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RESEARCH ARTICLE

Immune and hormonal modulation in the postprandial period of bullfrogs (*Lithobates catesbeianus*)

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ABSTRACT

Mammals show immune up-regulation and increased plasma and local (gastrointestinal tract) concentrations of some immunoregulatory hormones, such as corticosterone and melatonin, after feeding. However, little is known about the endocrine and immune modulation in the postprandial period of ectothermic animals. This study investigated the effects of feeding on endocrine and immune responses in the bullfrog (*Lithobates catesbeianus*). Frogs were fasted for 10 days and divided into two groups: fasted and fed with fish feed (5% of body mass). Blood and gastrointestinal tract tissues (stomach and intestine) were collected at 6, 24, 48, 96 and 168 h to measure neutrophil/lymphocyte ratio, plasma bacterial killing ability, phagocytosis of blood leukocytes, plasma corticosterone and melatonin, and stomach and intestine melatonin. Feeding increased plasma corticosterone at 24 h and decreased it at 168 h, and increased neutrophil/lymphocyte ratio at 6, 24 and 96 h. We also observed decreased bacterial killing ability 48 h after feeding. Stomach melatonin increased after 17 days of fasting. We show that feeding activates the hypothalamic–pituitary–interrenal axis and promotes transient immunosuppression, without stimulating an inflammatory response. Increased corticosterone may mobilize energy to support digestive processes and melatonin may protect the stomach during fasting. We conclude that feeding modulates secretion of immunoregulatory hormones, initially increasing plasma corticosterone levels, followed by a decrease at the end of meal digestion, and causes systemic immune cell redistribution, increasing neutrophil/lymphocyte ratio for almost the entire period of meal digestion in bullfrogs. Also, fasting modulates secretion of melatonin in the stomach.

KEY WORDS: Anuran, Corticosterone, Fasting, Feeding, Melatonin, Immune response

INTRODUCTION

After meal intake, the switch from fasting to the postprandial period is marked by a set of alterations in morphology and physiology of the gastrointestinal tract (GIT), as well as by local (GIT) and systemic immune and endocrine modulation (Hansen et al., 1997; Bubenik, 2001; Secor, 2003; Crespi et al., 2004; Lignot et al., 2005; Luoma et al., 2016). All these processes are accompanied by an energy investment (specific dynamic action or SDA; Kleiber, 1961) that demands increased aerobic metabolism (Secor and Diamond, 2000; Secor, 2005, 2009; Ott and Secor, 2007). In ectothermic animals, SDA modulation is especially pronounced as a result of the low basal metabolism cost and ingestion of large preys (Andrade et al., 2005). Bullfrogs (*Lithobates catesbeianus*) fed with 5% of body mass, for example, double aerobic metabolism in the postprandial period compared with basal levels, with SDA lasting for 2.5 days (Secor et al., 2007). In some ectothermic animals such as pythons (*Python molurus*), most SDA is invested in GIT performance, but part of the energy is invested in other activities concomitant with meal digestion (Secor, 2003). Furthermore, essential functions not directly related to digestion are up-regulated during the absorptive period, including the innate immune system and the release of hormones, such as glucocorticoids, which, among other functions, are immunomodulators (Hansen et al., 1997; Crespi et al., 2004; Luoma et al., 2016; Lemay et al., 2019).

During the absorptive meal period, the GIT mucosa is selective to the passage to the internal environment of potential pathogens ingested with food (Acheson and Luccioli, 2004; Hooper et al., 2012). In the GIT of most vertebrates, the gut-associated lymphoid tissue provides the first line of defense against pathogenic microorganisms, preventing their penetration into the internal environment, in addition to providing the biological signals that guide the adaptive immune response (Goldstine et al., 1975; Perdigo'n et al., 2001; Hooper et al., 2012). In addition to gut-associated lymphoid tissue, epithelial cells are an essential component of the intestinal immune system. Similar to immune cells, intestinal epithelial cells express receptors that recognize pathogen-associated molecular patterns (Spahn and Kucharzik, 2004). These receptors activate signaling cascades that finely control the production of antimicrobial substances and chemokines by gut-associated lymphoid tissue, depending on the signals that are delivered by the microbiota (Spahn and Kucharzik, 2004). However, the meal itself seems to be a sufficient stimulus to activate these epithelial receptors and, consequently, up-regulate the immune function. In humans, for example, circulating immune cells such as neutrophils and blood platelet count increase 45 min after feeding, returning to baseline values within 4 h of meal ingestion (Hansen et al., 1997). Ectothermic animals seem to present a similar reaction but with a longer time of response. According to Luoma et al. (2016), cornsnakes (*Pantherophis guttatus*) enhance the innate immune function during the food absorptive period, showing greater hemagglutination 24 h after food intake than after digestion (7 days after feeding). However, the temporal pattern of immune modulation during the postprandial period has been poorly investigated in ectothermic animals.

The release of immunoregulatory hormones, such as glucocorticoids (cortisol and corticosterone, CORT) and

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melatonin, is also modulated during the postprandial period. Humans enhance cortisol release in the first hour after feeding (Hansen et al., 1997), and anurans (*Xenopus laevis*) increase plasma CORT levels 6 h after meal intake (Crespi et al., 2004). Glucocorticoids show complex bi-directional immunomodulatory effects, depending on the intensity and duration of the immune stimulus (Fernandes et al., 2009; Markus et al., 2018). Regarding immunostimulatory effects, CORT enhances blood lymphocyte proliferation in rats and blood phagocytosis percentage in anurans and mammals, for example (Assis et al., 2017; Cain and Cidlowski, 2017). Moreover, high CORT plasma levels can increase gastrointestinal permeability (Meddings and Swain, 2000) and decrease the secretion of pineal melatonin (Demisch et al., 1988; Kellner et al., 1997) in mammals. Decreased melatonin secretion by the pineal gland enables the inflammatory response assemblage in mammals (Markus et al., 2018) and increases the phagocytosis of circulating leukocytes in snakes, for example (Singh and Singh, 2012). The GIT is one of the most significant sources of extra-pineal melatonin and, unlike pineal production, melatonin released by intestinal mucosa is not related to photoperiod (Mukherjee et al., 2014) but to the frequency of feeding – increasing in mammals after food intake (Bubenik et al., 1996; Bubenik, 2001). In the GIT, melatonin protects the mucosa and helps to synchronize the digestive processes (Bubenik, 2001). Similarly, ectothermic animals (fish, snakes and anurans) also exhibit melatonin production in the GIT, even during fasting (Bubenik and Pang, 1997). Yet, modulation of systemic glucocorticoids, pineal melatonin, GIT melatonin production and immune variables in response to feeding remains to be explored in ectothermic animals.

This study aimed to investigate the effects of feeding on endocrine and innate immune function in male bullfrogs (*Lithobates catesbeianus*). We hypothesized that: (1) feeding activates systemic innate immune function and modulates the systemic and local (GIT) hormonal profile; and (2) the immune and endocrine modulation is more pronounced 24 h post-feeding, coinciding with the maximum metabolic rate of bullfrogs in response to meal corresponding to 5% of body mass (Secor et al., 2007). In order to test these hypotheses, adult male bullfrogs were divided into two groups, fasted and fed with fish feed (5% of body mass), with blood and GIT tissues (stomach and proximal intestine) collected 6, 24, 48, 96 and 168 h after feeding. To assess systemic innate immune function, we measured: the proportion of blood immune cells (leukocytes) through neutrophil/lymphocyte (NL) ratio; the ability of non-cellular immune components (complement, antibody and lysozyme) to kill bacteria through plasma bacterial killing ability (BKA); and the ability of blood leukocytes to identify and engulf a foreign particle through phagocytosis percentage. To assess systemic and local (GIT) hormonal profile, we measured plasma CORT and melatonin levels, and stomach and proximal intestine melatonin. We predicted that feeding would increase NL ratio, BKA, phagocytosis percentage, plasma CORT and GIT melatonin, with a concomitant decrease in plasma melatonin levels. We also predicted that all these effects would be more pronounced 24 h after feeding.

MATERIALS AND METHODS

Frog husbandry

Adult males of the bullfrog *Lithobates catesbeianus* (Shaw 1802) (Anura: Ranidae) were used in this study. Frogs (N=75) were purchased from Rãs' World - SP and shipped to the facilities of the University of São Paulo in September/2019. Rãs' World is a commercial supplier of bullfrogs where the animals are bred in captivity under controlled feeding (fish feed; Poli-Peixe 400 Intensivo, Nutrição Animal - Poli-Nutri, #400 E) and environmental conditions (24±4°C and humidity >80%). In the laboratory, the frogs were individually kept in opaque plastic containers (37 cm height and 30 cm diameter) for 10 days before experimental procedures in a climate chamber at 25±1°C with an 11 h:13 h light:dark cycle (lights on at 06:00 h and off at 17:00 h), fasted but with free access to water according to previous studies (Secor, 2005; Claësson et al., 2015). Four days before the experimental procedures, all individuals had their body mass (mean±s.d. 260.58±36.86 g, empty bladders) and snout–vent length (141.01±5.31 mm) measured. All experimental procedures were performed in accordance with the 'Comissão de Ética no Uso de Animais', CEUA (protocol number 321/2018, 21 August 2018), Instituto de Biociências from Universidade de São Paulo.

Experimental procedure

Frogs were divided into two treatments: fasting (N=35) and fed (N=40). After a fasting period of at least 10 days, the animals of the fed treatment were force fed (5% of body mass, dry food) with the habitual fish feed (Poli-Peixe, #400 E). The fish feed was weighed dry but moistened before feeding. The meal was inserted into bullfrog's mouth and swallowed normally. The animals of the fasting treatment had their mouths manipulated (to mimic the force fed manipulation of the fed group) at 10:00 h. See Table S1 for nutritional information. Seven fasting animals and eight fed animals were sampled by cardiac puncture at 6, 24, 48, 96 and 168 h after treatment. After the blood samples were taken, the animals were euthanized by decapitation, and GIT organ samples (stomach and proximal intestine) were taken and stored at −80°C for tissue melatonin quantification.

Blood collection

Blood samples (900 µl) were taken 6, 24, 48, 96 and 168 h after treatment by cardiac puncture using a heparinized 1 ml syringe and 26 G×1/2 inch needle within 3 min of handling (Romero and Reed, 2005). A volume of 300 µl was used for the phagocytosis assay, 2 µl for leukocyte profile, and the remainder (~600 µl) was centrifuged (600 ***g***, 4 min) to isolate the plasma. Plasma samples were stored at −80°C for further hormone quantification and BKA assay.

CORT assays

For hormone assay, steroids were extracted with ether from 10 µl of plasma, according to Assis et al. (2015). Samples were then resuspended in EIA buffer. The plasma concentration of CORT was determined using EIA kits (Cayman Chemical, CORT#501320) according to the manufacturer's instructions and previous studies conducted with anurans (Assis et al., 2015, 2019), including this same species (Lima et al., 2020; Figueiredo et al., 2021). Intra- and inter-assay coefficients of variation were 11.52% and 8.57%, respectively, and assay sensitivity was 30.08 pg ml^{-1}.

NL ratio

The leukocyte profile was assessed from a 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).

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BKA

The bacterial killing ability assay was performed according to Assis et al. (2013), with slight modifications. Plasma samples (10 µl) were incubated with 10 µl of an *Aeromonas hydrophila* solution (2.5×10^7 ml^{-1}) in sterile Ringer's solution (190 µl) for amphibians (NaCl 6.5 g, KCl 1 g, NaH_2PO_4 0.1 g, $CaCl_2$ 1.125 g, $NaHCO_3$ 0.2 g, $C_6H_{12}O_6$ 2 g, diluted in 1.3 l of distilled water) for 1 h at 37°C. The positive control consisted of Ringer's solution (200 µl)+10 µl of *A. hydrophila* solution, and the negative control consisted only of Ringer's solution (210 µl). After incubation, tryptic soy broth (TSB; Kasvi, #K25-610053) solution (500 µl) was added, and 300 µl of the samples was transferred in duplicate to 96-well microplates and kept at 37°C. The plate was read every hour for 3 h in a spectrophotometer (595 nm). To account for plasma antimicrobial activity, BKA was quantified at the beginning of the exponential phase of bacterial growth according to the formula: 1−(average of duplicates for each sample/optical density of the positive control), representing the proportion of killed microorganisms in the samples compared with the positive control (Assis et al., 2013).

Phagocytosis assay

Phagocytosis assays from blood leukocytes were performed as described by Titon et al. (2017, 2019) and Figueiredo et al. (2021). Blood subsamples (300 µl) were decanted (30 min, 23°C) in a 600 µl microcentrifuge tube. By density differences, we had three different layers in the microtube: the erythrocytes in the bottom layer, the leukocytes (monocytes and neutrophils) in the middle layer (white cloud) and the plasma in the upper layer. Only the leukocytes were collected with a micropipette and transferred to a 15 ml conical tube with 10 ml of Anuran phosphate-buffered saline (APBS; NaCl 8 g, KCl 0.2 g, Na_2PO_4 1.44 g, KH_2PO_4 0.24 g, diluted in 1.3 l of distilled water). This process allows ≥95% erythrocyte exclusion. We stained 10 µl of each sample with Blue Trypan dye 0.4% (Biotecnologia, #BR30084-01) and calculated the average number of viable cells in the four quadrants of a Neubauer chamber under a microscope (400× magnification). We estimated the total number of viable cells in each sample, multiplying the average value by 10×10^4. Then, we centrifuged (260 ***g***, 4°C, 9 min) and resuspended each sample in an appropriate volume of APBS to standardize cell concentration between samples. Then, we added 100 µl of zymosan (Sigma, #Z4250) labeled with green fluorescence (CFSE Sigma, #21888F) suspension (1×10^6 particles) in 1000 µl of APBS containing 2×10^5 cells (monocytes and neutrophils). Samples were incubated under agitation for 60 min, at 25°C. After incubation, 2 ml of EDTA (6 mmol l^{-1}) was added, samples were centrifuged (260 ***g***, 4°C, 7 min), then the supernatant was discarded. After that, 200 µl paraformaldehyde (PAF, 1%) was added at 4°C for cell fixation. After incubating for 60 min (4°C), 500 µl of APBS was added and then centrifuged (260 ***g***, 4°C, 7 min). The supernatant was discarded, and 30 µl of APBS was added for flow cytometry. Samples were analyzed on an image cytometer (Flowsight AMNIS, Merck-Millipore, Germany) connected to a Dell computer. Data from 10,000 events were recorded using the 488 nm laser at 20× magnification using INSPIRE software. Phagocytes were identified by gates assembled from acquired images plotted on brightfield versus side scatter graphs. The quantification of phagocytosis was estimated by phagocytosis percentage, representing the percentage of cells that engulfed at least one zymosan particle. All analyses were performed using IDEAS software (EMD Millipore) version 6.1 for Windows.

Melatonin assay

For melatonin quantification, 60 mg of each tissue (stomach and proximal intestine) was pulverized in liquid nitrogen. The sprayed samples were homogenized in 400 µl of Tris-HCl buffer (Trizma base, Sigma #T6066-500G; HCl, EDTA and EGTA; pH 7) and centrifuged (20,000 ***g***, 4°C, 5 min; adapted from Pereira, 2016). After centrifugation, the supernatant was transferred to amber microtubes and stored at −80°C for melatonin measurement. Plasma melatonin levels and the stomach and proximal intestine (PI) melatonin were quantified using EIA kits (IBL International, #RE54021), according to the manufacturer's instructions and previous studies with anurans (Barsotti et al., 2017; Figueiredo et al., 2021). Intra- and inter-assay coefficients of variation were 2.41% and 1.78%, and assay sensitivity was 9.88 pg ml^{-1}. Tissue proteins were quantified with 1 µl of tissue supernatant in a Nanodrop apparatus, and the final stomach and PI melatonin values in pg mg^{-1} of tissue were calculated as the melatonin value (pg ml^{-1}) from the EIA kit divided by the protein values (mg ml^{-1}) obtained with the Nanodrop.

PI melatonin parallelism test

A parallelism test for PI melatonin was performed using a sample pool made up of 4 different animals from the fed group, 48 h post-treatment, to validate the use of *L. catesbeianus* proximal intestine tissue in the melatonin kit (IBL International). Tissue samples were extracted and diluted in Tris-HCl buffer according to the protocol described above ('Melatonin assay'). Top standard (standard of highest concentration) for hormone kit and pooled proximal intestine samples were serially diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) and subsequently assayed on the same plate. Standard and sample curves were plotted on the same *x*–*y* axes, and the 50% binding point was considered to be indicative of the best dilution factor to run the samples in future assays. Curves were parallel (Fig. 1), corroborating the functionality of the tissue melatonin assay for bullfrogs. The 50% binding point corresponded to 1:8 dilution for baseline (Fig. 1).

Statistical analysis

Except for CORT and NL ratio, data fitted the assumptions of normality and homogeneity, so we performed parametric tests

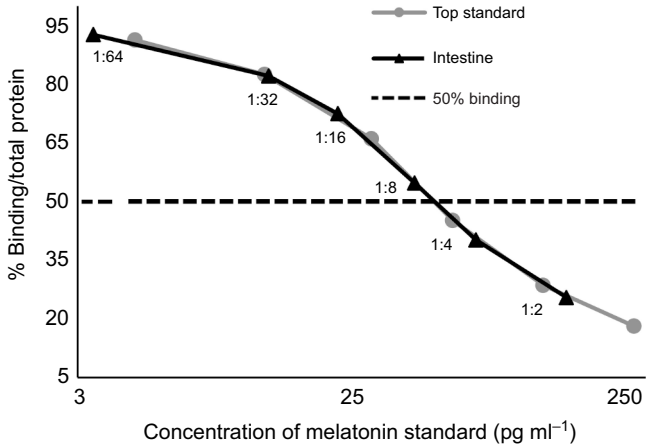

Fig. 1. Binding displacement curves of melatonin from the proximal intestine of bullfrogs. Binding displacement curves of serially diluted *Lithobates catesbeianus* pooled proximal intestine, against the standards used in the melatonin enzyme immunoassays. The *y*-axis shows the percentage hormone bound/total binding measured at 412 nm. The dashed line indicates 50% binding, which determined the best dilution factors for the extracted proximal intestine samples.

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(ANOVA and ANCOVA). CORT and NL ratio data were $\log_{10}$ transformed to fit normality and homogeneity prerequisites and then subjected to the same parametric tests. To test whether morphological measures (body mass, snout–vent length and body index) affected the variables, we performed an ANCOVA test in which studied variables (logCORT, logNL ratio, BKA, phagocytosis percentage, plasma melatonin, stomach melatonin and PI melatonin) were used as dependent variables, treatment (fasting and fed) and time post-treatment (6, 24, 48, 96 and 168 h) as factors and morphological measures as covariates (independently). As morphological measurements did not affect the studied variables, we performed ANOVA tests in which each studied variable was used as a dependent variable and treatment and time post-treatment as factors. Interactions between treatment and time post-treatment were also analyzed. The ANOVA were followed by tests for mean multiple pairwise comparisons with Bonferroni adjustments. We used a significance level of $P \leq 0.05$. Analyses were performed using the IBM SPSS Statistics 22 program. We illustrate the results without any transformation and generated graphics using the '*ggplot2*' package (Wickham, 2016).

RESULTS

No studied variable was influenced by morphological measures (body mass, snout–vent length and body index) under any conditions: CORT ($F_{1,63}$=3.036, $P \geq 0.088$); NL ratio ($F_{1,71}$=1.087, $P \geq 0.301$); BKA ($F_{1,73}$=3.464, $P \geq 0.067$); phagocytosis percentage ($F_{1,70}$=0.483, $P \geq 0.490$); plasma melatonin ($F_{1,57}$=0.739, $P \geq 0.394$); stomach melatonin ($F_{1,54}$=2.133, $P \geq 0.151$); PI melatonin ($F_{1,59}$=0.086, $P \geq 0.771$). We were able to observe the time frame of GIT distention and increased blood supply during the postprandial period, as well as follow the path of a meal in the GIT of dissected individuals over time after feeding (Fig. 2).

Plasma CORT levels were affected by time post-treatment×treatment interaction (Table 2) and were higher in fed than in fasting animals 24 h post-treatment (P=0.023; Fig. 3). Meanwhile, plasma CORT levels were lower in fed frogs than in fasted ones at 168 h post-treatment (P=0.003) and were also lower than in fed frogs at 24 h post-feeding (P=0.002; Fig. 3). The immune variables NL ratio and BKA were not affected by time post-treatment or the time post-treatment×treatment interaction, only by treatment (Table 1). Bonferroni adjustments highlighted that fed frogs exhibited a higher NL ratio than fasting frogs at 6 h (P=0.012), 24 h (P=0.002) and 96 h (P=0.028) post-treatment (Table 1, Fig. 4A). Fed animals exhibited lower BKA than fasting animals 48 h post-treatment (P=0.038; Table 1, Fig. 4B). In contrast, phagocytosis percentage was not affected by treatment, time post-treatment or the time post-treatment×treatment interaction (Table 1, Fig. 4C).

Table 1. ANOVA results for effect of feeding on immunological variables in *Lithobates catesbeianus* bullfrogs

Variable	d.f.	*F*	*P*
NL ratio			
Corrected model	9	2.966	**0.005**
Intercept	1	522.866	**<0.001**
Time post-treatment	4	0.319	0.864
Treatment	1	20.444	**<0.001**
Time post-treatment×treatment	4	1.425	0.236
Error	62		
Total	72		
Corrected total	71		
BKA (%)			
Corrected model	9	1.883	0.071
Intercept	1	16,705.811	**<0.001**
Time post-treatment	4	1.406	0.242
Treatment	1	6.836	**0.011**
Time post-treatment×treatment	4	0.919	0.459
Error	64		
Total	74		
Corrected total	73		
PP			
Corrected model	9	0.938	0.500
Intercept	1	153.211	**<0.001**
Time post-treatment	4	1.008	0.410
Treatment	1	3.690	0.059
Time post-treatment×treatment	4	0.076	0.989
Error	61		
Total	71		
Corrected total	70		

Treatment: fasting and fed (5% of body mass), and time post-treatment: 6, 24, 48, 96 and 168 h. Immunological variables neutrophil/lymphocyte (NL) ratio ($\log_{10}$), bacterial killing ability (BKA) and phagocytosis percentage (PP) were used as dependent variables and treatment (fasting and fed 5% of body mass) and time post-treatment (6, 24, 48, 96 and 168 h) as factors. NL ratio: fed N=33 and fasting N=39); BKA: fed N=34 and fasting N=40; PP: fed N=31 and fasting N=40. Significant P-values (≤0.05) are highlighted in bold.

Plasma melatonin levels did not differ between fed and fasting frogs at any time post-treatment (Table 2, Fig. 5A). Stomach melatonin was affected by the time post-treatment×treatment interaction (Table 2) and was higher in fasting than in fed animals 168 h post-treatment (P<0.001; Fig. 5B). Fasting frogs at 168 h also had higher stomach melatonin than fasting frogs at 24 h (P=0.005), 48 h (P=0.009), and 96 h (P=0.006; Fig. 5B). PI melatonin was not

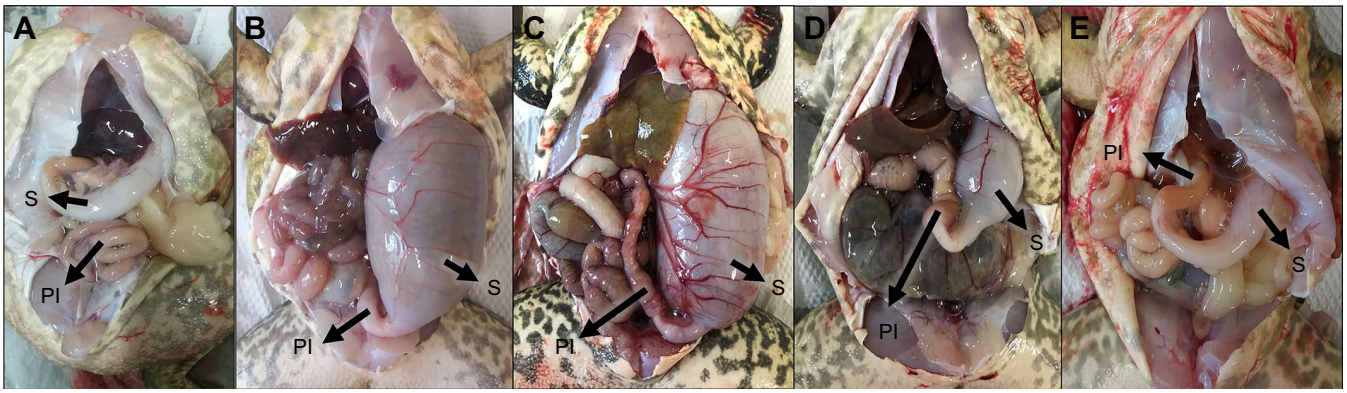

Fig. 2. The gastrointestinal tract of bullfrogs, *L. catesbeianus*. (A) Fasting, (B) 24 h after feeding, (C) 48 h after feeding, (D) 96 h after feeding and (E) 168 h after feeding (5% of body mass in meal). S, stomach; PI, proximal intestine.

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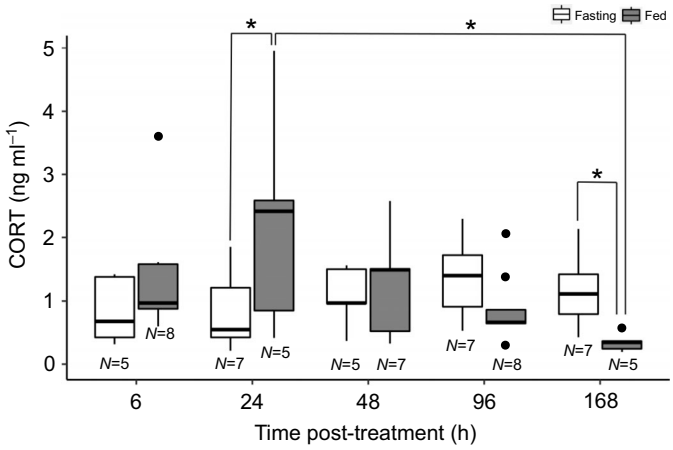

Fig. 3. Plasma corticosterone (CORT) levels in response to feeding in *L. catesbeianus*. Bullfrogs were subjected to two treatments: fasting and fed (5% of body mass). Measurements were taken 6, 24, 48, 96 and 168 h after treatment. For box plots, the box is delimited by the first quartile (lower bar), third quartile (upper bar) and the median (central bar); whiskers indicate the minimum (lower) and maximum (upper) non-outlier values; black circles represent outliers. Asterisks represent differences between groups ($P \leq 0.05$; ANOVA followed by Bonferroni adjustment).

affected by treatment, time post-treatment, or the time post-treatment×treatment interaction (Table 2, Fig. 5C).

DISCUSSION

Feeding increased CORT and NL ratio, and decreased plasma BKA. In this way, the first hypothesis of the present study was partly corroborated, as feeding activated the hypothalamic–pituitary–interrenal axis, increasing CORT and NL ratio. However, feeding did not activate a systemic innate immune response. Otherwise, the reduced BKA 48 h after feeding suggests a transient immunosuppression, which might be due to increased CORT levels. Feeding increased plasma CORT levels in the short term (24 h post-feeding) and led to a decrease in the long term (168 h post-feeding). Increased plasma CORT levels were more pronounced 24 h after feeding, in accordance with our second hypothesis. Still, we observed immune and endocrine modulation at all sampling points, depending on the variable. Moreover, we were able to observe that meal digestion duration of our bullfrogs (5–6 days; Fig. 2) was longer than that previously described (2.5 days) for bullfrogs fed 5% of body mass (Secor et al., 2007). The main reason for this discrepancy might be the difference between the temperature used here (25°C) and in Secor and colleagues' (2007) study (30°C), as higher temperatures decrease the duration of digestion and improve digestive efficiency (Wang et al., 2002; Toledo et al., 2003). Our results contribute to understanding of the digestion–immunity interactions in ectotherm animals, illustrating the time course of endocrine and innate immune modulation concomitant with meal digestion.

As expected, bullfrogs increased plasma CORT levels in the short term in response to feeding. Humans enhanced mean plasma cortisol levels by 2-fold a few minutes after feeding, returning to basal levels within 2 h (Hansen et al., 1997); and *Xenopus laevis* frogs (fed *ad libitum* at 23°C) enhanced mean CORT plasma levels by 3-fold, 6 h after meal intake, returning to basal levels within 24 h (Crespi et al., 2004). Interestingly, although mean values for CORT increased 6 h after feeding in our study – indicating that CORT modulation is initiated a few hours after meal intake – increased plasma CORT levels in response to feeding were more pronounced

Table 2. ANOVA results for effect of feeding on hormone variables in *Lithobates catesbeianus* bullfrogs

Source	d.f.	*F*	*P*
CORT (ng ml^{-1})			
Corrected model	9	2.541	**0.016**
Intercept	1	2.932	0.093
Time post-treatment	4	1.552	0.200
Treatment	1	0.086	0.771
Time post-treatment×treatment	4	4.696	**0.003**
Error	54		
Total	64		
Corrected total	63		
Plasma melatonin (ng ml^{-1})			
Corrected model	9	0.370	0.944
Intercept	1	161.268	**<0.001**
Time post-treatment	4	0.427	0.078
Treatment	1	0.949	0.335
Time post-treatment×treatment	4	0.203	0.936
Error	48		
Total	58		
Corrected total	57		
Stomach melatonin (pg mg^{-1} of tissue)			
Corrected model	9	2.892	**0.009**
Intercept	1	116.539	**<0.001**
Time post-treatment	4	2.475	0.058
Treatment	1	3.254	0.078
Time post-treatment×treatment	4	3.595	**0.013**
Error	45		
Total	55		
Corrected total	54		
PI melatonin (pg mg^{-1} of tissue)			
Corrected model	9	1.338	0.242
Intercept	1	164.187	**<0.001**
Time post-treatment	4	0.829	0.513
Treatment	1	3.199	0.080
Time post-treatment×treatment	4	1.382	0.253
Error	50		
Total	60		
Corrected total	59		

Treatment: fasting and fed (5% of body mass), and time post-treatment: 6, 24, 48, 96 and 168 h. Hormonal variables corticosterone (CORT; $\log_{10}$) and melatonin in the plasma, stomach and proximal intestine (PI) were used as dependent variables and treatment (fasting and fed 5% of body mass) and time post-treatment (6, 24, 48, 96 and 168 h) as factors. CORT: fed N=31 and fasting N=33; melatonin-plasma: fed N=27 and fasting N=31; stomach melatonin: fed N=28 and fasting N=27; PI melatonin: fed N=30 and fasting N=30. Significant P-values (≤0.05) are highlighted in bold.

24 h after treatment (2.6-fold enhancement). It is possible that, as for the SDA response, the timing and scope of the CORT response to feeding are related to various determinants, such as the quantity and quality of meal intake. Further studies are necessary to identify possible patterns of CORT modulation during the postprandial period and its modulatory factors. However, activation of the hypothalamic–pituitary–adrenal/interrenal axis resulting in glucocorticoid release after feeding has been observed in mammals and anurans independently of the scope and timing of the response.

CORT plays essential roles in GIT absorption, enhancing GIT permeability in mice, especially in the stomach, besides regulating intestinal ion uptake and osmolarity of intestine epithelia in amphibians (Collie and Hirano, 1987; Meddings and Swain, 2000). CORT also stimulates gluconeogenesis and protein catabolism, facilitating the mobilization of energy resources necessary to increase metabolic rate in the postprandial period and sustain SDA (Odedra et al., 1983; Wack et al., 2011; Yang et al., 2019a). The catabolic role of CORT may be critical in the first hours

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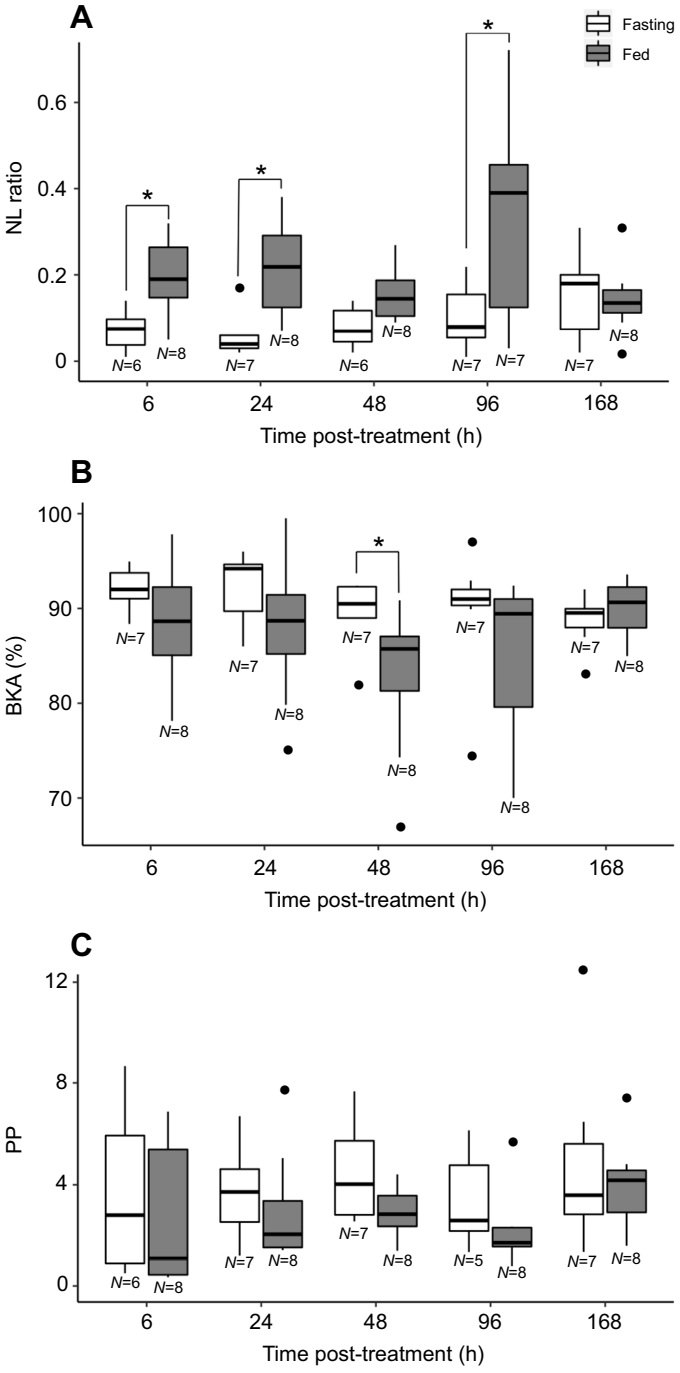

Fig. 4. Systemic immune parameters in response to feeding in *L. catesbeianus*. Bullfrogs were subjected to two treatments: fasting and fed (5% of body mass). (A) Neutrophil/lymphocyte (NL) ratio, (B) bacterial killing ability (BKA) and (C) phagocytosis percentage (PP). Measurements were taken 6, 24, 48, 96 and 168 h after treatment. For box plots, the box is delimited by the first quartile (lower bar), third quartile (upper bar) and the median (central bar); whiskers indicate the minimum (lower) and maximum (upper) non-outlier values; black circles represent outliers. Asterisks represent differences between groups ($P \leq 0.05$; ANOVA followed by Bonferroni adjustment).

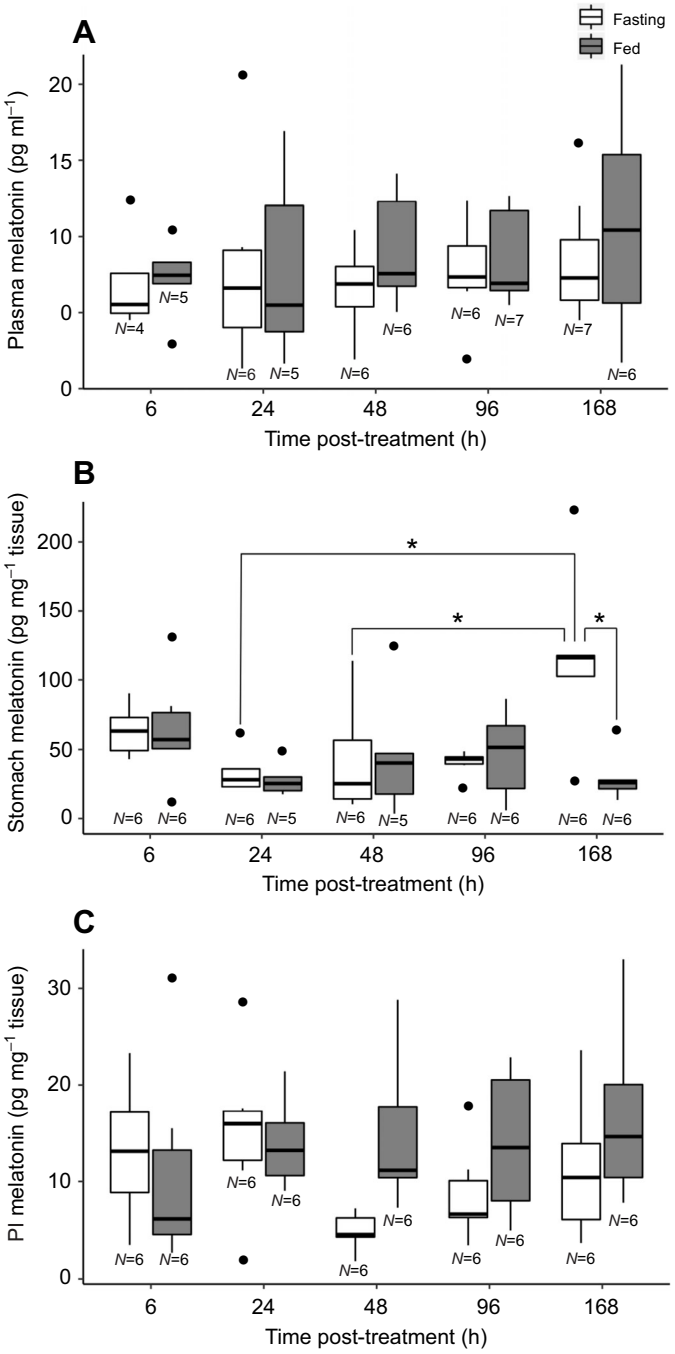

Fig. 5. Systemic and local (stomach and proximal intestine) melatonin parameters in response to feeding in *L. catesbeianus*. Bullfrogs were subjected to two treatments: fasting and fed (5% of body mass). (A–C) Melatonin levels in (A) plasma, (B) stomach and (C) proximal intestine (PI). Measurements were taken 6, 24, 48, 96 and 168 h after treatment. For box plots, the box is delimited by the first quartile (lower bar), third quartile (upper bar) and the median (central bar); whiskers indicate the minimum (lower) and maximum (upper) non-outliers values; black circles represent outliers. Asterisks represent differences between groups ($P \leq 0.05$; ANOVA followed by Bonferroni adjustment).

after feeding and unnecessary at the end of digestion, when energy sources are high, which might explain why our fed bullfrogs at the end of meal digestion (168 h post-feeding) decreased CORT levels compared with the fasting ones. At this point, fasted bullfrogs may rely on the catabolic effects of CORT, while fed ones no longer do so, possibly decreasing CORT levels back to baseline. In accordance, fed humans also decreased plasma glucocorticoid (cortisol) levels compared with fasting ones at the end of meal digestion (4 h post-feeding; Hansen et al., 1997). However, if feeding does not occur prior to depletion of body lipid storage,

fasting reaches phase III (Secor and Carey, 2016). At this phase of fasting, an elevated CORT release is required to mobilize energy via protein catabolism, when energy sources switch from lipids to amino acids (Secor and Carey, 2016). As our fasting bullfrogs did not show enhanced CORT levels at any time point, the animals probably did not reach that fasting phase even after 17 days (10 days prior to the experiment plus 168 h post-treatment) of food deprivation.

The increased NL ratio after feeding agrees with previous results on humans, in which the NL ratio increased by a mean of 1.5-fold, 3 h after feeding, returning to basal levels within 4 h (Hansen et al., 1997). Our bullfrogs increased NL ratio by a mean of 1.7-fold at 6 h post-feeding, but reached an increase of 3.6-fold 24 h after feeding, indicating that the immune cell redistribution was more pronounced at this time point. The differences in maximum scope and duration of increased NL ratio between humans and bullfrogs might, at least partially, be explained by the relative amount of food intake. Usually, meals for humans range from 0.5% to 1.5% of body mass and, compared with most ectothermic animals, humans exhibit a very modest postprandial metabolic response that lasts for 3–6 h (Secor, 2009). In contrast, after ingesting 5% of body mass in a meal, some of our bullfrogs exhibited an exacerbated and lasting response of increased NL ratio up to 96 h after feeding, while in other individuals the NL ratio had returned to basal levels at this time point (Fig. 4A). Therefore, ectotherms and mammals seem to show different scopes and temporal dynamics of changes in circulating NL ratio after feeding, with ectotherms showing more pronounced and lasting immune cell redistribution. The increased NL ratio is probably a consequence of increased CORT in our fed frogs, given that increased plasma glucocorticoid levels induce changes in NL ratio in several vertebrate models (including anurans) in different physiological contexts (Hansen et al., 1997; Falso et al., 2015; Assis et al., 2019; Figueiredo et al., 2021; Titon et al., 2021).

Interestingly, the BKA of fed bullfrogs decreased 48 h post-treatment. This transient immunosuppression could also be a consequence of increased CORT, as acute stressors associated with higher plasma CORT levels decreased BKA in *Rhinella marina* and *Rhinella icterica* toads, for example (Assis et al., 2015; Graham et al., 2012). Additionally, in mammals, the components of the meal are able to modulate the expression of blood receptors that recognize gram-negative bacteria pathogen-associated molecular patterns, such as the toll-like receptor 4 (TLR4; Sagaya et al., 2012; Lemay et al., 2019). After consuming high-fat meals, humans up-regulated blood TLR4 expression, for example (Lemay et al., 2019). However, blood TLR4 expression down-regulation is observed after the ingestion of dairy products (Sagaya et al., 2012), as well as in the presence of some micronutrients (vitamins and minerals) *in vitro* (Faghfouri et al., 2020). The expression of TLR4 has also been previously detected in frogs (Lau et al., 2018). If the modulation of TLR4 receptors in frogs resembles the pattern observed in mammals, the fish feed enriched with micronutrients offered to our frogs (Table S1) might have down-regulated blood TLR4 expression. Therefore, the blood of our fed bullfrogs could be more susceptible than the blood of fasting ones to the gram-negative bacteria used in the BKA assay. The expression of TLR receptors in the postprandial period and its interaction with diet remains to be explored in ectotherms. It is also important to consider that cornsnakes (*P. guttatus*) up-regulated the blood immune response when fed with mice that could contain ectoparasites and endoparasites (Luoma et al., 2016), but when fed with industrialized fish feed, our bullfrogs down-regulated BKA. Thus, the modulation of some immune functions might be dependent on several factors, including the species, the meal type and its nutritional characteristics, as well as on the presence of pathogens in the ingested food.

In mammals, the immune–pineal axis activation by an inflammatory stimulus inhibits central melatonin synthesis and secretion (Markus et al., 2018). Decreased plasma melatonin levels have been observed in anurans in response to lipopolysaccharide (Titon et al., 2021), indicating that central melatonin inhibition by the inflammatory stimulus is evolutionarily conserved. Interestingly, increased phagocytosis percentage of blood leukocytes in bullfrogs is associated with decreased plasma melatonin levels after immune challenges (Figueiredo et al., 2021). In the feeding context, as plasma melatonin levels and phagocytosis percentage were not modulated in our fed bullfrogs, the results indicate the absence of postprandial inflammation. In fact, humans decrease the production of pro-inflammatory cytokines after the consumption of a balanced meal (Hansen et al., 1997). Together, these results suggest that the ingestion of a balanced meal (such as the fish feed used here) did not induce an inflammatory response in frogs, as in mammals.

Contrary to expected, stomach melatonin did not increase after feeding. Instead, stomach melatonin increased in fasting animals after a 17 day fasting period (at 168 h post-treatment). Although GIT melatonin has been described as a digestion-facilitating hormone, it also performs essential protective functions in the GIT, and elevated GIT melatonin concentrations have been found in food-restricted mammals (Huether, 1994; Bubenik et al., 1992; Bubenik, 2001). Mice increase melatonin levels in most parts of the GIT after 48 h fasting periods, and – as in our bullfrogs – stomach melatonin enhancement is more pronounced (2-fold enhance) than in other parts of the GIT (Bubenik et al., 1992). GIT melatonin is produced by the enterochromaffin cells present in stomach mucosa and acts as a paracrine hormone in other segments of the GIT (Bubenik, 2001). High stomach melatonin protects the GIT, reducing the incidence and severity of tissue damage, such as gastric ulcers in pigs, for example (Ayles et al., 1996). Additionally, at 168 h post-treatment, fasting bullfrogs may still be adjusting and elevating stomach melatonin production, which could explain the large variance in stomach melatonin values (26.95–223.31 pg mg^{-1} tissue) at this time point. However, we did not observe increased PI melatonin after feeding or fasting. The pH is around 7 and 4, respectively, in the intestines and stomach of bullfrogs (Yang et al., 2019b). Thus, melatonin production in different parts of the GIT might be associated with the probability of tissue damage during fasting.

Overall, we conclude that feeding (5% of body mass) can modulate the systemic innate immune response and the hormonal profile in male bullfrogs, *Lithobates catesbeianus*, and fasting can modulate stomach hormonal profile. Shortly after feeding (6 h), the NL ratio increased followed by an increase in CORT (24 h post-feeding), and then a reduction in BKA (48 h post-feeding). The short-term increase in CORT was followed by decreased values in the long term (168 h post-feeding), indicating acute activation of the hypothalamic–pituitary–interrenal axis at the beginning of meal digestion. At this point, increased CORT may be important to mobilize energy to support digestive processes. Additionally, decreased plasma BKA could be due to increased CORT levels and meal composition. However, feeding did not promote changes in plasma melatonin levels or phagocytosis percentage of blood leukocytes, suggesting the absence of a postprandial inflammatory response. Our study also showed that fasting (17 day period) increased stomach melatonin levels, which might have a protective function under such conditions. Collectively, our results indicate

that feeding and fasting responses are assembled from a set of interactions between the immune and endocrine variables and that balanced-meal feeding activates the hypothalamic–pituitary–interrenal axis but does not stimulate an inflammatory response.

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Competing interests
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Author contributions
Conceptualization: A.C.d.F., S.C.M.T., F.R.G.; Methodology: A.C.d.F., S.C.M.T., J.C.C., L.A.K.N.; Formal analysis: A.C.d.F., S.C.M.T.; Investigation: A.C.d.F., S.C.M.T., J.C.C., F.R.G.; Resources: F.R.G.; Data curation: A.C.d.F., S.C.M.T.; Writing - original draft: A.C.d.F., S.C.M.T., F.R.G.; Writing - review & editing: A.C.d.F., S.C.M.T., A.C.d.F., S.C.M.T., J.C.C., L.A.K.N., F.R.G.; Visualization: S.C.M.T., J.C.C., L.A.K.N., F.R.G.; Supervision: S.C.M.T., F.R.G.; Project administration: F.R.G.; Funding acquisition: F.R.G.

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