Do changes in body mass alter white blood cell profiles and immune function

in Australian cane toads (*Rhinella marina*)?

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Abstract

Variation in food resources can result in dramatic fluctuations in the body condition of animals dependent on those resources. Decreases in body mass can disrupt patterns of energy allocation and impose stress, thereby altering immune function. In this study we investigated links between changes in body mass of captive cane toads (*Rhinella marina*), their circulating white blood cell populations, and their performance in immune assays. Captive toads that lost weight over a 3-month period had increased levels of monocytes and heterophils and reduced levels of eosinophils. Basophil and lymphocyte levels were unrelated to changes in mass. Because individuals that lost mass had higher heterophil levels but stable lymphocyte levels, the ratio of these cell types was also higher, partially consistent with a stress response. Phagocytic ability of whole blood was higher in toads that lost mass, due to increased circulating levels of phagocytic cells. Other measures of immune performance were unrelated to mass change. These results highlight the challenges faced by invasive species as they expand their range into novel environments which may impose substantial seasonal changes in food availability that were not present in the native range. Individuals facing energy restrictions may shift their immune function towards more economical and general avenues of combating pathogens.

Keywords: *Bufo marinus*, ecoimmunology, stress, energy budget, leukogram

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Introduction

The availability and/or quality of food is rarely constant over time for any organism, with substantial

variation induced by factors such as seasonal variation in environmental conditions or the abundance of

prey [1-5]. Reflecting that variation, individuals of many taxa exhibit considerable fluctuations in body

mass through time, with decreases during periods of resource limitation and fattening during periods of

resource abundance. Given the near-ubiquity of fluctuations in body condition through time, we expect

local adaptation and /or phenotypic plasticity to have fine-tuned responses to that situation. For example,

animals that encounter low food availability may respond by reducing their rate of metabolic expenditure

via reducing activity levels or selecting lower body temperatures [in ectotherms: 6] and by going into

torpor [in endotherms: 7]. In some species, loss of body mass may be an adaptive tactic to reduce

locomotor or maintenance costs [8].

The magnitude of mass loss varies substantially among species, driven by issues such as the stochasticity

and seasonality of food availability. Many of the most spectacular cases occur among ectothermic

animals, perhaps because large fat bodies compromise rates of thermal exchange across the body surface

in endotherms [9]. In contrast, an ectotherm can store large amounts of energy without affecting its

thermal biology [9]. High storage capacity provides an opportunity for the organism to accumulate energy

during brief periods of resource abundance, and then slowly expend that energy for maintenance over

the following months or years [e.g. 10]. Large fluctuations in body mass may also be common among

migratory or invasive species, because of the high energy demands of sustained dispersal [11]. Body mass

fluctuations in invasive species may also depend how close the population is to the invasion front.

Processes such as spatial sorting for enhanced dispersal among frontal populations may alter energy

demands and associated trade-offs [12]. The effects of unpredictable resource availability, and temporal

variation in rates of feeding, may be especially pronounced in invasive species if they did not evolve under

these conditions but encounter them in the invaded range [3].

In addition, the spatio-temporal shifts in population density that occur during range expansion influence

competition for resources among conspecifics and thus, energy intake [13-15]. Temporal fluctuations in

feeding rates and body condition may be especially likely as a species invades increasingly seasonal or

stochastically variable environments.

Regardless of the cause, variation in body mass inevitably affects energy-allocation decisions [16].

Patterns of investment into competing compartments - growth, storage, reproduction, maintenance etc.-

must be altered during periods when energy intake is low. The immune system is one such component,

likely to be reconfigured when energy is scarce [17, 18]. Immunocompetence is a strong target of

selection because pathogens can adversely affect the host's health and fitness. However, mounting a

robust immune response can require substantial energy [19, 20]. In addition to the cost of mounting a

full-scale immune reaction, manufacturing and maintaining the cells and substances required for robust

immune surveillance can be high [21] . An individual's immune configuration is therefore likely to be

tightly linked to the amount of energy it has available, in light of competing demands such as growth and

reproduction [19]. Energetically expensive immune components may need to be sacrificed and sparse

resources redirected in favour of less expensive mechanisms [19]. One way to fine-tune investment is to

allocate resources differentially to components of the immune system that provide general and relatively

low-cost defence *versus* components that can protect more effectively against specific challenges but are

costly to induce [22-24].

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Another means by which reduced energy intake could affect immune function is if it elicits a stress response and triggers the release of glucocorticoids such as corticosterone (CORT) [25, 26]. Although the links between CORT and stress are complex [27, 28], CORT plays a role in metabolism, mobilization and redirection of energy stores, and levels often increase during periods of food deprivation [25]. Another effect of glucocorticoids is alteration of the immune system. For example, increased CORT levels reconfigure the population of circulating white blood cells. Lymphocytes migrate out of circulation and are sequestered in the blood stream and into tissues, while heterophils are recruited into circulation and retained there. As a result of this redistribution of different cell types into and out of circulation, the ratio of heterophils to lymphocytes in the blood can be used as an index of circulating CORT concentration, and hence stress levels [29]. CORT can also modulate immune responses by altering the distribution and activity of cytokines and other immune agents [30-32]

The abundance, wide geographic distribution, and large body size of cane toads (*Rhinella marina*) have encouraged researchers to use these toads as a model species for examining physiological issues, including ecoimmunology. The aim of the present study was to determine whether the immune defenses of cane toads are correlated with their energy balance. We quantified changes in body mass in captive-reared cane toads and measured immune parameters at the time of their final weighing.

If the production and maintenance of immune products is constrained by energy balance, we predicted that toads in positive energy balance (i.e., individuals that gained mass over the study period) would exhibit higher performance in standardized immune assays than would conspecifics in poorer energy balance (i.e., that lost mass over the same period). However, if mass lose imposed stress, thereby increasing circulating heterophil concentration, immune functions performed by heterophils (i.e., phagocytosis) might be enhanced among individuals that lost weight.

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Materials and Methods

Study species. – Native to South America, cane toads (*Rhinella marina*, formerly *Bufo marinus*) have been

translocated to many countries for control of insect pests [33]. The climate in the toads' native range and

in serial translocation sites (Caribbean and Hawai'i) prior to their translocation to Australia is wet-tropical,

with rain falling during every month of the year [3]. Toads were released in Queensland on Australia's

east coast in 1935 and have spread >2,000 km westwards since then, experiencing progressively more

dramatic seasonality in rainfall (including sequences of rain-free months each year) as they advanced [3].

Our study was carried out at a field station 60 km east of the city of Darwin on the Adelaide River

floodplain (12.579S, 131.314E) in the Northern Territory. The area experiences a wet–dry tropical climate,

with maximum daily air temperatures > 32° C year-round. Precipitation is highly seasonal, with rainfall

largely restricted to the November–April wet season each year [34].

Mass change and immune measures in captive cane toads. – Our studies at the field station 60 km east of

the city of Darwin included captive breeding of parental toads from sites across the Australian range and

raising the progeny to adulthood under standardized "common garden" conditions. The advantages of

using captive-reared toads to study immune performance is that factors such as relatedness and age are

known and prior exposure to pathogens and parasites can be minimized. Offspring from captive

breedings were reared in large (110 X 110 x 70 cm) open-topped outdoor enclosures and used in studies

to evaluate heritability of various traits [for further details on common garden rearing see 35]. All

enclosures provided access to water and shade, and food was provided by artificial lights hanging above

each enclosure that attracted nocturnal insects. Commercially-purchased cockroaches and mealworms

dusted with vitamin powder were occasionally added to each enclosure as supplemental food. Nocturnal

insects are present year-round, but especially abundant during the wet season, when this study took

place. At the time of this study the number of toads in each enclosure ranged from 6 -17, grouped by

similar body sizes.

We periodically recaptured toads from the enclosures to measure them and/or conduct performance

assays [36-38]. The present paper is based data from 154 adult toads that had been bred in captivity and

reared all their lives in outdoor enclosures. The toads were weighed during the wet season in January

2016, several months after they had last undergone any performance trials. Then, two to three months

later (when the toads were 17-24 months old), they were weighed a second time. The difference in mass

between the first and second weighing was divided by the number of days elapsed to calculate the rate of

weight change. Immediately after their second weighing toads underwent a series of immunological

assays (see below).

We obtained a 0.2ml blood sample from each toad via cardiocentesis using a heparinized syringe. To

minimise the effects of handling time on blood components used for immune assays, samples were

obtained within three minutes of a toad being removed from its enclosure [28]. The toad was then

weighed and its snout-urostyle length (SUL) was measured. For immune measures (see below), we used

40 ul of whole blood to perform a phagocytosis assay, 5 ul for hemocytometry and 5 ul to prepare a blood

smear. The remaining blood sample was centrifuged for 4 min at 13000 G to provide plasma for a

bactericidal assay.

We performed three challenge-based immune assays, whereby immune function is stimulated by the

introduction of a pathogen signal. A bacteria-killing assay and phagocytosis chemiluminescence assay

assessed components of the innate immune system, which is fast-acting and non-specific. A

phytohemagglutinin (PHA) skin-swelling assay assessed the proliferation of lymphocytes at a site where a

T-cell mitogen (PHA) was injected [39]. The PHA test is a commonly used assay of cell-mediated immune

responses. As a component of the acquired arm of the immune system, such responses are slower to

initiate than innate responses but can target specific pathogens [39].

PHA skin-swelling assay

Immediately after each toad was blood-sampled and weighed, we measured the thickness of the webbing

between the second and third toes of each hind foot three times in succession using a dial thickness

gauge (Peacock G1-A; Ozaki Manufacturing Ltd, Tokyo, Japan). We then injected 0.05 ml of a PHA (Sigma

L7854) solution (2mg/ml in sterile phosphate buffered saline, = PBS) into the webbing between the

second and third toes of the right hind foot using a 0.3ml syringe with a 30g needle. As a procedural

control, to measure swelling caused by needle-damage alone, we injected 0.05 ml of sterile PBS into the

webbing of the left hind foot.

Toads were then placed into individual damp cotton bags inside a plastic container and held indoors in an

air-conditioned room. After 24 h, we removed toads from their cloth bag and the thickness of both

injected hind toe-webs was remeasured to quantify the amount of swelling. Toads were then returned to

their outdoor enclosures. We calculated the percentage swelling caused by injection of both PBS and PHA

as: 100 x ((24hr thickness - initial thickness) / initial thickness). We also calculated the relative difference

by subtracting the % swelling caused by PBS from the % swelling caused by PHA.

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Phagocytosis assay

This assay measures tested the capacity of phagocytes (mainly heterophils) in toad whole blood to engulf

zymosan particles. We added 40ul of whole blood to 760ul of sterile amphibian Ringers to produce a 1:20

dilution. We then added 240 mL of the diluted blood to duplicate wells of a 96-well microassay plate

along with 30 ul of luminol (A8511 Sigma) solution and 10 ul of zymosan (Z4250 Sigma) solution. A control

well for each sample contained 240 ul of diluted blood, and 30 ul luminol but with 10 ul of phosphate

buffered saline instead of zymosan. Immediately upon addition of zymosan to the wells, the plate was

placed in a luminometer (FluoroStar Optima; BMG Labtech, Ortenberg, Germany) and luminescence was

read at 5-min intervals over 180 min. Higher luminescence values indicate higher rates of phagocytosis

occurring in the sample.

Bacteria killing ability (BKA) assay

We tested the ability of toad plasma to kill *Escherichia coli* using a modification of Matson et al.'s method

[40]. A lyophilized pellet of *E. coli* (ATCC 8739) was dissolved in sterile (PBS), such that 10 ul contained

approximately 900 colony-forming units. Toad plasma was diluted 1:10 in sterile CO_2-independent media

enriched with L-glutamine. We added 10 ul of the diluted *E. coli* solution with 140 ul of diluted plasma

from each toad and immediately spread 50 ul of the solution onto a tryptic soy agar plate. The remaining

plasma-bacteria mixture was held at 25° C for 60 min and then another 50 ul sample was spread onto an

agar plate. To measure bacterial growth in the absence of toad plasma, we also prepared triplicate

control samples, where 10 ul of dilute *E. coli* solution was added to 140 mL of sterile CO_2-independent

media. As with the toad samples, 50 ul aliquots of control samples were plated out at 0 and 60 min. All

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plates were then incubated at 37° C for 24 h and the number of colonies on each plate was counted. To

quantify bactericidal ability of toad plasma we used the method of [41] which indexes the 24-hr %

changes in counts of bacteria incubated with toad plasma against 24-hr % changes in control samples.

Hemocytometry and white blood cell differentials

We used hemocytometry to determine the concentration of blood cells in circulation and used blood

smears to make differential counts of white blood cells. Blood smears were air-dried overnight, fixed in

methanol and stained using modified Giemsa solution. Slides were fitted with a cover glass and examined

at 1000X. The first 100 white blood cells encountered were identified as either basophil, eosinophil,

monocyte, heterophil, or lymphocyte (Fig. 1). For hemocytometry, 5 ul of whole blood was added to 995

ml of Natt Herrick solution. The solution was refrigerated for 60 min and then vortex-mixed to resuspend

the cells. A 10 ul aliquot was then placed onto a hemocytometer and examined in the chamber under

100X magnification to count total red blood cells (RBC) and total white blood cells (WBC) [42]. Using the

differential white cell counts taken from blood smears, the volume of the haemocytometer and the

dilution factor (1:200) of blood used for hemocytometry, we calculated the concentration of each type of

white blood cell per ml of whole blood [42].

Statistical analysis. – We used mixed model analyses to assess links between seven measures of immune

performance (PBS %swelling, PHA %swelling, PHA-PBS %swelling, mean luminescence, maximum

luminescence, time to maximum luminescence, Allen BKA index) and the rate of mass change of each

toad. Sex and body size (SUL) were included as covariates in all models. Litter ID, enclosure ID, and assay

date were included as random effects to incorporate levels of similarity among siblings, cage mates, and

assay groups. For graphical purposes, we calculated percentage change in body mass by dividing mass

change by initial mass and multiplying by 100. However, in analyses, rate of mass change (g/day) was

corrected for initial body size by including SUL as a covariate in the models (see below).

We used the same mixed model structure to assess the relationships between mass change and

concentrations of different blood cell types and H:L ratio. We log- transformed concentrations of the five

white blood cell types and H:L ratio prior to analyses to satisfy normality and variance assumptions.

To establish which types of white blood cells most affected phagocytic performance, we used a model

that included concentration of the cell types as independent variables and mean luminescence (from the

phagocytosis assay) as the dependent variable. Clutch ID, enclosure ID, and assay date were included as

random effects. All mixed model analyses were carried out using Proc Mixed in SAS 9.4 (SAS Institute,

Cary NC).

We used residuals from a regression of log-transformed body mass on log-transformed body length (SUL)

to index body condition (mass corrected for body size). To determine if body condition was related to the

degree of mass change over the preceding period, we calculated a Pearson correlation between the two

variables.

Results

Changes in mass of captive toads

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Changes in mass of the 154 captive toads re-weighed after 63 to 107 days ranged from a loss of 36 g to a gain of 78 g (-0.5% to +2.0% of initial mass per day). Fifty-six toads lost weight and 98 gained weight. At the time of second measure, when the immune assays performed, toads that had gained more mass were in better body condition (r = 0.45, P < 0.0001).

Relationships between mass change and immune measures.

None of our challenge-induced immune measures were significantly related to sex or body size (SUL) (all P > 0.10, Table 1). Immune responses related to toe-web swelling and BKA were also unrelated to the change in mass of toads over three months prior to the assays (all P > 0.05, Table 1). However, change in mass negatively affected phagocytosis activity, causing a decrease in both average luminescence (estimate = -54292, $F_{1, 104}$ = 4.78, P = 0.031; Table 1, Fig. 2) and maximum luminescence (estimate = -128721, $F_{1, 104}$ = 5.41, P = 0.022; Table 1, Fig. 2). Toads that lost mass had higher phagocytic ability than did toads that gained mass.

Hematological parameters did not differ significantly between male and female toads (all P > 0.07, Table 2). Body size was positively related to both erythrocyte concentration and eosinophil concentration (both P < 0.037, Table 2). Change in mass affected concentration of three of the five types of white blood cells (Fig 3, Table 2). Toads that gained mass prior to the second census had higher concentrations of eosinophils in circulation (estimate = 0.585, $F_{1, 104}$ = 4.88, P = 0.029), but lower levels of monocytes (estimate = -1.35, $F_{1, 104}$ = 12.85, P = 0.0005) and heterophils (estimate = -0.47, $F_{1, 104}$ = 21.07, P < 0.0001). Levels of basophils and lymphocytes were unaffected by changes in mass (Fig 3, Table 2). As a result of heterophils decreasing with weight change while lymphocytes levels remained stable, the ratio of heterophils to lymphocytes was negatively related to mass change (estimate = -0.59, $F_{1, 104}$ = 29.96, P <

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0.0001; Fig 3, Table 2). Toads that had lost mass prior to assays had more heterophils relative to

lymphocytes than did toads that had gained mass.

In a model with the concentrations of all five types of white blood cells included as independent variables,

only heterophils significantly affected the mean luminescence of whole blood (estimate = 109828, $F_{1, 102}$ =

40.97, P < 0.0001). Unsurprisingly, blood with higher concentrations of phagocytic heterophils exhibited

higher levels of phagocytosis. Significance levels of the other four blood cell types in the model were all

above P =0.13.

Discussion

Although total numbers of both red and white blood cells were unaffected by mass change, the

composition of the white cell population was substantially altered. Toads that lost mass rapidly had

higher levels of heterophils and monocytes, but lower levels of eosinophils, compared to toads that

gained mass. When glucocorticoids such as CORT are released, heterophils are moved into circulation

while lymphocytes move out of circulation. In the present study, we observed the former pattern but not

the latter. That is, heterophils increased among toads that lost mass while lymphocyte numbers remained

constant. The increase in heterophil numbers alone was enough to cause a significant increase in H:L

ratio. Only one of the two cell types expected to reflect stress was affected by mass loss, suggesting that

the stress was not severe. Stress can also induce a reduction in eosinophils [29], as we observed among

toads that lost mass.

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Toads that gained more mass also had higher levels of eosinophils in circulation, suggesting that production of these cells may impose too high an energy cost for individuals in poor energy balance. Toads in negative energy balance may invest in more economical defenses such as heterophils, which enhance a general immune response (phagocytosis). Although the roles of eosiniophils in ectotherms are not fully understood [43] they may enhance tissue remodeling [44]. Thus, the higher levels of eosinophils seen in individuals that gained mass may facilitate increased structural growth.

Because toads that lost mass had higher levels of heterophils and monocytes (both of which are phagocytic), they demonstrated superior performance in the phagocytosis assay. Performance in the other assays, that reflect different components of the immune system, were unaffected by body mass changes. Thus, we found no evidence to support the prediction that weight loss would weaken immune performance. Components of the immune system may be affected differentially when an organism experiences reduced energy availability [18]. Future studies could assay additional components of the immune system to look for other relationships.

We observed decreases in body mass over two to three months in 37% of captive cane toads. Major changes in body mass likely reflect foraging success and nutrition. Some minor changes in mass were undoubtedly influenced by differences in gut and bladder fullness at the time of weighing; however, this source of error should be as likely to increase as to decrease mass. Large decreases in mass among wild female toads can indicate oviposition [45] but this was not the case among captive toads, as no breeding was observed in enclosures. The fluctuations in body mass we observed in toads are likely to be largely a result of feeding rates, in turn driven by prey availability, and competition within enclosures.

Why did some captive toads in this study lose mass while others gained? This is an important issue for

interpreting our immunological results because it can clarify the direction of causation for the link

between changes in mass and in immune responses. That is, either a pathogen might activate immune

responses that subsequently cause weight loss; or weight changes may arise through variation in food

intake, and subsequently cause reconfiguration of immune cell populations. The latter scenario is more

likely. Because all toads were reared in captivity under identical conditions, exposure to pathogens and

parasites was minimized, or at least standardized [46]. All captive animals in this study appeared healthy,

and without wounds. Variation among individuals in in body mass trajectories likely reflect differences in

feeding success. Within each enclosure, toads competed for flying insects that were attracted by

overhead lights. Some individuals doubtless obtained more or larger prey than did others.

Does losing body mass act as a stressor in cane toads? We did not measure levels of corticosterone in our

study so are unable to use that as an index of stress. The changes in mass we recorded occurred over

periods up to three months, and thus an accompanying stress response would presumably be chronic

rather than acute. Among wild toads, CORT levels are lowest during the dry season, when fat stores and

rates of feeding, mass growth and activity are also low [3], indicating that periods of low food availability

may not always be stressful, especially if they are predictable and not severe [25, 47]. Studies on toads

have found no correlation between CORT levels and body condition [48-50] suggesting that energy

balance is not usually a strong stressor. However, a threshold could potentially exist whereby a prolonged

period without food, accompanied by sustained mass loss, does eventually trigger a stress response.

In the absence of a pronounced stress response or systemic immune reactions to infection or

inflammation, the relative abundance of different white blood cell types may be affected by their costs of

production and maintenance [22, 51]. Phagocytic cells (heterophils, monocytes) offer rapid and general

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pathogen protection as part of the innate immune system, and are considered less costly than are cells

such as lymphocytes which primarily play roles in acquired immunity [52]. An individual's relative

investment into innate *versus* acquired immunity can be affected by age, sex, size, or condition [18, 22,

51]. Under this scenario, cane toads that lose mass strategically reorganize immune investment rather

than indiscriminately downregulating all immune functions. Thus, some components might be down-

regulated while others are up-regulated, based on the costs and benefits of each under current

energetic/nutritional conditions and pathogen pressures. For toads that lost mass, increasing the

production of heterophils and monocytes while reducing numbers of eosinophils could reflect an

economic rationalization that best served their present circumstances.

Regardless of the mechanisms linking immune performance to mass change in cane toads, there are

important ecological implications. If immune function shifts in response to climatic or biotic

circumstances, then risk of pathogen infection may shift as well. If reduced energy intake and altered

immune function are coupled with other disease risk factors, such as increased conspecific density or sub-

optimal climatic conditions [53, 54], there may be strong seasonal patterns in health status.

Our results are preliminary in nature, and our interpretations speculate on complex underlying

interactions. A more focused and diverse set of experiments could clarify the patterns that we observed.

For example, future work could experimentally manipulate food intake rates to generate changes in body

mass, thus removing any ambiguity about causal links between immune responses and changes in body

mass. Density and competition effects could also be controlled or eliminated. It would be straightforward

to also measure changes in CORT levels, and thus clarify its relationships both with mass change and

immune function. In particular, such work could answer the question as to the extent to which mass loss

acts as a stressor in this system, and hence the degree to which shifts in immune function are attributable

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to stress responses rather than (or as well as) energy-driven prioritization of specific components of

immune function in the face of limited energy availability.

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Captions to Figures.

Figure 1. Representative cane toad (*Rhinella marina*) blood cell types. Basophil (B), eosinophil (E),

heterophil (H), lymphocyte (L), thrombocyte (t) and erythrocyte (r). Bar represents 10um. Geimsa stain.

Figure 2. Relationship between rate of mass change (%/day) and phagocytic ability of whole blood.

(Upper) Mean luminescence over 180 minutes and (**lower)** maximum luminescence recorded over 180

minutes. Y-axis scale is relative luminescence units (RLU).

Figure 3. Relationship between rate of mass change (%/day) and log-transformed white blood cell

concentrations and heterophil:lymphocyte (H:L) ratios.

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Table 1. Mixed model analyses of the relationships between sex, body size (snout-urostyle length) and recent change in body mass and immune assay performance of captive-reared cane toads (*Rhinella marina*). See text for assay descriptions. Family ID, Enclosure # and assay date were included as random effects in all models.

	PBS toe-web % swelling		PBS toe-web % swelling		PBS-PHA toe-web % swelling		Mean luminescence		Maximum luminescence		Time to max. luminescence		Allen BKA	
	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P
Sex	1.24	0.2683	0.66	0.4201	1.85	0.1763	0.06	0.8085	0.58	0.4495	2.84	0.0951	1.06	0.3053
Body size	0.59	0.4425	0.94	0.3352	2.60	0.1099	1.13	0.2908	1.30	0.2565	2.15	0.1460	2.67	0.1054
Mass change rate	0.39	0.5317	0.60	0.4385	0.30	0.5863	**4.78**	**0.0310**	**5.41**	**0.0220**	1.02	0.3142	1.06	0.3062

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Table 2. Mixed model analyses of the relationships between sex, body size (snout-urostyle length) and recent change in body mass and

circulating blood cell populations of captive-reared cane toads (*Rhinella marina*). See text for assay descriptions. Family ID, Enclosure # and

assay date were included as random effects in all models.

	RBC concentration		Total WBC concentration		Basophil concentration		Eosinophil concentration		Monocyte concentration		Lymphocyte concentration		Heterophil concentration		H:L ratio	
	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P	$F_{1,104}$	P
Sex	2.20	0.1411	1.52	0.2208	0.03	0.8645	0.39	0.5325	0.84	0.3627	2.48	0.1186	0.14	0.7138	3.22	0.0757
Body size	**4.48**	**0.0367**	0.01	0.9152	0.41	0.5249	**4.83**	**0.0303**	2.78	0.0986	0.08	0.7826	0.58	0.4464	2.15	0.1452
Mass change rate	1.43	0.2348	0.13	0.7193	0.48	0.4909	**4.88**	**0.0294**	**12.85**	**0.0005**	1.47	0.2274	**21.07**	**<0.0001**	**29.96**	**<0.0001**

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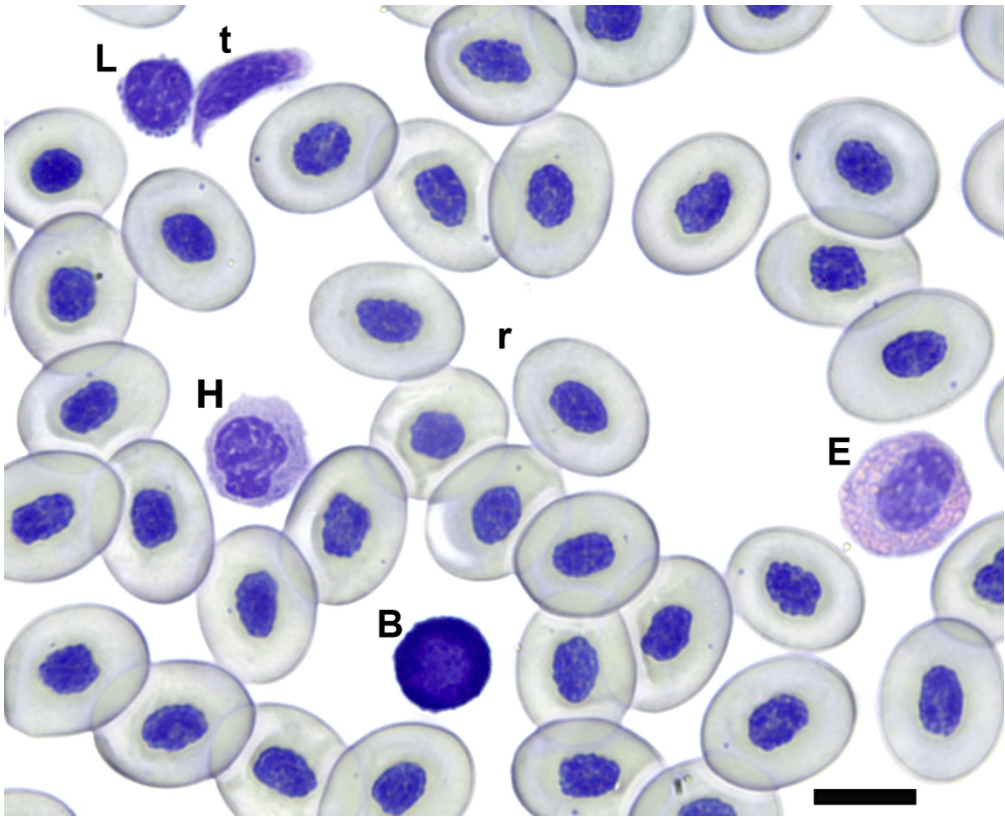
Figure 1. Representative cane toad (Rhinella marina) blood cell types. Basophil (B), eosinophil (E), heterophil (H), lymphocyte (L), thrombocyte (t) and erythrocyte (r). Bar represents 10um. Geimsa stain.

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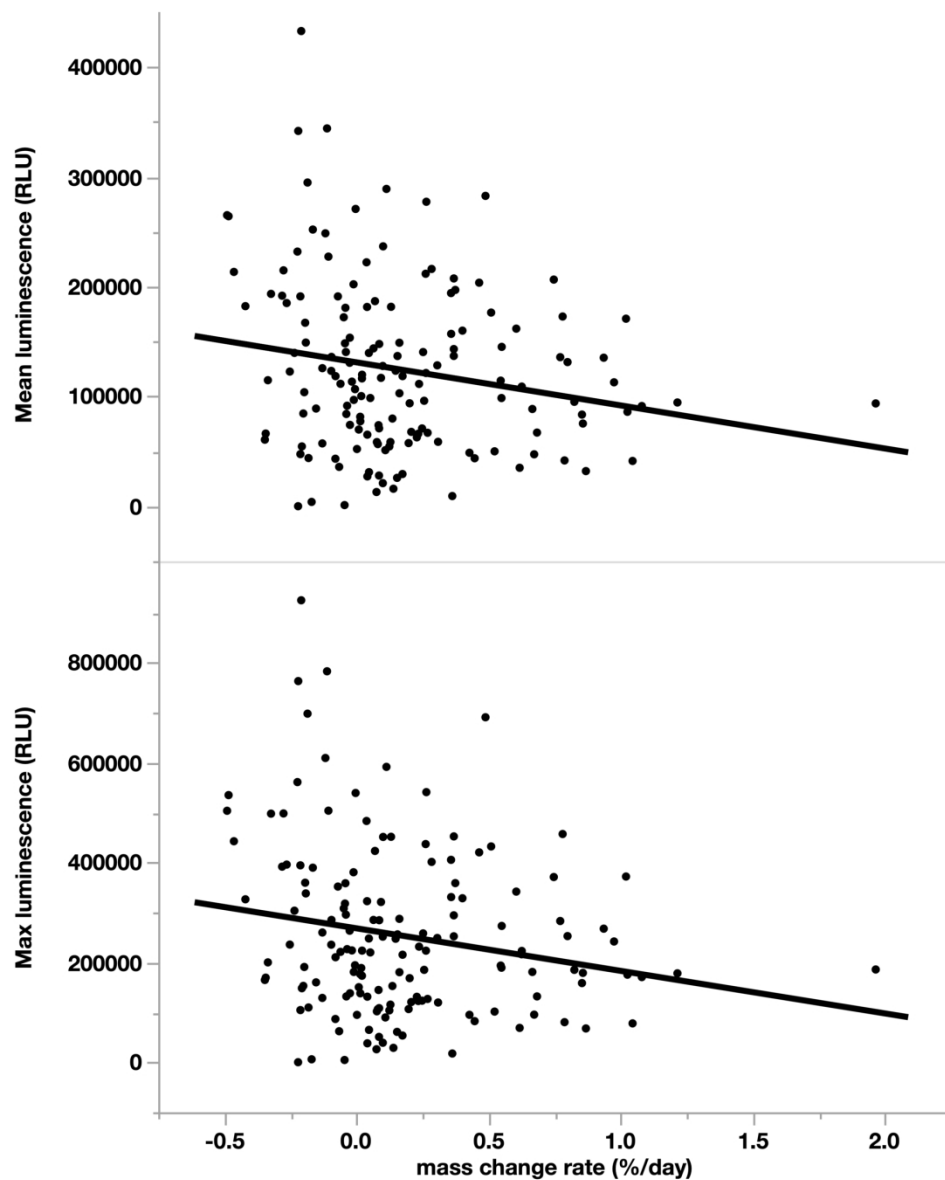51
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Figure 2. Relationship between rate of mass change (%/day) and phagocytic ability of whole blood. (Upper) Mean luminescence over 180 minutes and (lower) maximum luminescence recorded over 180 minutes. Y-axis scale is relative luminescence units (RLU).

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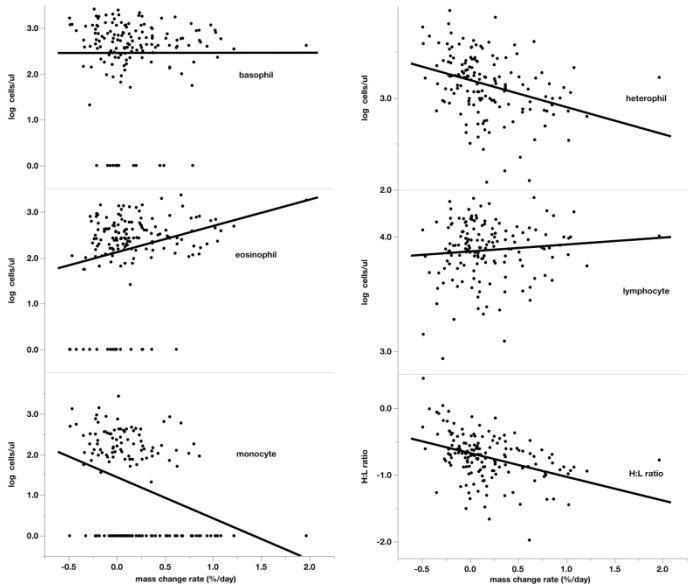51
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Figure 3. Relationship between rate of mass change (%/day) and log-transformed white blood cell concentrations and heterophil:lymphocyte (H:L) ratios.

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