CONTROL OF THE COQUI FROG, *Eleutherodactylus coqui*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

in

Biomedical Sciences (Physiology)

December 2003

By
Robert B. Hutchison

Thesis Committee:

Harry Ako, Chairperson
David A. Lally
G. Causey Whittow

ACKNOWLEDGEMENTS

There is an Ashanti proverb that goes, "if you climb a good tree we will give you a push". The proverb is meant to symbolize how individual success can be accomplished when other people help. In the case of this thesis, it would not have been possible without others giving me a "push" and I want to say thank you to those of you who really made a difference. To some, their individual contributions might not have seemed significant but to me they were.

I want to thank the members of my committee. Dr. Harry Ako, my mentor for the last two years. I cannot over emphasize how much of an impact he has had on me and how much I have learned and achieved here at the university under his tutelage. Dr. David Lally, my graduate advisor, who has patiently guided and worked with me on both my thesis and my degree and Dr. Causey Whittow for his support and input.

I want to thank the College of Tropical Agriculture and Human Resources and all of the people in the school that make it so great, from the office staff to my instructors and peers. The school has been a wonderful environment in which to learn and grow and I'm very thankful for all the opportunities it has afforded me.

I want to thank my friends and family. Your support and encouragement was invaluable and has kept me sane. Your faith in me kept me going despite my own self-doubts. My mother, Janelle Hutchison, who has always believed in me and been a pillar of strength and my wife, Rose Hutchison, who is my heart and my inspiration. I'm a better person because you are a part of my life.

ABSTRACT

Eleutherodactylus coqui is an invasive species whose unchecked population growth is having environmental and social impacts on the Hawaiian islands. One focus was to fine tune doses of possible toxicants to control the frogs. It was found that applied as a spray, a 1% caffeine and 0.01% pyrethrin cocktail yielded complete mortality in a single application. These concentrations could be tested in field trials. Animals treated with the caffeine/pyrethrin cocktail experienced decreases in liver and muscle glycogen and severe hyperglycemia. This is consistent with known phosphodiesterase inhibition triggering enzyme inhibitions that ultimately lead to lethality. IBMX, a caffeine analogue and potent phosphodiesterase inhibitor, when combined with pyrethrin, had a similar effect. Drugs blocking other possible modes of action such as adenosine antagonism and ryanodine receptor opening had no effect. It was therefore suggested that caffeine in combination with pyrethrin might be an effective method for controlling frog populations and the lethality of the treatment may be due to phosphodiesterase inhibition followed by eventual hyperkalemia.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

Because of Hawai`i's isolation as an oceanic archipelago and amphibians' intolerance of salt water, the Hawaiian Islands lack any native terrestrial species of amphibians (Krauss et al., 1999). With human occupation, Hawai`i has seen a number of amphibian and reptile introductions and a recent introduction of the frog species, *Eleutherodactylus coqui,* or the coqui frog. Since the early 90's, the coqui has established itself on four of the eight major islands (Krauss et al., 1999). The suspected routes of entry were unintentional introductions via the horticulture trade (Krauss et al., 1999). New frog populations often occur intrastate when potted plants have been moved to a location. This suggests that frogs hitchhike with potted plants and also suggests a route of entry into the state as well. Hawai`i lacks natural controls and has an environment conducive to coqui frog survival and breeding. Coqui populations have been estimated as high as 8000 per acre (Stewart and Pough, 1983; Stewart, 1995) or ten times the population density in their native Puerto Rico (Krauss et al., 1999). While methods have been investigated to control the frog, relatively small populations have increased such that populations once thought to be eradicable just four years ago (Table 1) may not be today (Krauss et al., 2002).

Table 1. Verified Coqui Frog Populations

	1998	2002
Hawai`i	8	101
Kauai	0	2
Maui	12	36
Oahu	1	14

There are some serious environmental concerns surrounding the establishment of large frog populations (Krauss et al., 1999). The coqui diet is composed primarily of arthropods and other insect species (Michael, 1995). The coqui can crop an average of 114,000-prey items/night/ha (Krauss et al., 1999). This may lead to coquis competing for food with other insectivorous species such as endangered native Hawaiian birds. Coquis might also act as a nutrient sink. They can also act as food sources for predators such as mongoose and rats, which in turn prey on endangered birds. From an anthropocentric point of view, one of the biggest concerns surrounding the coqui is the noise they create (Krauss et al., 1999). At night, male species of the frog emit a high frequency screech in order to attract females of the species (Michael, 1995). When several thousand of these frogs are grouped together, the noise levels produced by the frog is said to exceed 75dB and may be as high as 100dB. This is equivalent to the noise made by a lawnmower. This is bothersome when in residential or resort areas (Krauss et al., 1999).

One of the difficulties in controlling the coqui is that there are no known pesticides that can be legally used against them. Earl Campbell (manuscript in preparation) of the USDA tested several potential pesticides. The State Department of Agriculture also did preliminary testing. They found that caffeine used at the 2% level and some pyrethrins were promising. For 2% caffeine to be effective frogs needed to sit in a caffeine bath or receive multiple treatments if applied as a spray. For practical reasons this made its use in the field problematic. Pyrethrins had an effect on frogs. They paralyzed frogs but the effect was only temporary. The use of caffeine and pyrethrin needed to be refined and alternative pesticides such as citric acid looked at.

Citric acid was chosen because its use as a pesticide is exempt from Environmental Protection Agency regulations whereas caffeine is not.

It is known that caffeine will penetrate membranes and it is thought that when frogs are sprayed, they absorb caffeine directly through their skin. Once absorbed by the frogs though, it is not clear why caffeine and pyrethrin are toxic. Caffeine has several known physiological activities, and there are therefore several possible mechanisms. The first is that caffeine acts on ryanodine receptors and mobilizes intracellular calcium as occurs in muscle contraction. The second is that caffeine acts as an adenosine receptor agonist. The third is that caffeine acts as a phosphodiesterase inhibitor (Figure 1). The hormones glucagon and epinephrine initiate metabolic responses such as glycogen mobilization. Caffeine and theophylline inhibit down-regulation of the cAMP cascade.

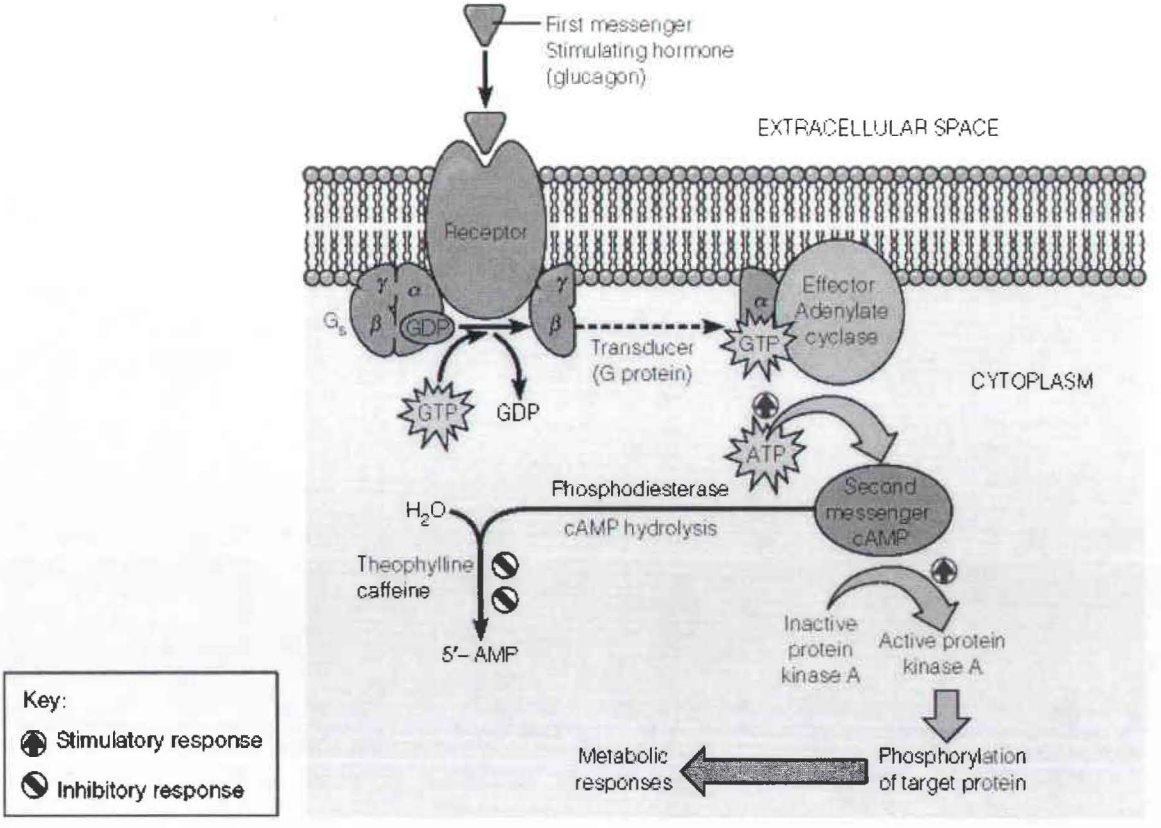

Fig. 1. Hormonal control of cAMP (Matthews et al., 2000).

Pyrethrin is a natural insecticide that is extracted from the plant *Chrysanthemum cinerariaefolium*. Pyrethrin kills by disrupting nerve conduction. Pyrethrins bind to and open sodium channels in nerve membranes. Increases in membrane permeability to sodium cause the nerve membrane to depolarize and a cessation of action potentials (Henk et al., 1979; Henk et al., 1982).

Given that phosphodiesterase is a down regulator of cyclic nucleotide second messengers, cAMP and cGMP, and that cyclic nucleotides are involved in several signal transduction pathways, the implications of phosphodiesterase inhibition can be quite profound. One known pathway is glycogen mobilization (Figure 2). In animals, most glucose is stored as the polysaccharide glycogen and represents the most immediate large-scale source of energy. It is abundant in muscle tissue and particularly in the liver. Glycogen mobilization or glycogenolysis proceeds via cascade and signal amplification. Since cAMP comes at the beginning of the cascade, small increases in cAMP will be amplified in their overall effect and an increase in glycogenolysis. At the same time, cAMP inhibits glycogen synthesis through a separate but related regulatory cascade. Hormones such as glucagon and epinephrine stimulate cAMP production whereas phosphodiesterase down regulates cAMP by converting it to 5'-AMP. Inhibition of phosphodiesterase with caffeine reduces this down regulation. Hypothetically, after a hormonal stimulus such as glucagon and epinephrine and in the presence of caffeine, cAMP levels would rise above normal levels and one of its effects would be an increase in glycogenolysis and a decrease in liver and muscle glycogen.

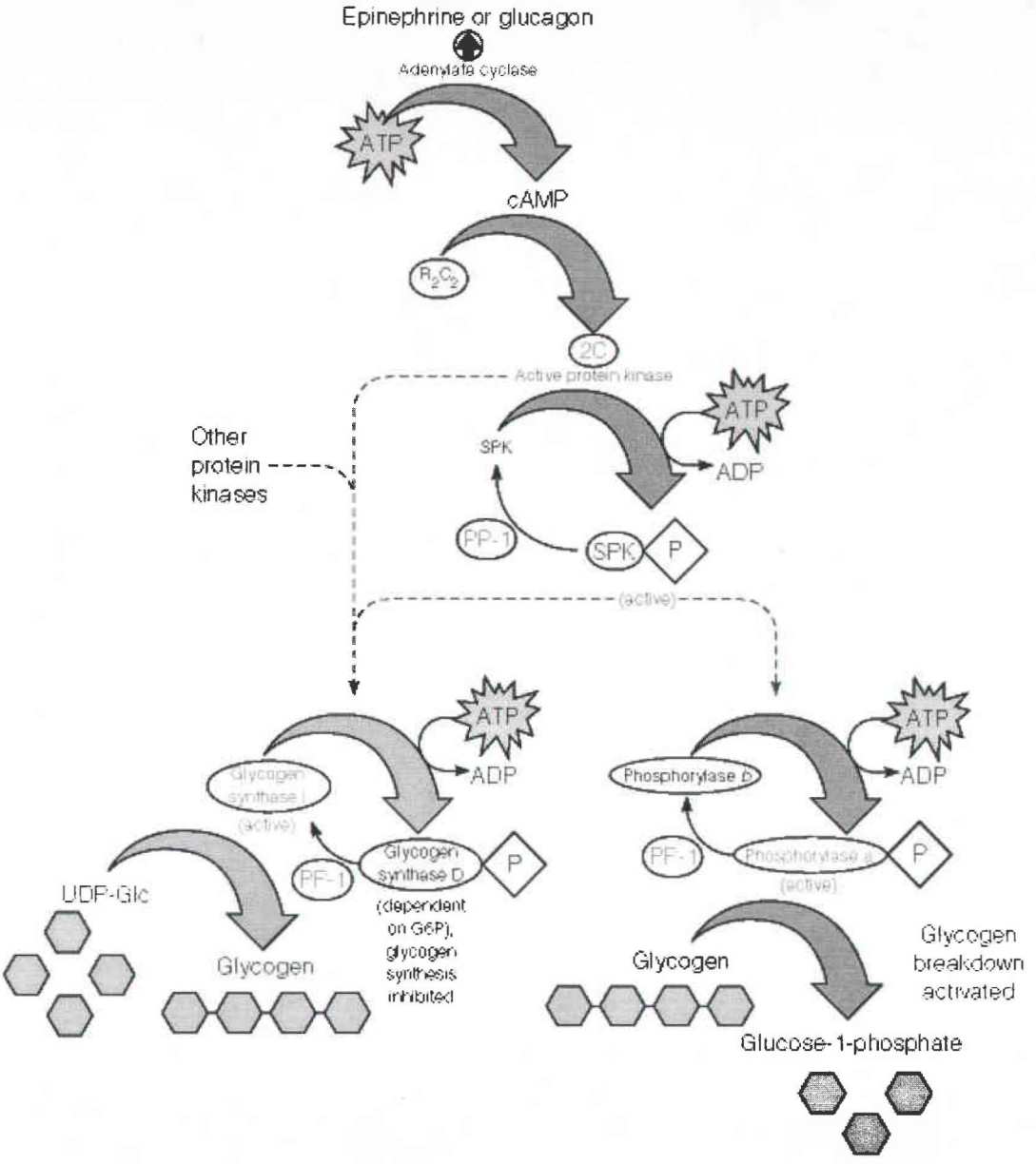

Fig. 2. Glycogenolytic cascade (Matthews et al., 2000).

The caffeine analogue IBMX (1-isobutyl methylxanthine) is a potent and specific phosphodiesterase inhibitor (Fort et al., 1997). IBMX is a caffeine analogue that mimics only some of the effects caffeine has. IBMX has a strong specificity and potency for phosphodiesterase inhibition and has been used as a caffeine analogue in toxicology studies of the frog species, *Xenopus* (Fort et al., 1997). IBMX does not affect ryanodine

mediated calcium release. Further evidence for phosphodiesterase inhibition's involvement with the killing mechanism of caffeine would be significant increases in cAMP following treatment with the caffeine and pyrethrin cocktail.

Caffeine has been linked with adenosine antagonism and renin secretion from the kidney (Brown et al., 1990). Renin is normally secreted in response to a decrease in renal blood pressure. Renin is secreted directly into the blood whereupon it catalyzes the conversion of angiotensinogen to angiotensin I. Angiotensin I is carried to the lungs where angiotensin conversion enzyme (ACE) converts it to angiotensin II. Angiotensin II is the most potent vasoconstrictor known. Caffeine is a known adenosine antagonist (Lee et al., 2001). Adenosine inhibits the release of renin from the kidney whereas caffeine increases renin secretion. It is thought that via this mechanism caffeine induced renin secretion increases blood pressure. In humans with chronic hypertension one treatment is the use of ACE inhibitors to block this pathway. Two ACE inhibitors currently in use are captopril and enalapril. If severe hypertension is related to frog mortality due to caffeine exposure, then an ACE inhibitor should have a protective effect on frogs exposed to a lethal dose of the caffeine and pyrethrin cocktail.

Changes in intracellular Ca^{2+} concentrations cause several physiological responses from muscle contraction to nerve conduction (Ganong, 1999; Williams, 1997). Increases in intracellular Ca^{2+} can be achieved either by entry of Ca^{2+} from the extracellular space through voltage and ligand-gated calcium channels or by release of Ca^{2+} from intracellular stores. Ryanodine receptors are calcium channels involved with muscle contraction. Caffeine increases the outpouring of Ca2+ by opening ryanodine receptors. This enhances muscle contraction. The muscle relaxant, dantrolene, is a Ca^{2+}

release blocker that acts on ryanodine receptors and closes calcium channels. It is capable of reducing the mobilization of calcium from both intracellular and extracellular stores (Hasko et al., 1998; Tayeb, 1990). Dantrolene would have an antagonistic effect to the Ca^{2+}mobilizing effects of caffeine and would have a protective effect if this mechanism is significant in caffeine toxicity.

CHAPTER 2. OBJECTIVES

1. Develop a simple and cost-effective pesticide for *Eleutherodactylus coqui.* Current control methods are limited in their effectiveness and the number of verified *Eleutherodactylus* populations continues to increase.
2. Understand the biochemistry and physiology of why a caffeine/pyrethrin cocktail kills. Providing toxicology data for caffeine and any treatment developed could suggest wiser, more effective, and less environmentally damaging uses.
3. Contribute to the knowledge base on control methods for amphibians and reptiles. In general, the information for amphibian and reptile control is limited although problems associated with invasive species of amphibians and reptiles are on the rise. On the other hand a knowledge base on pesticides and frogs will contribute to understanding the global decline in amphibian populations. Such declines have been blamed, in part, on agricultural pesticide use.
4. Testing possibly more effective chemicals reported to be toxic to amphibians to see whether there are cheaper and/or more effective alternatives.

CHAPTER 3. METHODS

Chemicals: Pyronyl Crop Spray (Prentiss Inc., active ingredients: 6.0% natural pyrethrins and 60% piperonyl butoxide {PBO}) and chemically synthesized caffeine (from a Chinese manufacturer) were generously provided by the Hawai`i State Department of Agriculture. IBMX, dantrolene, captopril, and enalapril were purchased from Sigma Chemical Company.

Frogs: Animals were collected by hand from established populations on the islands of Oahu and Hawai`i. The frogs were housed in 40 L aquaria containing moss, soil and tree bark. Population density was 30-50 frogs/aquarium. The frog habitat was wetted daily with about 42 mL of fresh water and fed every three to five days on a diet of fruit flies. Feeding rates were approximately 20 flies/frog/feeding. The frogs appeared normal and healthy at the time of the experiments, but holding and generous feeding had an effect (to be described later).

Exposure experiments: An application consisted of 3 puffs from a thin layer chromatography sprayer. Spray volume was determined by weighing the sprayer before and after applications to single frogs. Unless otherwise indicated animals were sprayed with 200 μL, which tended to form a 5 cm circle which was larger than the frog. The frogs sometimes walked through residual spray. Animals appeared to be thoroughly wetted by this application. Later sprays were 450 μL with animals removed from the spraying container after 15 min. The nutritional state of the animals was more important than spraying method. Well-fed animals were more resistant to treatment.

Glycogen extractions: Liver and muscle samples were placed into tared 1.5 mL centrifuge tubes containing 0.5 mL of 30% potassium hydroxide solution. They were heated for 60 min at 100°C and then centrifuged for 10 min at 3000 **g**. The supernatant was collected into tared sample tubes.

Saturated sodium sulfate (83 μL) was added to the supernatant and the glycogen precipitated by the addition of 0.5 mL of 95% ethanol. Tubes were reheated until the mixture began to boil, then cooled and centrifuged 10 min at 3000 **g**. The supernatant was discarded and the sample tubes drained on a paper towel. Precipitated glycogen was re-suspended in 1.0 mL of doubly deionized water.

Glycogen hydrolysis: HCl (6 M) was added to sample tubes at a final concentration of 1.2 M. Sample tubes were capped and heated for 1 hr at 100°C. Samples (10 µL aliquots) were subjected to Trinder glucose assay (Sigma Chem. Co.).

Blood Sampling: Blood glucose was measured with the One Touch Ultra blood glucose monitor. Immediately after their death (determined by a cessation of respiratory activity), chest cavities were opened to expose the heart. The exposed heart was lanced with a needle and blood allowed to flow and pool in a small reservoir below the heart. Using the glucose monitor, the test strip was placed next to the pooled blood and drawn into the sampling window via capillary action. The instrument automatically reported blood glucose levels.

Cyclic AMP extraction: Liver and muscle samples were dropped into tared, 1.5 mL centrifuge tubes containing 0.5 mL of 0.4 N perchloric acid and homogenized. Samples were centrifuged for 10 min at 20,000 **g** and the supernatant collected and neutralized with 1 N KOH to pH 7.0-7.5 (as determined on the pH meter) and then

applied to a Dowex 1 X 8 column (0.5 X 4cm, chloride form). The column was washed with 5 mL of doubly deionized water and the cAMP eluted with 3 mL of 0.5 N HCl. This fraction was lyophilized and then dissolved in 300 μL of 10mM PBS (phosphate buffered saline, 10 mM potassium phosphate, 0.15 M sodium chloride, pH 7.5).

Acetylation of Samples: Samples (300 μL) including standards were added to 10 μL of a 2:5 acetic anhydride/triethylamine mixture and immediately vortexed. Samples were ready to be assayed immediately following acetylation.

Cyclic AMP ELISA assays: The kits (NeoGen Corp.) consist of microplates containing immobilized antibodies attached to wells. Equal volumes of unknown cAMP containing samples and horseradish peroxidase attached to cAMP are incubated in the wells for 1 hour at room temperature. The nucleotides and the nucleotides attached to peroxidase compete for antibody binding sites. They are then removed after an hour and each well washed with wash buffer. Peroxidase substrate (150 µL) is added to each well and shaken and incubated for 30 min. The blue color generated is read on a microplate reader (Bio-Rad). The extent of color change is proportional to the amount of peroxidase containing bound cAMP attached to the well. The percent of maximal binding ($\%B/B_0$) is found by dividing the sample absorbance by the absorbance of maximal binding of the conjugate or B_0 (cAMP B_0=1.15). Concentration is determined by comparing sample $\%B/B_0$ to a standard curve.

Cyclic AMP Assay: Using the NeoGen cAMP assay kit, 50 μL of each sample and 50 μL of the diluted conjugate base were added into a well of the microplate, shaken, covered and incubated for 1 hour at room temperature. After incubation the contents of the plate were dumped out and each well washed with 300 μL of the diluted wash buffer.

Substrate (150 μL) was added to each well, shaken and allowed to incubate at room temperature for 30 min. The plate was read in a microplate reader at 650 nm.

Dosage Selection: Frog dosages for dantrolene, captopril and enalapril were ten times the maximum recommended dosages for humans. The maximum recommended human dosages (gathered from the manufacturer's website) for dantrolene, captopril and enalapril are 4 mg/kg, 6 mg/kg and 6 mg/kg respectively. The oral LD_{50} for captopril and enalapril are 4245 mg/kg and 2973/mg/kg respectively. The average male frog is 2.3 g and the average female frog is 5.5 g. Although female frogs tend to be larger than their male counterparts sex was not considered in any of the experiments. Therefore, dosage was based on the average mass of both male and female frogs or 3.9 g. Drugs were applied dermally as a spray. Due to the fact that not all of the sprayed drug actually gets absorbed by the frog (i.e. excess solution and limited dermal absorption) the final concentrations used were ten fold higher. This meant that for captopril to be delivered at a dosage of 6 mg/kg to a 3.9 g frog in 450 μL of solution and all of the solution is absorbed, the concentration of captopril would need to be 40 μg/mL or 0.004%. For captopril the ten fold excess meant a solution concentration of 400 μg/ml or 0.04%.

Statistical analysis: To determine whether different concentrations of glycogen, glucose and cAMP differed from tested animals and controls a two-sample *t*-test was used.

CHAPTER 4. RESULTS

Preliminary results

Caffeine/pyrethrins

Preliminary results suggested that caffeine and pyrethrins were especially toxic to the coqui. Thus "range-finding" experiments were conducted to refine practical concentrations. Frogs were sedentary when left alone and only become active when being handled or fed. A 0.02% pyrethrin solution (Pyronyl Crop Spray in which the pyrethrin was diluted to a 0.02% final concentration) was sprayed (Table 2). Immediately after spraying, the animals became very active (i.e. jumping and crawling). After about 40 min they flipped on their backs, paralyzed, with limbs extended. They periodically twitched. About at this time they were treated again (recorded as application 2). There was no change in the animals' behavior after the second treatment. After about 5-6 hr they appeared to be normal. No frogs were killed with pyrethrin.

Single applications of caffeine sprays caused the frogs to crawl around, jump on the walls of the aquarium, and wipe their eyes with their front legs (Table 2). Their breathing was exaggerated and pupils dilated. A second application applied 40 min later rendered the frogs incapable of resting on the vertical walls of the aquarium though their activity did not change as much as during the first application. The 0.5% treatment did not kill any of the 4 test animals even after two treatments. A fifth animal died later and was found to have an open wound on its leg and was therefore excluded from consideration. The 1% treatment killed 2 of 5 test frogs with two applications (but a third application applied 40 min after the second killed all five). The 2% treatment killed 5 out of 5 frogs after two applications.

Pyrethrin and caffeine diluted to 0.02% and 1.0% respectively, paralyzed the frogs after inducing a period of extreme activity. Prior to death at 0.5 hr, frogs would roll onto their backs and did not right themselves. The same crop spray at 0.01%/1.0% level yielded complete mortality in 1.5 hr. Lowering the caffeine and pyrethrin concentrations (0.01%pyrethrin/0.5% caffeine and 0.02%pyrethrin/0.5% caffeine) decreased frog mortality. It was concluded that, from a practical point of view, 0.01% pyrethrin and 1% caffeine was the best compromise. In all of these laboratory tests, surviving animals appeared normal within 24 hr after being treated and survived until used for another test.

Table 2. Results of caffeine and pyrethrin trials

Treatment	Application	Mortalities	Notes
0.02% pyrethrin	1	0 of 5	Paralyzed initially but recovered later
	2	0 of 5	No change
0.5% caffeine	1	0 of 4	Increased activity
	2	1of 5	Brief increased activity
1% caffeine	1	0 of 5	Increased activity, breathing heavy
	2	2 of 5	Increased activity, breathing heavy, unable to stay on walls
	3	5 of 5	Unable to crawl
2% caffeine	1	2 of 5	Increased activity, increased breathing, unable to remain on walls
	2	5 of 5	2 of 5 died when handled for returning to home container
0.02%pyrethrin 1% caffeine	1	5 of 5	Increased activity, bounced off walls, lay on backs and died
0.01%pyrethrin 1%caffeine	1	5 of 5	Increased activity, unable to stay on walls, increased breathing and died
0.01%pyrethrin 0.5%caffeine	1	1 of 5	Increased activity
0.02%pyretrhin 0.5%caffeine	1	4 of 5	Increased activity

Effects of Feeding

An early trial (7/29/02) was conducted on frogs held in captivity for 1-2 weeks. These animals generally died within an hour after treatment with the 1.0% caffeine/0.01% pyrethrin cocktail as described previously and in Table 3. As time progressed (12/21/02), time to death increased to as much as 3 hr (Table 3 and data not presented). Frogs used in these later trials were kept in captivity for at least 2-3 months and during that time were regularly fed (50 animals would crop 750 fruit flies every three days). It was suspected that animals became hardier and less susceptible to pesticide treatment. To test this a fasting trial was done. In the fasting trial (2/26/03) animals were fasted for two weeks prior to exposure of 1.0% caffeine/ 0.01% pyrethrin. All animals (4 of 4) died after a single application and time to death was less than an 1hr. In 3 of 4 cases a follow up experiment (4/1/03) was done on a separate group of frogs that was tested within 1 week of their capture. All animals were killed (5 of 5) and time to death was less than 1 hr.

Table 3. Comparison of different groups of frogs treated with caffeine and pyrethrin

Date of Experiment	Date Frog(s) Received (# of frogs)	Concentration caffeine/ pyrethrin (%)	Frogs tested	Mortalities after 2 hr	Total mortalities
7/29/02	7/16/02	1.0/0.01	5	5	5
12/21/02	8/14/02	1.0/0.01	3	1	3
2/26/03	8/14/02	1.0/0.01	4	3	4
4/1/03	3/26/03	1.0/0.01	5	5	5

Citric Acid

In support of a USDA Fish and Wildlife project, citric acid was tested as a possible chemical control (Table 4). The 16% citric acid treatment killed 3 of 5 and 4 of

5 animals in two replicates when applied in ample amounts (450 μL) and with animals sitting in the excess spray after treatment. Neutralizing the pH of 16% citric acid to 7.0 resulted in reduced kill rates (1 of 5 animals tested in two replicates). Removing animals so that they were not continually exposed to citric acid after spraying reduced kill rates as well. Animal activity increased briefly following exposure to unadjusted citric acid (pH 1.8) but not to the adjusted (pH 7.0).

Table 4. Results of citric acid

Treatment	Application	Mortalities	Notes
16% citric acid, pH unadjusted	1	3 of 5	Brief increased activity, 1st replicate
16% citric acid, pH 7.0	1	1 of 5	No increased activity, 1st replicate
16% citric acid, pH unadjusted	1	4 of 5	Brief increased activity, 2nd replicate
16% citric acid, pH 7.0	1	1 of 5	No increased activity, 2nd replicate

Effects of PBO, IBMX

As caffeine and pyrethrin were lethal an experiment was done to verify that pyrethrins and not the synergist PBO enhanced caffeine efficacy. Trials were made with PBO at 0.1%, 0.2% and 0.4% concentrations. This is equal to the PBO concentrations in 0.01%, 0.02% and 0.04% dilutions of pyrethrin. Kill rates were the same as 1.0% caffeine alone and data were between 1-4 mortalities of 5 animals tested when double treatments of pesticide were tested.

In IBMX exposure trials (Table 5) animals were sprayed with 450 μL of either 1.0% or 0.2% IBMX in 1.0% DMSO and 0.010% pyrethrin solution. Control frogs were unaffected by 1.0% DMSO. All IBMX/pyrethrin treated frogs displayed behavior

identical with those of a caffeine/pyrethrin treatment. Frog mortality was 100% (5 of 5) at either concentration of IBMX (Table 5). It is noted that the 0.2% IBMX treatment was as effective as the 1.0% treatment.

Table 5. Results of IBMX

Treatment	Application	Mortalities	Notes
1.0% IBMX/0.01% pyrethrin	1	5 of 5	Response to treatment similar to caffeine/pyrethrin
0.2% IBMX/0.01% pyrethrin	1	5 of 5	Response to treatment similar to caffeine/pyrethrin
1.0% DMSO	1	0 of 5	No response to treatment

Mode of action

Liver and muscle glycogen

The following experiments have a different goal and tested the mode of action of the caffeine/pyrethrin cocktail. Frog liver glycogen levels were determined for untreated frogs and animals killed as a result of treatment (Table 6). Frogs treated with caffeine/pyrethrin or IBMX/pyrethrin had lower but not significantly lower liver glycogen levels. Liver glycogen levels were lower for fasted frogs either treated or untreated with the caffeine/pyrethrin cocktail but the levels were not significantly lower compared with control frogs. For all of the treatments, the variances were high and if a much larger number of animals were tested, there might have been a significant difference. There were usually 5 animals per group.

Table 6. Liver glycogen levels following various treatments

Sample	Average ± sd (μg/mg tissue)
Control	123.0 ± 27.9a
1.0% caffeine/0.01% pyrethrin	94.3 ± 26.7a
1.0% IBMX/0.01% pyrethrin	87.4 ± 29.4a
0.2% IBMX/0.01% pyrethrin	94.0 ± 18.0a
Fasting, control	75.5 ± 19.9a
Fasting, treated	96.1 ± 34.0a

Alphabetical suffixes that are different indicate statistical differences.

Frog muscle glycogen levels were determined for untreated frogs and animals killed as a result of treatment. Muscle glycogen decreased after treatment with caffeine or IBMX and pyrethrin but was only significantly lower in animals treated with caffeine/pyrethrin (Table 7). Fasted animals treated or untreated with caffeine and pyrethrin had significantly lower levels of muscle glycogen.

Table 7. Muscle glycogen levels following various treatments

Sample	Average ± sd (μg/mg tissue)
Control	1.99 ± 0.81a
1.0% caffeine/0.01% pyrethrin	0.65 ± 0.29b
1.0% IBMX/0.01% pyrethrin	1.23 ± 0.53a
0.2% IBMX/0.01% pyrethrin	1.83 ± 0.83a
Fasting, control	0.84 ± 0.38b
Fasting, treated	0.93 ± 0.52b

Alphabetical suffixes that are different indicate statistical differences.

Blood glucose

The most dramatic response to the caffeine/pyrethrin cocktail was the increase in blood glucose. Frog blood glucose levels were determined for untreated frogs and animals killed as a result of treatment (Table 8). The blood glucose level of control (untreated) animals was very low but was not different from resting blood glucose levels of fasting animals. Blood glucose levels increased dramatically and significantly over controls in all cases where the animals were treated with either the caffeine/pyrethrin or the IBMX/pyrethrin cocktail. IBMX/pyrethrin caused the largest increase in blood glucose, significantly higher than did caffeine/pyrethrin.

Table 8. Blood glucose following various treatments

Sample	Average ± sd (mg/dL)
Control	22 ± 2a
1.0% caffeine/0.01% pyrethrin	100 ± 21c
1.0% IBMX/0.01% pyrethrin	182 ± 7d
Fasting, control	24 ± 6a
Fasting, treated	50 ± 26b

Alphabetical suffixes that are different indicate statistical differences.

Cyclic AMP

Cyclic AMP was measured to throw some light on the role of phosphodiesterase inhibition in the killing mechanism of caffeine. Cyclic AMP was present in all samples for both muscle and liver. Liver cAMP was significantly higher than muscle cAMP for both controls and treated animals (Table 9). No significant difference was observed between controls and treated animals for either liver or muscle.

Table 9. Cyclic AMP in muscle and liver as a function of treatment

Sample	cAMP in muscle (ng/mg tissue)	cAMP in liver (ng/mg tissue)
Control average ± sd	31.2 ± 6.01	180.8 ± 119.9
1.0% caffeine/0.01% pyrethrin average ± sd	34.2 ± 6.22	183.33 ± 69.15

ACE inhibitors were tested to see if they protected the animals from the toxic effects of a caffeine/pyrethrin treatment. No significant change was observed in frog behavior or mortality after treatment with ACE inhibitors (captopril and enalopril). Nor was a protective effect observed. Animals pretreated with the inhibitors and then treated with the caffeine/pyrethrin cocktail died (3 out of 3 for each group) as a result of and showed signs consistent with, caffeine/pyrethrin toxicity (Table 10).

Table 10. Results of ACE inhibitors

Treatment	Application	Mortalities	Notes
0.06% enalapril/1.0% caffeine/0.01% pyrethrin	1	3 of 3	Response to treatment similar to caffeine/pyrethrin
0.06% enalapril	1	0 of 3	No response to treatment
0.05% captopril/1.0% caffeine/0.01% pyrethrin	1	3 of 3	Response to treatment similar to caffeine/pyrethrin
0.05% captopril	1	0 of 3	No response to treatment

Dantrolene

The skeletal muscle relaxant dantrolene was used to protect the animals from excessive calcium mobilization following treatment with caffeine and pyrethrin. No significant change was observed in frog behavior or mortality after treatment with

dantrolene, nor was a protective effect observed. Animals pretreated with dantrolene and then treated with the caffeine/pyrethrin cocktail died (5 out of 5) and showed signs consistent with caffeine/pyrethrin toxicity.

CHAPTER 5. DISCUSSION

Our initial work focused on empirically testing various toxicants. This included the spraying of frogs with varying concentrations of caffeine, pyrethrins and citric acid and observing frog mortalities. In the caffeine trials, frog mortalities increased as caffeine dosages increased. However, multiple treatments were required raising questions as to labor costs in a theoretical field treatment. Empirical trials were also done with the pesticide Pyronyl Crop Spray containing natural pyrethrins. When used alone, Pyronyl Crop Spray was non-toxic to the coqui. While it elicited temporary paralysis it is not a frog pesticide. It was then found that caffeine and pyrethrin concentrations that were unacceptable when used separately were 100% lethal when used together. On the basis of our tests alone, 1.0% caffeine and 0.01% pyrethrin seem very attractive. When combined they are very toxic and are 100% lethal after a single application. The 0.01% pyrethrin proposed for use is the maximum legal concentration. The state of Hawai`i originally proposed to treat infested areas with 2.0% caffeine, and two treatments were proposed to ensure a good kill. Our method requires only one treatment with half as much caffeine thus decreasing the costs of caffeine 4-fold and reduces labor 2-fold. The overall decrease in cost is 8-fold.

From the mechanistic point of view it is likely that pyrethrin and not PBO in the Pyronyl Crop Spray enhances the lethality of caffeine. PBO is an oxidase inhibitor (Cole and Casida, 1983) and may impede catabolism of caffeine. Used alone it was non-toxic and it did not enhance the efficacy of caffeine as did the pyrethrin/PBO mixture, which is the Pyronyl Crop Spray.

A 16% citric acid solution is also lethal to coqui frogs when applied as a spray in ample amounts. In our hands citric acid was problematic. Probably acid pH played a role in the toxicity because citric acid was more toxic than citrate that was acid neutralized. Perhaps citric acid is toxic because it is both an irritating acid and because it is a chelator. A whiff of citric acid burns the eyes and the lungs. Unfortunately citric acid is not as toxic as desired and must be rinsed off plants because of phytotoxicity. The advantage of citric acid is that it is generally regarded as safe and requires no pesticide clearance. Caffeine on the other hand is an uncleared pesticide at present.

According to the MSDS (material safety data sheet) for caffeine there is no known LD_{50} or LC_{50} for humans. The MSDS does however consider the consumption of 10 g of caffeine sufficient to kill a normal person. If the average person weighs 66 kg (150 lb) this would be a LD_{50} of 150 mg/kg. This is comparable to the oral LD_{50} of caffeine for rats or 192 mg/kg. For 1.0% caffeine solution there is 4.5 mg of caffeine in 450 μL. If the average frog (3.9 g) were treated with 450 μL of a 1.0% caffeine solution and all of the caffeine was absorbed by the frog this would mean the dosage of caffeine relative to the frog's mass would be 1153 mg/kg. This is nearly six fold higher than the LD_{50} of caffeine for both rats and humans, making it seem unlikely that the frogs are particularly sensitive to caffeine. If frogs are sensitive to comparable dosages then the frog absorbs approximately only 16% of the caffeine in 450 μL.

The question may then be further raised as to mode of action. Three possible modes of action of caffeine were investigated. Calcium mobilization via ryanodine receptors in skeletal muscle, adenosine antagonism in renal tissue and the subsequent vasoconstriction via angiotensin II, and glucose mobilization via phosphodiesterase

inhibition are possibilities. If ryanodine receptors in skeletal muscle were the major site of action for caffeine's toxicity then dantrolene should have had a protective effect. Dantrolene had no protective effect. Dantrolene results are consistent with a mechanism other than ryanodine receptors being involved in the killing mechanism. ACE inhibitors similarly had no protective effect. ACE inhibitors should basically block caffeine stimulated hypertensive effects due to angiotensin II via an adenosine antagonism effect. ACE inhibitor experiments are consistent with vasconstriction not being significant in the killing mechanism of caffeine.

Our data suggest that hyperglycemia plays a major role in the killing mechanism. There were large and significant increases in blood glucose levels as a consequence of caffeine/pyrethrin treament. We observed a significant decline in muscle glycogen concentrations as a consequence of caffeine treatment. There is also a trend for liver glycogen to decrease even if not significantly as a consequence of caffeine treatment. Since the liver is the primary exporter of endogenous glucose it was probably the source of blood glucose increases. Blood glucose increased nearly 5 fold from 20 mg/dl to 100 mg/dl after treatment with caffeine and pyrethrin and increased nearly 9 fold to 182 mg/dl after treatment with IBMX and pyrethrin.

The observed glycogen depletions are consistent with increased glycogenolysis due to phosphodiesterase inhibition and resulting in an increase in blood glucose as mobilized glucose is exported out of the liver and into the blood. Although it has been reported that frogs experience hyperglycemia as a result of the stress of captivity and gentle handling (Harri, 1980), reported changes in blood glucose due to stress alone are modest when compared to our results and it is unlikely that the observed changes we saw

are due to stress alone. The significance of phosphphodiesterase inhibition is supported by our results with IBMX and pyrethrin. IBMX, the most potent of the phosphodiesterase inhibitors used, had the most dramatic effect. IBMX is about 100 times more potent a phosphodiesterase inhibitor *in vitro* and about 5 times more potent *in vivo* than caffeine (Fort et al., 1997). A 1.0% IBMX used in conjunction with 0.01% pyrethrin applied dermally as a spray was lethal to the frogs. When IBMX is reduced 5 fold to 0.2%, it loses none of its efficacy as a toxin and this also matches known sensitivities. The IBMX/pyrethrin cocktail elicited blood glucose levels significantly greater than caffeine/pyrethrin.

Further support for phosphodiesterase's role in caffeine toxicity would have been significant increases in cAMP in both the muscle and particularly the liver. Although cAMP was present in all samples, the significance of its similar concentration in the various tissues as a function of treatment remains unclear. Rapid changes in cAMP concentration can occur (Beavo et al., 2002) and it has been reported that cAMP levels increase in muscle samples following treatment with caffeine and theophylline, a caffeine derivative, during heavy exercise (Greer, et al., 200). We found no significant change indicating that our hypothesis is wrong. Perhaps elevated levels of cAMP occurred in an initial burst and we missed it or perhaps our extraction of cAMP from tissues was faulty.

The significance of hyperglycemia and frog mortality can be speculated upon at this time. In humans, hyperglycemia can be fatal (Montoliu, 1985). Excessive blood glucose can lead to hyperosmotic gradients that passively pull electrolytes from surrounding tissues (Goldfarb et al.,1976). A resulting hyperkalemia, or the passive diffusion of potassium out of cells, can lead to cardiac arrhythmias and death. It may be

the case with these frogs that hyperglycemia in the animals leads to an ion imbalance that results in death. If this is the case then the opening of sodium channels with pyrethrin may exacerbate this effect and act synergistically with the caffeine.

A physiological phenomenon unrelated to caffeine toxicity but associated with one of caffeine's effects is glycogen sparing during exercise in humans. For years it has been thought that caffeine has a glycogen sparing effect and that this enhances athletic performance during endurance events (Brooks et al., 2000). Several theories have been proposed as to why this may be true. One is that caffeine stimulates lipolysis via a protein-linked activation of lipase and as plasma free fatty acid concentration increases, lipid is preferentially used over glucose (Spriet, 1992). Caffeine is thought to stimulate lipolysis in one of two ways, either by the caffeine induced epinephrine response (Greer, 2000) or by direct stimulation as an adenosine antagonist (Mohr, 1998). The data are sometimes contradictory where caffeine does not always have a glycogen sparing effect even in the presence of increased lipid mobilization and elevated epinephrine (Chesley, 1995). It may be that increases in lipid mobilization and epinephrine secretion are related to caffeine consumption but do not play a significant role in glycogen sparing. Our data seem to suggest that large doses of caffeine result in elevated levels of blood glucose, most certainly exported from the liver. In the case of athletes the massive export of glucose from the liver could be masked by its continuous uptake by working muscle. A glycogen sparing effect may have more to do with caffeine's effect on the export of glucose from the liver than lipid mobilization.

Fasting data suggests frogs are resistant to significant depletion of their liver glycogen reserves but not muscle glycogen. This is the opposite of what happens in man.

It may be that the frogs selectively metabolize lipid over glycogen when fasting and/or they have significantly lower basal metabolic rates. If anything, it seems that fasted frogs are less able to mobilize glucose though it is unclear what these changes have to do with toxicity. Fasting also appears to make the frogs more vulnerable, meaning that their time to death after exposure was relatively short (<1 hr) compared to well fed frogs (>1 hr). Fasted results also compared favorably with tests done on animals recently brought back from the wild. Recently captured animals also had relatively short time to death (<1 hr) suggesting that animals in the wild may be nutritionally marginal.

These experiments have laid the groundwork for future field trials. Efficacy of the use of the 1.0% caffeine and 0.01% pyrethrin cocktail in the field needs to be evaluated. Fieldwork will likely be done on infested sites on the Big Island of Hawai`i. The effect the cocktail has on both the coqui and non-target organisms will be looked at. Some interesting data have also been generated as to why caffeine kills. Future work will continue to study the biochemistry and physiology to narrow down the toxicology .

CHAPTER 6. CONCLUSION

The coqui frog is just one of many invasive species in Hawai`i and the number of new species introduced grows every year. It is an ongoing struggle for the State of Hawai`i to block new introductions and to control or eradicate those already here. The coqui frog is particularly challenging since no established methods exist to deal with amphibians as a pest. The scope of this research was to see if a simple cost-effective method to control the frog could be developed. Our work suggests that the 1.0% caffeine/0.01% pyrethrin cocktail would be an effective pesticide to control the coqui. It can be applied as a spray and is 100% lethal after a single application.

Our work is just one part of a cooperative effort involving local, state and federal agencies to control the coqui. The final solution to the coqui problem will probably require an integrated approach involving different methods from hand capture, to the use of pesticides. We believe our cocktail can be a valuable too in the effort to control the coqui and it is our hope that the work we have done will have value that goes beyond this paper.

REFERENCES

Beavo, J.A., Brunton, L.L., 2002. Cyclic nucleotide research-still expanding after a half century. Nat. Rev. Mol. Cell. Biol., 3, 710-8.

Brooks, G., Fahey, T., White, T., Baldwin, K., 2000. Exercise physiology: human bioenergetics and its applications, 3rd Edition. Mayfield Publishing Company, Mountain View, CA.

Brown, N.J., Porter, J., Ryder, D., Branch, R.A., 1990. Caffeine potentiates the renin response to Diazoxide in man. Evidence for a regulatory role of endogenous adenosine. J. Pharmacol. Exp. Ther., 256, 56-61.

Chesley, A. Hultman, E., Spriet, L.L., 1995. Effects of epinephrine infusion on muscle glycogenolysis during intense aerobic exercise. Am. J. Physiol. Endicronol. Metab., 268, 127-134.

Cole, L.M., Casida, J.E., 1983. Pyrethroid toxicology in the frog. Pestic. Biochem. Physiol., 20, 217-224.

Fort, D.J., Stover, T.L., Propst, T.L., Faulkner, B.C., Vollmuth, T.A., Murray, F.J., 1997. Evaluation of the developemental toxicity of caffeine and caffeine metabolites using the frog embryo teratogenesis assay-Xenopus (FETAX). Food Chem. Toxicol., 36, 591-600.

Ganong, W., 1999. Review of medical physiology, 19th Edition. Appleton & Lange, Stamford, CT.

Godafarb S., Cox, M., Singer, I., Goldberg, M., 1976. Acute hyperkalemia induced by hyperglycemia: hormonal mechanisms. Ann. Intern. Med., 84, 426-32.

Greer, F., Friars, D., Graham, T.E., 2000. Comaparison of caffeine and theophylline ingestion: exercise metabolism and endurance. J. Appl. Physiol., 89, 1837-1844.

Harri, M.N.E., 1980. Hyperglycaemia and hyperlalactacidemia as stress indicators in the frog. Comp. Biochem. Physiol., 69C, 371-374.

Hasko, G., Szabo, C., Nemeth, Z. H., Lendvai, B., Vizi, E. S., 1998. Modulation by dantrolene of endotoxin-induced inerleukin-10, tumour necrosis factor-α and nitric osxide production *in vivo* and *in vitro*. Br. J. Pharmacol., 124(6), 1099-106.

Henk, P.M., Vijverberg, G., Van Der Bercken, J., 1979. Frequency-dependent effects of the pyrethroid insecticide decamethrin in frog myelinated nerve fibers. Eur. J. Pharmacol., 58, 501-504.

Henk, P.M., Vijverberg, G., Ruigt, Van Der Bercken, j., 1982. Structure related effects of pyretrhroid insecticides on the lateral-line sense organ and on peripheral nerves of the clawed frog, Xenopus laevis. Pestic. Biochem. Physiol., 18, 315-324.

Kraus, F., Campbell, E.W., Allison, A., and Pratt, T., 1999. Eleutherdactylus frog introductions to Hawai`i. Herpetol. Rev., 30, 21-25.

Krauss, F., Campbell, E.W., 2002. Human-mediated escalation of a formerly eradicable problem: the invasion of Caribbean frogs in the Hawai`ian Islands. Biological Invasions, 4, 327-332.

Lee, T. H., Emala, C.W., 2001. Systemic adenosine given after ischemia protects renal function via A_{2a} adenosine receptor activation. Am. J. Kidney Dis., 38, 610-618.

Mathews, C.K., vanHolde, K.E., and Ahern, K.G., 2000. Biochemistry, Third Edition. Addison Wesley, Longman, San Francisco, CA.

Michael, S.F., 1995. Captive breading of two species of Eleutherodactylus (Anura: Leptodactylidae) from Puerto Rico, with notes on behavior in captivity. Herpetol. Rev., 26, 27-29.

Mohr, T., Van Soeren, M., Graham, T.E., Kjaer, M., 1998. Caffeine ingestion and metabolic responses of tetraplegic humans during electrical cycling. J. Appl. Physiol., 856, 979-985.

Montoliu, J., Revert, L., 1985. Lethal hyperkalemia associated with severe hyperglycemia in diabetic patients with renal failure. Am J. Kidney Dis., 5, 47-48.

Spriet, L.L., MacLean, D.A., Dyck, D.J., Hultman, E., Cederblad, G., Graham, T.E., 1992. Caffeine ingestion and muscle metabolism during prolonged exercise in humans. Am. J. Phsiol. Endocrinol. Metab., 262, 891-898.

Stewart, M.M., Pough, F.H., 1983. Population density of tropical forest frogs: relation to retreat sites. Science, 221, 570-572.

Stewart, M.M., 1995. Climate driven population fluctuations in rain forest frogs. J. Herpetology, 29, 437-446.

Tayeb, O., 1990. A serious interaction of dantrolene and theophylline. Vet. Hum. Toxicol., 32,442-444.

Williams, J., 1997. Contractile apparatus and sarcoplasmic reticulum function: effects of fatigue, recovery and elevated calcium. J. Appl. Physiol., 83, 444-450.