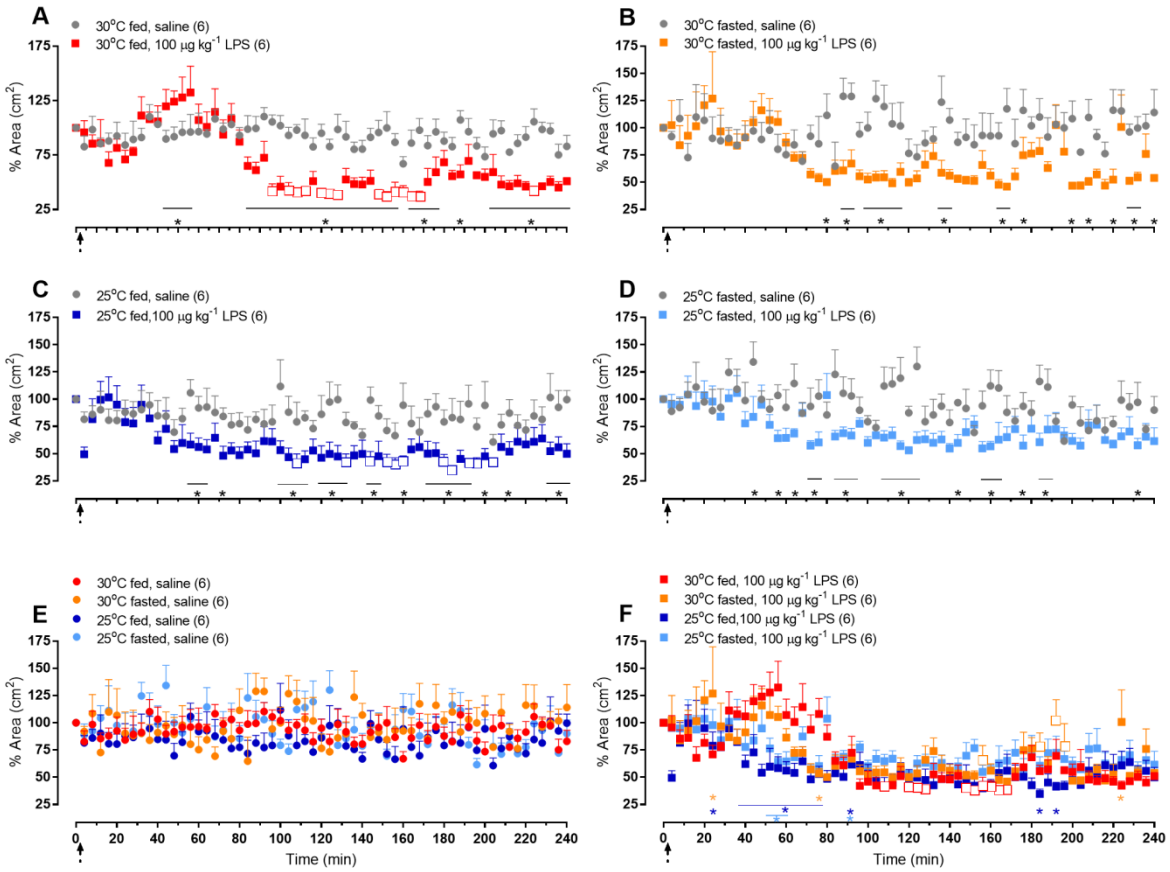

Figure 6. Time course effect of changes in the area occupied by chicks (% from pre-injection values) treated with lipopolysaccharide (LPS) when exposed to different environmental trade-offs. A) Fed chicks at 30°C. B) Fasted chicks at 30°C. C) Fed chicks at 25°C. D) Fasted chicks at 25°C. E) Saline injected chicks at different ambient conditions. F) LPS injected chicks exposed to different ambient conditions. Data are means ± SEM. Number of groups (of 5 individuals) is shown in parenthesis. Dashed arrows indicate injection time. Open symbols represent statistical differences from initial values within the same group. * represents difference between treatments at the same time (A to D). For the bottom panels *, * and * respectively represent differences from the groups 30°C fasted, 25°C fed and 25°C fasted to the group 30°C fed (control for ambient conditions).

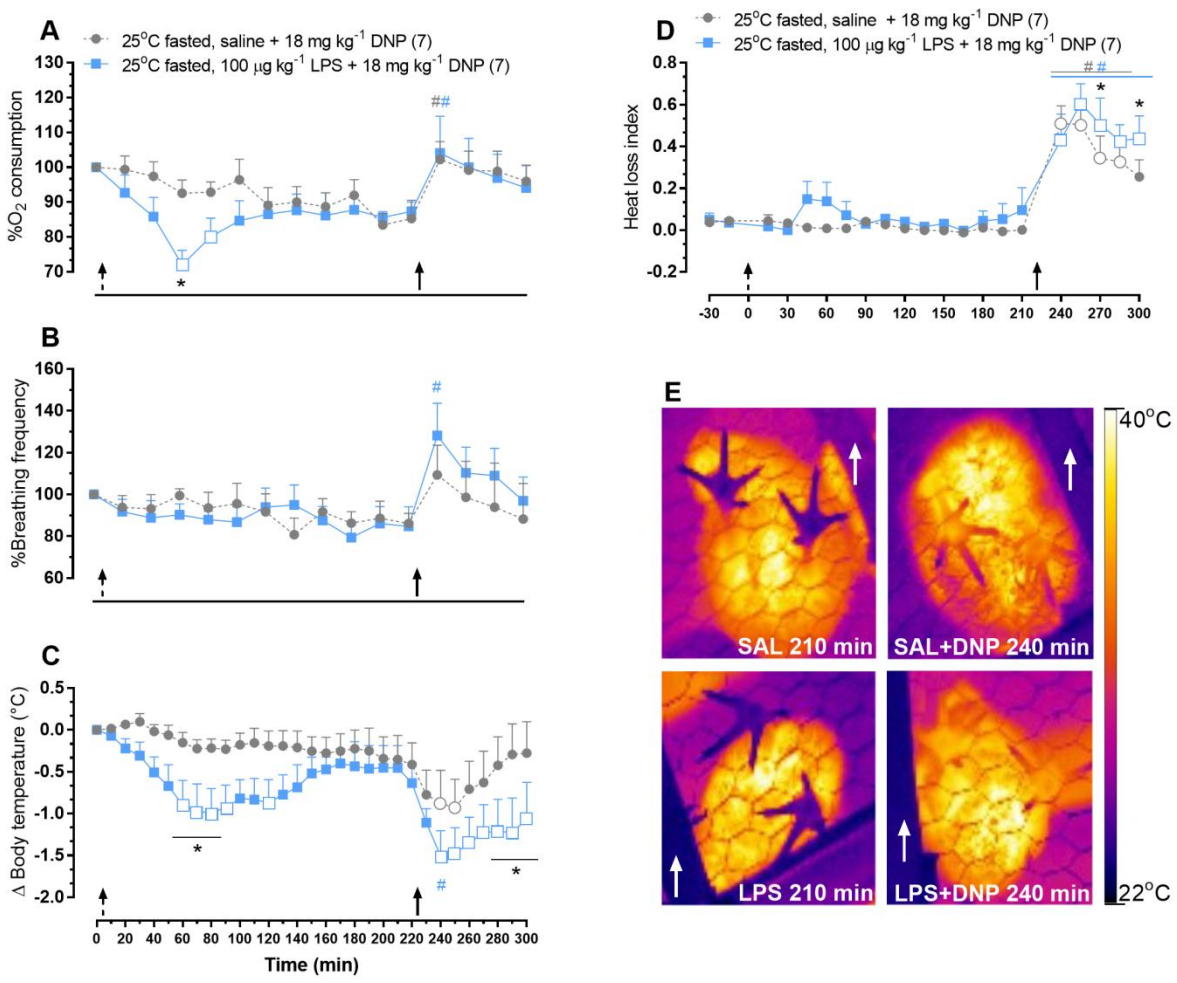

Figure 7. 2.4 Dinitrophenol (DNP) effect on body temperature and autonomic thermoregulatory responses to lipopolysaccharide (LPS) or saline in fasted chicks at 25 °C. A) % Changes (from pre-injection values) in oxygen consumption. B) % Changes in breathing frequency C) Changes in body temperature (delta from the pre-injection value). D) Heat loss index. E) Representative thermographic images from before (210 min) and after (240 min) DNP treatment in chicks injected at 60 min with saline (top, SAL) and LPS (bottom). Data are means ± SEM. Number of subjects is shown in parenthesis. Dashed arrows indicate the time of saline/LPS injection. Arrows indicate the time of DNP injection. Open symbols represent statistical differences from initial values (0 min) within the same group. * represents difference from saline treatment. # and # represent differences from DNP injection overtime compared to the last values before DNP injection (at 220/210 min) respectively for saline+DNP and LPS+DNP groups.

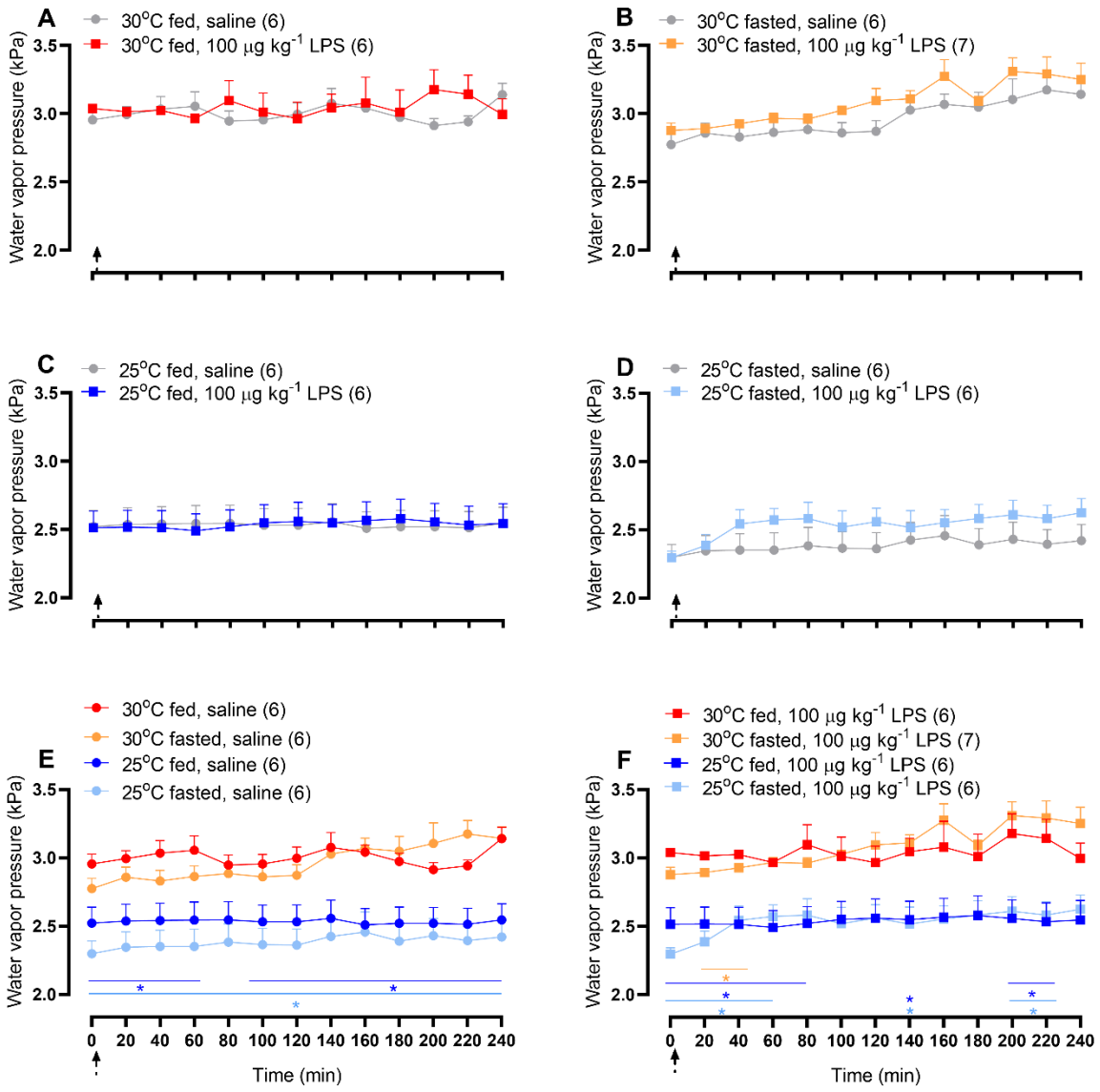

Fig. S1. Water vapor pressure in the chamber of immune challenged chicks exposed to different ambient conditions. Top and middle panels – LPS and saline injected chicks fed at 30ºC (A), fasted at 30ºC (B), fed at 25ºC (C), and fasted at 25ºC (D). Bottom panels - different ambient conditions effects in chicks treated with saline (E) or LPS (F). Data are means ± SEM. Number of subjects is shown in parenthesis. Dashed arrows indicate injection time. No difference was observed between

LPS and saline treatments or due to feeding or fasting protocol. Differences in water vapor pressure induced by temperature can be observed in the bottom panels. Two-way RM ANOVA followed by Tukey post hoc test was used for comparisons. *, * and * respectively represent differences from the conditions 30ºC fasted, 25ºC fed, and 25ºC fasted to fed chicks at 30ºC (control for ambient conditions).