Acid-base balance in the developing marsupial: from ectotherm to endotherm

Sarah J. Andrewartha,1,2 Kevin J. Cummings,3 and Peter B. Frappell1,2
1University of Tasmania, Hobart, Tasmania, Australia; 2CSIRO Marine and Atmospheric Research, Hobart, Tasmania, Australia; and 3Department of Biomedical Sciences, University of Missouri, Columbia, Missouri

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Andrewartha SJ, Cummings KJ, Frappell PB. Acid-base balance in the developing marsupial: from ectotherm to endotherm. J Appl Physiol 116: 1210–1219, 2014. First published March 13, 2014; doi:10.1152/japplphysiol.00996.2013.—Marsupial joeys are born ectothermic and develop endothermy within their mother’s thermally stable pouch. We hypothesized that Tammar wallaby joeys would switch from α-stat to pH-stat regulation during the transition from ectothermy to endothermy. To address this, we compared ventilation (Ve), metabolic rate (V̇O₂), and variables relevant to blood gas and acid-base regulation and oxygen transport including the ventilatory requirements (Ve/V̇O₂ and Ve/V̇CO₂), partial pressures of oxygen (Pao₂), carbon dioxide (Paco₂), pH(-a), and oxygen content (CaO₂) during progressive hypothermia in ecto- and endothermic Tammar wallabies. We also measured the same variables in the well-studied endotherm, the Sprague-Dawley rat. Hypothermia was induced in unrestrained, unanesthetized joeys and rats by progressively dropping the ambient temperature (Ta). Rats were additionally exposed to helox (80% helium, 20% oxygen) to facilitate heat loss. Respiratory, metabolic, and blood-gas variables were measured over a large body temperature (Tb) range (~15–16°C in both species). Ectothermic joeys displayed limited thermogenic ability during cooling; after an initial plateau, V̇O₂ decreased with the progressive drop in Tb. The Tb of endothermic joeys and rats fell despite V̇O₂ nearly doubling with the initiation of cold stress. In all three groups the changes in V̇O₂ were met by changes in Ve, resulting in constant Ve/V̇O₂ and Ve/V̇CO₂, blood gases, and pH(-a). Thus, although thermogenic capability was nearly absent in ectothermic joeys, blood acid-base regulation was similar to endothermic joeys and rats. This suggests that unlike some reptiles, unanesthetized mammals protect arterial blood pH with changing Tb, irrespective of their thermogenic ability and/or stage of development.

Tammar wallaby (Macropus eugenii); Sprague-Dawley rat; thermoregulation; ventilatory requirement; acid-base balance

THE NEWBORN MARSUPIAL JOEY is ectothermic, relying entirely on the thermally stable pouch of its mother for thermal homeostasis (7, 22, 42, 52, 58). The development of endothermy occurs as a continuum of events rather than a step-wise change (see Fig. 2 in Ref. 19) with the transition beginning at least halfway through pouch life (19, 42, 52, 58). The transition zone between ectothermy and endothermy can be clearly defined by examining the resting mass-specific rate of oxygen consumption (V̇O₂) at pouch body temperature (Tb), which gradually changes from values close to the predicted V̇O₂ for an adult ectotherm (e.g., lizard) of the same mass to values predicted for an adult marsupial (see Fig. 2 in Ref. 19, 27). In the Tammar wallaby, this transition occurs between postnatal days 55 and 200 at a mass of 70–300 g (see Fig. 2 in Ref. 19). A myriad of morphological, physiological, and behavioral changes that allow for endothermy and the maintenance of Tb occurs across this time period (21, 48). The marsupial joey is easily accessible via the external pouch and therefore provides an insightful model for the study of homeostatic mechanisms that develop in parallel with endothermy (see Ref. 62 for a review).

At pouch vacation, most joeys are able to successfully defend Tb through increased shivering and nonshivering thermogenesis (NST) (19, 36, 42). Shivering in marsupials has been first observed at approximately two-thirds of the way through pouch development (54, 65), although quantitative measurements are lacking. Brown adipose tissue (BAT) is the major site of NST in newborn placental mammals and plays an important role in the maintenance of Tb during Ta fluctuations (11). The presence of BAT in marsupials and its role in NST is an issue that has a contentious history (23, 29, 37, 49), and there is evidence that skeletal muscle may be the primary NST site in some marsupials (56). Although the precise thermogenic mechanisms may differ between placentals and marsupials, species from both orders of mammals are prone to hypothermia during cold stress in the early stages when thermogenic ability is still developing.

Changes in V̇O₂ with temperature must be met with proportional changes in Ve if arterial acid-base status is to be maintained constant. Indeed, Ve/V̇O₂ is maintained constant in postnatal day 18 Tammar wallaby joeys during Tb challenges (38), suggesting that blood gases and pH(a) remain stable with changing Tb, at least at this age (i.e., a “pH-stat” mechanism of regulation) In contrast, many ectotherms increase pH(a) with decreasing Tb. This strategy is hypothesized to maintain constant the fractional α-imidazole ionization, thereby conserving charge status and protein conformation (i.e., α-stat regulation: ΔpHa/ΔTb ~ 0.16 U/°C) (see Refs. 14, 24, 45; for review, e.g., Ref. 50).

How neonatal endotherms regulate acid-base status during Tb changes is still uncertain. Some studies show a strategy that approaches α-stat (e.g., 66), whereas others suggest pH-stat (e.g., 64). Discrepancies between different studies are likely the result of methodological differences with respect to the induction of hypothermia; some, for example, have used anesthetics (e.g., pentobarbital and halothane) with known effects on Ve, thermogenesis, and acid-base status (1, 2, 40, 57). Further discrepancies arise with the use of heterothermic adult mammals. Some torpid mammals adopt a pH-stat strategy, maintaining constant Paco₂ and pH(a) over a wide Tb range (e.g., 30, 32; see Ref. 39 for a review). Conversely, awake ground squirrels show a hyperventilation (i.e., increased Ve/V̇O₂) during hypothermia (8, 69) and a decrease in Paco₂, that results in a ΔpHa/ΔTb of ~0.012 (9), approximating α-stat regulation.

Whether unanesthetized marsupials adopt an α- or pH-stat strategy of blood acid-base regulation over pouch life, when the control systems regulating respiratory and metabolic homeostasis are undergoing rapid development, have not been
investigated. This study aims to characterize the regulation of metabolic, respiratory and blood acid-base variables in hypothemic Tammar wallaby joeys (Macropus eugenii) during the transition from ecotothermy to endothermy. We hypothesize that acid-base regulation is linked with thermoregulatory ability with ecotothermic joeys (in which Tb is essentially unregulated) utilizing an α-stat strategy for acid-base regulation (i.e., similar to some ectothermic reptiles) and endothermic joeys adopting a pH-stat strategy similar to rat pups. To test this hypothesis we will utilize two groups of joeys on either side of the ecotothermic-endothermic transition and compare their ventilatory, metabolic, and acid-base responses during forced hypothermia to those of the juvenile rat, a known endotherm.

**METHODS**

**Animals**

*Marsupials.* Experiments were performed on four ecotothermic Tammar wallaby joeys (Macropus eugenii) (aged ~90 days, mean body mass of 97.71 ± 2.06 g) and five endothermic joeys (aged ~125 days, mean body mass of 253.64 ± 49.79 g). Different animals were used for endothermic and ecotothermic joey measurements. Animals were selected by mass to fall at the lower and upper ends of the transition zone between ecotothermy and endothermy (19). Animals were obtained from an established colony maintained outdoors at La Trobe University (Bundoora, Australia) with unrestricted access to irrigated pasture and water. Experiments were conducted under La Trobe University animal ethics permit [LTU AEC 04/37(3)].

*Rodents.* Experiments were performed on 13 Sprague-Dawley rats (Rattus norvegicus; aged 27 ± 0.5 days, mean body mass of 77.64 ± 3.22 g). One-week postweaned rats of ~80 g were targeted because they were the smallest rats that could be cannulated and have blood sampled repeatedly. Animals were obtained from a colony at La Trobe University Central Animal House where they were maintained in a temperature-controlled holding facility at 22°C with a 12:12-h light/dark cycle and had access to water and rat pellets ad libitum.

*Cannulation and Blood Variables*

*Marsupials.* Animals were removed from the pouch and anesthetized with halothane (5% induction, 2–1% maintenance, as appropriate). A small midventral incision (~1.5 cm) was made in the neck, and the left internal carotid artery was oclusively cannulated with cannula tubing (ID 0.50 mm, OD 0.90 mm, Microtube extrusions, North Rocks, NSW). The tip of the cannula was positioned so that it rested as close to the heart as possible. The cannula was sutured into place and coiled to allow some movement, and a subcutaneous tunnel used to externalize the cannula in a middorsal position on the neck, where it was reinforced with a suture. The cannula was filled with heparin (1,000 units/ml) to ensure it remained patent while the animal was breathing. A small midventral incision (~0.5 cm) was made, and a small cannula tubing (ID 0.50 mm, OD 0.90 mm, Microtube extrusions, North Rocks, NSW) was inserted to a depth of 0.5 cm. The cannula tubing was reinforced with a suture. The cannula was filled with heparin (1,000 units/ml) to ensure it remained patent while the animals recovered in the mother’s pouch for at least 24 h. All young were observed to have reattached to the teat after surgery.

Arterial samples (0.5 ml) were collected at Tb = 36.5, 28, and 20°C and analyzed immediately (see protocol), and volume replacement with 0.5 ml heparin-saline (20 units/ml) ensured that the animals did not dehydrate or hemodilute. PO₂, PCO₂, and pH were determined from each blood sample using blood gas analyzers (models PHM 73 and BMS 3 MK 2, Radiometer, Denmark) maintained at each experimental Tb and calibrated before and after every sample. pH was immediately measured using ~7 μl of the sample to obtain an initial reading, then subsequently moving ~10-μl increments of the sample through the electrode until the readings varied less than 0.005 units. PO₂ and PCO₂ were measured using ~0.45 ml of the sample (including the blood used for pH measurement, which was still hermetically preserved) every 30 s over a 3- to 7-min period (temperature dependent) with PO₂ regressed linearly back to time zero. Oxygen content (CO₂) was determined on 10 μl of each sample using a galvanic cell (Oxycon, University of Tasmania, Australia). Approximately 20 μl of each sample was used to determine lactate concentration (Accuscript analyser, Boehringer Mannheim, Mannheim, Germany) and hemoglobin concentration (HemoCue, Angelholm, Sweden).

*Rodents:* cannulation and blood variables. As detailed above for the joeys, except recovery was at 26°C (within thermoneutral zone = 22–30°C).

![Figure 1. A: rate of oxygen consumption (VO₂, ml·min⁻¹·kg⁻¹) against changing body temperature (Tb) in ecotothermic (gray diamonds, n = 4) and endothermic (●, n = 5) Tammar wallaby joeys and 27 ± 0.5-day-old Sprague-Dawley rats, initially in normoxia (●) and during exposure to helox (■). Rate of carbon dioxide production (VCO₂, ml·min⁻¹·kg⁻¹) (B) and minute ventilation (Ve, ml·min⁻¹·kg⁻¹) (C) in the same joeys and rats. * Values for rats as reported in Table 3. Error bars are ±SE and some errors are too small to visualize. *Significant difference (Dunnett’s) from the control values (where Tb = 36.5°C for the joeys and Tb = 37.5°C in helox for the rats).
26–28°C in air; S. Andrewartha, personal observation; Ref. 12) for at least 24 h.

Respiratory Variables and Aerobic Metabolism

*Marsupials.* To obtain respiratory gases, a mask constructed from either the base of a 50-ml centrifuge tube or 25-ml syringe was sealed over the mouth and nostrils of the joey with a nontoxic polyether material (Impregum F ESPE, Henry Schein Halas, Melbourne, Victoria, Australia). Air was pushed through the mask, via a Y-connector sealed into the top of the mask, at a known rate of 0.2–0.8 l/min (mass dependent) and monitored by a mass-flow controller (840L-2, Sierra Instruments, Monterey, CA). Air leaving the mask was subsampled, dried (Drierite, W.A. Hammond drierite, Xenia, OH), and analyzed for fractional O₂ and CO₂ concentration (S-3A/1 and CD-3A, respectively, Applied Electrochemistry, Pittsburgh, PA). VO₂ and VCO₂ were calculated from airflow though the chamber and the difference between the incoming and excurrent fractional concentrations of the dry gases as detailed in Frappell et al. (18).

Ve was determined using a pneumotachograph (LT110L, AD Instruments, Bella Vista, NSW, Australia) placed upstream from the mask. Pressure changes resulting from alterations in respiratory-related airflow were measured using a differential pressure transducer (MP45-1 Validyne Engineering, Northridge, CA) and integrated to yield volume. The system was calibrated by injecting known volumes of air into the sealed mask (before the animal was connected). Up to 60 breaths were analyzed at each target Tb for tidal volume (VT), expiratory time (TE), inspiratory time (TI), inspiratory pause (TP, present in young marsupials, see MacFarlane and Frappell (38) for review), total breath time (TTOT, = TI + TE + TP), breathing frequency (f = 1/TTOT × 60), and minute ventilation (VE = VT × f) as outlined by Ref. 18.

Rodents. Air was pushed through the 1-liter respirometry chamber at a known rate of ~0.9 l/min and monitored by a mass-flow controller (840L-2, Sierra Instruments, Monterey, CA). Air leaving the chamber was subsampled, dried (Drierite, W.A. Hammond drierite), and analyzed for fractional O₂ and CO₂ concentration (S-3A/1 and CD-3A, respectively, Applied Electrochemistry, Pittsburgh, PA), and VO₂ and VCO₂ were calculated as above. It was not necessary to instantaneously correct VO₂ and VCO₂ for chamber washout considerations, because the average deviation of measured to corrected raw values was less than 3%.

Ve was obtained using the barometric method (for a comprehensive review of the technique, see Ref. 44). The chamber was sealed by activating solenoids for a duration of ~60 breaths at each target Tb (see protocol below). A moist cloth in the chamber ensured a saturated environment. Pressure oscillations caused by inspiration could be measured by a differential pressure transducer (MP45-1 Validyne Engineering, Northridge, CA). The system was calibrated by injecting a known volume of air (0.1 ml) into the chamber and monitoring the resulting pressure oscillation. The relatively large animal chamber ensured that potential overestimations of calibration volumes were avoided, because any potential pressure spike was absorbed by the chamber volume.

Experimental Protocol

*Marsupials.* Young were removed from the pouch (after 24 h postsurgery recovery), fitted with a mask (see above) and a thermocouple inserted into their urogenital sinus, and placed into a dark constant-temperature (CT) chamber (modified freezer). A settling period of at least 1 h was allowed to ensure complete equilibration of Tb with Ta (Tb = 36.5°C). For both ectothermic and endothermic joeys, Ta was adjusted to ensure that Tb reached 20°C in ~2 h. Thus, for the endothermic joeys, the Ta was changed to 10°C immediately after the 36.5°C blood sample was taken, and for the ectothermic joeys, Ta was initially adjusted to 20°C after the 36.5°C blood sample was taken and then to 10°C after the 28°C blood sample was taken. Ta within the CT chamber declined at a rate of ~0.2°C/min after each adjustment.

Arterial blood samples were collected at Tb = 36.5, 28, and 20°C. VO₂, VCO₂, Tb, Ta, and ventilatory variables were monitored continuously throughout the experimental period and collected with a Powerlab (AD Instruments, Bella Vista, NSW, Australia) at a rate of 1,000 Hz (see above). When Tb reached 20°C and blood samples had been taken, the animal was removed from the experiment, anesthetized, the cannula removed via surgical procedure, and the joey returned to the pouch.

Rodents. Each animal (after at least 24 h postsurgery recovery at 26°C) was placed into the 1-liter respirometry chamber inside a constant-temperature chamber (modified refrigerator) at 28°C and allowed at least 30 min to settle. The incoming air was then changed to helox (80% helium, 20% oxygen), which facilitates heat loss because helium has a conductance for heat ~6 times greater than nitrogen (at 27°C; Refs. 31, 59). A further 0.5-h settling period was allowed at 30°C (because of difference in lower critical temperature in helox, see Ref. 12). The chamber Ta was then adjusted to 7°C (cooled at a rate of ~0.2°C/min), and over ~2.5 h Tb decreased from 37.5 to 22°C. Arterial blood samples were collected at Tb = 37.5 (normoxia), 37.5 (helox), 30, and 22°C. The respiratory chamber was sealed for ventilation measurements at Tb = 37.5 (normoxia), 37.5 (helox), 34, 30, 26, and 22°C. VO₂, VCO₂, Tb, and Ta were monitored continuously

### Table 1. Respiratory variables during dynamic Tb changes in ectothermic (mass = 97.7 ± 2.06 g) Tammar wallaby joeys

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tb = 36.5°C</th>
<th>Tb = 32°C</th>
<th>Tb = 28°C</th>
<th>Tb = 24°C</th>
<th>Tb = 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tl; s†</td>
<td>0.32 ± 0.07 (4)</td>
<td>0.26 ± 0.02 (4)</td>
<td>0.33 ± 0.04 (4)</td>
<td>0.53 ± 0.08 (4)</td>
<td>0.67 ± 0.05 (4)*</td>
</tr>
<tr>
<td>Tp; s†</td>
<td>0.57 ± 0.15 (4)</td>
<td>0.40 ± 0.04 (4)</td>
<td>0.45 ± 0.07 (4)</td>
<td>0.93 ± 0.05 (4)</td>
<td>1.25 ± 0.12 (4)*</td>
</tr>
<tr>
<td>V̇E/Tl; s†</td>
<td>0.24 ± 0.24 (2)</td>
<td>0.13 ± 0.08 (3)</td>
<td>0.35 ± 0.06 (4)</td>
<td>0.34 ± 0.23 (3)</td>
<td>0.27 ± 0.15 (3)</td>
</tr>
<tr>
<td>V̇E/TI; s†</td>
<td>1.07 ± 0.26 (4)</td>
<td>0.76 ± 0.07 (4)*</td>
<td>1.14 ± 0.13 (4)*</td>
<td>1.72 ± 0.27 (4)</td>
<td>2.12 ± 0.13 (4)*</td>
</tr>
<tr>
<td>V̇E/ml/kg†</td>
<td>5.26 ± 0.58 (4)</td>
<td>5.98 ± 0.65 (4)*</td>
<td>9.01 ± 0.37 (4)*</td>
<td>8.37 ± 0.46 (4)*</td>
<td>9.76 ± 0.42 (4)*</td>
</tr>
<tr>
<td>f, breaths/min†</td>
<td>58.48 ± 10.75 (4)</td>
<td>83.47 ± 8.42 (4)*</td>
<td>58.11 ± 6.39 (4)</td>
<td>38.69 ± 5.64 (4)</td>
<td>29.61 ± 1.88 (4)*</td>
</tr>
<tr>
<td>V̇T/Tl,T; ml/kg†</td>
<td>0.34 ± 0.04 (4)</td>
<td>0.36 ± 0.04 (4)</td>
<td>0.30 ± 0.02 (4)</td>
<td>0.32 ± 0.02 (4)</td>
<td>0.32 ± 0.03 (4)</td>
</tr>
<tr>
<td>V̇E/V̇Tl†</td>
<td>1.24 ± 0.19 (4)</td>
<td>1.72 ± 0.48 (4)</td>
<td>2.10 ± 0.56 (4)*</td>
<td>1.25 ± 0.30 (4)</td>
<td>1.01 ± 0.15 (4)</td>
</tr>
<tr>
<td>V̇E, ml/min-1kg-1†</td>
<td>362 ± 101 (4)</td>
<td>385 ± 158 (4)</td>
<td>372 ± 98 (4)</td>
<td>212 ± 48 (4)*</td>
<td>146 ± 20 (4)*</td>
</tr>
<tr>
<td>VO₂, ml/min-1kg-1†</td>
<td>8.01 ± 0.42 (4)</td>
<td>9.03 ± 1.33 (4)</td>
<td>8.28 ± 1.20 (4)</td>
<td>5.44 ± 1.00 (4)*</td>
<td>3.37 ± 0.39 (4)*</td>
</tr>
<tr>
<td>V̇CO₂, ml/min-1kg-1†</td>
<td>5.87 ± 0.26 (4)</td>
<td>7.27 ± 0.07 (4)*</td>
<td>7.13 ± 0.13 (4)*</td>
<td>4.61 ± 0.29 (4)*</td>
<td>2.44 ± 0.60 (4)*</td>
</tr>
<tr>
<td>RER†</td>
<td>0.74 ± 0.06 (4)</td>
<td>0.80 ± 0.05 (4)</td>
<td>0.86 ± 0.03 (4)</td>
<td>0.85 ± 0.05 (4)</td>
<td>0.11 ± 0.04 (4)*</td>
</tr>
<tr>
<td>V̇E/V̇O₂</td>
<td>45.35 ± 11.86 (4)</td>
<td>40.33 ± 10.17 (4)</td>
<td>45.64 ± 9.56 (4)</td>
<td>42.92 ± 10.07 (4)</td>
<td>44.26 ± 4.68 (4)</td>
</tr>
<tr>
<td>V̇E/V̇CO₂</td>
<td>59.32 ± 11.90 (4)</td>
<td>49.05 ± 10.13 (4)</td>
<td>52.68 ± 10.88 (4)</td>
<td>5.14 ± 13.87 (4)</td>
<td>45.10 ± 6.61 (4)</td>
</tr>
</tbody>
</table>

Values are means ± SE (n). Tb, body temperature; Tl, inspiratory time; Tp, expiratory time; TlTOT, total breathing time; TI, inspiratory pause; VT, tidal volume of breathing; f, breathing frequency; V̇T/TlTOT, inspiratory timing index; V̇E/Tl, mean inspiratory flow; V̇E, minute ventilation; VO₂ rate of oxygen consumption; V̇CO₂, rate of carbon dioxide production; RER, respiratory exchange ratio (VO₂/V̇CO₂); V̇E/V̇O₂ and V̇E/V̇CO₂, ventilatory requirement ratios. †Significant effect of temperature on the variable; *difference from control, Tb = 36.5°C values (Dunnnett’s comparison to a control).
throughout the experimental period. All variables were collected by Powerlab (AD Instruments, Bella Vista, NSW, Australia) at a rate of 1,000 Hz.

Sixty breaths at each target Tb were analyzed for VT according to the equation described by Drorbaugh and Fenn (17). The same 60 breaths were also analyzed for breathing frequency (f = 1/TfTOT × 60) and minute ventilation (Ve = VT × f) as outlined by Frappell et al. (18).

Data Analysis and Statistics

Technical limitations prevented us from obtaining measurements for some animals at certain Tb setting. Thus, rather than using general linear models, a mixed model approach—valid when missing values are at random (35, 68)—was adopted. The effects of Tb on ventilatory, metabolic, and blood gas variables were tested for using a mixed model approach (PROC MIXED) with repeated measures (i.e., using animal as a random factor) and Dunnett’s post hoc comparison (with Tb = 36.5°C as control for joeys and Tb = 37.5°C in helox as control for rats) using SAS (v 9.1) software (34). The variance structures were determined following the recommendations for repeated-measures analyses by Littell et al. (34), and in all cases an “unstructured” variance structure was used that makes no assumption regarding equal variances or correlations. Initially, all models were run with all interaction terms included, but as no interactions were statistically significant only the main effects are reported. Statistical significance was assumed at P < 0.05.

RESULTS

Metabolic Variables

At the initial Tb of 36.5°C, VO2 and VCO2 in ectothermic joeys were 8.01 ± 0.42 and 5.87 ± 0.46 ml-min⁻¹·kg⁻¹, respectively (Fig. 1, A and B, Table 1). VO2 and VCO2 were maintained at the start of cooling, but both reduced significantly compared with initial values when Tb reached 26°C and 23°C, respectively (P < 0.05 for both). Overall, there was a significant effect of reducing Tb on both VO2 and VCO2 (P < 0.001), with Q10 values of 2.41 ± 0.28 and 2.47 ± 0.39 respectively.

In contrast to ectothermic joeys, VO2 and VCO2 in endothermic joeys increased during initial cooling, from initial values of 17.42 ± 1.94 and 11.78 ± 1.53 ml-min⁻¹·kg⁻¹, respectively, to Tb = 36.5°C to maximal values of 32.51 ± 6.61 and 25.54 ± 6.32 ml-min⁻¹·kg⁻¹, respectively, at Tb = 31°C (P < 0.01 for both, Fig. 1, A and B, Table 1). From its maximum value, VO2 returned to initial (36.5°C) value at Tb = 28°C, falling slightly but significantly below initial value by Tb = 20°C (P = 0.037), VCO2 changed in parallel to VO2 with falling Tb, but was not significantly lower than the initial VCO2 (at

Table 3. Respiratory variables during a dynamic body temperature changes in 27 ± 0.05-day-old Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tb = 37.5°C</th>
<th>Tb = 37.5°C</th>
<th>Tb = 37.5°C</th>
<th>Tb = 37.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc, s†</td>
<td>0.21 ± 0.01 (13)</td>
<td>0.21 ± 0.01 (13)</td>
<td>0.15 ± 0.01 (13)</td>
<td>0.15 ± 0.01 (13)*</td>
</tr>
<tr>
<td>Tc, s†</td>
<td>0.29 ± 0.01 (13)</td>
<td>0.26 ± 0.01 (13)</td>
<td>0.16 ± 0.01 (8)*</td>
<td>0.15 ± 0.01 (13)*</td>
</tr>
<tr>
<td>TfTOT, s†</td>
<td>0.50 ± 0.02 (13)</td>
<td>0.47 ± 0.01 (13)</td>
<td>0.30 ± 0.01 (13)*</td>
<td>0.28 ± 0.01 (13)*</td>
</tr>
<tr>
<td>VT/TTOT</td>
<td>0.04 ± 0.02 (13)</td>
<td>0.45 ± 0.01 (13)</td>
<td>0.48 ± 0.01 (13)</td>
<td>0.47 ± 0.01 (13)</td>
</tr>
<tr>
<td>VVT/TVI, ml/kg†</td>
<td>11.22 ± 0.66 (13)</td>
<td>13.39 ± 1.17 (13)</td>
<td>20.32 ± 1.09 (13)*</td>
<td>18.62 ± 1.00 (13)*</td>
</tr>
<tr>
<td>f, breaths/min†</td>
<td>121.96 ± 3.53 (13)</td>
<td>130.72 ± 4.25 (13)</td>
<td>201.16 ± 6.44 (13)*</td>
<td>219.33 ± 10.28 (13)*</td>
</tr>
<tr>
<td>V˙O2</td>
<td>0.42 ± 0.01 (13)</td>
<td>0.45 ± 0.01 (13)</td>
<td>0.48 ± 0.01 (13)</td>
<td>0.47 ± 0.01 (13)</td>
</tr>
<tr>
<td>V˙CO2</td>
<td>0.03 ± 0.01 (13)</td>
<td>0.02 ± 0.01 (13)</td>
<td>0.02 ± 0.01 (13)</td>
<td>0.02 ± 0.01 (13)</td>
</tr>
<tr>
<td>V˙CO2</td>
<td>37.14 ± 1.62 (11)</td>
<td>40.71 ± 1.66 (9)</td>
<td>87.97 ± 3.98 (9)*</td>
<td>81.15 ± 4.19 (13)*</td>
</tr>
<tr>
<td>V˙O2</td>
<td>28.09 ± 1.57 (11)</td>
<td>31.04 ± 1.99 (13)</td>
<td>63.26 ± 2.30 (13)*</td>
<td>57.16 ± 2.34 (13)*</td>
</tr>
<tr>
<td>V˙CO2</td>
<td>0.76 ± 0.02 (9)</td>
<td>0.73 ± 0.02 (13)</td>
<td>0.72 ± 0.03 (13)</td>
<td>0.72 ± 0.03 (13)</td>
</tr>
<tr>
<td>V˙O2/V˙CO2</td>
<td>38.04 ± 3.35 (11)</td>
<td>41.10 ± 5.07 (9)</td>
<td>46.59 ± 2.29 (13)</td>
<td>51.51 ± 2.76 (13)</td>
</tr>
<tr>
<td>V˙O2/V˙CO2</td>
<td>51.92 ± 5.69 (11)</td>
<td>53.57 ± 5.40 (9)</td>
<td>64.37 ± 3.15 (13)</td>
<td>73.06 ± 3.26 (13)</td>
</tr>
</tbody>
</table>

Values are means ± SE (n). †Significant effect of temperature on the variable; *difference from control, Tb = 37.5°C helox values (Dunnett’s comparison to a control).
From Tb
Ventilation and Respiratory Requirement

0.14, respectively.

The increased V˙E at Tb
reaching a maximum value of 4,089
P
37.5 to 34°C (doubled during cooling when exposed to helox; Tb fell from
for all; Fig. 1,
Tb
487 ml·min

(36.5°C in joeys, 37.5°C helox in rats) for the 3 groups.

Tb
31–20°C was 1.83
P
34 –25°C, both V˙O2 and V˙CO2 decreased, falling to
values initially observed at Tb = 37.5°C values. Q10 for V˙O2 and V˙CO2 from Tb
28°C (Fig. 1,
C
31–20°C was 2.42
P
34 –22°C was not different than the

A

B

Fig. 3. Arterial partial pressure of oxygen (Pao2, kPa) (A), partial pressure of carbon dioxide (PCO2, kPa) (B), and pH (pHa) during dynamic body temperature (Tb) cooling in endothermic Tammar wallaby joeys (●, n = 3), ectothermic Tammar wallaby joeys (gray diamonds, n = 4), and Sprague-Dawley rats (■, n as reported in Table 5) initially in normoxia (○) and during exposure to helox (■). Error bars are ±SE. A summary of the joey and rat data can be found in Tables 4 and 5. The dashed regression in C indicates predicted α-stat regulation (ΔpHa/ΔTb = 0.16 U/°C) from pHa of ectothermic joeys at 37°C (Tbstart).

Blood Gases

Blood gases were unaltered by changes in Tb in all groups. Ectothermic joeys were relatively hypoxic and hypercapnic relative to the endotherms. Pao2 and PCO2 averaged 16.95 ± 0.35 and 5.39 ± 0.18 kPa in ectothermic joeys, 18.59 ± 0.51

to overall V˙E, V˙T/Ti more than doubled with initial cooling, from 5.10 ± 0.53 ml/s at Tb = 37.5°C to 10.79 ± 0.49 ml/s at Tb = 34°C. This was the result of a decrease in Ti and an increase in V˙T (P < 0.01 for both).

In all groups, V˙E/V˙O2 and V˙E/V˙CO2 remained unchanged as Tb fell. V˙E/V˙O2 averaged 43.70 ± 9.27 in ectothermic joeys, 51.45 ± 4.25 in endothermic joeys, and 47.90 ± 2.79 in rats. V˙E/V˙CO2 averaged 51.58 ± 10.68, 68.00 ± 3.76, and 65.87 ± 3.17 in ecto- and endothermic joeys and rats, respectively, across the Tb range (Fig. 2, Tables 1 and 3).

Blood Gases

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A

B

Fig. 2. V˙E (ml·min⁻¹·kg⁻¹) vs. V˙CO2 (ml·min⁻¹·kg⁻¹) during changes in Tb in ectothermic (gray diamonds, n = 4) and endothermic (●, n = 5) Tammar wallaby joeys and 27 ± 0.5-day-old Sprague-Dawley rats, initially in normoxia (○) and when exposed to helox (■). n Values for rats as reported in Table 3. The numbers inside (or next to) the symbols represent Tb. Error bars are ±SE. The isopleth represents the average V˙E/V˙CO2 value at control Tb (36.5°C in joeys, 37.5°C helox in rats) for the 3 groups.

Tb = 36.5°C at the coldest Tb. Q10 for V˙O2 and V˙CO2 from Tb = 31–20°C was 1.83 ± 0.13 and 1.78 ± 0.15, respectively.

For rats, no difference was observed in V˙O2 or V˙CO2 measured in room air or helox at the initial Tb of 37.5°C (P > 0.10 for all; Fig. 1, A and B, Table 3). Both V˙O2 and V˙CO2 more than doubled during cooling when exposed to helox; Tb fell from 37.5 to 34°C (P < 0.001 for both; Fig. 1, A and B, Table 3). From Tb = 34–25°C, both V˙O2 and V˙CO2 decreased, falling to values initially observed at Tb = 37.5°C values. Q10 for V˙O2 and V˙CO2 from Tb = 34–22°C was 2.42 ± 0.22 and 2.45 ± 0.14, respectively.

Ventilation and Respiratory Requirement

In ectothermic joeys, V˙E fell significantly with progressive hypothermia, from 362 ± 101 ml·min⁻¹·kg⁻¹ at Tb = 36.5°C to 212 ± 48 and 146 ± 20 ml·min⁻¹·kg⁻¹ at Tb = 24–20°C (P = 0.011; Fig. 1C, Table 1). The decrease in V˙E with falling Tb resulted from a significant fall in f (P < 0.001), whereas VT progressively increased (P = 0.003). VT/Ttot was maintained constant across the Tb range (Table 1). VT/Tt was nearly twice as great at Tb = 28°C (2.10 ± 0.56 ml/s) compared with Tb = 36.5°C (P = 0.004; Table 1).

In the endothermic joeys, V˙E doubled with initial cooling, from 737 ± 149 ml·min⁻¹·kg⁻¹ at Tb = 36.5°C to 1,553 ± 487 ml·min⁻¹·kg⁻¹ at Tb = 28°C (P = 0.011; Fig. 1C, Table 2). The increased V˙E at Tb = 28°C was produced via an increase in f (P = 0.038, Table 2). Paralleling the increase in overall V˙E, V˙T/Tt doubled as Tb was lowered to 28°C (P = 0.041; Table 1).

Similar to endothermic joeys, V˙E more than doubled in rats with initial cooling (parallel to changes in V˙O2 and V˙CO2), reaching a maximum value of 4,089 ± 199 ml·min⁻¹·kg⁻¹ at Tb = 30°C (Fig. 1C, Table 3). Again, in parallel to the metabolic effects, V˙E at Tb = 22°C was not different than the initial level at Tb = 37.5°C (Fig. 1C). Changes in V˙E in the rats were achieved with increases in V˙T and f (Table 3). In addition

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DISCUSSION

In contrast to our initial hypothesis, young Tammar wallaby joeys with little ability to thermoregulate, did not utilize an acid-base regulation occurs in younger animals and we may have detected this if we measured even younger animals. Given the preservation of Ve/V02 previously observed with cooling in younger joeys (38), this seems unlikely. Rather, our data suggest that marsupial joeys adopt a pH-stat strategy of acid-base regulation during hypothermia, regardless of stage of development and thermogenic ability.

Our resting V02 values are comparable with previously published values. Metabolic data obtained for ecto- and endothermic joeys at their normal, euthermic Tb of 36.5°C fall on their prediction lines (Fig. 1, see Fig. 2 in Ref. 19). Similarly, the resting values for V02 and VCO2, Ve/V02, and Ve/VCO2 of normothermic (Tb = 37.5°C) rats (in normoxia and helox) are expected for ~80 g rats (46). Importantly, although some of these variables have been measured on cold-tolerant species that undergo torpor (e.g., 69) this study provides simultaneous respiratory, metabolic, and blood acid-base data that have been obtained without anesthetics that have potent effects on thermoregulatory and respiratory control and has previously complicated data interpretation (e.g., 2, 40). The use of helox to facilitate heat loss in the rats had no effect on initial metabolic, ventilatory, or blood variables, as has been shown in other species (26).

To assess the relatively small sample sizes, post hoc power analysis (analysis of variance with repeated measures, within factors) was conducted on Ve, which was a factor that changed significantly in all animal groups. The analysis revealed statis-

and 5.17 ± 0.29 kPa in endothermic joeys, and 16.17 ± 0.53 and 4.60 ± 0.36 kPa in rats (Fig. 3, Tables 2 and 5). Pa02 was maintained constant in all groups during hypothermia, averaging 7.22 ± 0.05, 7.31 ± 0.01, and 7.4 ± 0.03 in ecto- and endothermic joeys and rats, respectively (Tables 4 and 5). Similarly the other blood variables remained unchanged across the Tb range (Tables 4 and 5). CaO2 averaged 3.34 ± 0.29 mmol/l in ectothermic joeys, 4.52 ± 0.01 mmol/l in endothermic joeys and 5.22 ± 0.33 mmol/l in rats across the Tb range. [Hb]a averaged 52.17 ± 10.00 g/l in endothermic joeys, 72.67 ± 4.67 g/l in ectothermic joeys, and 87.06 ± 1.53 g/l (P = 0.520) in rats. [La] averaged 2.35 mmol/l in endothermic joeys and 0.57 ± 0.24 in rats across the Tb range (Tables 2 and 5).

### Methodological Considerations

During the initial stages of hypothermia, our “ectothermic” joeys maintained V02 and VCO2, demonstrating some (albeit very limited) ability to mount a thermogenic response, most likely through NST because shivering was not observed in these young animals (Fig. 4). Indeed, despite this plateau in the V02 response, these animals were unable to defend Tb to any measurable degree during ambient cooling. Nevertheless, it is possible that a transition from an α- to a pH-stat mechanism of acid-base regulation occurs in younger animals and we may have detected this if we measured even younger animals. Given the preservation of Ve/V02 previously observed with cooling in younger joeys (38), this seems unlikely. Rather, our data suggest that marsupial joeys adopt a pH-stat strategy of acid-base regulation during hypothermia, regardless of stage of development and thermogenic ability.

Our resting V02 values are comparable with previously published values. Metabolic data obtained for ecto- and endothermic joeys at their normal, euthermic Tb of 36.5°C fall on their prediction lines (Fig. 1, see Fig. 2 in Ref. 19). Similarly, the resting values for V02 and VCO2, Ve/V02, and Ve/VCO2 of normothermic (Tb = 37.5°C) rats (in normoxia and helox) are expected for ~80 g rats (46). Importantly, although some of these variables have been measured on cold-tolerant species that undergo torpor (e.g., 69) this study provides simultaneous respiratory, metabolic, and blood acid-base data that have been obtained without anesthetics that have potent effects on thermoregulatory and respiratory control and has previously complicated data interpretation (e.g., 2, 40). The use of helox to facilitate heat loss in the rats had no effect on initial metabolic, ventilatory, or blood variables, as has been shown in other species (26).

### Table 5. Blood gas variables during a dynamic body temperature changes in 27 ± 0.05 day-old Sprague Dawley rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tb = 37°C</th>
<th>Tb = 37°C</th>
<th>Tb = 30°C</th>
<th>Tb = 22°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa02, kPa</td>
<td>16.30 ± 0.93 (7)</td>
<td>16.51 ± 0.86 (7)</td>
<td>16.26 ± 0.54 (8)</td>
<td>17.26 ± 0.61 (5)</td>
</tr>
<tr>
<td>PaCO2, kPa</td>
<td>5.42 ± 0.60 (8)</td>
<td>4.68 ± 0.27 (8)</td>
<td>3.71 ± 0.41 (8)</td>
<td>4.23 ± 0.74 (5)</td>
</tr>
<tr>
<td>pH</td>
<td>7.42 ± 0.04 (7)</td>
<td>7.42 ± 0.05 (8)</td>
<td>7.36 ± 0.02 (8)</td>
<td>7.39 ± 0.01 (5)</td>
</tr>
<tr>
<td>CaO2, mmol/l</td>
<td>5.10 ± 0.64 (6)</td>
<td>4.86 ± 0.26 (6)</td>
<td>5.50 ± 0.44 (7)</td>
<td>4.61 ± 0.67 (5)</td>
</tr>
<tr>
<td>[Hb]a, g/l</td>
<td>93.3 ± 3.50 (4)</td>
<td>86.6 ± 4.71 (4)</td>
<td>90.6 ± 5.56 (4)</td>
<td>85.00 ± 9.00 (2)</td>
</tr>
<tr>
<td>[La]a, mmol/l</td>
<td>0.60 ± 0.32 (4)</td>
<td>0.7 ± 0.40 (4)</td>
<td>1.0 ± 0.43 (4)</td>
<td>LO (2)</td>
</tr>
</tbody>
</table>

Values are means ± SE (n). LO = below detectable levels. Temperature exerted no significant effect on any variable.
As reported in Table 3) initially in normoxia (assuming a respiratory exchange ratio of 0.85 and a Q10 of 2.5).

cooling in endothermic Tammar wallaby joeys (straight line with a slope of Q10, which remains permanently attached to the teat until near pouch vacation. Our experiments required us to remove joeys from the teat, surgically implant catheters, return the joey to the pouch, reattach them to the teat, and trust the mother would not reject the joey (as occurred in a number of cases).

$$\dot{V}_E, \dot{V}_{O_2}, \text{and pH}a \text{ Regulation during Hypothermia: Comparative and Developmental Aspects}$$

In euthermic conditions, an increase in the prevailing $\dot{V}_{O_2}$ and $\dot{V}CO_2$ of mammals is generally met by an increase in $\dot{V}_E$ for the maintenance of blood gases (e.g., during moderate exercise, see Ref. 67 for review). There are, however, exceptions, as is the case during sleep when there is a relative hypventilation and subsequent increase in $P_{aCO_2}$ (e.g., 53).

In all three groups there was no significant effect of Tb on $\dot{V}_E/\dot{V}_{O_2}$ or $\dot{V}_E/\dot{V}CO_2$. Alterations in $\dot{V}_E$ were achieved by different means, however, among the different groups. In parallel with the increase in $V_{O_2}$ with initial cooling, rats increased $\dot{V}_E$ via an increase in both $V_T$ and $f$ before $V_T$ fell once Tb reached 22°C. This pattern is similar to immature rodents, where the decrease in $V_T$ was attributed to failure at the level of the central rhythm generator for breathing (60). In contrast, endothermic joeys initially increased $\dot{V}_E$ by increased $f$ only. Both $V_T$ and $f$ decreased in ectothermic joeys during hypothermia, similar to previous observations in younger joeys during severe hypothermia (38). There was no alteration in postinspiratory pause in ectothermic joeys in contrast to neonatal rats where postinspiratory (and postexpiratory) pauses become larger during cooling (60).

Broadly speaking, whatever the thermoregulatory ability or species, inspiratory flow rates ($VT/TI \equiv \text{inspiratory drive}$) are directly correlated with $\dot{V}_E$, i.e., an increase in $\dot{V}_E$ was associated with an increase in $VT/TI$. $\dot{V}_E$ is tightly correlated with $V_{O_2}$ and the increase in $V_{O_2}$ that accompanied thermogenesis occurred when Tb declined. Therefore, although direct effect of Tb on inspiratory drive is expected because of the direct effect of temperature on chemo- and stretch receptors (41, 61), changes in drive were presumably masked by the effects of thermogenesis and the need to ensure $\dot{V}_E$ remain matched to metabolic rate. A similar conclusion was drawn for newborns, where the increase in metabolism during cold exposure masked the effects that Tb per se has on vagal ventilatory mechanisms (38). It thus appears that the predominant driver in endothermic (and those that will become endothermic) animals is the preservation of $\dot{V}_E, \dot{V}_{O_2}, \text{and } \dot{V}_E/\dot{V}CO_2$ that results in constant blood gases (Figs. 2 and 3).

Unlike our animals, awake, nonanesthetized ground squirrels show a relative hyperventilation (and thus an increase in pHa) during hypothermia (Tb = 37.5–31°C) when measurements were taken immediately after the animals were awakened from a daily torpor bout (9). During “forced” hypothermia (used in the current study), the drop in Tb is unregulated, whereas the Tb changes occurring in torpid animals is regulated. Furthermore, animals capable of torpor appear to be more intrinsically cold tolerant, at least at the cellular level (47), and therefore may not respond to hypothermia in a similar manner to a homeotherm.

Despite their relative immaturity, $\dot{V}_E/\dot{V}_{O_2}$ (and $\dot{V}_E/\dot{V}CO_2$) was preserved across the Tb range in our younger joeys, resulting in stable blood gases and pHa. The findings of this study are supported by the constant $\dot{V}_E/\dot{V}_{O_2}$ observed in ectothermic day 18 Tammar wallaby joeys (average mass = 2.66 ±
0.17 \text{ g}) across the same Tb range (38). Thus neither ectothermic nor endothermic joeys conform to the values predicted by the α-stat hypothesis (50) and often observed in reptiles (e.g., 10, 16, 20). The constant pHα (ΔpHα/ΔTb ~ 0.002 U/°C) of the rats in this study differs from previous studies using unanesthetized rats during forced hypothermia, where ΔpHα/ΔTb has been shown to range from ~0.0015 to ~0.009 U/°C (2, 3, 63, 66). Discrepancies may be related to methodologies used such as restraint with potential stress-related effects on blood gases and acid-base balance (63) or shaving, which can alter behavioral adaptations to cold stress (2, 3), such as gross movement, piloerection, and postural adjustments that reduce surface area. In addition, restraint can further confound findings by causing increased V˙E (15), increased V˙O2 with no corresponding increase in V˙E (55), and increased plasma corticosterone levels, heart rate, and arterial blood pressure (33).

Acid-base regulation in unanesthetized, unrestrained ectothermic joeys is more similar to unanesthetized, unrestrained endothermic rats and joeys than other ectothermic vertebrates that hyperventilate with reduced Tb (28, 45). If indeed an α-stat strategy serves to preserve protein conformation during hypothermia (50) why do some vertebrates (unanesthetized mammals in this study) appear to conform to a pH-stat strategy for acid-base regulation, irrespective of thermoregulatory ability, whereas other ectothermic vertebrates (see Ref. 13 for review; 51) conform to an α-stat strategy? The answer may be related to the trade-offs that exist between acid-base regulation and thermoregulation. To achieve α-stat regulation during lowered Tb, a relative hyperventilation (i.e., increase in V˙E/V˙CO2) must be achieved through either an increase in V˙E or a decrease in V˙CO2 (or a combination of both). Heat loss in many homeothermic mammals is primarily across the respiratory surface. An increase in V˙E is in direct conflict with the drive to decrease V˙CO2 in an attempt to minimize heat loss and maintain Tb. Similarly a decrease in V˙CO2 (and V˙O2) is in direct conflict with the drive to increase thermogenesis (and therefore increase V˙O2 and V˙CO2) during lowered Tb. Thus, irrespective of ability to generate heat or stage of development, it appears mammals (at least those used in this study) do not sacrifice thermoregulatory ability in an effort to maintain protein charge status. It may be that a lower Tb during reductions in Ta has more deleterious effects for growth and development compared with alterations in protein charge status. Whatever the reason, unanesthetized, homeothermic vertebrates (joeys and rats) do not regulate acid-base status in the same fashion as many ectothermic vertebrates (reptiles).

### Potential Clinical Relevance

These data potentially provide some insight into the acid-base regulation in mammals (including humans; e.g., newborns) that may be susceptible to hypothermia. In clinical settings (e.g., during cardiopulmonary bypass and other surgeries), there is still much debate over the most appropriate approach (i.e., α- or pH-stat) for regulating pH (see Refs. 4, 25 for reviews). A recent, comprehensive clinical review suggests that age may be important and that adverse effects are best avoided by adopting a pH-stat strategy for pediatric and an α-stat strategy for adult patients (6). Our data obtained in unanesthetized animals suggest that a pH-stat strategy during clinical hypothermia will result in an acid-base status that is more similar to accidental hypothermia.

### Conclusions

Constant ventilatory requirement resulted in constant blood gases and hence, constant pHα in endothermic and ectothermic joeys and rats. Thus, across the transition zone between ectothermy and endothermy, the regulation of blood gases and blood-acid base balance in marsupial joeys is more akin to the regulation seen in endothermic and heterothermic mammals (constant pHα, e.g., rats in this study) than that often observed for ectothermic vertebrates (α-stat). Although young marsupial joeys, similar to ectothermic vertebrates (e.g., reptiles), are poikilothermic, the regulation of blood acid-base status is more akin to that observed in homeothermic mammals (older joeys and rats). Constant pHα regulation while the young marsupial joey is still essentially poikilothermic (similar to a reptile) suggests that pHα regulation is established early (and perhaps independently) during the development of endothermy in marsupials. Furthermore, these data have potential implications for clinical management of pHα of newborns with highly labile body temperatures, situations of accidental hypothermia, and clinical hypothermia that may occur during surgery.

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### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

### AUTHOR CONTRIBUTIONS

Author contributions: S.J.A. and P.B.F. conception and design of research; S.J.A. performed experiments; S.J.A., K.J.C., and P.B.F. interpreted results of experiments; S.J.A. prepared figures; S.J.A. drafted manuscript; S.J.A., K.J.C., and P.B.F. edited and revised manuscript; S.J.A., K.J.C., and P.B.F. approved final version of manuscript.

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