RESEARCH ARTICLE

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

Lara do Amaral-Silva[1,2,*], Welex Cândido da Silva[1], Luciane Helena Gargaglioni[1] and Kênia Cardoso Bícego[1,*]

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 facing 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 day old broiler chicks exposed to fasting, cold (25°C), and fasting combined with cold with a control group fed under thermoneutral conditions (30°C). The chicks were injected with saline or with a high dose of endotoxin known to induce a biphasic thermal response composed of a drop in body temperature (T_b) followed by fever. Then T_b, 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 chicks fed/fasted 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.

KEY WORDS: Birds, Endotoxin, Cold, Fasting, Fever, Regulated hypothermia

INTRODUCTION

The 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 (T_b) and activity in the cold, or alternatively decrease T_b (torpor) to save

energy during the cold season or food shortage (Bicego et al., 2007; Hohtola, 2012; McKechnie, 2008; Ruf and Geiser, 2015). Independent of the conditions 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 resistance towards a pathogen infection is particularly significant because they already present an intrinsic energetically costly lifestyle, with the highest T_b 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 in a bird's energetic budget when the response launched is fever (Marais et al., 2011). Fever is characterized as an elevated T_b actively established and defended by heat-producing and/or -conserving mechanisms, which consist of an important aide 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 is the opposite of a thermal response of fever; that is, it depends on thermolytic effectors to actively defend a lower T_b (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, 2021; Dantonio et al., 2016). Likewise, several species of small passerines are reported to present only a T_b decrease after an

[1]Department of Animal Morphology and Physiology, São Paulo State University (FCAV-UNESP), Jaboticabal 14884-900, SP, Brazil. [2]Department of Biology, University of North Carolina at Greensboro (UNCG), Greensboro, NC 27412, USA.

*Authors for correspondence (lara.amaral-silva@unesp.br; l_doamarals@uncg.edu; k.bicego@unesp.br)

L.d., 0000-0001-6273-3240; W.C.d., 0000-0002-2912-4651; L.H.G., 0000-0003-4097-5286; K.C.B., 0000-0002-1180-1132

immune stimulus, even though none of the studies addressed the possible regulated nature of this 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 especially challenged in their energy budget because their small size entails a large relative surface area for passive heat loss, in addition to their limited fasting capacity or tolerance of anorexia (Hohtola, 2012). In contrast, adult chickens, which present larger energy stores, seem to need much higher doses of LPS to challenge their energy resources, resulting in decreased T_b (Leshchinsky and Klasing, 2001). More evidence of 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 never considered 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 an immune challenge with a biphasic thermal response. This treatment model is commonly used to simulate severe inflammation. Within the first hour after injection, circulating LPS concentration is high and hypothermia occurs; over time, circulating LPS concentration decreases and a mild inflammation triggers fever ~4–5 h 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 environmentally challenged chicks (*Gallus gallus*), we analyzed T_b, O2 consumption (thermogenesis index), cutaneous temperature (vasoconstriction/vasodilation index), breathing frequency (respiratory heat loss) and huddling (thermoregulatory behavior) as thermoeffectors. An additional protocol was followed to ascertain thermogenic capacity during fever elimination by measuring T_b and the autonomic thermoeffectors in immune-challenged chicks exposed to 2,4-dinitrophenol, a mitochondrial uncoupler that increases metabolic rate.

MATERIALS AND METHODS
Animals and housing
Hatchlings and fertile eggs from *Gallus gallus domesticus* (Linnaeus 1758), lineage Cobb 500, were supplied by a local commercial hatchery (Pluma, Descalvado, SP, Brazil). Fertile eggs were incubated at 37.5°C with 60% relative humidity and were rotated every 2 h in an automatic incubator (Premium Ecologica, Belo Horizonte, MG, Brazil). On day 19 of embryonic development, eggs were transferred to a hatcher (Premium Ecologica) kept at 37.5°C and 70% humidity. A 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 use in all other procedures. All hatchlings were housed in temperature-controlled brooders (Premium Ecologica) at 33°C, a temperature that was progressively decreased to 30°C until the 5th day post-hatching. The brooders were placed in a room with a 14 h:10 h 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 thermogenesis is known to be fully established and they are able to display regulated hypothermia and fever in response to endotoxin (Amaral-Silva et al., 2020, 2021; Dantonio et al., 2016; Tazawa et al., 2004; Tzschentke and Nichelmann, 1999).

All procedures were 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 began, chicks were anesthetized with isoflurane (3% for induction, 1% for maintenance in 100% O2) and a temperature sensor (biotag: BioTherm13, ~13 mm, 134.2 kHz FDX-B, Biomark, Boise, ID, USA) was inserted into the coelomic 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^{-1}, i.m.) and anti-inflammatory (flunixin meglumine, 2.5 mg kg^{-1}, s.c.) drugs to avoid infection and pain. On the day of the experiment, T_b was measured by telemetry using a reader antenna, and data were recorded (Biomark HPR Plus™, Biomark).

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} through the respirometer, into a water vapor pressure (WVP) analyzer (RH300, Sable Systems). The outflow was then subsampled (160 ml min^{-1}; SS4, Sable Systems), and sequentially pulled through a drying column (Drierite, Sigma Aldrich, St Louis, MO, USA), into a calibrated O2 analyzer (PA-10, Sable Systems). All equipment was connected to an analog–digital converter and signals were recorded using PowerLab (LabChart, ADInstruments, Dunedin, Otago, New Zealand). 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: $\dot{V}_{O_2}=[FR_E(F_{I_{O_2}}-F_{E_{O_2}})]/[1-F_{I_{O_2}}(1-RER)]$, where FR_E is the excurrent flow rate (outflow), $F_{I_{O_2}}$ is the incurrent fractional concentration of oxygen, $F_{E_{O_2}}$ is the excurrent fractional concentration of oxygen and RER is the respiratory exchange ratio (considered to be 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 baseline $\dot{V}_{O_2}$, the chamber was closed and f measured using a pressure transducer (ADInstruments) connected to the experimental chamber. The pressure signal was then converted by an analog–digital converter (Powerlab, ADInstruments), and recorded inline using LabChart (ADInstruments). f 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 (T_s) 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 (T_a) was measured from thermal images of a black tape (emissivity 0.95) attached to the chamber bottom close to the chick's feet (Amaral-Silva et al., 2021; Tattersall, 2016).

The thermal images were analyzed using ThermaCam (FLIR), and T_a, T_s and T_b were used to calculate the heat loss index (HLI) as: HLI=$(T_s-T_a)/(T_b-T_a)$ (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. The next morning, the brooders were uncovered and the chicks were photographed using the time-lapse function of HandyAvi (AZcendant, Tempe, 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 occupied before injection was considered to be 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 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) 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, 2021; Dantonio et al., 2016). All experiments were conducted during the light phase between 07:00 h and 19:00 h. The experiments were performed in four different conditions: (1) fed in thermoneutrality (30°C; control for ambient conditions); (2) fasted in thermoneutrality; (3) fed in cold (25°C); and (4) fasted in cold. For fasting, food was taken from the brooder on the day preceding the experiments at lights off (20:00 h) as chicks naturally stop eating in the dark phase. The following day, experiments started always at 07:30 h, standardizing 11:30 h of food deprivation prior to the experiment (established after pilot experiments seeking a fasting effect whilst maintaining chick welfare). Chicks in the fed groups started eating at 06:00 h when the lights turned on in the chick facility, and food was offered *ad libitum* throughout the light phase. All ambient conditions were maintained during the experiments for every chick; for example, chicks in the fasting groups were 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°C) started 90 min before and lasted the entire duration of the experiment. As 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, with a total of 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 ensure death.

Influence of trade-offs on the LPS effect on T_b

Chicks previously implanted with a temperature sensor were subjected to the different ambient conditions described above. T_b was recorded once by telemetry, the chicks were injected with LPS or saline and T_b was measured hourly for an additional 6 h after injection. Sixty-two chicks were used for this protocol

Influence of trade-offs 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, and the chick was injected with LPS or saline and immediately returned to the chamber. $\dot{V}_{O_2}$ and f were then measured for an additional 240 min. A total of 49 chicks were used for this protocol.

As 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 as a result of feeding compared with the fasting protocol at 25 or 30°C. As expected, the only factor affecting WVP was T_a, 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. Because WVP is directly affected by temperature, we believe that the small difference in WVP (~0.5 kPa) is intrinsic to T_a and may not affect any comparisons made in this study.

Influence of trade-offs on the LPS effect on HLI

Chicks previously implanted with a temperature sensor were habituated in the chambers under experimental conditions for at least 40 min. After that, thermal images of the feet and T_b were recorded every 15 min during the whole experiment. Two initial measurements preceded the injection of LPS or saline, which was followed by continuous T_s and T_b measurements for the next 240 min. This protocol was applied to 62 chicks.

Influence of trade-offs on the LPS effect on huddling behavior

Groups of 5 chicks kept at one of the ambient conditions described above were photographed every minute for 1 h. 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, totaling 240 chicks. All 5 individuals in each group received the same treatment, LPS or saline.

Induction of mitochondrial uncoupling for testing thermogenic capacity during trade-off influence on LPS effects

Only chicks challenged with fasting combined with cold were used in this protocol. 2,4-Dinitrophenol (DNP, Sigma), 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 min after the LPS/saline injection for two reasons: (i) chicks in a control environment (fed at 30°C) present fever at about 240 min (see Results); and (ii) DNP affects $\dot{V}_{O_2}$ about 20 min after its injection into 5 day old chicks (Amaral-Silva et al., 2021).

For this protocol, two sets of experiments were performed. First, chicks previously implanted with a temperature sensor were placed in a respirometer and $\dot{V}_{O_2}$, f and T_b were concomitantly measured.

An intramuscular injection of LPS or saline was carried out after two initial measurements and the chicks were returned to the respirometer for an additional 220 min of analysis. The chicks were then intraperitoneally injected with 18 mg kg^{-1} of DNP dissolved in saline, resulting in two groups: saline+DNP and LPS+DNP. After DNP injection, chicks were placed back in the respirometer for a further 80 min of $\dot{V}_{O_2}$, f and T_b measurements (300 min after LPS/saline injection). Second, chicks from another group previously implanted with a temperature sensor were acclimated for 40 min in the chambers for HLI measurements. Thermal images of the feet and T_b were then recorded with a 15 min interval. Two initial measurements preceded the injection of LPS or saline, and an injection of 18 mg kg^{-1} of DNP was administered 220 min after LPS/saline injection. Twenty-eight chicks were used for this protocol.

Statistical analysis

Data are shown as means±s.e.m. The number of animals used in each experiment (n) is indicated in the figure legends. The effects of the ambient conditions alone on T_b, $\dot{V}_{O_2}$, HLI, f and the area occupied by the chicks were analyzed using one-way ANOVA. As $\dot{V}_{O_2}$ 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 chick T_b, $\dot{V}_{O_2}$, HLI, f and behavioral responses to LPS or saline was analyzed using two-way repeated measures ANOVA considering treatment×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. Two-way repeated measures ANOVA was also performed for DNP experiments considering the effects of treatment (saline+DNP and LPS+DNP)×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 chick T_b and thermoeffectors

We first evaluated how the ambient conditions alone affected chick T_b and thermoeffectors. T_b (Fig. 1A) was not significantly affected by the competing environmental demands ($P=0.063$). Challenged chicks also presented a similar $\dot{V}_{O_2}$ (Fig. 1B) to that of 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 a difference between these two groups ($P=0.033$). Breathing frequency (Fig. 1C) was slightly decreased by fasting in general and fasted chicks at 25 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 with 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, at 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 with that of fed chicks at 30°C ($P<0.001$ for all). Fasted chicks at 25°C also occupied a smaller area compared with 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 change in T_b and thermoeffectors ($\dot{V}_{O_2}$, f, HLI and huddling behavior) in response to an immune challenge in 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 T_b decreased 60 min after injection compared with that prior to injection (up to −0.6°C, $P=0.011$) and at 60 and 120 min after injection compared with that in saline-injected chicks ($P=0.005$ for both). The T_b drop was followed by an increase in T_b at 240 and 360 min compared with the initial values (up to 0.6°C, $P=0.019$ and 0.004) and from 240 to 360 min compared with saline-injected chicks ($P<0.001$ to 0.014). For fasted chicks at 30°C (Fig. 2B), LPS injection also caused a decrease in T_b at 60 min compared with that prior to injection (−0.8°C, $P=0.001$), and T_b was higher from 180 to 360 min compared with the saline treatment ($P<0.001$ for all) but not higher than the initial values. This probably occurred because fasting per se decreased T_b from 120 to 360 min compared with initial values ($P=0.001$ to 0.009) as observed in saline-injected chicks at 30°C. Fed chicks at 25°C (Fig. 2C) decreased T_b in response to LPS at 60 and 180 min compared with that prior to injection (up to −1.3°C, $P<0.001$ to 0.008), and T_b was lower than in the saline group from 60 to 180 min ($P=0.002$ to 0.045) with no subsequent fever, differing from fed chicks at 30°C. Finally, fasted chicks at 25°C (Fig. 2D) also had only a sharp T_b drop in response to LPS from 60 to 180 min compared with initial values (up to −2°C, $P<0.001$ for all) and with the saline group at 60 and 120 min ($P<0.001$ and 0.006), with no fever response. Fasted 25°C chicks injected with saline decreased T_b at 300 and 360 min compared with that prior to injection ($P<0.001$ to 0.032). When saline-injected chicks from different ambient conditions were compared, the fasting effect on T_b at both T_a was clear as fasted chicks reduced T_b from 60 to 360 min whether at 30°C ($P<0.001$ to 0.032) or 25°C ($P<0.001$ to 0.012) compared with fed chicks at 30°C (Fig. 2E). Among LPS-treated chicks, the fasted ones at 25°C had a greater decrease of T_b (−1.5°C) than control chicks (fed at 30°C) at 60 and 120 min ($P=0.008$ and 0.016) (Fig. 2F).

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

Concurrently with the decrease in $\dot{V}_{O_2}$, LPS-injected chicks had a higher f than the saline group at 60 min when fed at 30°C ($P=0.039$; Fig. 4A), fasted at 30°C ($P=0.018$; Fig. 4B) and fed at 25°C ($P=0.045$; Fig. 4C). Additionally, fed chicks at 30°C had higher f at 100 min when injected with LPS compared with saline ($P=0.041$). 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).
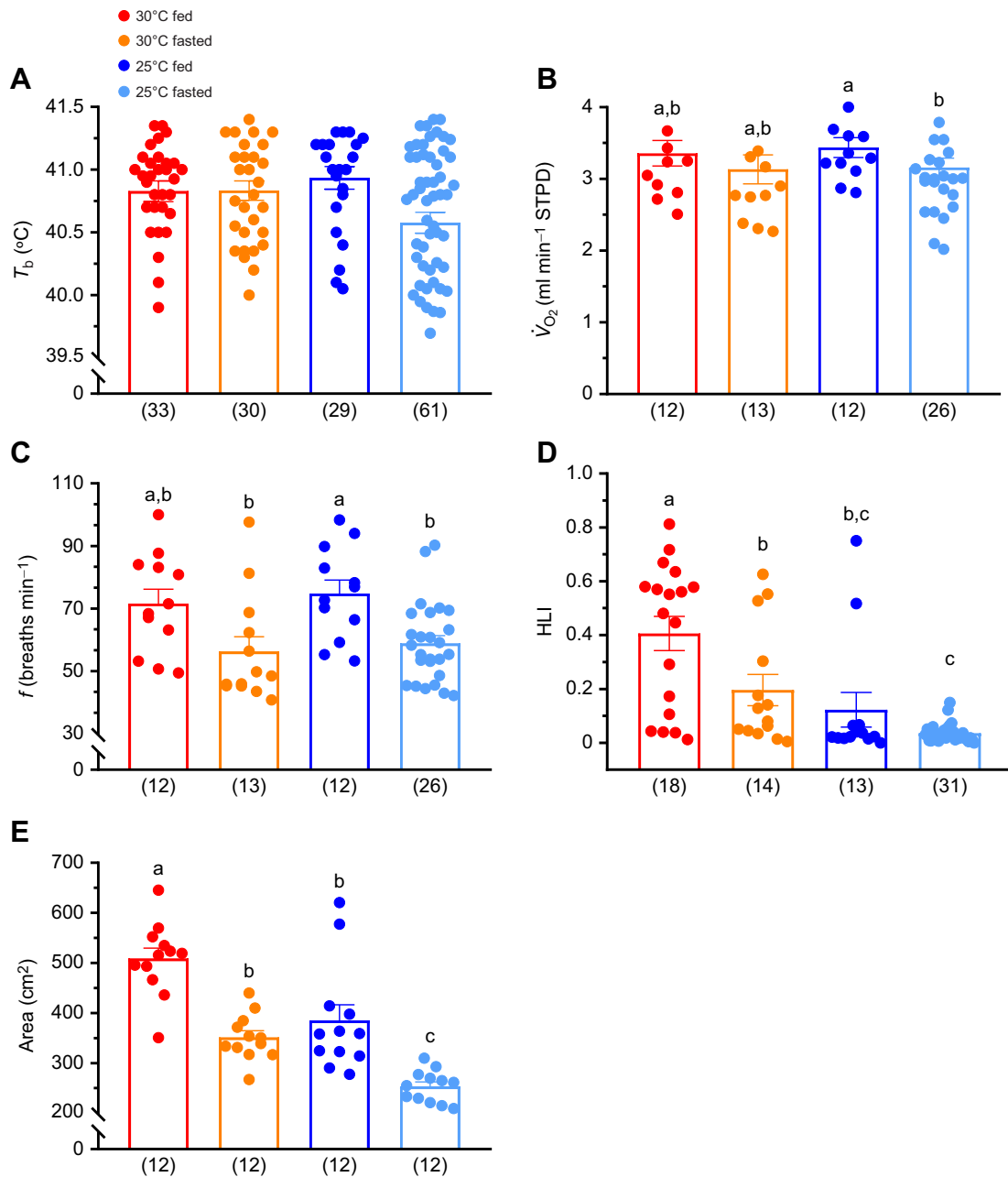
Fig. 1. Effect of trade-offs on body temperature and thermoeffectors. (A) Body temperature (T_b), (B) oxygen consumption rate ($\dot{V}_{O_2}$), (C) breathing frequency (f), (D) heat loss index (HLI) and (E) area occupied by chicks exposed to competing environmental demands before saline or lipopolysaccharide (LPS) injection. Data are means±s.e.m. The number of subjects (A–D) or groups of 5 individuals (E) is shown in parentheses. Different letters indicate significant differences among treatments ($P\leq0.05$).

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 in the feet (lower HLI) during most time points between 90 and 240 min, compared with the saline group ($P=0.008$ to 0.047) and at 180 and 210 min compared with initial values ($P=0.038$ and 0.041; Fig. 5A,B). Fasted chicks injected with LPS at 30°C also presented lower HLI than saline-injected ones from 135 to 175 min ($P=0.003$ to 0.025; Fig. 5C,D). In contrast, LPS-treated fed chicks at 25°C showed peripheral vasodilation (higher HLI) at 45 and 60 min compared with saline-injected chicks ($P<0.001$ and 0.004; Fig. 5E,F). Similarly, fasted chicks at 25°C injected with LPS increased HLI at 45 min (395%, $P<0.001$; Fig. 5G,H)

compared with initial values, and at 15 and 45 min compared with the saline group ($P=0.038$ and $P<0.001$). The ambient conditions alone affected the HLI of chicks injected with saline (Fig. 5I). Compared with 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 with that of 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).
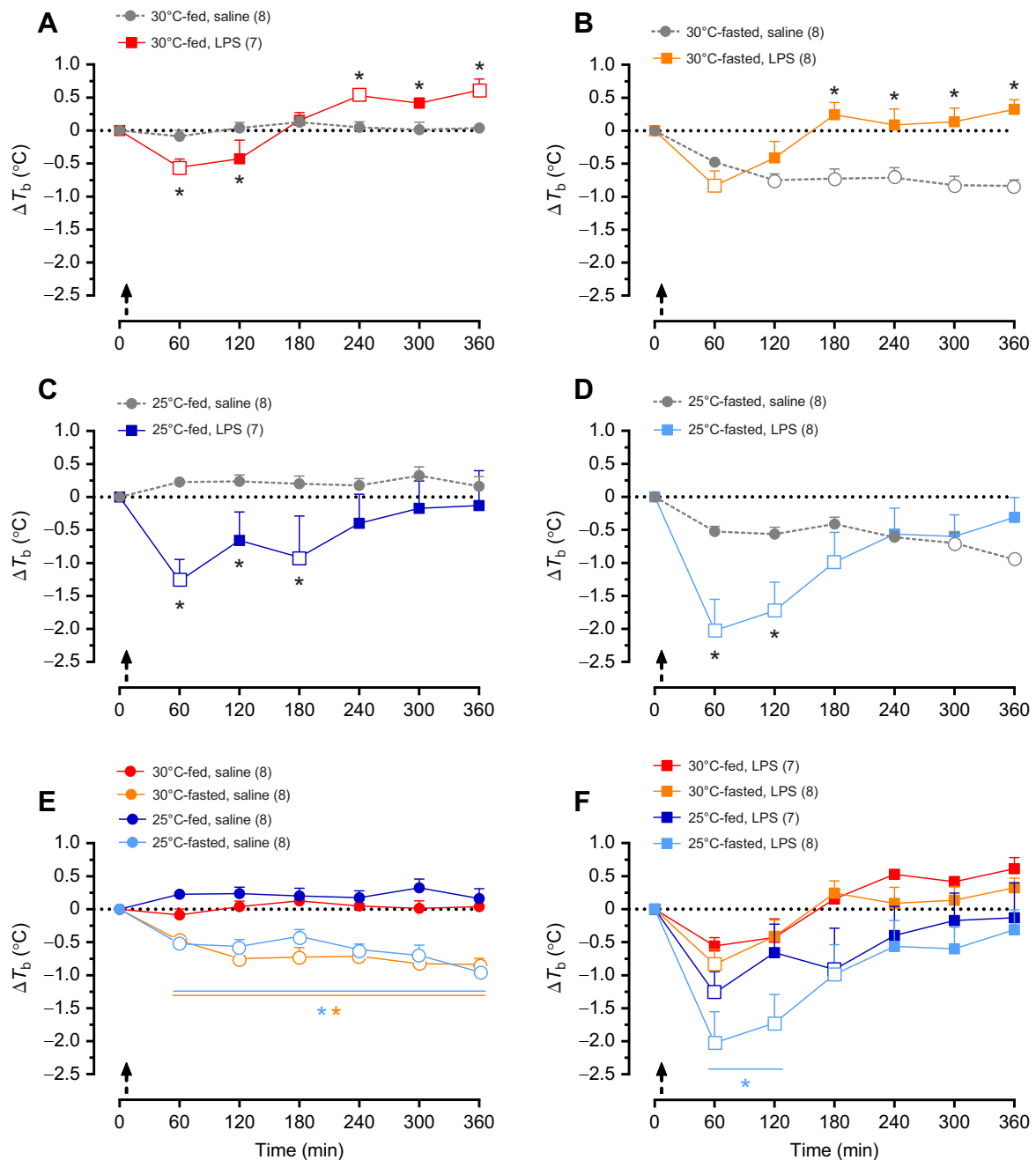
Fig. 2. Ambient conditions affect chick thermal responses to an immune challenge. (A–D) LPS effects on T_b of fed chicks at 30°C (A), fasted chicks at 30°C (B), fed chicks at 25°C (C) and fasted chicks at 25°C (D). (E,F) Effect of ambient conditions on T_b of chicks injected with saline (E) or LPS (F). Data are mean±s.e.m. difference from pre-injection temperature. Number of subjects is shown in parentheses. Dashed arrows indicate injection time. Open symbols represent statistical differences from initial values (0 min) within the same group. *Significant difference from saline-injected chicks in A–D or differences from the 30°C fasted, 25°C fed and 25°C fasted groups to the 30°C fed group (control for ambient conditions).

Huddling behavior was used as a heat conservation mechanism in chicks exposed to all environmental conditions but chick 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 with saline-injected chicks from 44 to 56 min (P=0.11 to 0.037) (Fig. 6A), preceding 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, and subsequently remained huddled for 85% of the time until the end of the experiment (P<0.001 to 0.036; percentage calculated from the first significant

reduction in area until the end of the experiment, at 240 min). Compared with the initial values, the area occupied by fed chicks at 30°C injected with LPS also decreased during 35% of this same period (first time 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 group 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
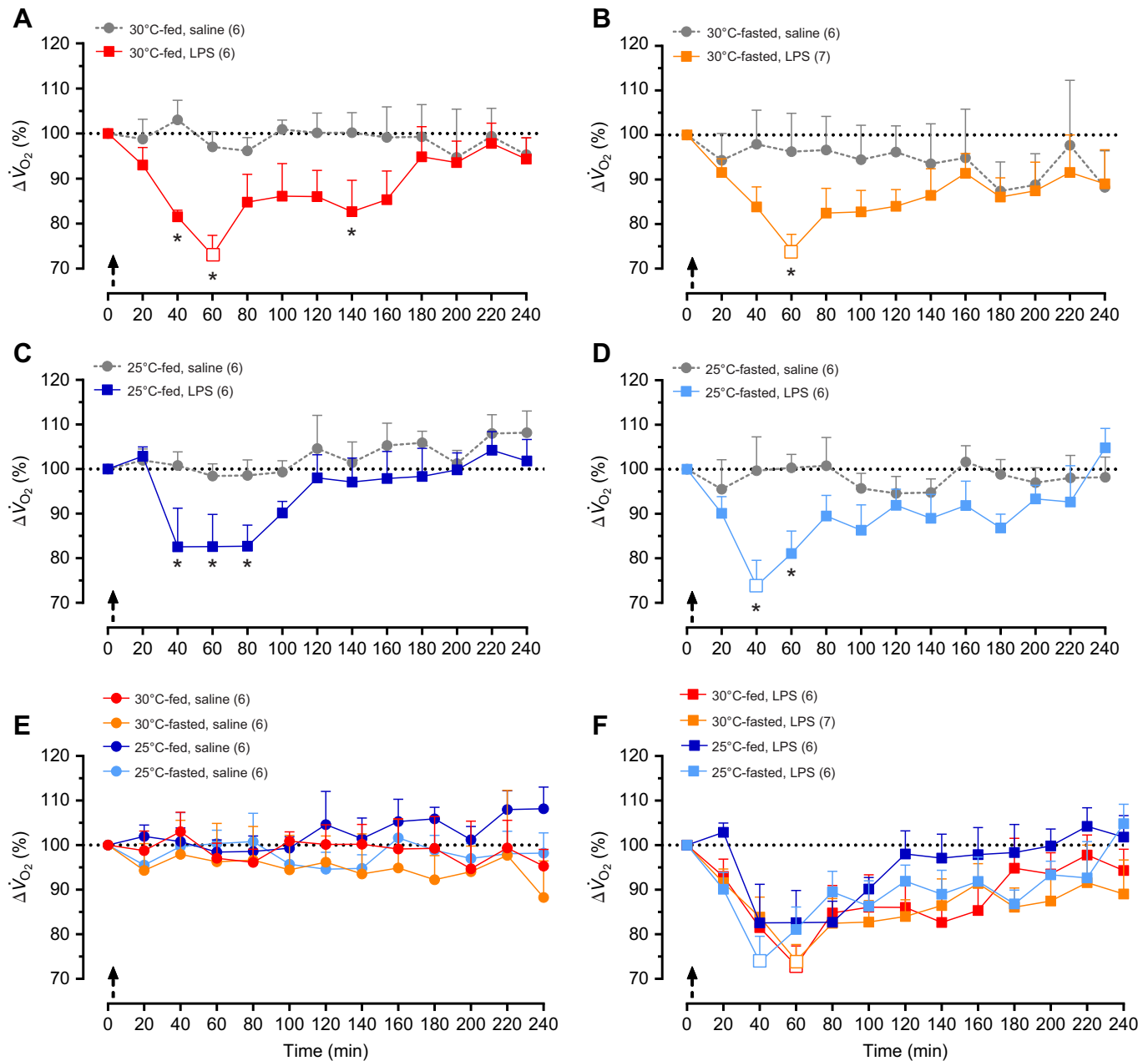
Fig. 3. Changes in oxygen consumption in response to LPS in chicks exposed to different ambient conditions. (A–D) LPS effects on $\dot{V}_{O_2}$ of fed chicks at 30°C (A), fasted chicks at 30°C (B), fed chicks at 25°C (C) and fasted chicks at 25°C (D). (E,F) Effect of ambient conditions on $\dot{V}_{O_2}$ of chicks treated with saline (E) or LPS (F). Data are mean±s.e.m. percentage difference from pre-injection values. Number of subjects is shown in parentheses. Dashed arrows indicate injection time. Open symbols represent statistical differences from initial values within the same group. *Significant difference between treatments at the same time.

occupied compared with 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-injected counterparts 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 seemed to cause a similar response among the different conditions, except for when 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 whether the chicks that eliminated the fever response to LPS were able to increase metabolic rate for thermogenesis, chicks in 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 out 220 min after the saline/LPS injection, the time at which 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% 60 and 80 min after LPS injection compared with initial values (P<0.01 and 0.004) and at 60 min compared with the saline group (P=0.007;
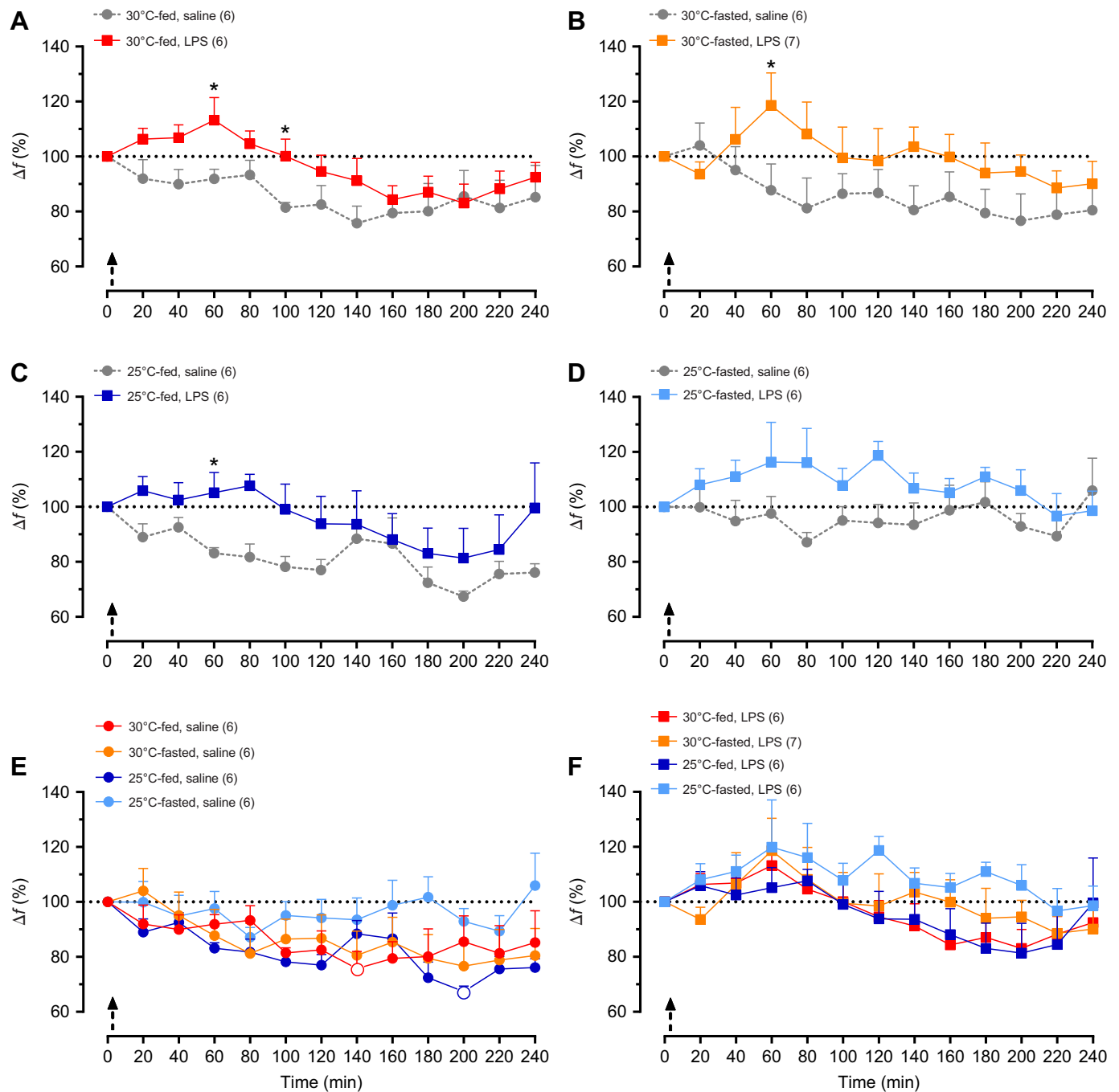
Fig. 4. Changes in breathing frequency of LPS-challenged chicks exposed to different ambient conditions. (A–D) LPS effects on f in fed chicks at 30°C (A), fasted chicks at 30°C (B), fed chicks at 25°C (C) and fasted chicks at 25°C (D). (E,F) Effect of ambient conditions on f of fed chicks treated with saline (E) or LPS (F). Data are mean±s.e.m. percentage difference from pre-injection values. Number of subjects is shown in parentheses. Dashed arrows indicate injection time. Open symbols represent statistical differences from initial values within the same treatment. *Significant difference between treatments at the same time.

Fig. 7A). T_b also decreased from 60 to 80 min compared with the saline group (P=0.036 to 0.043) and from 60 to 90 and 120 min compared with initial values (P=0.006 to 0.047; Fig. 7C). Breathing frequency was unchanged by the LPS treatment (P=0.807; Fig. 7B), as was HLI, which did not significantly differ from that of the saline group despite a clear increase after LPS injection (Fig. 7D). DNP was injected after the measurement of $\dot{V}_{O_2}$ and f at 220 min; thus, the 220 min measurements were used as the reference for DNP effects on $\dot{V}_{O_2}$, f and T_b. 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 minutes after DNP injection (240 min of the experiment), chicks increased $\dot{V}_{O_2}$ by 20% for both the group pre-injected with saline (P=0.041) and the LPS group (P=0.048) compared with values before DNP injection (220 min) (Fig. 7A). 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 with that at 220 min (Fig. 7B). As a result of DNP injection, chicks also presented a dramatic increase in HLI
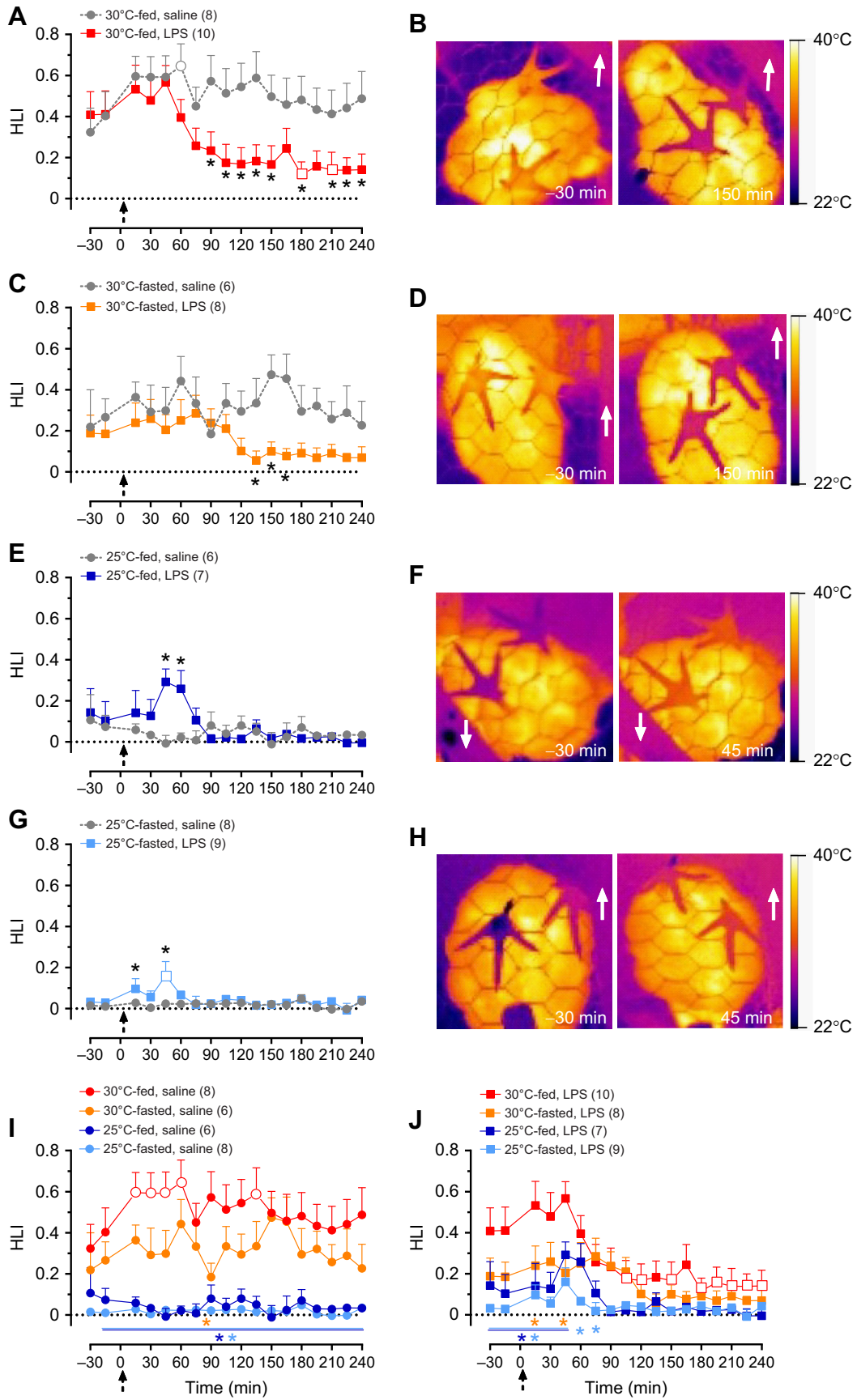
Fig. 5. See next page for legend.

Fig. 5. Heat loss index (HLI) under different ambient conditions in immune challenged chicks. (A–H) Left: HLI of chicks treated with LPS or saline. Right: corresponding representative thermographic images from before (−30 min) and after 150 min (B,D) or 45 min (F,G) of LPS treatment. (A,B) Fed chicks at 30°C, (C,D) fasted chicks at 30°C, (E,F) fed chicks at 25°C and (G,H) fasted chicks at 25°C. (I,J) Comparison of HLI from saline-injected (I) and LPS-injected (J) chicks under the different environmental demands. Data are means±s.e.m. Number of subjects is shown in parentheses. Dashed arrows indicate injection time. White arrows in the thermal images indicate the 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. *Significant difference between treatments at the same time in A–G or differences of the 30°C fasted, 25°C fed and 25°C fasted groups to the fed chicks at 30°C (control for ambient conditions).

(Fig. 7D). Saline-injected chicks increased HLI from 240 to 285 min compared with values before DNP injection (210 min, $P<0.001$ to 0.002), and also compared with initial values ($P<0.001$ to 0.016), while chicks pre-treated with LPS increased HLI from 240 to 300 min compared with that at 210 min ($P<0.001$ to 0.009) and compared with initial values ($P<0.001$ for all). T_b was lower than the initial values at 240 and 255 min for the saline+DNP group ($P=0.016$ and 0.007) and from 240 to 300 min for the LPS+DNP

group ($P<0.001$ to 0.002). The LPS+DNP group also presented a decrease in T_b at 240 min (20 after DNP, $P=0.044$; Fig. 7C) compared with the last measurement before DNP injection at 220 min. The saline+DNP group seemed to recover faster from the DNP-induced T_b drop and had higher T_b than the LPS+DNP group 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 the 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 alone and cold combined with fasting presented pronounced regulated hypothermia by suppressing thermogenesis and promoting thermolysis. Even though fasted chicks in the cold are capable of increasing metabolic rate (demonstrated with DNP) over a period corresponding to the fever phase observed in the chicks at 30°C, no additional energy was used for thermogenesis and the fever phase was completely eliminated in those groups at 25°C.
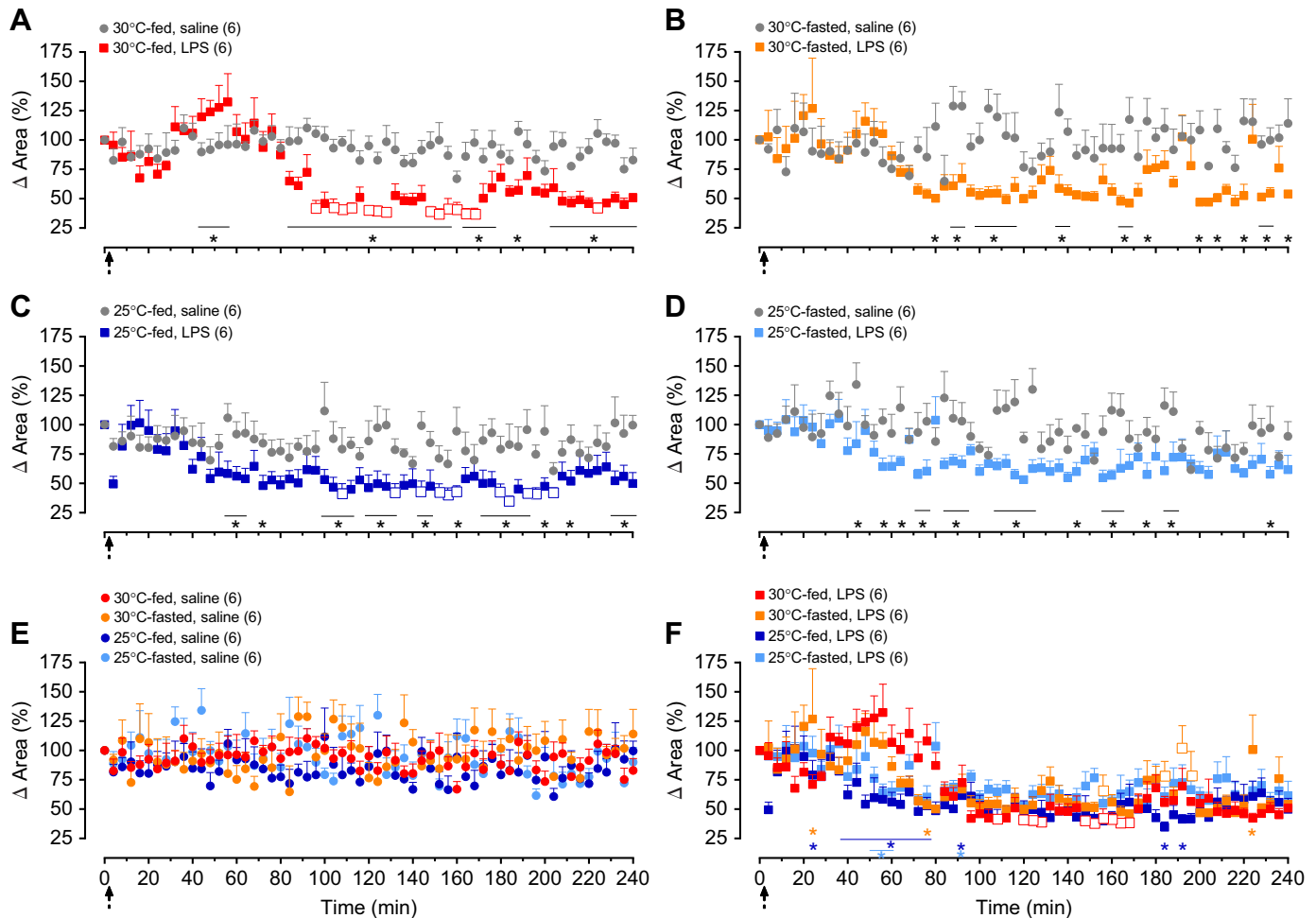

Fig. 6. Time course of changes in the area occupied by chicks treated with LPS exposed to different environmental trade-offs. (A–D) LPS effects on area occupied in fed chicks at 30°C (A), fasted chicks at 30°C (B), fed chicks at 25°C (C) and fasted chicks at 25°C (D). (E,F) Effect of ambient conditions on area occupied by chicks injected with saline (E) or LPS (F). Data are mean±s.e.m. percentage change from pre-injection values. Number of groups (of 5 individuals) is shown in parentheses. Dashed arrows indicate injection time. Open symbols represent statistical differences from initial values within the same group. *Significant difference between treatments at the same time in A–D or differences of the 30°C fasted, 25°C fed and 25°C fasted groups to the 30°C fed group (control for ambient conditions).
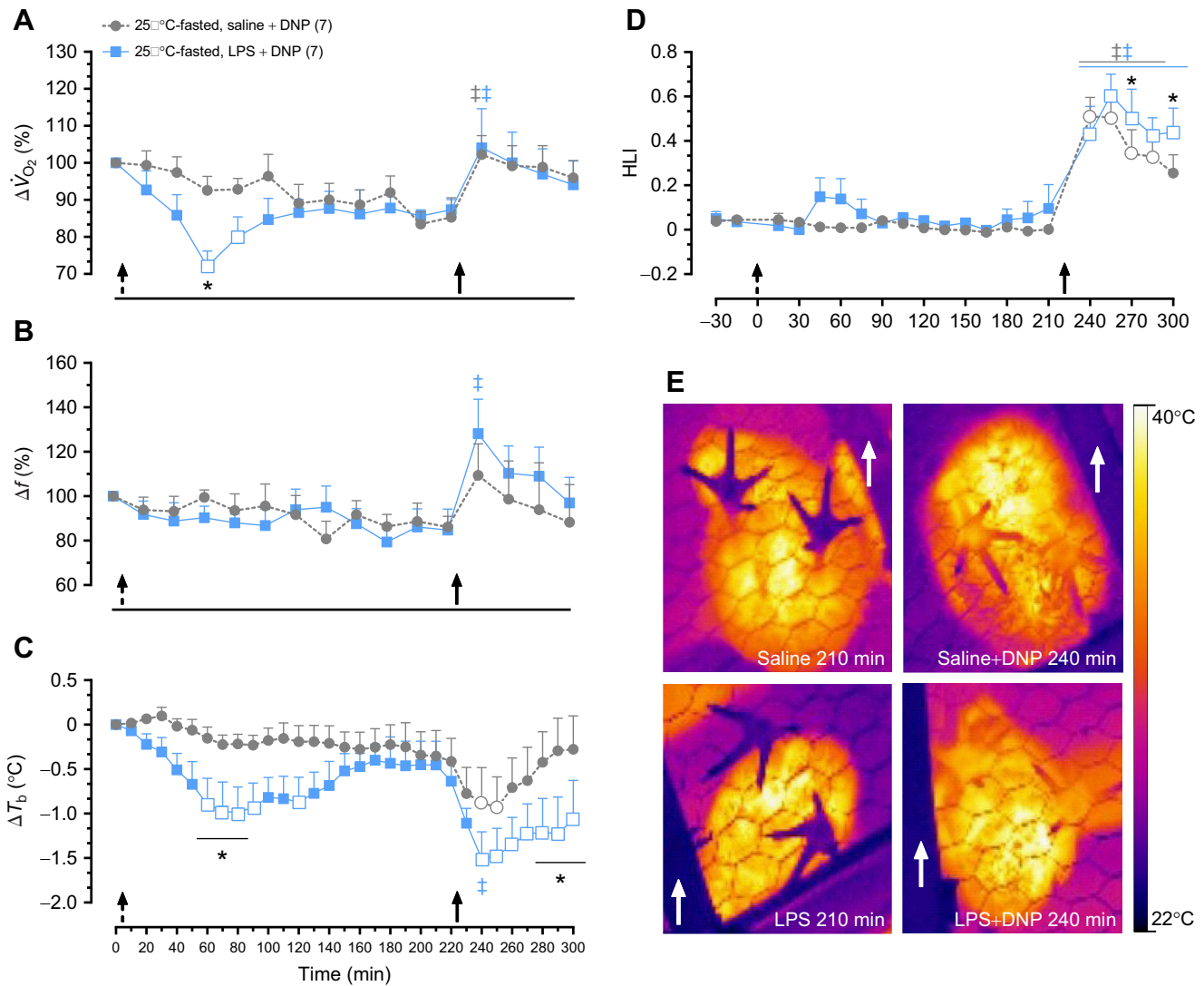
Fig. 7. Effect of 2,4-dinitrophenol (DNP) on T_b and autonomic thermoregulatory responses to LPS or saline in fasted chicks at 25°C. (A–D) Effect on $\dot{V}_{O_2}$ (percentage change from pre-injection values; A), f (percentage change from pre-injection values; B), T_b (change from pre-injection value; C) and HLI (D). (E) Representative thermographic images from before (210 min) and after (240 min) DNP treatment in chicks injected at 60 min with saline (top) and LPS (bottom). Data are means±s.e.m. Number of subjects is shown in parentheses. 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. *Significant difference from saline treatment. ‡Significant difference after DNP treatment compared with the last values before DNP injection (at 220/210 min) for saline+DNP and LPS+DNP groups.

The changes in the ambient conditions applied in the present study represented a challenge for our chicks. Despite the fact that chicks in challenging ambient conditions had similar T_b and $\dot{V}_{O_2}$ to those of 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 a 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. However, the decrease in HLI shows that all the trade-offs applied triggered activation of vasoconstriction to impair peripheral heat exchange. This vasoconstriction response progressively increased from that in fasted chicks at 30°C (relatively mild vasoconstriction), to that in fed chicks at 25°C, and finally fasted chicks at 25°C (completely vasoconstricted), reflecting a progression in the intensity of the challenge 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 exposed to the surrounding temperature conserves heat among the individuals in the group, contributing to significant energy saving during cold exposure (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 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 with some components of the acute phase response to an immune challenge in birds, suggesting that the energetic outcome invested for pathogen resistance depends on metabolic status and reserves (Ashley and Wingfield, 2011; Evans et al., 2017). Our study integrates the idea 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 the T_b drop in immune-challenged chicks, which occurred similarly for chicks at all experimental conditions. Indeed, we have shown that chicks are able to display a T_b and $\dot{V}_{O_2}$ drop in response to high doses of LPS in both warm and cold conditions and that these responses are regulated, rather than a result of metabolic failure (Amaral-Silva et al., 2021). Here, besides confirming the key role of a $\dot{V}_{O_2}$ drop during a T_b 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 T_b, even when heat loss is already facilitated by the cold environment. An increase in breathing frequency was also observed during the T_b drop in 30°C fed and 30°C fasted chicks injected with LPS compared with 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 cooling, which seems to be unnecessary to decrease T_b in chicks at 25°C. In the same way, only chicks fed at 30°C occupied a larger area during the regulated hypothermia caused by LPS, a thermolytic behavior in the case of heat stress (Alsam and Wathes, 1991) that also seems to be activated only when needed. In contrast, the non-evaporative thermolytic effector peripheral vasodilation (McCafferty, 2013; Scott et al., 2008; Tattersall et al., 2009) was activated to support the decrease of T_b for chicks exposed to cold (fed or fasted) but not for chicks in thermoneutral conditions. These responses corroborate our previous study in which a high thermal gradient between body and environment is required to activate peripheral vasodilation during the LPS-induced T_b 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 the absence of an 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. Regardless, peripheral vasodilation seems to be an important mechanism for LPS-induced heat loss as fasted chicks at 30°C displayed smaller decreases in T_b than chicks in the 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 T_b 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 T_b in all ambient conditions to which they were exposed, even when fed at thermoneutrality. The chicks' ability to drop T_b in thermoneutral conditions may then be related to the higher basal metabolic rate and T_b of birds compared with mammals, as well as the early life costs for growth in chicks compared with adults, which implies 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, as observed by the clear reduction in HLI. At thermoneutrality, peripheral vasoconstriction seems to have a predominant role to increase T_b 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 pre-LPS injection values, and no fever was observed in these groups. Even though peripheral vasoconstriction was not an option available to increase T_b 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 as 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 corresponding to fever in controls; thus, the absence of fever seems to be a regulated event. In fact, the chicks in which $\dot{V}_{O_2}$ was increased by DNP displayed a hypothermic response, resulting from the activation of thermolytic responses such as tachypnea (Fig. 7B) and cutaneous vasodilation (Fig. 7D,E) facilitating the T_b 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 the case of competing energy demands. We believe that hypothermia was caused by the DNP treatment because maintaining (saline group) or increasing the metabolic rate towards the initial value (LPS group) for fasted chicks in the 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 400 times higher than the dose used to cause regulated hypothermia, which resulted in a second decrease in T_b 180 min after the injection (Krall et al., 2010). For these rats, hypothermia seems also to occur as a result of the unfavorable metabolic conditions caused by the high LPS dose combined with the environmental challenges.

When chicks had the opportunity to express behavioral thermoregulation, it became a significant mechanism for fever

induction. This corroborates our previous results showing the essential participation of huddling in fever as LPS-injected chicks in thermoneutrality may not increase T_b when alone, but do so 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 T_b increase for fever. Noticeable, however, was that the higher severity of environmental challenge resulted in an earlier huddle. Chicks fed at 30°C started huddling 84 min after LPS injection, while fasted chicks at 30°C showed this response 76 min after LPS injection, 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 plays a bigger role than the instant thermoregulatory component. The return to euthermia after T_b reduction is considered an energetically expensive event for rodents after a LPS-induced T_b 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 initiated to prevent extra energy expenditure to return $\dot{V}_{O_2}$ and T_b to a 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 had the urge to use behavior to return T_b to euthermy, which seems to be crucial for survival as 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 from other thermoeffectors. For example, in rats, lethargy and loss of appetite occur during LPS-induced hypothermia, which seems to be driven by a different pathway from the thermoregulation considering that Tlr4 knockout mice still present such responses to LPS, while the decrease in T_b and metabolic rate is 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 a T_b 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). 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 T_b when food is restricted every other day (Cornelius et al., 2017).

Conclusion and perspectives

Our data support that metabolic status and 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 the 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 the wild and in captivity (Bonneaud et al., 2003; Nord et al., 2020; Owen-Ashley and Wingfield, 2006; Owen-Ashley et al., 2006, 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 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.

Additionally, 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 development has not been 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. Still, our study can aid in understanding the thermal response to immune challenge in animals that T_b is more directly influenced by the environment as even ectotherms seem to regulate the preferred T_b 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 T_a 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 the 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.

References

Almeida, M. C., Steiner, A. A., Branco, L. G. S. and Romanovsky, A. A. (2006). Cold-seeking behavior as a thermoregulatory strategy in systemic inflammation. *Eur. J. Neurosci.* **23**, 3359-3367. doi:10.1111/j.1460-9568.2006.04854.x

Alsam, H. and Wathes, C. M. (1991). Thermal preferences of chicks brooded at different air temperatures. *Br. Poult. Sci.* **32**, 917-927. doi:10.1080/00071669108417418

Amaral-Silva, L., Tazawa, H., Bicego, K. C. and Burggren, W. W. (2020). Metabolic and hematological responses to endotoxin–induced inflammation in chicks experiencing embryonic TCDD exposure. *Environ. Toxicol. Chem.* **39**, 2208-2220. doi:10.1002/etc.4832

Amaral-Silva, L., Gargaglioni, L. H., Steiner, A. A., Oliveira, M. T. and Bicego, K. C. (2021). Regulated hypothermia in response to endotoxin in birds. *J. Physiol.* **11**, 2969-2986. doi:10.1113/JP281385

Arad, Z. and Marder, J. (1982). Comparative thermoregulation of four breeds of fowls (Gallus domesticus), exposed to a gradual increase of ambient temperatures. *Comp. Biochem. Physiol. A Physiol.* **72**, 179-184. doi:10.1016/0300-9629(82)90029-9

Ashley, N. T. and Wingfield, J. C. (2011). Sickness behavior in vertebrates: allostasis, life-history modulation, and hormonal regulation. In *Ecoimmunology* (ed. G. E. Demas and R. J. Nelson), pp. 45-91. New York, NY: Oxford University Press.

Bennett, I. L. and Beeson, P. B. (1953). Studies on the pathogenesis of fever. I. The effect of injection of extracts and suspensions of uninfected rabbit tissues upon the body temperature of normal rabbits. *J. Exp. Med.* **98**, 477-492. doi:10.1084/jem.98.5.477

Bennett, C. E., Thomas, R., Williams, M., Zalasiewicz, J., Edgeworth, M., Miller, H., Coles, B., Foster, A., Burton, E. J. and Marume, U. (2018). The broiler chicken as a signal of a human reconfigured biosphere. *R. Soc. Open Sci.* **5**, 1-11. doi:10.1098/rsos.180325

Bícego, K. C. and Mortola, J. P. (2017). Thermal tachypnea in avian embryos. *J. Exp. Biol.* **220**, 4634-4643. doi:10.1242/jeb.171702

Bicego, K. C., Barros, R. C. H. and Branco, L. G. S. (2007). Physiology of temperature regulation: comparative aspects. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **147**, 616-639. doi:10.1016/j.cbpa.2006.06.032

Blatteis, C. M. (2003). Fever: Pathological or beneficial, injurious or beneficial? *J. Therm. Biol.* **28**, 1-13. doi:10.1016/S0306-4565(02)00034-7

Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B. and Sorci, G. (2003). Assessing the cost of mounting an immune response. *Am. Nat.* **161**, 367-379. doi:10.1086/346134

Branco, L. G. S., Soriano, R. N. and Steiner, A. A. (2014). Gaseous mediators in temperature regulation. *Compr. Physiol.* **4**, 1301-1338. doi:10.1002/cphy.c130053

Cabanac, M. and Aizawa, S. (2000). Fever and tachycardia in a bird (Gallus domesticus) after simple handling. *Physiol. Behav.* **69**, 541-545. doi:10.1016/S0031-9384(00)00227-4

Clarke, A. and Pörtner, H. O. (2010). Temperature, metabolic power and the evolution of endothermy. *Biol. Rev.* **85**, 703-727. doi:10.1111/j.1469-185X.2010.00122.x

Coon, C. A. C., Warne, R. W. and Martin, L. B. (2011). Acute-phase responses vary with pathogen identity in house sparrows (Passer domesticus). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, 1418-1425. doi:10.1152/ajpregu.00187.2010

Cornelius, E. A., Vézina, F., Regimbald, L., Hallot, F., Petit, M., Love, O. P. and Karasov, W. H. (2017). Chickadees faced with unpredictable food increase fat reserves but certain components of their immune function decline. *Physiol. Biochem. Zool.* **90**, 190-200. doi:10.1086/689913

Corrigan, J. J., Fonseca, M. T., Flatow, E. A., Lewis, K. and Steiner, A. A. (2014). Hypometabolism and hypothermia in the rat model of endotoxic shock: independence of circulatory hypoxia. *J. Physiol.* **592**, 1-16. doi:10.1113/jphysiol.2014.277277

Craig, M. C., Silva, L. O. and Swoap, S. J. (2021). Behavioral thermoregulation in the fasted C57BL/6 mouse. *J. Therm. Biol.* **96**, 102821. doi:10.1016/j.jtherbio.2020.102821

Cristina-Silva, C., Gargaglioni, L. H. and Bicego, K. C. (2021). A Thermoregulatory role of the Medullary Raphe in birds. *J. Exp. Biol.* **224**, jeb234344. doi:10.1242/jeb.234344

Dantonio, V., Batalhão, M. E., Fernandes, M. H. M. R., Komegae, E. N., Buqui, G. A., Lopes, N. P., Gargaglioni, L. H., Carnio, É. C., Steiner, A. A. and Bícego, K. C. (2016). Nitric oxide and fever: immune-to-brain signaling vs. thermogenesis in chicks. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **310**, 896-905. doi:10.1152/ajpregu.00453.2015

Deen, C. M. and Hutchison, V. H. (2001). Effects of lipopolysaccharide and acclimation temperature on induced behavioral fever in juvenile Iguana iguana. *J. Therm. Biol.* **26**, 55-63. doi:10.1016/S0306-4565(00)00026-7

Dias, M. B., Almeida, M. C., Carnio, E. C. and Branco, L. G. S. (2005). Role of nitric oxide in tolerance to lipopolysaccharide in mice. *J. Appl. Physiol.* **98**, 1322-1327. doi:10.1152/japplphysiol.01243.2004

Ederstrom, H. E. and Brumleve, S. J. (1964). Temperature Gradients in the Legs of Cold-Acclimatized Pheasants. *Am. J. Physiol.* **207**, 457-459. doi:10.1152/ajplegacy.1964.207.2.457

Evans, J. K., Buchanan, K. L., Griffith, S. C., Klasing, K. C. and Addison, B. A. (2017). Ecoimmunology and microbial ecology: Contributions to avian behavior, physiology, and life history. *Horm. Behav.* **88**, 112-121. doi:10.1016/j.yhbeh.2016.12.003

Ganeshan, K., Nikkanen, J., Man, K., Leong, Y. A., Sogawa, Y., Maschek, J. A., Van Ry, T., Chagwedera, D. N., Cox, J. E. and Chawla, A. (2019). Energetic trade-offs and hypometabolic states promote disease tolerance. *Cell* **177**, 399-413.e12. doi:10.1016/j.cell.2019.01.050

Garami, A., Steiner, A. A. and Romanovsky, A. A. (2018). Fever and hypothermia in systemic inflammation. In *Handbook of Clinical Neurology* (ed. J. Aminoff Michael and D. F. S. François Boller), pp. 565-597. Amsterdam, NH, Netherlands: Elsevier.

Geiser, F. (2010). Hibernation, daily torpor and estivation in mammals and birds: Behavioral aspects. In *Encyclopedia of Animal Behavior* (ed. M. D. Breed and J. Moore), pp. 77-83. Oxford: Academic Press.

Gilbert, C., McCafferty, D., Le Maho, Y., Martrette, J. M., Giroud, S., Blanc, S. and Ancel, A. (2010). One for all and all for one: the energetic benefits of huddling in endotherms. *Biol. Rev.* **85**, 545-569. doi:10.1111/j.1469-185X.2009.00115.x

Gleeson, M. (1986). Respiratory adjustments of the unanaesthetized chicken, Gallus domesticus, to elevated metabolism elicited by 2,4-dinitrophenol or cold exposure. *Comp. Biochem. Physiol. A Physiol.* **83**, 283-289. doi:10.1016/0300-9629(86)90575-X

Gray, D. A., Marais, M. and Maloney, S. K. (2013). A review of the physiology of fever in birds. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **183**, 297-312. doi:10.1007/s00360-012-0718-z

Hiebert, S. M. (1990). Energy costs and temporal organization of torpor in the rufous hummingbird (Selasphorus rufus). *Physiol. Zool.* **63**, 1082-1097. doi:10.1086/physzool.63.6.30152634

Hillman, P. E., Scott, N. R. and Van Tienhoven, A. (1982). Vasomotion in chicken foot: dual innervation of arteriovenous anastomoses. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **11**, 582-590. doi:10.1152/ajpregu.1982.242.5.R582

Hohtola, E. (2012). Thermoregulatory adaptations to starvation in birds. In *Comparative Physiology of Fasting, Starvation and Food Limitation* (ed. M. D. McCue), pp. 155-170. Springer.

IUPS Thermal Physiology Commission (2001). Glossary of terms for thermal physiology. *Jpn. J. Physiol.* **51**, 245-280.

Johansen, K. and Bech, C. (1983). Heat conservation during cold exposure in birds (vasomotor and respiratory implications). *Polar Res.* **1**, 259-268. doi:10.3402/polar.v1i3.6993

King, M. O. and Swanson, D. L. (2013). Activation of the immune system incurs energetic costs but has no effect on the thermogenic performance of house sparrows during acute cold challenge. *J. Exp. Biol.* **216**, 2097-2102. doi:10.1242/jeb.079574

Kluger, M. J., Kozak, W., Conn, C., Leon, L. and Soszynski, D. (1996). The adaptive value of fever. *Infect. Dis. Clin. North Am.* **10**, 1-20. doi:10.1016/S0891-5520(05)70282-8

Koteja, P. (1996). Measuring energy metabolism with open-flow respirometric systems: which design to choose? *Funct. Ecol.* **10**, 675. doi:10.2307/2390179

Krall, C. M., Yao, X., Hass, M. A., Feleder, C. and Steiner, A. A. (2010). Food deprivation alters thermoregulatory responses to lipopolysaccharide by enhancing cryogenic inflammatory signaling via prostaglandin D2. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, 1-14. doi:10.1152/ajpregu.00158.2010

Laurila, M., Pilto, T. and Hohtola, E. (2005). Testing the flexibility of fasting-induced hypometabolism in birds: effect of photoperiod and repeated food deprivations. *J. Therma* **30**, 131-138. doi:10.1016/j.jtherbio.2004.09.002

Legendre, L. J. and Davesne, D. (2020). The evolution of mechanisms involved in vertebrate endothermy. *Philos. Trans. R. Soc. B Biol. Sci.* **375**, 1-12. doi:10.1098/rstb.2019.0136

Le Maho, Y., Delclitte, P. and Chatonnet, J. (1976). Thermoregulation in fasting emperor penguins under natural conditions. *Am. J. Physiol.* **231**, 913-922. doi:10.1152/ajplegacy.1976.231.3.913

Leshchinsky, T. V. and Klasing, K. C. (2001). Divergence of the inflammatory response in two types of chickens. *Dev. Comp. Immunol.* **25**, 629-638. doi:10.1016/S0145-305X(01)00023-4

Liu, E., Lewis, K., Al-Saffar, H., Krall, C. M., Singh, A., Kulchitsky, V. A., Corrigan, J. J., Simons, C. T., Petersen, S. R., Musteata, F. M. et al. (2012). Naturally occurring hypothermia is more advantageous than fever in severe forms of lipopolysaccharide- and Escherichia coli-induced systemic inflammation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **302**, 1-15. doi:10.1152/ajpregu.00250.2011

Lochmiller, R. L., Deerenberg, C. (2000). Trade-offs in evolutionary immunology : just what is the cost of immunity? *Oikos* **88**, 87-98. doi:10.1034/j.1600-0706.2000.880110.x

Marais, M., Maloney, S. K. and Gray, D. A. (2011). Ambient temperature modulates the magnitude of LPS-induced fevers in Pekin ducks. *J. Therm. Biol.* **36**, 121-127. doi:10.1016/j.jtherbio.2010.12.003

Mashaly, M. M., Webb, M. L., Youtz, S. L., Roush, W. B. and Graves, H. B. (1984). Changes in serum corticosterone concentration of laying hens as a response to increased population density. *Poult. Sci.* **63**, 2271-2274. doi:10.3382/ps.0632271

McCafferty, D. J. (2013). Applications of thermal imaging in avian science. *Ibis (Lond. 1859)* **155**, 4-15. doi:10.1111/ibi.12010

McKechnie, A. E. (2008). Phenotypic flexibility in basal metabolic rate and the changing view of avian physiological diversity: a review. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **178**, 235-247. doi:10.1007/s00360-007-0218-8

McKechnie, A. E. and Lovegrove, B. G. (2001). Thermoregulation and the energetic significance of clustering behavior in the white-backed mousebird (Colius colius). *Physiol. Biochem. Zool.* **74**, 238-249. doi:10.1086/319669

McKechnie, A. E., Whitfield, M. C., Smit, B., Gerson, A. R., Smith, E. K., Talbot, W. A., McWhorter, T. J. and Wolf, B. O. (2016). Avian thermoregulation in the heat: Efficient evaporative cooling allows for extreme heat tolerance in four southern hemisphere columbids. *J. Exp. Biol.* **219**, 2145-2155. doi:10.1242/jeb.146563

Merchant, M., Fleury, L., Rutherford, R. and Paulissen, M. (2008). Effects of bacterial lipopolysaccharide on thermoregulation in green anole lizards (Anolis carolinensis). *Vet. Immunol. Immunopathol.* **125**, 176-181. doi:10.1016/j.vetimm.2008.04.014

Mortola, J. P. (2021). Social interaction and the thermogenic response of chicken hatchlings. *Physiol. Behav.* **232**, 113317. doi:10.1016/j.physbeh.2021.113317

Mortola, J. P. and Maskrey, M. (2011). Metabolism, temperature, and ventilation. *Compr. Physiol.* **1**, 1679-1709. doi:10.1002/cphy.c100008

Nord, A., Hegemann, A. and Folkow, L. P. (2020). Reduced immune responsiveness contributes to winter energy conservation in an Arctic bird. *J. Exp. Biol.* **223**, 1-11. doi:10.1242/jeb.219287

O'Connor, R. J. (1975). The influence of brood size upon metabolic rate and body temperature in nestling Blue tits Parus caeruleus and House sparrows Passer domesticus. *J. Zool.* **175**, 391-403. doi:10.1111/j.1469-7998.1975.tb01407.x

Owen-Ashley, N. T. and Wingfield, J. C. (2006). Seasonal modulation of sickness behavior in free-living northwestern song sparrows (Melospiza melodia morphna). *J. Exp. Biol.* **209**, 3062-3070. doi:10.1242/jeb.02371

Owen-Ashley, N. T., Turner, M., Hahn, T. P. and Wingfield, J. C. (2006). Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (Zonotrichia leucophrys gambelii). *Horm. Behav.* **49**, 15-29. doi:10.1016/j.yhbeh.2005.04.009

Owen-Ashley, N. T., Hasselquist, D., Råberg, L. and Wingfield, J. C. (2008). Latitudinal variation of immune defense and sickness behavior in the white-crowned sparrow (Zonotrichia leucophrys). *Brain. Behav. Immun.* **22**, 614-625. doi:10.1016/j.bbi.2007.12.005

Romanovsky, A. A., Shido, O., Sakurada, S., Sugimoto, N. and Nagasaka, T. (1996). Endotoxin shock: Thermoregulatory mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **270**, 693-703. doi:10.1152/ajpregu.1996.270.4.R693

Romanovsky, A. A., Ivanov, A. I. and Shimansky, Y. P. (2002). Ambient temperature for experiments in rats: a new method for determining the zone of thermal neutrality. *J. Appl. Physiol.* **92**, 2667-2679. doi:10.1152/japplphysiol.01173.2001

Ruf, T. and Geiser, F. (2015). Daily torpor and hibernation in birds and mammals. *Biol. Rev.* **90**, 891-926. doi:10.1111/brv.12137

Ruhs, E. C., Vézina, F. and Karasov, W. H. (2019). Physiological and immune responses of free-living temperate birds provided a gradient of food supplementation. *Physiol. Biochem. Zool.* **92**, 106-114. doi:10.1086/701389

Sakurada, S., Shido, O., Sugimoto, N., Hiratsuka, Y., Yoda, T. and Kanosue, K. (2000). Autonomic and behavioural thermoregulation in starved rats. *J. Physiol.* **526**, 417-424. doi:10.1111/j.1469-7793.2000.00417.x

Scott, G. R., Cadena, V., Tattersall, G. J. and Milsom, W. K. (2008). Body temperature depression and peripheral heat loss accompany the metabolic and ventilatory responses to hypoxia in low and high altitude birds. *J. Exp. Biol.* **211**, 1326-1335. doi:10.1242/jeb.015958

Shah, S. W. A., Chen, D., Zhang, J., Liu, Y., Ishfaq, M., Tang, Y. and Teng, X. (2020). The effect of ammonia exposure on energy metabolism and mitochondrial dynamic proteins in chicken thymus: Through oxidative stress, apoptosis, and autophagy. *Ecotoxicol. Environ. Saf.* **206**, 111413. doi:10.1016/j.ecoenv.2020.111413

Sköld-Chiriac, S., Nord, A., Tobler, M., Nilsson, J.-A. and Hasselquist, D. (2015). Body temperature changes during simulated bacterial infection in a songbird: fever at night and hypothermia at day. *J. Exp. Biol.* **218**, 2961-2969. doi:10.1242/jeb.122150

Steen, I. and Steen, J. B. (1965). The importance of the legs in the thermoregulation of birds. *Acta Physiol. Scand.* **63**, 285-291. doi:10.1111/j.1748-1716.1965.tb04067.x

Steiner, A. A. and Romanovsky, A. A. (2019). Energy trade-offs in host defense: immunology meets physiology. *Trends Endocrinol. Metab.* **30**, 875-878. doi:10.1016/j.tem.2019.08.012

Stier, A., Massemin, S. and Criscuolo, F. (2014). Chronic mitochondrial uncoupling treatment prevents acute cold-induced oxidative stress in birds. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **184**, 1021-1029. doi:10.1007/s00360-014-0856-6

Tattersall, G. J. (2016). Infrared thermography: A non-invasive window into thermal physiology. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **202**, 78-98. doi:10.1016/j.cbpa.2016.02.022

Tattersall, G. J., Andrade, D. V. and Abe, A. S. (2009). Heat exchange from the toucan bill reveals a controllable vascular thermal radiator. *Science (80-.)* **325**, 468-470. doi:10.1126/science.1175553

Tazawa, H., Chiba, Y., Khandoker, A. H. H., Dzialowski, E. M. M. and Burggren, W. W. (2004). Early development of thermoregulatory competence in chickens: Responses of heart rate and oxygen uptake to altered ambient temperatures. *Avian Poult. Biol. Rev.* **15**, 166-176. doi:10.3184/147020604783638056

Tickle, P. G., Hutchinson, J. R. and Codd, J. R. (2018). Energy allocation and behaviour in the growing broiler chicken. *Sci. Rep.* **8**, 1-13. doi:10.1038/s41598-018-22604-2

Tzschentke, B. and Nichelmann, M. (1999). Development of avian thermoregulatory system during the early postnatal period: Development of the thermoregulatory set-point. *Ornis Fenn.* **76**, 189-198.

Winder, L. A., White, S. A., Nord, A., Helm, B. and McCafferty, D. J. (2020). Body surface temperature responses to food restriction in wild and captive great tits. *J. Exp. Biol.* **223**, 1-8. doi:10.1242/jeb.220046

Yoda, T., Crawshaw, L. I., Yoshida, K., Su, L., Hosono, T., Shido, O., Sakurada, S., Fukuda, Y. and Kanosue, K. (2000). Effects of food deprivation on daily changes in body temperature and behavioral thermoregulation in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, 134-139. doi:10.1152/ajpregu.2000.278.1.R134

Zbikowska, E. and Cichy, A. (2012). Symptoms of behavioural anapyrexia – Reverse fever as a defence response of snails to fluke invasion. *J. Invertebr. Pathol.* **109**, 269-273. doi:10.1016/j.jip.2011.12.006
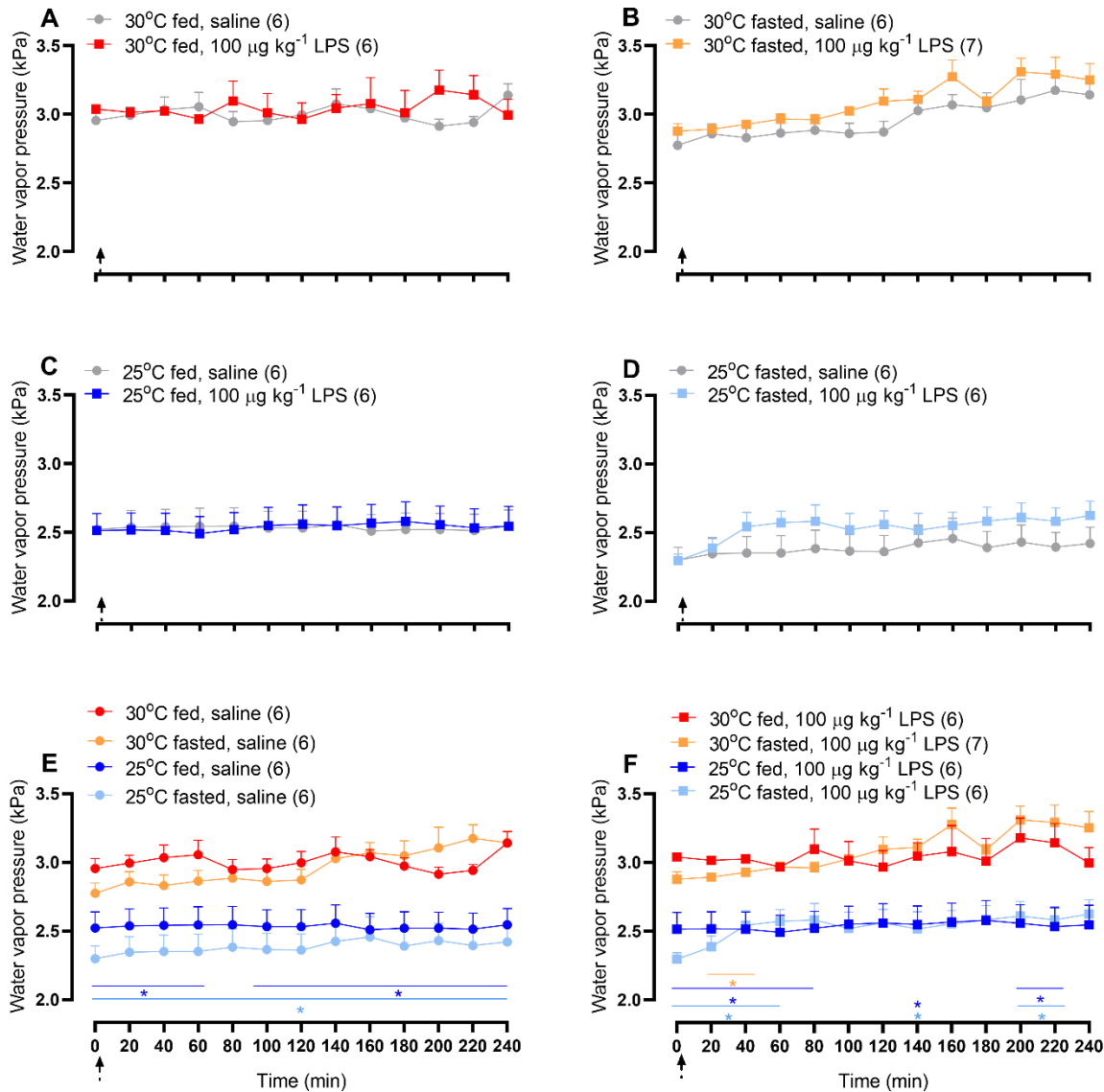
Fig. S1. Water vapor pressure in the chamber of immune challenged chicks exposed to different ambient conditions. Top and middle panels – LPS and saline injected chicks fed at 30ºC (A), fasted at 30ºC (B), fed at 25ºC (C), and fasted at 25ºC (D). Bottom panels - different ambient conditions effects in 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).