Comparative Biochemistry and Physiology, Part A 306 (2025) 111873

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Fasting elicits immune modulation and leukocyte redistribution in bullfrogs (*Lithobates catesbeianus*)

Aymam C. de Figueiredo [a,*], Débora M.A. Prado [a], Felipe R. Floreste [a], Patrício G. Garcia Neto [a], Fernando R. Gomes [a], José E. de Carvalho [b]

[a] Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, Travessa 14, 321, Cidade Universitária, CEP 05508-900 São Paulo, SP, Brazil
[b] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, Campus Diadema, R. Artur Riedel 275, Jd. Eldorado, CEP 09972-270 Diadema, SP, Brazil

ARTICLE INFO

Editor name: Dr. Michael Hedrick

Keywords:
Anuran
BKA
Food shortage
Hemagglutination
Neutrophil/lymphocyte ratio
Plasma corticosterone

ABSTRACT

An integrated range of behavioral, physiological, and biochemical adjustments reduces metabolic expenditure and energy utilization during fasting to maintain homeostasis. In order to cope with these adjustments, several vertebrates can modulate immune function and corticosterone secretion while fasting to save energy. However, in ectothermic animals that can fast for longer periods due to their low metabolic rate, the underlying corticosterone and immune modulation is still not well understood. This study aimed to investigate corticosterone and immune modulation during fasting in the bullfrog (*Lithobates catesbeianus*). Bullfrogs were divided into two treatments: a 'control group', fed twice a week with fish feed for 31 days; and a 'fasting group', which remained fasting for 31 days. On the 31st day of experiment, blood was collected to measure corticosterone plasma levels and immune function (neutrophil/lymphocyte ratio, plasma bacterial killing ability - BKA, and hemagglutination) variables. Fasting did not affect corticosterone plasma levels but increased neutrophil/lymphocyte ratio and hemagglutination, while BKA was decreased. In this way, fasting modulates the innate immune function in male bullfrogs by increasing the NL ratio and hemagglutination activity while reducing BKA, without affecting plasma CORT levels or fat body mass content. Thus, 31 days of fasting induces blood immune cell redistribution and an immunomodulatory response without significant activation of the HPI axis or depletion of energy stores.

1. Introduction

In some species, fasting is present in life cycle events such as development, reproduction, estivation, and migration period of several animals (Secor and Carey, 2016). Mainly, fasting can be divided into three phases: phase I is the short period of food deprivation after meal ingestion (energy source switches from the last meal to body storage of glycogen, lipids, and protein); phase II is usually the longest phase, with lipids accounting for the massive amount of the metabolized substrate; and phase III, when lipid storage reaches a threshold level and energy source switches from lipid to amino acid catabolism (Secor and Carey, 2016). The depletion of fat storage along with the atrophy of some gastrointestinal tract tissues during fasting leads to the loss of body mass (Cramp et al., 2005; Secor, 2005; Secor and Carey, 2016). Fasting may drive animals toward a new state of energetic homeostasis as they respond to the stress of food deprivation, gastrointestinal morphological adjustments, and the depletion of endogenous energy reserves. To maintain homeostasis, a coordinated suite of behavioral, physiological, and biochemical adjustments works to reduce metabolic expenditure and optimize energy utilization (McCue, 2012; Secor and Carey, 2016). In this context, it is particularly intriguing how endocrine and immune functions - key processes in biological regulation and defense - are modulated during the challenges imposed by fasting. For example, hormones such as thyroid T3 and T4, that increase metabolism and support some physiological processes (e.g. immune function) are downregulated, while hormones that increase the endogenous substrates mobilization (e.g. glucocorticoids) are upregulated (Secor and Carey, 2016). Ectothermic animals exhibit a greater capacity for energy saving due to their low metabolic rate, which allows these animals to fast for longer periods (Secor, 2005). Particularly in anurans, fasting is a

Abbreviations: BKA, bacterial killing ability; BM, body mass; CORT, corticosterone; HPI axis, hypothalamic-pituitary-interrenal axis; NL ratio, neutrophil/lymphocyte ratio; SRBC, sheep red blood cells; SVL, snout-vent length.

* Corresponding author.
E-mail address: aymam.figueiredo@gmail.com (A.C. de Figueiredo).

https://doi.org/10.1016/j.cbpa.2025.111873
Received 16 January 2025; Received in revised form 24 April 2025; Accepted 28 April 2025
Available online 29 April 2025

widespread phenomenon, often linked to critical life history stages. For instance, male European common frogs (*Rana temporaria*) cease feeding for days or weeks during breeding, redirecting energy toward reproduction (Ryser, 1989). Similarly, Andean toads (*Bufo spinulosus*) voluntarily fast during hibernation in cold months (Naya et al., 2009).

While fasting, the synthesis of glucocorticoid hormones such as cortisol and corticosterone (CORT, commonly found in anurans) is sometimes necessary to trigger mobilization of energetic substrates through gluconeogenesis, as well as protein and lipid catabolism (Neuman-Lee et al., 2015; Secor and Carey, 2016). During fasting phase II, low levels of cortisol/CORT support lipolysis; while in phase III a more pronounced elevation of glucocorticoid secretion is necessary for the protein catabolism and/or to support foraging behavior (Secor and Carey, 2016). In endothermic fasting animals (i.e., mammals and birds) the increased glucocorticoid production during the final stage of phase II and during phase III is vastly documented. For example, dark-eyed Juncos birds (*Junco hyemalis*) show increased CORT plasma levels after 36 h of food deprivation (Astheimer et al., 1992); while mice (*C57BL Mus musculus*) show increased CORT plasma levels after 48 h of food deprivation (Ahima et al., 1996). Similarly, some ectothermic animals such as the red salmon (*Oncorhynchus nerka*; Idler et al., 1959) and the watersnake (*Nerodia sipedon*; Webb et al., 2017) exhibit pronounced increase in plasma glucocorticoid levels after 5 and 2 weeks of fasting period, respectively. On the other hand, CORT levels were not affected by 40 days of fasting in non-stressed garter snakes (*Thamnophis elegans*; Neuman-Lee et al., 2015). For anurans, tadpoles of the western spadefoot toad (*Spea hammondii*) increase whole-body CORT levels after 5 days of fasting (Crespi and Denver, 2005), but CORT levels in adult African clawed frog (*Xenopus leavis*) remain unaltered after 31 days of food deprivation (Crespi et al., 2004a, 2004b). In this way, modulation of CORT levels during fasting remains to be better described in anurans.

Due to the limited endogenous energy sources during the fasting period, the available resources might be diverted from other physiological functions, such as immunity (Secor and Carey, 2016). In fact, during energetically costly processes, variables such as the ability of plasma non-cellular immune components to kill bacteria (plasma bacterial killing ability, BKA) decreases, as shown in pregnant pygmy rattlesnakes (*Sistrurus miliarius*; Lind et al., 2020); and during meal digestion in bullfrogs (*Lithobates catesbeianus*; Figueiredo et al., 2021b). When fasted for 12 and 30 days, respectively, ducks (*Anas plathyrhnchos*) and king penguins (*Aptenodytes patagonicus*) presented a decline on the plasma levels of natural antibodies (e.g. immunoglobulins; Bourgeon et al., 2007; Bourgeon et al., 2010). In both studies, decreased immune function was concomitant with the peak of plasma CORT levels. On the other hand, increased CORT levels induce blood immune cell redistribution by increasing the neutrophil/lymphocyte (NL) ratio in several vertebrate models (including anurans), in different physiological contexts (Falso et al., 2015; Assis et al., 2019; Figueiredo et al., 2021a; Figueiredo et al., 2021b). In humans, studies show elevated NL ratio and neutrophil activation after 72 h of intensive fasting, as an indication of inflammation or stress (Qian et al., 2021). Therefore, glucocorticoids may exert a complex immunomodulatory role during fasting; however, the systemic effects of these hormones and their influence on immune regulation during fasting remain poorly understood in non-mammalian vertebrates.

This study aimed to investigate the glucocorticoid and immune modulation during the fasting period in anurans, using bullfrogs (*Lithobates catesbeianus*) as a study system. We hypothesized that fasting would stimulate the glucocorticoid secretion, represented by elevated CORT plasma levels in fasting animals comparing to fed ones. We also hypothesized that fasting would lead to changes in white blood cell redistribution increasing NL ratio, along with decreasing BKA and the blood natural antibodies activity (hemagglutination) in comparison to fed animals.

2. Material and methods

2.1. Frog husbandry

Adult males of the bullfrog (*Lithobates catesbeianus*; Anura: Ranidae, $n = 21$) were purchased from a commercial frog farm (Ranakann, São Paulo/Brazil) and shipped to the facilities of the Universidade de São Paulo in November of 2021. In the frog farm, the animals are bred in captivity under controlled feeding with carnivorous fish dry food (Qualy Nutrição Animal, Maxi fish truta HP 42 10–12 mm, fed weekly according to the local farm's specifications - usually two pellets of dry food per day) and environmental conditions (24 ± 4 °C and humidity >80 %). In the laboratory, the frogs were kept in a climate chamber at 25 ± 1 °C with an 12:12 light: dark cycle (lights on at 6:00 and off at 18:00), individually placed in opaque plastic buckets (20L) partially filled with water (~1 L) and a plastic platform as a dry substrate option. All experimental procedures were performed in accordance with the Ethics Committee for Animal Use (CEUA protocol number 321/2018, 21 August 2018, Instituto de Biociências from the Universidade de São Paulo).

2.2. Experimental procedure

On the first day of the experimental procedures, all individuals had their body mass (BM, 198.29 ± 20.46 g; mean ± standard deviation, sd; empty bladders) and snout-vent length (SVL, 126.71 ± 6.15 mm) measured. Frogs were divided into two treatments: control group ($n = 10$) and fasting group ($n = 11$) with same mean body mass (mean ± sd; control: 200.4 ± 19.9 g, fasting: 198.6 ± 21.6 g; *t*-test, $p = 0.944$) and snout-vent length (mean ± sd; control: 12.6 ± 0.6 cm, fasting: 12.8 ± 0.6 cm; t-test, $p = 0.262$). Animals from both groups were fed (≅ 2 % of body mass, dry food) with the same fish feed used in the frog farm, at 10 am, to standardize the beginning of the experimental period. Animals were force-fed on all feeding events to ensure homogeneity in the amount of food ingested. The meal was inserted into the bullfrogs' mouth and swallowed normally. Then, during the subsequent 31 days, animals from 'control group' were fed (≅ 2 % of body mass) twice a week (minimum 48 h between meals) at 10:00, and animals from fasting group had their mouths manipulated (to mimic the force-fed manipulation of the control group) concomitant with the control group feeding events.

After 31 days of the beginning of the experimental procedure, 6 days after the last feeding/mouth manipulation events (enough time to empty the gastrointestinal tract of control group; Figueiredo et al., 2021b), blood samples were taken at 10:00. The blood samples (300 μL) were taken by cardiac puncture using a heparinized 1 mL syringe and 26 G × 1/2″ needle within 3 min of handling (Romero and Reed, 2005). The volume of 2 μL was reserved for leukocyte count, and the remaining blood was centrifuged (600*g*, 4 min) to isolate the plasma, which was subsequently stored at −80 °C for further CORT quantification, BKA and hemagglutination assays. After cardiac puncture, the animals were euthanized by decapitation and all the abdominal fat body was removed and weighted immediately.

2.3. Plasma CORT assay

CORT was extracted with ether from 15 μL of plasma, according to Assis et al. (2015). Samples were then resuspended in the enzyme immunoassay buffer (1 M phosphate solution containing 1 % bovine serum albumin, 4 M sodium chloride, 10 mM EDTA, and 0.1 % sodium azide). The plasma concentration of CORT was determined in duplicates using EIA kits (Cayman Chemical Company, #501320) according to the manufacturer's instructions and previous studies conducted with anurans (Assis et al., 2015; Assis et al., 2019), including this same species (Lima et al., 2020; Figueiredo et al., 2021a, 2021b). Intra- and inter-assay coefficients of variation were 14.90 % and 13.41 %,

respectively, and assay sensitivity was 3.96 pg/ml.

2.4. Neutrophil/lymphocyte ratio (NL ratio)

The leukocyte profile was performed from the smear of 2 μL of blood. The slide was fixed with methanol (5 min), flushed for 15 min with Giemsa 10 % (Cinética, #51811–82-6), and observed under an optical microscope (1000× magnification) with immersion oil (Laborclin, #570662) by the same investigator to count individual leukocyte types. One hundred leukocytes were counted and morphologically identified as neutrophils, lymphocytes, eosinophils, basophils, and monocytes. The NL ratio was calculated as the number of neutrophils divided by the number of lymphocytes, according to Campbell (2006).

2.5. BKA assay

The plasma bacterial killing ability assay (BKA) was performed according to Figueiredo et al. (2021a, 2021b). Plasma samples (10 μL) were incubated with 10 μL of a bacteria (*Aeromonas hydrophila*) solution (2.5×10^7/mL) in sterile Ringer's solution (190 μL) for amphibians (NaCl: 6.5 g, KCl: 1 g, NaH2PO4: 0.1 g, CaCl2: 1.125 g, NaHCO3: 0.2 g, C6H12O6: 2 g, diluted in 1.3 L of distilled water) for 1 h at 37 °C. The positive solution consisted of Ringer's solution (200 μL) + 10 μL of *A. hydrophila* solution, and the negative solution consisted only of Ringer's solution (210 μL). After incubation time, 500 μL of tryptic soy broth (TSB; Kasvi, #K25–610053) was added, and 300 μL of the samples were transferred in duplicate to 96 wells microplate and kept at 37 °C. The plate was read hourly for 3 h in a spectrophotometer ($\lambda = 595$ nm; Molecular Devices, SpectraMax 190). To account for plasma antimicrobial activity, the BKA was quantified at the beginning of the exponential phase of bacterial growth according to the formula: 1 - (average of the optical density to each sample/optical density of the positive control), representing the proportion of killed microorganisms in the samples compared to the positive control (Assis et al., 2013).

2.6. Hemagglutination assay

In a 96-well plate, 20 μL of phosphate-buffered saline (PBS) solution was added to wells 1–10. After, 20 μL of plasma was added to the first well of each row. Then, 20 μL of the plasma + PBS solution was transferred from well 1 to well 2. Subsequently, 20 μL was transferred from well 2 to well 3 and so on, until 20 μL was added to well 10. Then, the excess of 20 μL from the final solution was discarded. This created a serial dilution (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, to 1:1024). After, 20 μL of PBS was added to well 11 (negative control) and 20 μL of hemolysin to well 12 (positive control). Then, 20 μl of 2 % sheep red blood cells (SRBC) was added to all wells and the plate was incubated for 3 h at 25 °C. Finally, SRBC agglutination was visually scored after enough natural antibody activity was present (Matson et al., 2005). In this case, the antibodies agglutinate the SRBC, causing a visible spreading on the wells surface, otherwise the SRBC form a distinct "button" at the bottom of the V-shaped well. Higher natural antibodies activity or natural antibodies titers allowed more plasma dilutions before the agglutination stops. The hemagglutination titer was calculated from the $\log_2$ of the highest dilution (Assis et al., 2020; López-Pérez et al., 2020).

2.7. Statistical analysis

Except for fat body mass and BKA, none of the data fitted the assumptions of normality and homoscedasticity (Shapiro–Wilk and Levene tests), and therefore we transformed them to Log_{10}. As both fat body mass and BKA are proportional data (percentage) we performed the arcsine square root transformation prior to the statistical analysis (Lin and Xu, 2020). Herein, data was plotted untransformed. To test if morphological measures (body mass and snout-vent length) affected the variables, we performed an ANCOVA test in which studied variables (asin(sqrt(fat body mass), LogCORT, LogNL ratio, asin(sqrt(BKA)), and LogHemagglutination) were used as dependent variables, treatment (control and fasting) as a factor, and morphological measures (BM and SVL) at the end of treatments as cofactors (independently). As morphological measurements did not affect any studied variables, we performed parametric *t*-tests (to fat body mass and BKA) and nonparametric Mann-Whitney tests (to CORT, NL ratio, and hemagglutination) in which we used each studied variable as a dependent variable and treatment as a factor. To test if the animals lost body mass over the fasting period, we performed a general linear model (GLM) test in which body mass was used as dependent variable, and treatment and moment of treatment (before and after treatment) as factors. We used the significance level of $p \leq 0.05$. We performed analyses using the IBM SPSS Statistics 22 program.

3. Results

None of the studied variables were influenced by morphological measures (body mass or snout-vent length): fat body mass ($F_{1,20} = 0.125$, $p \geq 0.419$); CORT ($F_{1,19} = 0.416$, $p = 0.528$); NL ratio ($F_{1,20} = 0.078$, $p = 0.784$); BKA ($F_{1,19} = 0.261$, $p = 0.616$); hemagglutination ($F_{1,20} = 0.009$, $p = 0.926$).

Body mass was affected by fasting (Table 1); in which fasting bullfrogs lost ≅ 4 % of their body mass ($p = 0.005$), while control bullfrogs maintained their body mass over the 31 days of treatment ($p = 0.758$; Fig. 1). Meanwhile, both body and fat body mass were similar between groups after treatment (Tables 1, 2; Fig. 1, 2A).

Plasma CORT levels were similar between groups (U = 33.5, $p = 0.212$, Fig. 2B; Table 2). Regarding the immune variables, fasting bullfrogs showed 2-fold higher NL ratio (U = 26.0, $p = 0.041$; Fig. 3A), concomitant with lower BKA ($T = 18$, $p = 0.044$, Fig. 3B) and higher hemagglutination (U = 35.0, $p = 0.039$, Fig. 3C).

4. Discussion

We demonstrated that 31 days of fasting modulated the immune profile of bullfrogs, elevating NL ratio and blood hemagglutination, along with decreasing BKA. Simultaneously, fasted bullfrogs showed CORT levels similar to control animals at the end of the experiment, despite high individual variation within each group. Our results contribute to a better understanding of the fasting–CORT/immune interactions in ectothermic animals, illustrating especially some of the innate immune modulations during food restriction.

Declining body mass over fasting is well documented for both endothermic and ectothermic animals, being much slower for ectotherms though, due to their lower mass-specific metabolic rates (McCue et al., 2012). Prairie dogs (*Cynomys leucurus* and *Cynomys ludovicianus*), for example, lose ≅ 40 % of their initial body mass after five weeks of fasting, while frogs (*Rana esculenta*) lose ≅ 30 % of body mass after 20 months of fasting (Harlow, 1995; Grably and Piery, 1981). Even though the fasting bullfrogs in our study lost 4 % of their initial body mass over the 31 fasting days, there was no difference in body mass when compared to the control group at the end of the experiment. Body mass loss is a typical parameter used to distinguish the fasting phases in

Table 1
Effect of fasting (31 days) in *Lithobates catesbeianus* body mass through a GLM test in which body mass was used as dependent variable, treatment (control and fasting) and time (before and after treatment) as factors.

Variable	Source	DF	Z	*p*
Body mass (g)	Time	1	5.915	**0.025**
	Treatment ×Time interaction	1	3.917	0.062
	Error	19		

DF Degrees of freedom. Variables with *p* significant ≤0.05 are highlighted in bold.

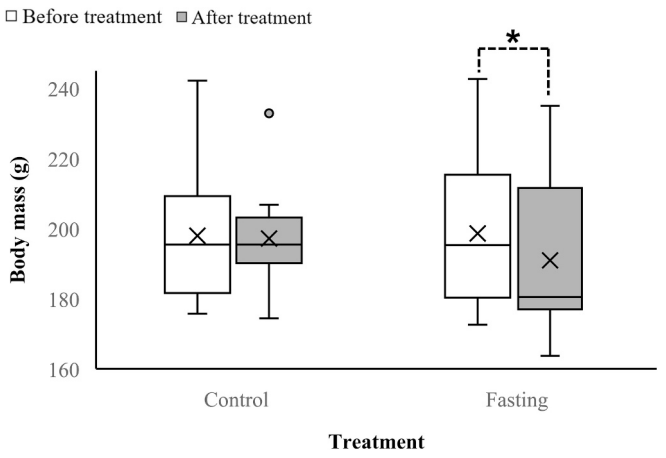

Fig. 1. Body mass (g) of *Lithobates catesbeianus* before and after treatments. Sample sizes were $n = 10$ for control group and $n = 11$ for fasting group. In Box Plot, the box is delimited by the first quartile (lower bar), third quartile (upper bar), and median (central bar). The variation bars are made up of the minimum (lower) and maximum (upper) non-Outliers values. Circles represent outliers' values, and the x letter represents the group average. Asterisks (*) represent differences between groups ($p \leq 0.05$).

Table 2
Effect of fasting (31 days) in *Lithobates catesbeianus* hormonal and immune variables through a set of T- and Mann-Whitney tests in which hormonal and immunological variables were used as dependent variables and treatment (control and fasting) as factors.

Variable	Test	DF/U	T/Z	*p*
Fat body mass (%)	Independent T-test	19	−1.698	0.106
CORT (ng/ml)	Mann-Whitney	33.500	−1.248	0.212
NL ratio	Mann-Whitney	26.000	−2.044	**0.041**
BKA (%)	Independent T-test	18	−2.167	**0.044**
Hemagglutination	Mann-Whitney	35.000	−2.068	**0.039**

DF Degrees of freedom; *CORT* corticosterone plasma levels; *NL ratio* neutrophil/lymphocyte ratio; *BKA* bacterial killing ability. Variables with *p* significant ≤0.05 are highlighted in bold.

endotherms, in which the body loss is greater during phases I and III and lower during phase II (Secor and Carey, 2016). However, in ectotherms, the loss of body mass during fasting is generally moderate and may, at times, primarily reflect a reduction in body water content rather than a significant depletion of energy reserves (Anderson et al., 2017). In this way, the fasted bullfrogs were probably not experiencing phase III on the 31st fasting day, since both treatment groups showed no difference in body mass on the last day of the experiment.

Contrary to our prediction, fasting did not alter the plasma glucocorticoid levels in bullfrogs. In fact, the plasma CORT values of the fasting bullfrogs shown here are relatively small (mean 2.36 ng/ml; 2-fold increase compared to control frogs) when compared to previous studies performed in endothermic animals. We recognize that individual variation within our experimental groups presents challenges for interpreting fasting's effects on CORT levels. Nevertheless, broader comparative analyses across taxa may help identify consistent biological patterns. For example, during fasting phase III, Mallard ducks (*Anas plathyrhnchos*) increased plasma CORT levels by 7-fold (Bourgeon et al., 2010), and Zucker rats (*Rattus norvegicus*) increased plasma CORT levels by 19-fold (Cherel et al., 1992). Therefore, the unaltered CORT levels reported here could suggest that fasting in bullfrogs takes longer than 31 days to activate the hypothalamus-pituitary-interrenal (HPI) axis. In fact, some ectothermic animals such as the Galapagos marine iguanas (*Amblyrhynchus cristatus*) only presented significantly elevated CORT levels (up to 2-fold) during fasting phase III, when body condition dropped below a critical threshold with muscle wastage (Romero and

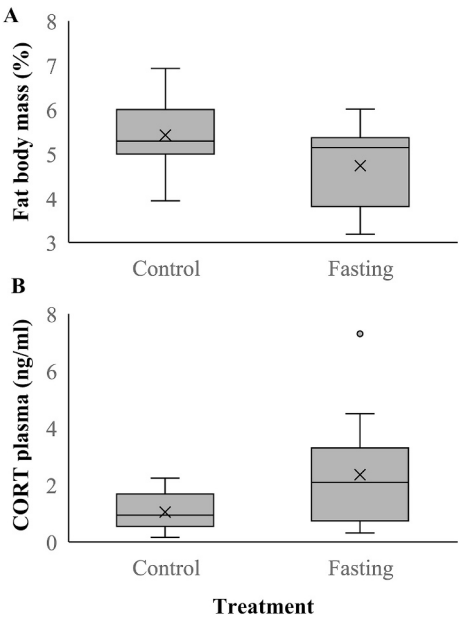

Fig. 2. (A), Fat body mass (%) and (B), plasma corticosterone (CORT) of *Lithobates catesbeianus* in response to fasting. Sample sizes were $n = 10$ for control group and $n = 11$ for fasting group. In Box Plot, the box is delimited by the first quartile (lower bar), third quartile (upper bar), and median (central bar). The variation bars are made up of the minimum (lower) and maximum (upper) non-outliers' values. Circles represent outliers' values, and the x letter represents the group average.

Wikelski, 2001). Similarly, the northern water snake (*Nerodia sipedon*) and the garter snake (*Thamnophis marcianus*) also show 1.5 to 2-fold increase in CORT plasma levels after 15 days and 13 weeks of food shortages, respectively (Webb et al., 2017; Holden et al., 2019). Moreover, plasma CORT levels remain unchanged after 31 days of fasting in adult clawed frogs (*Xenopus leavis*; Crespi et al., 2004a, 2004b). Hence, due to their low metabolic rates, it is possible that ectothermic animals require lower glucocorticoid adjustment (increasing up to 2-fold) to cope with energy storage mobilization during fasting when compared to endothermic animals. Glucocorticoid hormones also participate in behavior reorientation, promoting foraging by increasing locomotor activity and appetite (Crespi et al., 2004a, 2004b; Crespi and Denver, 2005). However, as a 'sit-and-wait' predator – which is an efficient energy-saving strategy (Hervant, 2012) - bullfrogs do not demand a great energy mobilization during foraging, which could also contribute to tolerate prolonged starvation without great glucocorticoid alterations. The adjustments in baseline CORT plasma levels are essential for energy reserve mobilization (Odedra et al., 1983; Yang et al., 2019), being necessary during fasting to fuel essential physiological functions and to maintain homeostasis in high energy demanding organisms. In bullfrogs, 31 days of fasting does not appear to pose a significant challenge for energy depletion or glucocorticoid modulation.

As expected, our fasting bullfrogs increased NL ratio (2-fold). Leukocyte redistribution in anurans is a dynamic process influenced by a complex interplay of factors including captivity stress (Davis et al., 2008; Assis et al., 2015; Davies et al., 2016), environmental condition (Rollins-Smith, 1998; Raffel et al., 2006; Butler et al., 2013), infection (Fites et al., 2013), and nutritional deficiency (Berger et al., 1998; Kiesecker et al., 2001). Despite elevated NL ratio in anurans being associated with higher glucocorticoids plasma levels in several physiological contexts (Falso et al., 2015; Figueiredo et al., 2021a; Titon Jr et al., 2021; Titon et al., 2021), evidence shows that leukocyte redistribution can occur even with minor plasma CORT levels modifications. Cane toads (*Rhinella marina*), for example, increased NL ratio following an immune challenge injection without great plasma CORT enhancement

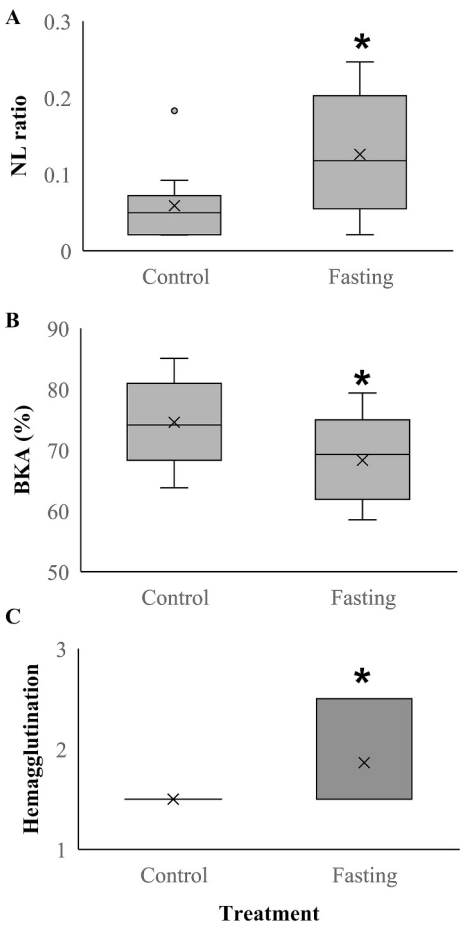

Fig. 3. Immune parameters of *Lithobates catesbeianus* in response to fasting, (A) neutrophil/lymphocyte (NL) ratio, (B) plasma bacterial killing ability (BKA), and (C) blood hemagglutination. Sample sizes were $n = 10$ for control group and $n = 11$ for fasting group. In Box Plot, the box is delimited by the first quartile (lower bar), third quartile (upper bar), and median (central bar). The variation bars are made up of the minimum (lower) and maximum (upper) non-outliers' values. Circles represent outliers' values, and the x letter represents the group average. Asterisks (*) represent differences between groups ($p \leq 0.05$).

(Gardner et al., 2020). Meanwhile, bullfrogs increased NL ratio after low-dose CORT implants and without great plasma CORT enhancement following lipopolysaccharide injection (Falso et al., 2015; Figueiredo et al., 2021a). In addition, other steroid hormones (e.g. estradiol and progesterone) also affects neutrophil/lymphocyte distribution and activation (Molloy et al., 2003; Butts and Sternberg, 2009). All the evidence suggests that the NL ratio may be influenced by a variety of intricated factors beyond hormonal signaling, particularly during stressful events (reviewed in Gomes et al., 2022). In the fasting context, humans and rats show elevation in neutrophil count after 72 h and 6 h, respectively (Nowland et al., 2011; Qian et al., 2021). Altogether, our results show that blood leukocyte redistribution during fasting occurs in anurans similarly to endothermic models but within distinct timeframe. In addition, like stress stimuli, changes in energy availability (e.g., hypoglycemia) can induce changes in the HPI axis output (Crespi and Denver, 2005; Harrell et al., 2016). In this regard, our study presents evidence that changes in NL ratio during fasting in bullfrogs may happen prior to a significant activation of the HPI axis, evidencing the sensitivity of leukocyte redistribution to other factors beyond changes in plasma CORT levels.

Consistent with our prediction, BKA decreased during fasting in the bullfrogs, suggesting a reduced capacity to eliminate bacteria and other injurious agents. The immune function suppression due to limited endogenous energy resources is well known in other vertebrates (Bourgeon et al., 2010; Holden et al., 2019). For instance, mallard ducks (*Anas plathyrhnchos*) fasted for 12 days present a decline in plasma levels of natural antibodies concomitant with the 7-fold CORT plasma levels increase (Bourgeon et al., 2010). Similarly, in fasted garter snakes (*Thamnophis marcianus*), higher CORT plasma levels are associated with lower BKA (Holden et al., 2019). These results suggest that energetic resources are diverted from the immune surveillance to other functions associated to immediate homeostasis maintenance under fasting conditions (Secor and Carey, 2016). In this way, similarly to some energetic demanding processes, as pregnancy and digestion (Lind et al., 2020; Figueiredo et al., 2021b), our results suggest that fasting has also the potential to induce trade-offs with BKA without alterations in CORT levels. Apart from the energy trade-off, fasting restructures gut microbiota of several animals, which can lead to alterations in host immune function (e.g., Sacristán et al., 2016; Eslamloo et al., 2017). Four weeks of fasting period, for example, decreased health and digestion beneficial microbiota, as well as increased pathogenic microbiota (causing dysbiosis) in the gut of crayfish (*Cherax cainii*; Foysal et al., 2020). This gut microbiota imbalance triggered a local downregulation of genes associated with crayfish gut innate immune response (Foysal et al., 2020), highlighting another possible pathway through which fasting can trigger immunosuppressive effects.

Interestingly, fasted bullfrogs exhibited higher hemagglutination compared to control individuals, suggesting an increased presence of agents (e.g., antibodies) capable of inducing red blood cell aggregation. In the lumen of the gastrointestinal tract, epithelial cells recognize patterns on the surface of gut microorganisms, activating signaling cascades that modulate systemic and local immune function in snakes, for example (Lyte et al., 2010; but see also Spahn, 2004). In fact, it is known that blood hemagglutination can be modulated by some stimuli in the gastrointestinal tract, such as probiotics (Haghighi et al., 2005). Chickens (*Gallus gallus*) treated with probiotics, for instance, exhibit greater hemagglutination when immunized than chickens that did not receive probiotics (Haghighi et al., 2005). Based on these findings, we propose that potential dysbiosis induced by fasting may be responsible for enhancing hemagglutination activity in the fasted bullfrogs observed in this study. Future studies are necessary to investigate if dysbiosis is able to modulate blood hemagglutination in anurans. Furthermore, with nutritional unbalance, hemagglutination usually decreases. When submitted to food restriction during early-life (growing and development stage), for example, garter snakes *(Thamnophis marcianus*) exhibit lower hemagglutination than normal-diet animals, being restored once nutritional stress was alleviated (Holden et al., 2019). In this way, we might conclude that 31 fasting days does not represent sufficient nutritional unbalance to decrease hemagglutination in bullfrogs.

5. Conclusions

In conclusion, fasting modulates the innate immune function in male bullfrogs by increasing the NL ratio and hemagglutination activity while reducing BKA, without affecting plasma CORT levels or fat body mass content. Thus, 31 days of fasting induces blood immune cell redistribution and an immunomodulatory response without significant activation of the HPI axis or depletion of energy stores.

CRediT authorship contribution statement

Aymam C. de Figueiredo: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Débora M.A. Prado:** Writing – review & editing, Methodology, Investigation. **Felipe R. Floreste:** Writing – review & editing, Visualization, Methodology, Investigation. **Patrício G. Garcia Neto:** Writing – review & editing, Methodology, Investigation. **Fernando R. Gomes:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Investigation, Funding acquisition,

Conceptualization. **José E. de Carvalho:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP 2014/16320. JEC is supported by FAPESP grant 2020/12962-5. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)- Finance Code 001.

Declaration of competing interest

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

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP 2014/16320. JEC is supported by FAPESP grant 2020/12962-5. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)- Finance Code 001.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2025.111873.

Data availability

Data will be made available on request.

References

Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., Flier, J. S., 1996. Role of leptin in the neuroendocrine response to fasting. Nature 382, 250–252.

Anderson, R.C.O., Bovo, R.P., Eismann, C.E., Menegario, A.A., Andrade, D.V., 2017. Not good, but not all bad: dehydration effects on body fluids, organ masses, and water flux through the skin of *Rhinella schneideri* (Amphibia, Bufonidae). Physiol. Biochem. Zool. 90 (3), 313–320. https://doi.org/10.1086/690189.

Assis, V.R., Titon, S.C.M., Barsotti, A.M.G., Spira, B., Gomes, F.R., 2013. Antimicrobial capacity of plasma from anurans of the Atlantic Forest. South Am. J. Herpetol. 8, 155–160.

Assis, V.R., Titon, S.C.M., Barsotti, A.M.G., Titon Jr., B., Gomes, F.R., 2015. Effects of acute restraint stress, prolonged captivity stress and transdermal corticosterone application on immunocompetence and plasma levels of corticosterone on the Cururu toad (*Rhinella icterica*). PLoS One 10, 1–21.

Assis, V.R., Titon, S.C.M., Gomes, F.R., 2019. Acute stress, steroid plasma levels, and innate immunity in Brazilian toads. Gen. Comp. Endocrinol. 273, 86–97.

Assis, V.R., Gardner, S.T., Smith, K.M., Gomes, F.R., Mendonça, M.T., 2020. Stress and immunity: field comparisons among populations of invasive cane toads in Florida. J. Exp. Zool. A. https://doi.org/10.1002/jez.2389.

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C., 1992. Interactions of corticosterone with feeding, activity and metabolism in passerine birds. Ornis Scand. 23, 355. https://doi.org/10.2307/3676661.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Parkes, H., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc. Natl. Acad. Sci. 95 (15), 9031–9036.

Bourgeon, S., Viera, V.M., Raclot, T., Groscolas, R., 2007. Hormones and immunoglobulin levels in king penguins during moulting and breeding fasts. Ecoscience 14, 519–528.

Bourgeon, S., Kauffmann, M., Geiger, S., Raclot, T., Robin, J.P., 2010. Relationships between metabolic status, corticosterone secretion and maintenance of innate and adaptive humoral immunities in fasted re-fed mallards. J. Exp. Biol. 213, 3810–3818.

Butler, M.W., Stahlschmidt, Z.R., Ardia, D.R., Davies, S., Davis, J., Guillette, L.J., DeNardo, D.F., 2013. Thermal sensitivity of immune function: evidence against a generalist-specialist trade-off among endothermic and ectothermic vertebrates. Am. Nat. 181 (6), 761–774.

Butts, C.L., Sternberg, E.M., 2009. Neuroendocrine factors Alter host defense by modulating immune function. Cell. Immunol. 252 (1–2), 7–15.

Campbell, T.W., 2006. Hematologia de anfíbios. In: Trall, M.A. (Ed.), Hematologia e Bioquímica Clínica Veterinária. Editora Roca São Paulo, pp. 291–300.

Cherel, Y., Robin, J.P., Heitz, A., Calgari, C., Le Maho, Y., 1992. Relationships between lipid availability and protein utilization during prolonged fasting. J. Comp. Physiol. B. 162, 305–313.

Cramp, R.L., Franklin, C.E., Meyer, E.A., 2005. The impact of prolonged fasting during aestivation on the structure of the small intestine in the green-striped burrowing frog, *Cyclorana alboguttata*. Acta Zool. 86, 13–24.

Crespi, E.J., Denver, R.J., 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. Comp. Biochem. Physiol. A 141, 381–390.

Crespi, E.J., Vaudry, H., Denver, R.J., 2004. Roles of corticotropin-releasing factor, neuropeptide Y and corticosterone in the regulation of food intake in *Xenopus laevis*. J. Neuroendocrinol. 16 (3), 279–288.

Davies, S., Noor, S., Carpentier, E., Deviche, P., 2016. Innate immunity and testosterone rapidly respond to acute stress, but is corticosterone at the helm? J. Comp. Physiol. B. 186 (7), 907–918.

Davis, A.K., Maney, D.L., Maerz, J.C., 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct. Ecol. 22, 760–772.

Eslamloo, K., Morshedi, V., Azodi, M., Akhavan, S.R., 2017. Effect of starvation on some immunological and biochemical parameters in tinfoil barb (*Barbonymus schwanenfeldii*). J. Appl. Anim. Res. 45, 173–178.

Falso, P.G., Noble, C.A., Diaz, J.M., Hayes, T.B., 2015. The effect of long-term corticosterone treatment on blood cell differentials and function in laboratory and wild-caught amphibian models. Gen. Comp. Endocrinol. 212, 73–83.

Figueiredo, A.C., Titon, S.C.M., Titon Jr., B., Vasconcelos-Teixeira, R., Barsotti, A.M.G., Gomes, F.R., 2021a. Systemic hormonal and immune regulation induced by intraperitoneal LPS injection in bullfrogs (*Lithobates catesbeianus*). Comp. Biochem. Physiol. A 253, 110872.

Figueiredo, A.C., Titon, S.C.M., Cyrino, J.C., Nogueira, L.A.K., Gomes, F.R., 2021b. Immune and hormonal modulation in the postprandial period of bullfrogs (*Lithobates catesbeianus*). J. Exp. Biol. 224, 243153.

Fites, J.S., Ramsey, J.P., Holden, W.M., Collier, S.P., Sutherland, D.M., Reinert, L.K., Rollins-Smith, L.A., 2013. The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. Science 342 (6156), 366–369.

Foysal, M.J., Fotedar, R., Tay, A.C.Y., Gupta, S.K., 2020. Effects of long-term starvation on health indices, gut microbiota and innate immune response of fresh water crayfish, marron (*Cherax cainii*, Austin 2002). Aquaculture 514, 734444.

Gardner, S.T., Assis, V.R., Smith, K.M., Appel, A.G., Mendonça, M.T., 2020. Innate immunity of Florida cane toads: how dispersal has affected physiological responses to LPS. J. Comp. Physiol. B. 190, 317–327.

Gomes, F.R., Madelaire, C.B., Moretti, E.H., Titon, S.C.M., Assis, V.R., 2022. Immunoendocrinology and ecoimmunology in brazilian anurans. Integr. Comp. Biol. 62, 1654–1670.

Grably, S., Piery, Y., 1981. Weight and tissue changes in long-term starved frogs *Rana esculenta*. Comp. Biochem. Physiol. A 69, 683–688.

Haghighi, H.R., Gong, J., Gyles, C.L., Hayes, M.A., Sanei, B., Parvizi, P., Sharif, S., 2005. Modulation of antibody-mediated immune response by probiotics in chickens. Clin. Vaccine Immunol. 12, 1387–1392.

Harlow, H.J., 1995. Fasting biochemistry of representative spontaneous and facultative hibernators: the white-tailed prairie dog and the black-tailed prairie dog. Physiol. Zool. 68, 915–934.

Harrell, C.S., Gillespie, C.F., Neigh, G.N., 2016. Energetic stress: the reciprocal relationship between energy availability and the stress response. Physiol. Behav. 166, 43–55.

Hervant, F., 2012. Starvation in subterranean species versus surface-dwelling species: crustaceans, fish, and salamanders. Comp. Physiol. Fasting, Starvation, and Food Limitation 91–102. https://doi.org/10.1007/978-3-642-29056-5_7.

Holden, K.G., Reding, D.M., Ford, N.B., Bronikowski, A.M., 2019. Effects of early nutritional stress on physiology, life histories and their trade-offs in a model ectothermic vertebrate. J. Exp. Biol. 222, jeb200220.

Idler, D.R., Ronald, A.P., Schmidt, P.J., 1959. Biochemical studies on sockeye salmon during spawning migration: vii. Steroid hormones in plasma. Can. J. Biochem. Physiol. 37, 1227–1238.

Kiesecker, J.M., Blaustein, A.R., Belden, L.K., 2001. Complex causes of amphibian population declines. Nature 410 (6829), 681–684.

Lima, A.S., Ferreira, L.F., Silva, D.P., Gomes, F.R., Titon, S.C.M., 2020. Thermal sensitivity of bullfrog' s immune response kept at different temperatures. J. Exp. Zool. A 333, 767–778.

Lin, L., Xu, C., 2020. Arcsine-b, ased transformations for meta-analysis of proportions: pros, cons, and alternatives. Health. Sci. Rep. 3.

Lind, C.M., Agugliaro, J., Farrell, T.M., 2020. The metabolic response to an immune challenge in a viviparous snake, *Sistrurus miliarius*. J. Exp. Biol. 223. https://doi.org/10.1242/jeb.225185.

López-Pérez, J.E., Meylan, P.A., Goessling, J.M., 2020. Sex-based trade-offs in the innate and acquired immune systems of *Sternotherus minor*. J. Exp. Zool. 333, 820–828.

Lyte, M., Vulchanova, L., Brown, D.R., 2010. Stress at the intestinal surface: Catecholamines and mucosa–bacteria interactions. Cell Tissue Res. 343, 23–32.

Matson, K.D., Ricklefs, R.E., Klasing, K.C., 2005. A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. Dev. Comp. Immunol. 29 (3), 275–286.

McCue, M.D., 2012. An introduction to fasting, starvation, and food limitation. In: McCue, M.D. (Ed.), Comparative Physiology of Fasting, Starvation, and Food Limitation. Springer-Verlag, Berlin, pp. 1–5.
McCue, M.D., Lillywhite, H.B., Beaupre, S.J., 2012. Physiological responses to starvation in snakes: Low energy specialists. In: McCue, M.D. (Ed.), Comparative Physiology of Fasting, Starvation, and Food Limitation. Springer-Verlag, Berlin, pp. 103–131.
Molloy, E.J., O'Neill, A.J., Grantham, J.J., Sheridan-Pereira, M., Fitzpatrick, J.M., Webb, D.W., Watson, R.W.G., 2003. Sex-specific alterations in neutrophil apoptosis: the role of estradiol and progesterone. Blood 102 (7), 2653–2659.
Naya, D.E., Veloso, C., Sabat, P., Bozinovic, F., 2009. The effect of short- and long-term fasting on digestive and metabolic flexibility in the Andean toad, *Bufo spinulosus*. J. Exp. Biol. 212, 2167–2175.
Neuman-Lee, L.A., Bobby Fokidis, H., Spence, A.R., Van der Walt, M., Smith, G.D., Durham, S., French, S.S., 2015. Food restriction and chronic stress alter energy use and affect immunity in an infrequent feeder. Funct. Ecol. 29 (11), 1453–1462.
Nowland, M.H., Hugunin, K.M.S., Karen, L., 2011. Rogers effects of short-term fasting in male Sprague–dawley rats. Comp. Med. 61, 138–137.
Odedra, B.R., Bates, P.C., Millward, D.J., 1983. Time course of the effect of catabolic doses of corticosterone on protein turnover in rat skeletal muscle and liver. Biochem. J. 214, 617–627.
Qian, J., Fang, Y., Yuan, N., Gao, X., Lv, Y., Zhao, C., Zhang, S., Li, Q., Li, L., Xu, L., Wei, W., Wang, J., 2021. Innate immune remodeling by short-term intensive fasting. Aging Cell 20, e13507.
Raffel, T.R., Rohr, J.R., Kiesecker, J.M., Hudson, P.J., 2006. Negative effects of changing temperature on amphibian immunity under field conditions. Funct. Ecol. 20 (5), 819–828.
Rollins-Smith, L.A., 1998. Metamorphosis and the amphibian immune system. Immunol. Rev. 166 (1), 221–230.
Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp. Biochem. Physiol. A 140, 73–79.
Romero, L.M., Wikelski, M., 2001. Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. Proc. Natl. Acad. Sci. USA 98, 7366–7370.
Ryser, J., 1989. Weight loss, reproductive output, and the cost of reproduction in the common frog, *Rana temporaria*. Oecologia 78, 264–268.
Sacristán, H.J., Ansaldo, M., Franco-tadic, L.M., Gimenez, V.F., Greco, L.S.L., 2016. Longterm starvation and posterior feeding effects on biochemical and physiological responses of midgut gland of *Cherax quadricarinatus* juveniles (Parastacidae). PLoS One 11, e0150854.
Secor, S.M., 2005. Evolutionary and cellular mechanisms regulating intestinal performance of amphibians and reptiles. Integr. Comp. Biol. 45, 282–294.
Secor, S.M., Carey, H.V., 2016. Integrative physiology of fasting. Compr. Physiol. 6, 773–825. https://doi.org/10.1002/cphy.c150013.
Spahn, T.W., 2004. Modulating the intestinal immune system: the role of lymphotoxin and GALT organs. Gut 53, 456–465.
Titon Jr., B., Titon, S.C.M., Assis, V.R., Barsotti, A.M.G., Vasconcelos-Teixeira, R., Fernandes, P.A.C., Gomes, F.R., 2021. LPS-induced immunomodulation and hormonal variation over time in toads. J. Exp. Zool. A. https://doi.org/10.1002/jez.2474.
Titon, S.C.M., Titon Jr., B., Gomes, F.R., Assis, V.R., 2021. Short-term stressors and corticosterone effects on immunity in male toads (*Rhinella icterica*): a neuroimmune-endocrine approach. Brain, Behavior, & Immunity – Health 13, 100230.
Webb, A.C., Chick, L.D., Cobb, V.A., Klukowski, M., 2017. Effects of moderate food deprivation on plasma corticosterone and blood metabolites in common watersnakes (*Nerodia sipedon*). J. Herpetol. 51, 134–141.
Yang, J., Kim, E., Beltran, C., Cho, S., 2019. Corticosterone-mediated body weight loss is an important catabolic process for poststroke immunity and survival. Stroke. https://doi.org/10.1161/strokeaha.119.026053.