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The value of chemical defense: the effects of toxin milking on the physical, physiological, and behavioral performance of cane toads (Rhinella marina)

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SIT Study Abroad

The value of chemical defense: the effects of toxin milking on the physical, physiological, and behavioral performance of cane toads (*Rhinella marina*)

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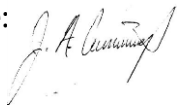
Signature: 

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Abstract

Amphibians have cutaneous glands on their skin which produce a number of toxic compounds that serve as protection against predators and microorganisms. Cane toads (*Rhinella marina*) have large parotoid glands located on their shoulders to store toxins, many of which are derived from lipids and are thus energetically expensive to produce. I used a combination of field and laboratory studies to investigate behavioral and physiological consequences of toxin loss and replenishment in cane toads. In a cross-sectional study, free-ranging toads were euthanized and dissected to identify correlations between toxin content and morphology /physiology. Experimental manipulations (manually expressing toxin from glands) were performed to mimic a predator encounter that resulted in toads expelling their toxin in defense. A control group for this manipulation consisted of individuals whose glands were squeezed in a manner that did not express the toxin. Manipulations were carried out in both captivity and in the field. In both settings, growth rate, behavior, activity and energy allocation (relative organs masses) were compared between de-toxined and control toads. A significant negative correlation between toxin content and liver mass was evident among wild toads. The experimental manipulation of removing toxin affected change in body mass in both captive and radio-tracked wild toads. However, the effect on mass changed differed between captive and wild settings. De-toxined toads that were fasted exhibited poorer locomotor performance than their control counterparts. However, toxin milking had no significant effect on personality traits, such as boldness, or foraging skills. Radio-tracking demonstrated that de-toxined toads also disperse more slowly than the control toads, preferring to stay closer to the water source at which they were originally captured. I found evidence of physiological and behavioral costs associated with toxin removal,

even over a relatively short period (5-20 days). Therefore, toxin may represent a precious commodity to its host and may only be deployed as a last line of defense.

Key words: *Bufo marinus*, parotoid glands, radio-telemetry, toxin production, toxin replenishment.

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1.0 Introduction

An organism's ability to defend itself against predators is vital to its survival (Regueira et al., 2015; Arbuckle and Speed, 2015). Because of this need, a variety of animals have evolved a range of strategies to reduce the risk of detection and attack by a predator (Regueira et al., 2015). Tactics include cryptic coloration and behaviors such camouflage and mimicry, warning colors, and chemical defenses (Arbuckle and Speed, 2015).

Nearly 50% of all phyla in the animal kingdom contain venomous species, the manufacture of chemicals is a common means used by organisms to defend themselves against predators, competitors, para-sites, microbes, and other potentially harmful organisms (Smith et al., 2014; Derby and Aggio, 2011; Nisani et al., 2012). Chemical toxins can either be poisonous or venomous. Poisonous animals produce chemicals that will kill a predator that attempts to consume it, while venomous animals would inject predators, or prey, with toxin via biting or stabbing. Toxins are produced by a wide array of taxa, ranging from cnidarians, arachnids, and amphibians to colubrid, viperid and elapid snakes (Pintor et al., 2010; Regueira et. al. 2015). Even some mammals produce toxin; notably male Platypuses (*Ornithorhynchus anatinus*), which develop venomous spurs during the mating season for use against competing males, and in hunting prey (Whittington and Belov, 2007).

Chemical defenses are common among amphibians (Regueira et. al. 2015). Cutaneous glands on amphibian skin produce a number of different toxic compounds which serve as protection against predators and microorganisms. These glands are typically dispersed across all skin surfaces but sometimes with local accumulations (Mailho-Fontana et al.). Many of the compounds that make up toxin in the skin of these animals consist of alkaloids, bufadienolides, proteins, peptides, salts, animo acids and other organic compounds (Caroll and Weldon, 2006;

Casewell et al., 2013). Defense by amphibians against other organisms is generally passive, and this is notably the case in toads (Regis-Alves et al., 2017; Kowalski et al., 2018). Though potent, amphibian toxin is only secreted from glands into the mouth of a predator attempting to bite it and is not injected into the predator (Jared et. al, 2014; Whittington and Belov, 2007; Nisani et al., 2012). In addition to deterring large predators, amphibian skin secretions also serve to deter small biting insects, such as ants and mosquitos (Carroll and Weldon, 2006).

Little is known about the energetic requirements involved in producing chemical defenses in amphibians. Many organisms are frugal with the use of their chemical compounds, in most cases leaving it for the last stage of antipredator defense after flight and other behavioral tactics have failed (McCue, 2006; Nisani et al., 2012; Arbuckle 2015; Regis-Alves et al., 2017). This frugality appears to be attributed to the high metabolic and energetic cost of toxin regeneration (Nisani et al., 2012). This also explains why venomous species will sometimes employ dry bites (bites that do not inject toxin). Studies on scorpions (*Parabuthus transvaalicus*) and North American pit vipers (*Crotalus atrox, Crotalus horridus, Agkistrodon contortrix*) have demonstrated that chemical defenses are metabolically costly to produce and represent a valuable asset to their hosts. The abundance of toxin an animal possesses often correlates to conditions such as water and food availability (Mailho-Fontana et al; and Nazareth e. al 2016). In the case of harvestmen (*Magnispina neptunus*), well fed individuals produce secretions with higher mass and concentration than fasted individuals do (Nazareth et al., 2016). This supports the hypothesis that a nontrivial energy investment is necessary to produce and replenish the chemicals contained in toxins. Conversely, other studies indicate that toxin production may not be as costly in terms of time and metabolism as are other functions, such as shedding and digesting, as is the case with the common death adder (*Acanthophis anticarcticus*) and centipedes (*Scolopendra polymorpha*)

(Pintor et al., 2010; Cooper et al., 2014). These contrasting findings may indicate that the cost of toxin compounds may increase with their complexity and that this may vary among taxa (Nisani et al., 2012).

Cane toads (*Rhinella marina*), like other bufonids, store toxin in paired parotoid glands located on their shoulders (Mailho-Fontana et al.). Although this toxin can be lethally potent it is typically the last of a series of defenses, including flight, puffing themselves up to appear larger, immobilization, and in some cases, head butting (Jared et al., 2014; Chen and Kovarikova, 1967; Kowalski et al., 2018). Normally, it is not until they are actually bitten by a predator that toads release their stored chemicals as a last-ditch defense. The process of regeneration of toxin by cane toads appears to be slow, estimated to take 11 weeks to restore only two thirds of the original volume (Jared et. al, 2014; Chen and Kovarikova 1967). This suggests that toxin production is constrained by time as well as by energy and that repeated replenishment of large quantities may not be possible. Another factor that may affect the amount of toxin that a toad produces is its perceived level of threat. Toads that frequently encounter predator cues may increase their toxin production in response to potentially higher risk of encounters (i.e. inducible chemical defense) (Bucciarelli et. al, 2017). Consequently, toxin content could be related to the toad's personality, bold vs shy, and/or the habitats they reside in, as some habitats may have more predators than others.

The toxins produced by amphibian glands also spread onto the skin and act as a first line of chemical immune defense against pathogens (Rollins-Smith and Woodhams 2011). Thus, removal of stored toxin could interfere with its immune role and result in upregulation of other immune components in compensation. Alternatively, the increased energetic demands required to replenish toxin stores could reduce the energy available to maintain other immune defenses.

The aims of this study are to investigate the costs associated with toxin production in cane toads. The study consisted of three parts:

i) a cross-sectional study of wild toads to assess morphological and physiological correlations of toxin volume.

Toxin milking is used as an experimental manipulation to mimic a predator encounter that caused a toad to utilize its chemical stores and hence to initiate the production of replacement toxin. This was carried out in two longitudinal studies:

ii) in a captive setting, on subadult toads maintained in the lab and

iii) in the wild, on free-ranging toads.

For parts ii and iii of the investigation I compared growth rates, various measures of energy allocation (relative organ masses), behavior, and survival between toxin milked and control toads.

Based on the hypothesis that the production of replacement toxin represents a major energetic expense that de-toxined toads face, but control toads do not, several predictions were made. It is predicted that, relative to control toads, de-toxined toads would:

a) grow less quickly, even under conditions of abundant food.

b) show lower levels of activity and physical performance

c) exhibit altered levels of immune performance

d) exhibit lower levels of survival in the wild

2.0 Methods

2.1 Study Site

Leaning Tree Lagoon Nature Park (12°71′33″S, 131°41′96″W) is adjacent to the Adelaide River floodplain, 80km south-east of Darwin in Australia's Northern Territory. This study was conducted during the end of the dry season in the month of November. The lagoon is a permanent waterbody and acts as a refugial area for many organisms during the dry season, including cane toads. Cane toads arrived in the area in 2005 (Shine, 2018) and can be found in high abundance at the park (Finnerty et al 2018).

2.2 Study Species

Cane toads (*Rhinella marina*) are an ancient and widely distrusted species of bufonid anurans from central and south America (Lever, 2001). They were initially brought into Australia from Hawaii, where they are also invasive, in 1935 as a biocontrol to reduce insect pests from sugar cane farms in Queensland. Since then, toads have spread to colonize several other states of Australia, including New South Wales, the Northern Territory, and Western Australia (Shine, 2010). As in several other species of amphibians, cane toads' glands possess potent toxins. They were chosen for this study because their toxins, which consist of expensive and complex compounds, are concentrated in two large paratoid glands located on their shoulders. Because their poison is so densely concentrated in one area, it could be easily and harmlessly milked as part of an experimental manipulation; and because they are found in such high abundance, a large sample size could be used.

2.3 Assessing the physiological cost of parotoid toxin

Toads were collected by hand and placed in damp cloth bags and left in the lab overnight. 43 individuals of various sizes were captured, 22 of which were female and 21 were male. The next

day, the toads were humanely euthanised by an overdose of Lethabarb, and the snout-urostyle length (SUL), tibia length and body mass were measured. The length and width of each parotoid gland was also measured, and their toxins were extracted by squeezing the gland between the thumb and forefinger (Figure 1). Extracted toxin was scrapped onto a glass microscope slide and weighed to 0.001g. The liver, gonads, spleen, and fat bodies were extracted, and individually patted and weighed to 0.001g accuracy to find relationships between toxin mass and organ mass. The lungs were examined for the presence of parasites (*Rhabdias pseudospaerocephala*). Blood samples were taken to conduct immune assays (phagocytosis, white blood count, hematocrit) (see Brown et al. 2015 for details of immune assay methods) to find any correlations between immune function and toxin production.

Figure 1. Milking toxin from a cane toad parotoid gland. Toxin can be expressed by squeezing the gland between thumb and forefinger under a pane of glass to prevent toxin from shooting upwards.

2.4 Studying Captive Toads

A total of 40 subadult toads (50-80mm snout-urostyle length) were captured at night from within 5 kilometers of the University of Sydney's Tropical Ecology Research Facility in Middle Point,

NT. They were individually toe clipped and had snout-urostyle length, tibia length, head width, and mass recorded. They were injected ivermectin at a dose of 0.02mg/100g to remove nematode lungworms which are known to affect toad growth rates (Finnerty et al., 2018). Toads were orally dosed with metranidazole (Flagyl, 10mg/100g) to remove potentially pathogenic protists from their guts (Shilton et al., 2018) from their intestines. The length and width of their parotoid glands were measured to find relationships between gland size and toxin content. The toads were placed in individual 12l bins (40 × 30 × 20 cm), equipped with newspaper floor covering, a water dish, and small shelter. Each toad was then randomly assigned to one of four treatment groups that were determined by toxin gland manipulation and feeding regime. Half of the toads had their glands squeezed in an outward radial pressure between thumb and forefinger that caused the toxin to be expressed from the gland. The other half endured "sham" treatments, which involved squeezing their glands with and inward pressure that did not cause the toxins to be expressed. This was done in order to prevent a difference in the stress levels that the captive toads may have experienced. The 20 toads that did not have their toxins milked acted as a control group.

Figure 2. A depiction of the two methods employed to squeeze cane toad parotoid glands. One method resulted in toxin being expressed and the other did not and was used as a procedural control manipulation.

A total of 20 toads (10 with toxins removed and 10 control) were maintained on a high feeding regime (5 crickets a day) for 20 days. The other 20 toads were fed 10 crickets before the start of the trial period, and another 10 crickets after a 20-day fasting period. The two feeding regimes represent the two ends of the spectrum faced by free ranging toads during the wet and dry seasons in Australia. During the wet season, when insects are abundant, cane toads may be able to satiate their hunger every day. However, they may rarely get an opportunity to feed in the dry season when competition is high, and insects are scare. Snout-urostyle length, tibia length, head width, and mass were measured again on day 10 and 20 toad to assess if there were any changes in growth or mass. At the end of the trial toads were humanely euthanized by overdose of Lethabarb. Liver, gonads, spleen, and fat bodies were patted dry and weighed. Immune assays (see above) were conducted on the 20 toads in the fed group to investigate relationships between toxin removal and immune function.

2.5 Evaluating Toad Behavior

2.5.1. Boldness and Activity Level

Toad boldness and activity levels were scored from videotaped trials conducted once at the beginning of the experiment before toxin extraction manipulations had taken place and repeated 20 days later at the end of the experiment. Toad activity was measured using boldness trials (González-Bernal, Brown and Shine, 2014). Toads were placed individually into 70l arenas (64 x 42 x 30 cm) marked with 10 x 10 cm grid lines on the floor. Each arena held a black 1l shelter box with a door that could be closed. Toads were placed into the closed shelters. After a 5-minute acclimation period, the door was opened, and the trial was filmed for 30 minutes. The following variables were scored from videotapes as measures of boldness and activity: the time elapsed until the toads head first emerged from the shelter, the time elapsed until it's body fully

emerged from the shelter, the number of grid lines crossed after emerging from the shelter, the number of jumping attempts to escape the arena, and the time it escaped the arena (if applicable). The trials were conducted after dark between the hours of 19.30-00.45. Trials were conducted under ambient outdoor weather conditions, between temperatures of 31-34.1°C and 52-60% humidity.

2.5.2 Locomotor Performance

Toad performance was measured using locomotor trials. Each toad was placed at the start of an indoor raceway (285 x 14.5x 8.5 cm) and encouraged to run along the track by gently tapping its hindquarters. Each toad was raced up 240cm central section of the track and then back and the time taken to traverse each 60cm section was recorded. The fastest time to cover any of the 60cm sections was used as a measure of sprint speed and the total time taken to traverse all 8 60cm sections was used as a measure of endurance. If a toad was tapped more than 10 times in a row without response, the trial was terminated. Trials were conducted at the beginning of the experiment before toxin extraction manipulations had taken place and repeated 20 days later. Trials were conducted during daytime under ambient weather conditions, between temperatures of 31.4-34.5°C and 41-68% humidity.

2.5.3 Foraging Success

Foraging success was measured by placing individual toads in 70l arenas (64 x 42 x 30 cm) with 10 crickets. The number of crickets eaten within 5 minutes was used as a measure of foraging ability. These trials were also conducted at the start of the experiment, before toxin manipulations were carried out, and again at the end to assess whether toxin removal affected foraging behaviour in either of the feeding regimes. Temperatures during feeding trials ranged from 31.0-35.5°C and relative humidity ranged from 37-56%.

2.6 Studying free-ranging toads

154 toads were collected from the perimeter surrounding the water at Leaning Tree Lagoon, between the hours of 20.30 and 21.30hr. They were placed in damp cloth bags and left in the lab overnight. Each toad has its snout-urostyle length, tibia length, head width, and mass measured and was sex was determined based on the presence of secondary characteristics (release call, rough texture, yellow colouration, and nuptial thumb pads in males). Toads were individually toe clipped and divided in two groups at random (control (C) and toxin milking (T)). 80 of the toads had their toxins milked and the remaining 74 had their glands massaged as the control. Toads were all released back at the point of capture with the intent of recapture at the end of the study.

2.6.1 Activity level

36 of the wild toads captured at Leaning Tree Lagoon as part of the mark-recapture study (see above) were also fitted with a bead chain belt bearing a 3g radio-transmitter (model PD2; Holohil Systems, Ottawa, Canada). Body mass of the 36 radio tracked toads ranged between 56 and 280g. Therefore, the radio transmitter and belt represented a burden of < 6% of body mass. Half were milked of toxin the remaining acted as control. They were released back at the point of capture 24hrs later. Radiotracked toads were located daily for 5 days and the co-ordinates of each toad's diurnal shelter site were determined using a hand-held GPS receiver, accurate to 3 m. After 5 days radio tracking, each toad was captured and returned to the lab where it was euthanized, dissected and toxin squeezed from its parotoid glands and weighed to 0.001g.

2.7 Statistical Analysis

A combination of parametric and non-parametric analyses was conducted, depending on the normality of the dependant variable. One-way analysis of variance (ANOVA) or Wilcoxon (nonparametric) tests were used to compare continuous variables between two treatments. Linear

regressions (parametric) or Spearman correlations (nonparametric) were used to assess relationships between two continuous variables. Analysis of covariance (ANCOVA) was used to compare between two treatment groups incorporating a continuous variable (e.g. body size) as a correction factor. Multiple regressions were conducted to simultaneously assess the effects of several independent variables. All analyses were conducted using JMP 13 software (Sas Institute, Cary NC) and significance was accepted at $p<0.05$.

2.8 Animals Ethics

This study was conducted under the approval of the University of Sydney Animal Care and Ethics Committee (permit numbers 2017/1195 and 2018/1441).

3.0 Results

3.1 Morphological correlation of toxin volume.

The 43 toads collected from Leaning Tree Lagoon ranged in body size from 88.1-126.8mm SUL (mean=104.7mm) and body mass from 53.5-236.4g (mean =129.4g). Larger toads contained significantly more toxin in their glands than smaller toads (linear regression $F_{1,42} = 13.68$, $p=0.0006$, **Figure 3**)

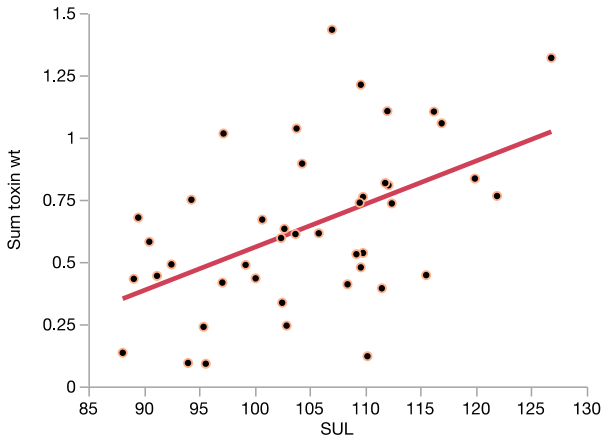

Figure 3. Linear regression of total toxin weight on toad body size (snout to urostyle length (SUL)).

After adjusting for body size differences between males and females, it was found that both sexes contain similar amounts of toxin (ANCOVA, SUL F1,40= 7.97, p=0.0074; Sex F1,40=1.52, p=0.23). They also contain identical volumes of toxin in their left and right glands (paired t-test; t=0.02, df=42, p=0.98). Toxin volume was significantly and positively correlated with both length and width of parotoid glands (**Figure 4).**

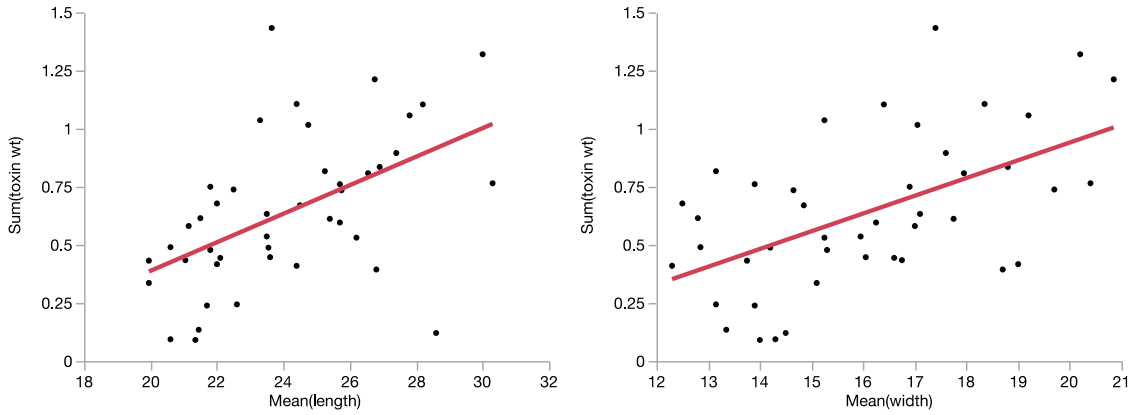

Figure 4. Linear regressions of toxin weight on length (F1,41=14.55, p=0.0005) and width (F1,41= 17.51, p= 0.0001) of a toad's parotoid glands.

To assess what other factors correlate with toxin volume a series of multiple regressions were performed to evaluate the combined effects of body size (SUL), and masses of fat bodies, spleen, liver, and gonads on toxin volume. Once again, it indicated a positive relationship between SUL and toxin production (Table 1). After adjusting for the body size effect, only liver mass showed a significant relationship with toxin volume (Table 1), demonstrating that individuals with relatively large livers had relatively lower amounts of toxin **(Figure 5).**

Table 1. Results of multiple regression analysis of the effects of body size and organ masses on amount of toxin contained by 43 cane toads.

Trait	df	F	P
SUL	1,37	13.6394	**0.0007**
Fat body	1,37	0.2440	0.6243
Liver	1,37	5.9133	**0.0200**
Gonad	1,37	0.7810	0.3826
Spleen	1,37	0.4974	0.4851

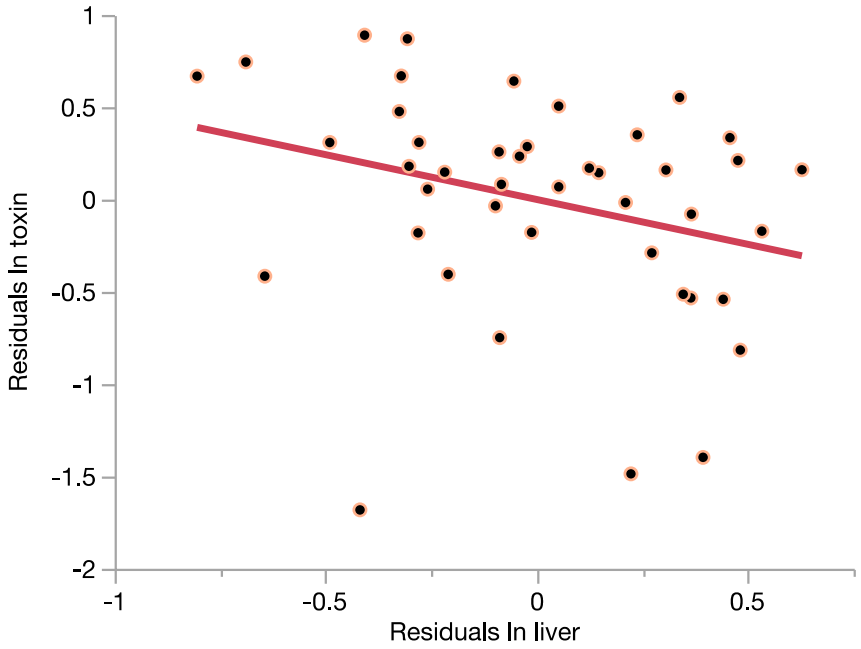

Figure 5. Relationship between relative toxin volume and relative liver mass of 43 cane toads. Relative values were calculated as residuals from linear regressions of each variable on SUL.

To test whether the amount of toxin a toad contains affects its immune performance, a series of regressions were conducted incorporating body size (SUL) and toxin mass as independent variables and various immune measures as response variables (Table 2). Body size and toxin had no effect on level of lungworm infection, white blood cell (wbc) concentration, maximum phagocytosis level or the time to reach maximum phagocytosis. Larger toads had high mean levels of phagocytosis, but this was not affected by toxin mass. Hematocrit level was affected by toxin mass- toads with relatively high levels of toxin had lower hematocrit.

Table 2. Results of multiple regression demonstrating the relationships between body size (SUL), toxin volume, and immune function in 43 wild cane toads.

Trait	Effect	DF	F	P
lungworm	Sum toxin wt	1,40	0.0550	0.8158
	SUL	1,40	0.8671	0.3573
hematocrit	Sum toxin wt	1,40	4.8034	**0.0343**
	SUL	1,40	2.8414	0.0997
wbc concentration	Sum toxin wt	1,40	0.0994	0.7542
	SUL	1,40	0.1255	0.7250
Mean phagocytosis	Sum toxin wt	1,40	0.5058	0.4811
	SUL	1,40	4.6575	**0.0370**
Maximum phagocytosis	Sum toxin wt	1,40	0.0729	0.7885
	SUL	1,40	3.8358	0.0572
Time to maximum phagocytosis	Sum toxin wt	1,40	0.4569	0.5030
	SUL	1,40	0.3442	0.5607

3.2 The effects of fasting on captive toads

The feeding regime caused a significant difference in the change in mass exhibited by toads over the 20-day experiment ($F_{1,38}= 54.16$, $p<0.0001$). Fasted toads lost an average of 21% mass over the trial period, while fed toads gained an average of .3% mass. Note that three toads in the fed treatment decreased substantially in mass (Figure 6). Although live crickets were provided daily, these individuals (all in the de-toxined treatment) rarely ate them.

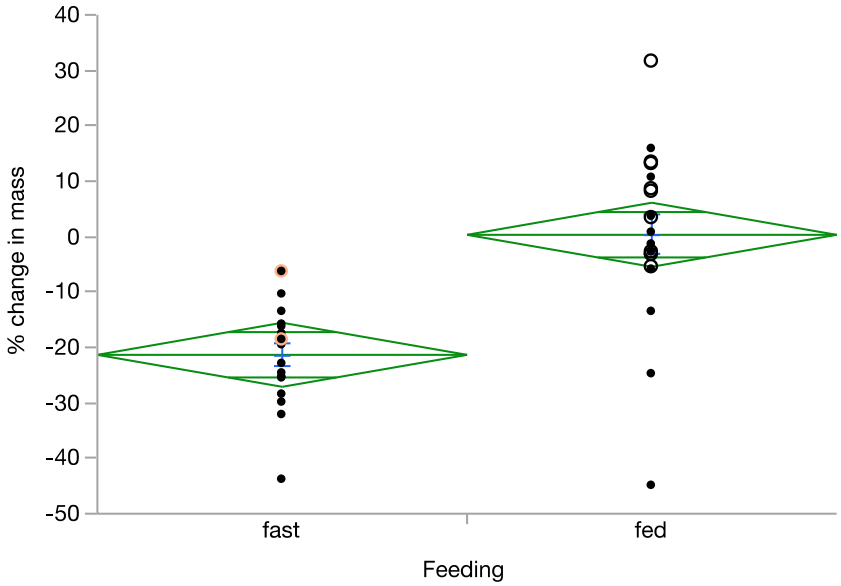

Figure 6. Results of feeding regime on changes of percentage of mass on toads.

Among the fasted toads, there was no effect of sex or toxin milking on changes in body mass (Table 3, Figure 7 left panel). Among fed toads however, the toxin milking treatment brought about a significant divergence in mass. De-toxined individuals lost an average of 1.7% in mass, while control individuals gained an average of 7% in mass (Table 3, Figure 7 right panel). Among fed individuals, females tended to increase mass more than males. However, the difference between sexes was only borderline significant (p=0.07).

Table 3. Result of multiple regression on the effects of sex and toxin removal on percent change in body mass of captive toads maintained under two feeding regimes for 20 days.

Feeding Regime	Effect	df	F	P
fast	Toxin	1,17	0.0454	0.8338
	Sex	1,17	0.9703	0.3384
fed	Toxin	1,17	4.7885	**0.0429**
	Sex	1,17	3.6897	0.0717

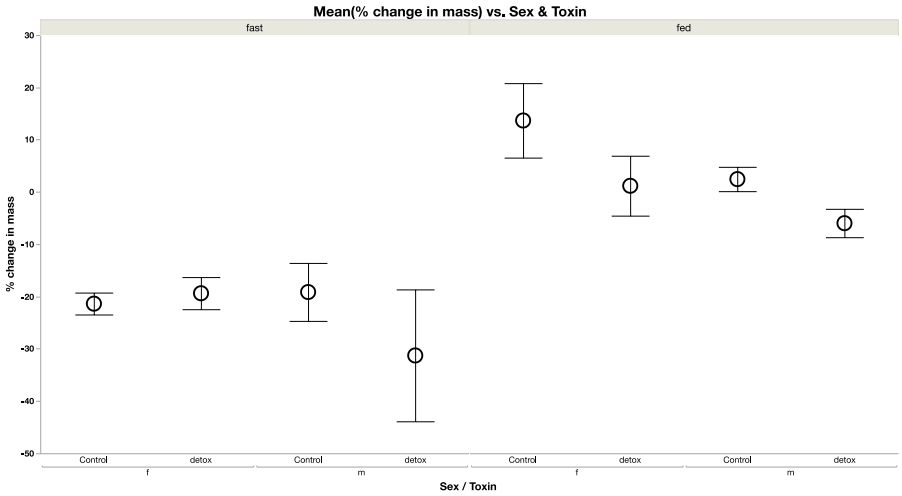

Figure 7. Result of multiple regression on effects of toxin removal and feeding treatment on changes in percentage of mass of captive toads.

Multiple regressions were used to simultaneously assess the effects of feeding regime and toxin treatment on organ sizes measured at the end of the experiment (Table 4). SUL was also included as an independent variable in the models to control for effects of body size on organ masses. After correcting for body size, there was no evidence of organ weights differing between de-toxined and control toads. However toads in the fed treatment had relatively larger livers and spleens than did individuals in the fasted group. The size of fat bodies was not related to body size nor to toxin treatment or feeding group.

Table 4. Results of multiple regressions on the effects of body size, feeding regime and toxin removal on relative organ weights of captive toads.

Trait	Effect	df	F	P
Liver	SUL	1,36	72.7982	**<.0001**
	Feeding	1,36	71.2120	**<.0001**
	Toxin	1,36	0.6884	0.4122
Spleen	SUL	1,36	4.1690	**0.0485**
	Feeding	1,36	5.1800	**0.0289**
	Toxin	1,36	0.8077	0.3748
Fat body	SUL	1,36	0.8719	0.3567
	Feeding	1,36	0.9035	0.3482
	Toxin	1,36	0.7607	0.3889

3.3. Changes in toad behavior

Body out time was chosen as the best measure of boldness as it reflects an individual's decision to fully expose itself to any potential risks outside the safe shelter box. Individuals that emerged from shelter quickly during the first trial also emerged quickly during the second trial 20 days later (Spearman r = 0.88, P< 0.0001, Figure 8). Therefore, boldness (as measured by time to fully emerge from shelter) may represent a personality trait that an individual demonstrates consistently over time.

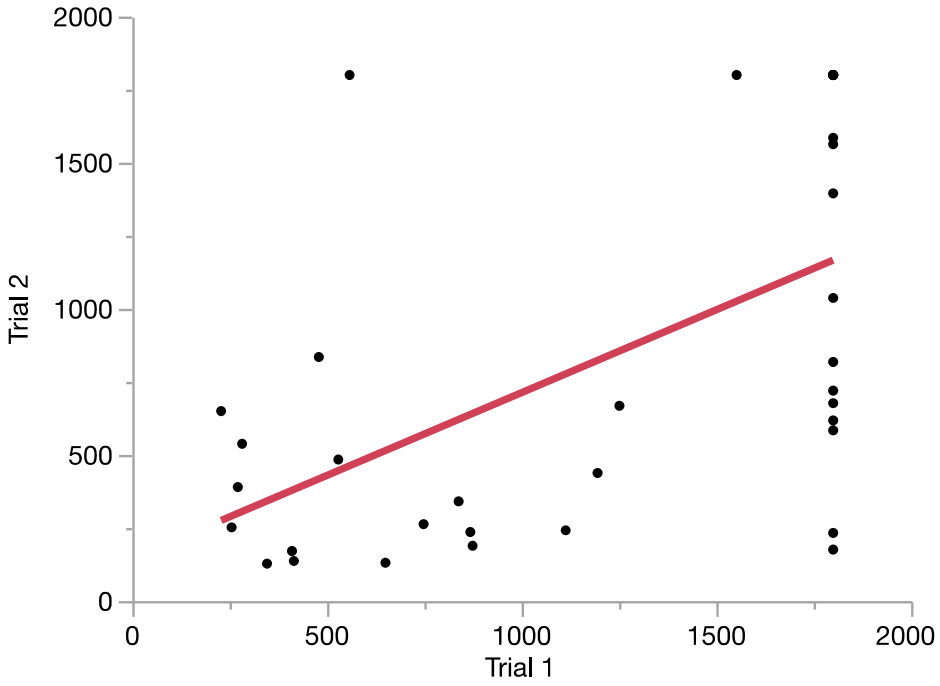

Figure 8. Comparison of body out times in seconds between the two trials conducted pre and post treatment. Trial 1 represents scores recorded before treatment and trial 2 represent scored recorded after the 20-day trial period.

Changes in boldness between the first (pre-treatment) and second (post treatment) trials was measured by subtracting the time taken for the body to emerge from the shelter in trial 1 from the time in taken in trial 2. Negative values indicate individuals that emerged from shelter more quickly in the second trial than the first (i.e. became bolder). Individuals that did not emerge

from shelter during a given trial were assigned a value of 1800 seconds, which represents the total duration of a trial. Change in boldness between trials was not related to the toxin manipulation in either the fed or fasted groups of toads (both Wilcoxon $\chi^2 < 0.69$, both $p > 0.41$, Figure 9).

Activity levels in the boldness trials was scored as the number of 10cm gridlines a toad crossed per minute after it emerged from the shelter. (Toads that did not emerge from shelter were given activity scores of 0). Individuals tended to show similar levels of activity between trials though the similarity was at the border of statistical significance (Spearman r= 0.32, p=0.056). To measure changes in activity between trials, each individual score in trial 1 was subtracted from its score in trial 2. Changes in activity between trials was not related to the toxin manipulation in either the fed or fasted groups of toads (both Wilcoxon $\chi^2 < 1.66$, both $p > 0.20$).

The data collected from locomotor trials were nonnormally distributed, so I used nonparametric methods to assess differences among groups. At the beginning of the experiment, sprint time and total locomotor time did not differ between toads that were subsequently assigned the different feeding groups or toxin treatment groups (Wilcoxon test, all $\chi^2 < 1.35$, all $P > 0.24$). At the end of the experiment, de-toxined individuals that were fasted had significantly higher sprint times (i.e. lower sprint speed) than control toads (Wilcoxon $\chi^2=3.88$, $P=0.049$). The total time it took to complete the locomotor trial distance did not differ between fasted toads in the two toxin treatment groups (Wilcoxon $\chi^2=1.34$, $p=0.25$, Fig). In the fed groups, neither sprint time or total time it took to complete the trial differed between de-toxined and control toads. (Wilcoxon χ^2 both <0.69, both $P > 0.41$).

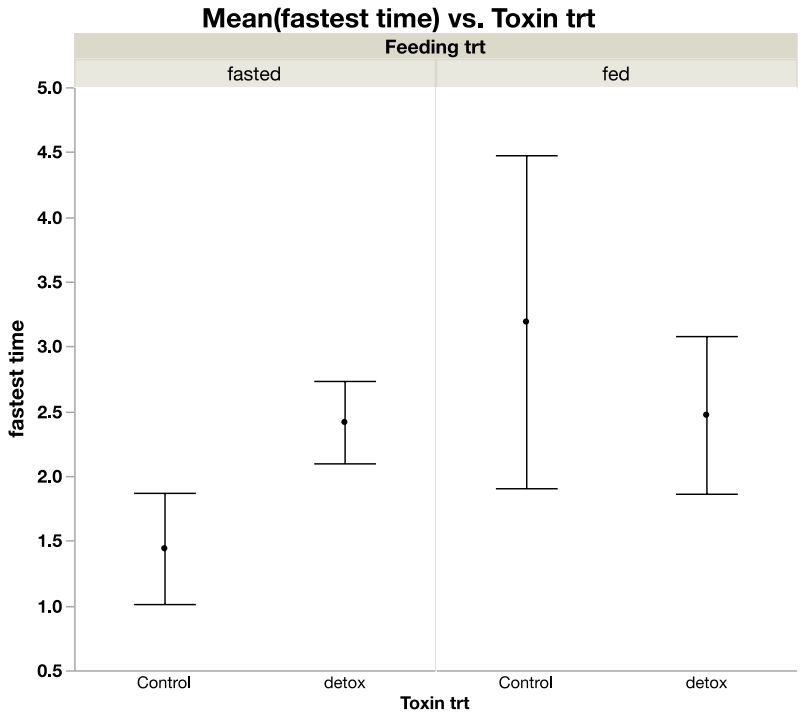

Figure 9. Average time (sec) for toads to sprint 60cm in a lab runway. The left panel shows the average times for toads in the fasted treatment, where detoxined toads were significantly slower than control toads. The right panel shows the results for control and de-toxined toads in the fed treatment.

There were no differences in numbers of crickets eaten between toads at the beginning of the experiment before they were assigned to their respective feeding groups (all Wilcoxon χ^2 <1.78, all P > 0.18). Similar data was collected at the end of the experiment, as there was no effect of toxin treatment on feeding performance for toads in either feeding treatment (all Wilcoxon χ^2 <0.29, all P > 0.59).

3.4. Behavior and growth of wild toads

3.4.1 Rates of movement

There was no significant difference between treatment groups in the mean distance moved between daily refugia sites ($F_{1,6}$=0.42, P=0.54), and no difference in mean distance moved from original capture location ($F_{1,9}$=2.70, P=0.14). However, the average movements over the course

of the 5-day telemetry period differed between control and de-toxined toads. Control toads increasingly moved increasingly further away from their original capture location, while toxin-milked toads did not move any further from their capture location over the 5-days (Table 5, Fig 10). The mean distances between daytime refugia also increased over time (P= 0.065, Table 5), nonetheless this did not differ between treatment groups (Table 5).

Table 5. ANCOVA results of the effects of toxin milking and day number on movements of radio tracked cane toads. Control toads moved increasingly further away from their initial capture location as the 5-day telemetry period progressed, while toads thatthat had their toxins milked did not.

Variable	Effect	df	F	P
Mean distance between refugia	day #	1,6	6.3542	0.0653
	Toxin	1,6	0.7484	0.4358
	Toxin*day #	1,6	0.1802	0.6931
Mean distance from capture site	day #	1,9	8.8181	**0.0250**
	Toxin	1,9	5.2374	0.0621
	Toxin*day #	1,9	6.8405	**0.0398**

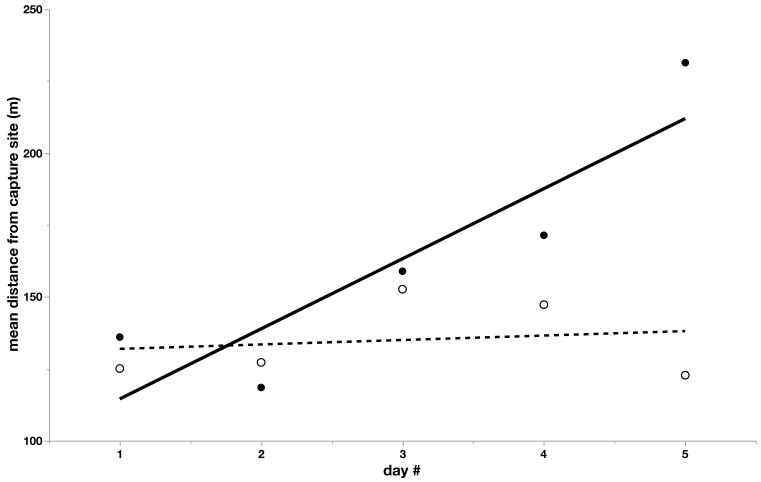

Figure 10. Distance moved from capture site during 5 days of radio telemetry. Control toads (solid line and circles) moved further away over time. De-toxined toads (dashed line, open circles) did not move further away over time.

3.4.2 Growth rates

Among the 36 toads used in the radio telemetry study, de-toxined toads gained significantly more weight during the 5-day tracking period than did control toads (5.6% vs -0.05%, Wilcoxon χ^2= 4.02, P=0.045). However, there was no detectable difference in the masses of organs between control and de-toxined toads (all Wilcoxon χ^2 < 1.07, all P >0.30).

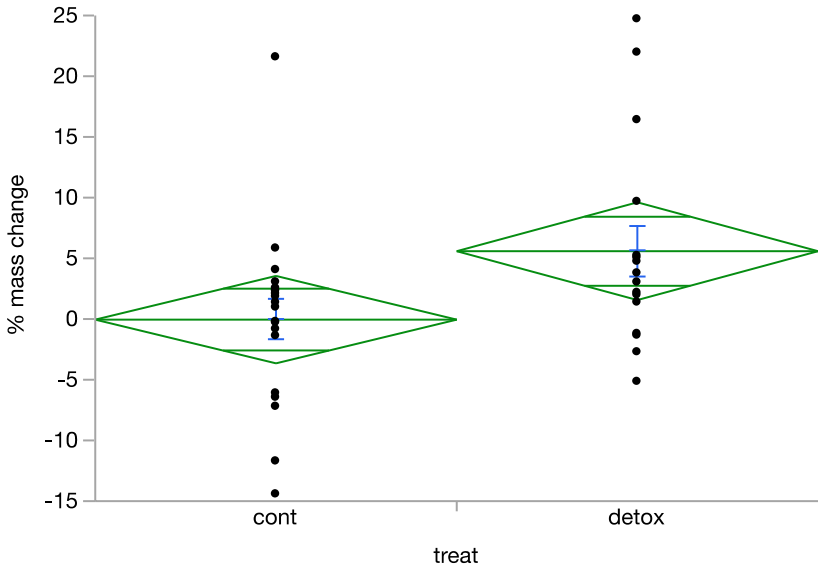

Figure 11. Comparison of the changes in body mass between control and de-toxined toads over the 5-day radio tracking period. Detoxined toads gained significantly more mass than control toads.

4.0 Discussion

The results of the cross-sectional study verified several logical expectations. Larger toads carry more toxin than smaller toads, and larger parotoid glands carry more toxin than smaller ones. The negative relationship between toxin volume and relative liver mass nevertheless is counter-intuitive. Typically, relatively larger livers are associated with increased capacity for energy storage, metabolism and synthesis (Withers and Hillman, 2001). It might be expected that individuals with larger livers also contained more toxin. However, because toxin is stockpiled in

the parotid glands for long-term storage, its actual synthesis would have occurred long before the toads were euthanized. The amount of toxin a toad contained would not represent a current metabolic demand, but rather reflect a past demand. It is possible that contemporary production of toxin would require a large and active liver. Yet, once toxin had been produced and stored, liver size might decrease and result in the observed negative correlation with toxin volume. Further experimental manipulation would help to clarify this relationship. Removal of toxin from parotoid glands may initiate production of more toxin and result in an immediate increase in liver mass.

The negative correlation between toxin content and hematocrit is also unexpected. Hematocrit represents the proportion of circulating blood that consists of red blood cells (as opposed to white blood cells and plasma). There is no obvious causal mechanism that would result in toads with more toxin to have fewer red blood cells. Perhaps this negative relationship could arise due to an underlying link between both factors and liver mass. Toads with large livers have less toxin but higher hematocrit. The apparent connection between toxin and hematocrit may be a result of both variables being affected by liver mass in different ways.

Although toxin content is significantly related to SUL (Figure 3) and relative liver size (Figure 5), there is still substantial variation evident in these scatterplots. For instance, at SUL of 110mm, individuals range more than tenfold in toxin content, from 0.12 to 1.4g. These toads were taken from the wild and may have encountered other risks prior to their capture. Conceivably, they may have used their toxin before-hand, due to some sort of predator encounter, and were in the process of replenishing it when captured for this study. Another possible explanation for the variation in toxin content, is behavioral. Toads have different personalities and habits, some may live in more risk-prone habitats than others, and therefore feel

that they need more toxin (Bucciarelli et al., 2017). The toads used for this study were all collected at the same capture point, so variation found in toxin content may be due to differing personalities and what individuals consider threats, rather than different densities of predators or their cues.

Previous studies found that male and female toads tend to have differing amounts of toxin and a variety in toxin distribution among sexes (Chen et al., 2017). However, the present study did not demonstrate a different in toxins amount. Chen et als' (2017) study did not quantify toxin volume but rather measured the basal area of parotoid glands. Gland thickness was not measured. Potentially, male toads could have somewhat thicker parotoid glands than females and store just as much toxin, despite the length and width of their glands being somewhat smaller.

Some of the variation in mass change observed between de-toxined and control toads in the fed treatment group arose because a few de-toxined individuals did not eat the crickets offered to them. Their refusal to eat their crickets might be attributable to their being more sensitive to the foreign conditions of captivity and become more stressed. This may have contributed to their apparent loss of appetite. These individuals also did not eat during their initial foraging trials, so toxin removal may not have been the only factor responsible for their weight loss.

Toxin replenishment was not estimated in the present study due to the potential for variation in milking success. In addition, it is easier to thoroughly squeeze toxin from the glands of a dead toad than a live one, because the latter may attempt to struggle during handling and could potentially be harmed if their glands are squeezed too hard. The toxin that was removed from previously milked toad at post-mortem could be a combination of leftover toxin from incomplete milking plus recently replenished compounds.

Toad behavior is difficult to analyze, since each toad is unique and has its own personality. However, some of the fed toads may have had higher sprint speeds in the locomotor trials because they didn't see me as a threat, as I was feeding them on a daily basis. Whereas the fasted toads did not interact with me as much over the course of the 20-day trial period and were probably much hungrier, and more defenseless in the case of the de-toxined toads, and therefore more eager to escape.

The observation that radio-tracked toads that were de-toxined toads did not as move far away from their capture site as control toads did (Figure 11), suggests that the experimental manipulation altered dispersal behavior. Potentially, the absence of toxin may alter an individual's assessment of predator risk. De-toxined toads may feel 'disarmed' and more susceptible to predator attack if it ventures away from familiar habitat. Alternatively, milked toads may be diverting energy towards replenishment of toxin stores and therefore have less energy to invest in increased dispersal. In either case, toxin removal appears to invoke a cost in terms of toad movement.

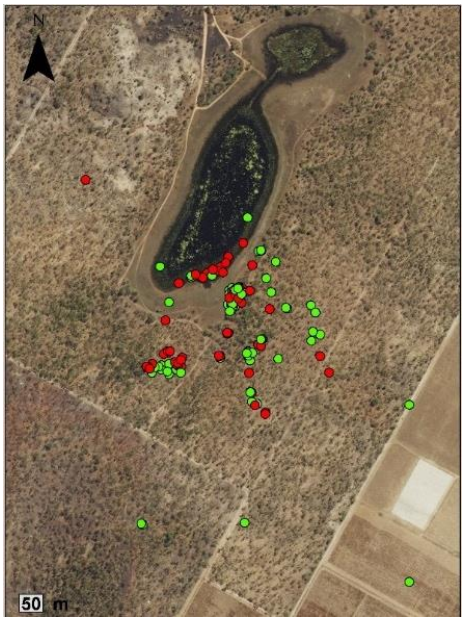

Figure 12. An aerial image of Leaning Tree Lagoon showing the daily locations of 36 toads radio-tracked over 5 days. The green dots represent the control toads, and the red

dots represent de-toxined toads. Control toads dispersed further from the lagoon where they were initially caught.

The contrasting findings between the changes in mass of the wild toads versus the captive toads could be attributed to their setting. In the wild, de-toxined toads tended to stay closer to the point of capture by the lagoon than control toads did, and it can be inferred that they were most likely trying to conserve more energy and forage more than the control toads did. They may need the extra food in order to replenish their toxins at a faster pace (Nazareth et al., 2016). Control toads may have been less preoccupied with conserving energy and more interested in dispersing, therefore loosing mass. The artificial setting of captivity has the benefit of allowing important conditions (e.g. temperature, moisture, food provision) to be standardized among individuals. However, a cost of captivity is its artificialness, which may invoke stress and lead to different effects than would be observed under natural less stressful conditions.

Cane toads, like other amphibians, are ectothermic ("cold-blooded"), meaning they function at environmental temperatures (Shine, 2018). In warmer temperatures, they are more active and consequentially expend more energy. The opposite occurs in colder climates, and they become slower, which helps them to conserve energy. Because of this, temperature plays a significant role in metabolic function. Further research that integrates temperature manipulations to assess thermal regulation toxin milked toads could provide more answers as to the effects of producing toxin on energy levels.

The short period of time allotted for the study posed a limitation for retrieving data for the mark-recapture portion. This study was conducted during the month of November, when the shift between the dry to wet season was just beginning. These weather changes caused many of the toads to start dispersing. Rather than have to return to the billabong every night, new sources of water were forming, allowing for more freedom of movement for many of the toads. A more

long-term study would allow for better mark recapture results. This does however suggest that toxin removal doesn't completely affect dispersal rates in toads. Further research would also provide more data on the effects of toxin removal on captive toads. As it takes a very long time for toads to replenish small amounts of toxin at a time (Jared et. al, 2014; Chen and Kovarikova 1967), another study would be useful in determining the long-term effects of toxin removal on physical and physiological performance. This does, however, support the hypothesis that the chemical compounds in cane toad toxin are metabolically costly to make and thus very precious to their hosts.

5.0 Conclusions

Though this was a short-term study, it demonstrated that experimental toxin milking in cane toads can be a useful means of gauging the value of chemical defenses. With results demonstrating relationships with physiology, altered locomotor performance, and changes in mass on both in the wild and captivity, the hypothesis that toxin milking would alter energy levels and growth rates was supported. However, a longer-term study is likely to clarify and reveal additional costs associated with toxin removal, such as growth, performance and survival rates among wild toads.

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