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Toxin-Induced Gustatory Conditioning in Rats: Examining the Effects of Low Dose Toxins in Food on Rat Feeding Behaviour and Avoidance Conditioning

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TOXIN-INDUCED GUSTATORY CONDITIONING IN RATS: EXAMINING THE
EFFECTS OF LOW DOSE TOXINS IN FOOD ON RAT FEEDING BEHAVIOR AND
AVOIDANCE CONDITIONING.

(Thesis format: Integrated Article)

by

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ABSTRACT

Foraging animals must learn which foods in their environment will maximize their nutritional needs but minimize the amount of ingested toxins. These animals rely on the integration of sensory and gustatory information and post-ingestive feedback from the foods they consume. Gustatory conditioning can be studied by using the conditioned taste avoidance paradigm and the toxin LiCl. This thesis first examined the dose related effects of low levels of LiCl on the ingestion of different palatable sucrose and salt solutions. The present findings support the hypothesis that rats use a behavioral tolerance mechanism to regulate their intake of foods containing low levels of toxins. Possible toxin-nutrient trade-off behaviors were also examined. It was found that when presented with a palatable sweetener and salt solution, rats will increase their consumption of the toxic food only when the calorie reward is high.

Keywords

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DEDICATION

I would like to dedicate this thesis to my parents, Dwayne and Mary Good. Knowing that you have supported my decisions, both academic and personal, means a great deal to me and I thank you for the opportunities you have given me. Here's where all your money went, enjoy.

TABLE OF CONTENTS

Chapter 1

Chapter 2

Chapter 3

Chapter 5

LIST OF FIGURES

Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

Organisms are in a continuous battle to maintain a state of equilibrium with their internal and external environments. Foraging animals are often faced with the task of learning which foods in their environment will meet their nutritional and energetic needs, and which will have harmful effects when ingested. However, this task is not a simple choice of "one or the other". Foraging animals are generally presented with a wide variety of food options, each with different nutrients and toxins that are combined in one food source. Homeostatic state can be upset by a diet with nutritional deficits or potentially toxic metabolites (Glenndinning, 2007; Provenza et al., 1998). It is this integration of nutrients and chemical contents into one food source that drives foraging behavior (see Forbey, et al., 2009). The goal is to learn which foods, and how much of those foods, are safe to consume for homeostatic purposes, without overdosing on any one toxin. To accomplish this, animals must learn to associate the tastes/flavours of food with their accompanying post-ingestive effects (Yearsley et al., 2006). These associations can develop into food preferences or aversions, depending on the beneficial or detrimental post-ingestive consequences of these foods (Yearsley et al., 2006).

Thus, a key step in learning which foods, or how much of a food, can be safely consumed, depends on the visceral feedback an animal receives after consumption. With the presentation of a new food or flavour, animals will exhibit neophobic behavior and limit their intake until post-ingestive feedback has been experienced (Corey, 1978; Lin, Amodeo, Arthurs, & Reilly, 2012). With no subsequent negative gastrointestinal or systemic effects, the food is deemed as safe and familiar, so intake will increase (Lin et al., 2012). On the other hand, if a novel taste is followed by negative post-ingestive effects, such as nausea or other gastrointestinal malaise and distress, the animal will acquire an aversion to or avoidance of that food (e.g., Bures et al., 1998; Cross-Mellor et al., 2004; Garcia et al., 1955; Garcia et al., 1985; Garcia and Koelling, 1967; Nachman and Ashe, 1973; Ossenkopp & Eckel, 1995).

A conditioned avoidance occurs when an animal ingests a novel food/flavour and then becomes ill, minutes to hours later. It may take only minutes for animals to learn the association between the novel food and negative post-ingestive effects, such as gastrointestinal malaise, as well as the visual or oral sensory input of that food (Bures et

al., 1998; Garcia et al., 1985; Garcia et al., 1955; Garcia & Koelling, 1967). Upon subsequent presentations of the food/flavour alone, animals will avoid consumption (Cross-Mellor et al., 2004; Garcia et al., 1985; Nachman & Ashe, 1973). If the reaction to the novel food is strong enough, it will only take one pairing for the animal to make the associations, which can last for long time periods (Bures et al., 1998; Garcia et al., 1955).

Most taste avoidance research has used high concentrations of toxins, such as lithium chloride (Cross-Mellor et al., 2004; Garcia & Koelling, 1967; Nachman & Ashe, 1973; Ossenkopp & Eckel, 1995). These high concentrations can result in complete future avoidance and are infrequently found in natural foraging situations. In nature, foraging herbivores or omnivores encounter plant secondary metabolites, which act as the plants defense mechanisms and produce negative systemic effects for the animals ingesting them. The effects of these metabolites may include weight loss, gastrointestinal distress, altered metabolic rates, or reduced digestibility of nutrients, all depending on the type and amount of toxin consumed (Dearing, Foley, & McLean, 2005; Sorenson, McLister, & Dearing, 2005). One plant may contain many of these toxins at low doses, but also many different nutrients, so the most successful foraging behavior involves a successful balance between the different nutrients and toxins, each at low doses (Villalba & Provenza, 2009). Behavioral strategies do not often include complete avoidance due to ubiquity and diversity of secondary metabolites (Forbey et al., 2009). For example, herbivores select a diet from an array of plants containing different nutrients and plant secondary metabolites, in order to avoid over consuming any one toxin (Freeland and Janzen, 1974; Launchbaugh, Burritt & Provenza, 1993; Provenza, 1996).

Avoidances and aversions to certain foods explain why animals limit or stop their intake, but taste-feedback interactions provide a mechanism for the association (Garcia et al., 1985; Ossenkopp & Eckel, 1995; Provenza, 1996). Animals use these conditioned associations to discriminate between the mixture of nutrients and toxins within a food, and can remember the associated olfactory cues across time, due to post-ingestive feedback (Provenza, 1995). Post-ingestive feedback is a neurally mediated interaction between the senses and viscera, which enables animals, such as ruminants, to sense the consequences of ingesting a food, which can include satiety or illness, and adjust intake

and preference according to the negative or positive feedback (Provenza, 1995). Post-ingestive feedback lets animals associate a food's' taste with its homeostatic utility and create automatic changes in perceived palatability for that food (Garcia et al., 1985; Provenza, 1995). Foods with adequate nutrients will increase in preference or hedonic value, whereas foods with excess toxins or inadequate nutrients will decrease in preference and these changes can occur within a meal or across meals (Garcia et al., 1985; Provenza, 1995). Toxins, such as LiCl, induce upper-gastrointestinal distress and are associated with negative shifts in the hedonic value of a food (cf. Ossenkopp & Eckel, 1995; Loy & Hall, 2002; Provenza et al., 1994).

Toxins can cause a decrease in preference for a food, but they do not necessarily prevent animals from consuming the food entirely, especially if the food contains needed nutrients (Wang & Provenza, 1996; 1997). Previous research studying the foraging behavior of ruminants has shown that the degree to which animals make associations between a food/flavor and its post-ingestive properties will depend on the impact of nutrients or toxins, as well as the state of the animal (Yearsley et al., 2006). For example, lambs offered different foods containing the toxin LiCl will continue to eat only the most nutritious ones (Wang & Provenza, 1997).

Animals have developed physiological and behavioral mechanisms for dealing with toxin-containing foods. Glendinning (2007) suggested three possible behavioral mechanisms that animals use for increasing their tolerance for toxin defended foods. These mechanisms have been demonstrated in many mammals such as voles, possums, and ruminants (Duncan et al., 2006; Provenza, 1996; Roy and Bergeron, 1990; Wiggins, McArthur, Davies, & McLean, 2006). One mechanism involves "processing" foods in order to reduce their toxin concentration (Glendinning, 2007). Voles were observed cutting branches off young conifers and returning to them days later to consume the branches, when concentrations of phenols and tannins were reduced (Roy & Bergeron, 1990). Similarly, a species of white-throated hoodrat utilized a food caching strategy to reduce the concentration of turpenes in plants (Torregrossa & Dearing, 2009). These hoodrats continuously added to their cache, but used the oldest store first, containing the lowest amount of toxin (Torregrossa & Dearing, 2009). Humans have also been shown to use this processing mechanism for plants such as cassava, which are cyanogenic and

require extended root soaking time before it can be consumed (Chiwona-Karltun et al., 2000).

A second behavioral tolerance mechanism, seen most often in ruminants and other roaming foragers, is to switch frequently between food sources (Freeland & Janzen, 1974; Glendinning, 2007). Different plants often contain different types or concentrations of toxins. This strategy of eating a varied diet helps to avoid toxicosis with any one toxin, but increases the acquisition of different toxins (Provenza, 1996). For example, when presented with two food options, each containing a different concentration of LiCl, lambs will consume both in small amounts to avoid overloading on either one (Burritt & Provenza, 2000).

The third mechanism by which organisms can regulate their toxin consumption is based on a threshold point. This behaviorally regulated tolerance mechanism has been observed in rats, birds, and marsupials. When post-ingestive aversive effects (illness and/or gastrointestinal malaise) reach a certain level, organisms will stop food consumption (Glendinning, 2007).

However, when animals have only one food source and it contains toxins, they must be able to regulate their intake to avoid toxicosis, yet maximize their caloric intake. Previous research has often employed a 'forced exposure' to toxin paradigm to investigate the resulting effects on behavior. There has been much less research on the voluntary intake of toxin containing foods. By presenting the toxin mixed into solution, rats are forced to control their own intake and regulate what toxin amount they are willing to consume.

The main objective of this thesis was to develop a possible model of foraging behavior, but using voluntary intake and regulation of toxin containing food, rather than forced toxin exposure. First, I wanted to examine the ability of rodents to process low levels of the toxin LiCl, and monitor how their behavior is regulated with a single toxic food source. In the first experiment (Chapter 2), rats were tested in a conditioned taste avoidance paradigm by means of oral ingestion of a sucrose solution containing various low levels of LiCl. Behavior was monitored by intake and lick number. After conditioning, rats were presented with corresponding sucrose solutions containing NaCl (instead of LiCl), and intake and licking behavior were again recorded. It was found that

rats regulate their behavior in a dose-dependent manner with the LiCl concentration present in solution.

The second aim of this thesis was to examine putative toxin-nutrient trade-off by rodents. Foraging animals often use an integrated representation of their foods' to decide how much to consume (i.e. caloric value, nutritional value, and toxic contents). Trade-off strategy here refers to the willingness of the rat to increase its toxin load, by consuming a greater amount of solution in to maximize its caloric intake. To determine which aspect of food contributed to trade-off behavior rodents were again tested using a paradigm that resembled that used in the first experiment by using the conditioned taste avoidance paradigm, this time using sucrose (Chapter 3) or saccharin (Chapter 4) and a single toxin concentration. After conditioning, rats were presented with the corresponding sweetener solution containing NaCl. Behavior in both phases was measured by intake, number of licks, and microstructural licking variables (cluster number and cluster size). Chapter 3 found a pronounced trade-off effect between calories and toxin, when the amount of calories available was high. Chapter 4 supported the idea of a toxin-nutrient trade-off strategy used by rats when consuming a toxin containing food, as no trade-off behavior was seen when drinking the calorie free sweetener saccharin.

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Chapter 2

MODELLING THE EFFECTS OF LOW TOXIN LEVELS IN FOOD ON FEEDING: DOSE-DEPENDENT REDUCTION OF FLUID INTAKE BY LOW LEVELS OF LITHIUM CHLORIDE.

2.1 Introduction

Organisms must be able to determine which foods available in their environment will meet both their energetic and nutritional needs. Foraging strategies and behaviors that underlie food selection have evolved so that organisms can learn to maximize nutrients and minimize toxins ingested in order to keep a balanced internal environment (e.g., Glendinning, 2007; Provenza et al., 1998). Foraging behavior is reinforced through feedback loops, from sensory input to motor output (Villalba & Provenza, 2007). The first inputs come from the visual, olfactory, and taste receptor systems, which allow for food discrimination and assessment. Visceral receptors then signal to the central nervous system information about nutrient value and toxin load, and whether or not consumption should continue (Janssen & Depoortere, 2013; Provenza et al., 1998; Villalba & Provenza, 2007). This feedback also helps to determine how certain foods will be limited in subsequent encounters and may alter the hedonic value of food (Pfister et al., 2010).

An important, behaviorally adaptive learning mechanism that helps animals avoid consuming potentially harmful foods is conditioned taste avoidance. This learning process allows organisms to make associations between illness and recently ingested novel flavors (Bures et al., 1998; Garcia et al., 1955; Garcia et al., 1985). If an organism becomes ill, within minutes to hours after ingesting a novel food or flavor, it will learn to associate the visual or oral sensory input of the food with the induced illness. If the reaction to the toxin is strong enough, an association between the sensory cues and the induced illness can be made with one pairing that will last for long time periods (Bures et al., 1998; Garcia et al., 1955, 1985).

Lithium chloride (LiCl) is a toxin frequently used to induce aversions or avoidances of novel tastes or places in rats and other animals (c.f. O'Donnell & Gould, 2007; Riley & Freeman, 2004; Tenk et al., 2005, 2006). When LiCl is paired with a highly palatable food or flavour, such as sucrose, the hedonic value of the flavour can become diminished as it becomes associated with the toxic effects of LiCl (Cross-Mellor et al., 2004; Ossenkopp & Eckel, 1995). In rodents, the toxic effects and illness associated with LiCl can manifest within 15 - 20 min of ingestion or systemic treatment (Garcia et al., 1985; Provenza, 1996). Nachman and Ashe (1973) first observed that the strength of a LiCl-induced conditioned taste avoidance, measured as decreased intake of

a food, is proportional to the dose administered with higher doses of LiCl resulting in a stronger avoidance. Effects of the route of administration on taste avoidance learning were also examined by comparing intraperitoneal (i.p.) injections, subcutaneous injections, and direct stomach infusion. As all of the animals tested learned the lithium-induced avoidance, it was concluded that route of administration had little effect on the strength of avoidance learning (Nachman & Ashe, 1973). Even with relatively low levels of ingested toxin, a rat can establish a conditioned taste avoidance that might otherwise not affect other behaviors, such as locomotor activity or fear responses (Nachman & Ashe, 1973). This suggests that the formation of a taste avoidance is an automatic response, and that gustatory conditioning is a useful paradigm in studying the phenomenon. Orally ingested LiCl can produce robust conditioned taste avoidance (Ladowsky & Ossenkopp, 1986; Loy & Hall, 2002) and even small quantities are able to decrease ingestive responses to the toxin-containing flavor (Cross-Mellor et al., 2004).

Glenndinning (2007) proposed three possible behavioral mechanisms that organisms can use to decrease toxin load and increase calories consumed. The first mechanism involves "processing" foods in ways that will reduce the toxin content. For example, some meadow voles will chew off small branches from plants containing a high concentration of phenolics and tannins and then return to them at a later time for consumption, as the toxins decay substantially across days (Roy & Bergeron, 1990). Humans have also been shown to use this processing mechanism for plants such as cassava, which are cyanogenic and require extended root soaking time before it can be consumed (Chiwona-Karltun et al., 2000). The second proposed mechanism involves switching between different foods sources having different chemical defenses. By doing this the organism can minimize the specific toxin load from each plant, and maximize nutrients available from the different plants. For example, ruminants will alternate food sources in one meal and eat from each source until a threshold level of toxin from each plant is reached (Provenza, 1996). The third mechanism by which organisms can regulate their toxin load is based on a threshold point. This behaviorally regulated tolerance mechanism has been observed in rats, birds, and marsupials. When post-ingestive aversive effects (illness and/or gastrointestinal malaise) reach a certain level, organisms will stop food consumption (Glendinning, 2007).

The behaviorally regulated tolerance mechanism was investigated in the current study by presenting rats with a highly palatable food (a solution of 0.3 M sucrose plus 0.12 M salt) containing differing low doses of the toxin LiCl. As food selection involves the interaction of sensory assessment (vision, taste and smell) and postingestive feedback, both positive and negative (Garcia, 1989; Glendinning, 2007; Janssen & Depoortere, 2013; Provenza 1995) the present study examined the dose relationship between the oral ingestion of different concentrations of LiCl in a standard sucrose solution on food consumption (drinking behavior). At equimolar concentrations the smell and taste of LiCl and NaCl solutions are indistinguishable to naïve rats (Keifer, 1978; Loy & Hall, 2002; Nachman, 1963; Strom et al., 1970; Ossenkopp et al., 1997) and upon initial presentation rats will consume equivalent amounts. With repeated presentation rats can eventually learn to discriminate between the effects of equimolar NaCl and LiCl solutions. Rats conditioned with a high dose of LiCl will also exhibit an extended extinction period when subsequently presented with a solution of NaCl (Kiefer, 1978; Loy & Hall, 2002; Nachman, 1963; Strom et al., 1970). This inability of rats to easily discriminate between the smell and taste of equimolar NaCl and LiCl solutions allowed for the present experimental design.

A highly palatable fluid (sucrose plus salt) can easily be manipulated in terms of toxin load. In a 0.3 M sucrose solution mixed with a standard salt concentration of 0.12M the salt component can be varied between 0.12 M NaCl to 0.12 M LiCl, as well as mixtures of these salts, to obtain low levels of toxin load in the fluids (e.g., 0.01 M LiCl plus 0.11 M NaCl). Note that for all solutions the total salt concentration is kept constant at 0.12 M. As the tastes of the solutions are highly similar, the effects of drinking these solutions on behavior relate to the post-ingestive effects of the differing levels of LiCl.

In the present experimental design the negative control solution (no toxin) consisted of 0.3 M sucrose plus 0.12 M NaCl. A positive control solution (high toxin) of sucrose plus 0.12 M LiCl, previously shown to condition a robust taste avoidance (Cross-Mellor et al., 2004; Loy & Hall, 2002; Nachman & Ashe, 1973), was used to verify the toxin induced taste avoidance effect. Of particular interest was the effect of ingesting low levels of toxin, and these were represented by sucrose solutions that contained low concentrations of LiCl ranging from 0.005 M to 0.02 M. Different groups of adult male

rats, maintained on a water deprivation schedule, were tested with these different fluid solutions. Amount of fluid consumed and licking behavior were recorded for each rat and subsequently analyzed and compared. On the basis of the behaviorally regulated tolerance mechanism hypothesis (Glendinning, 2007) a LiCl dose-related reduction in fluid intake and lick frequency was hypothesized to occur over days of testing in the groups of rats drinking the various sucrose plus salt solutions.

2.2 Methods

2.2.1 Animals

Fifty-eight naïve adult male Long-Evans rats (Charles River, Quebec, Canada), weighing 250-300 g at the start of the experiment were used. Rats were individually housed in polypropylene cages, in a temperature controlled colony room maintained at 21 ± 1° C, with a 12-h light: 12-hr dark schedule (lights on at 07:00 h). Food (Prolab rat chow) and water was available ad libitum unless otherwise noted. The experimental methodology was carried out according to the Canadian Council on Animal Care guidelines and was approved by the Institutional Animal Care Committee.

2.2.2 Apparatus

An automated lickometer was used during testing (Contact 108 lick analysis system, Dilog Instruments, Tallahassee, Fl). Eight clear Plexiglas chambers (31cm X 31cm X 40 cm) with Plexiglas lids containing air holes were used. Each chamber had a 100 mL graduated drinking tube mounted and centered on the front, with the spout being 8 cm above chamber floor. The drinking tube was accessible through an oval opening, in which only the rats tongue could make contact, and would not alter the tongue and jaws natural movements. Each time the rat's tongue came into contact with drinking spout, a non-detectable current (~60 nA) passed through the spout to complete an electric circuit, registering one lick. Signals were amplified before being stored on a desktop computer for compilation of licking measures (QLick, version 4.0).

2.2.3 Procedure

2.2.3.1 Water Restriction and Habituation. Over a period of five days, a split water deprivation schedule was established. After 24h with no access to water, animals had access to water for 30 minutes, twice daily. For the first five days water access occurred in their home cages, first between 0900 h–1100 h, and then again between 1400

h–1600 h. Over the next 3 days, animals were habituated to the general testing procedure and lickometer testing chambers. For 20 minutes a day, access to water was given in these boxes (between 0900 h – 1100 h). After each test session chambers were cleaned and deodorized using Alconox and baking soda. The habituation phase lasted until drinking levels were stable (approximately 7 mL/session) for all animals. Baseline drinking levels were recorded to ensure groups did not differ in their initial drinking levels. The additional 30 minutes of water access was given in the home cages between 14:00 – 16:00 h.

2.2.3.2 Acquisition Phase. Once habituated, all animals were randomly assigned to one of the six different groups drinking 0.3 M sucrose solutions with differing levels of LiCl. On 5 days of acquisition training rats were presented with a 0.3 M sucrose plus 0.12 M salt solution for 20 min in the lickometer chambers with one of the following salt combinations: 0.12 M NaCl (negative control; $n = 10$); 0.005 M LiCl + 0.115 NaCl ($n = 10$); 0.01 M LiCl + 0.11 NaCl ($n = 10$); 0.015 M LiCl + 0.105 M NaCl ($n = 10$); 0.02 M LiCl + 0.10 M NaCl ($n = 10$); and 0.12 M LiCl (positive control; $n = 8$). "Intake volume" and "number of licks" were the dependent variables. An additional 30 minutes of water access was again given in the home cages between 14:00 – 16:00 h.

2.2.3.3 Extinction Phase. Over the subsequent five days of testing all rats were given 20 minutes of access to a 0.3 M sucrose + 0.12 M NaCl solution in the lickometer chambers. Intake volume and number of licks were again recorded. 30 minutes of water access was again provided at the usual time in the home cages.

2.2.4 Data Analysis

The lickometer system recorded and stored number of licks. Intake volume was recorded by noting the starting and finishing volumes in the graduated tubes, recorded to the nearest half mL. Data were analyzed with a mixed design analysis of variance (ANOVA) procedure. In all analyses the between subjects factor was LiCl concentration (at 6 levels; 0, 0.005, 0.01, 0.015, 0.02, 0.12 M) and the within subjects factor was Day (at five levels; 1, 2 …5; for each phase). Significant interactions were subsequently investigated with Tukey's HSD test of pairwise comparisons, and $\alpha = 0.05$ was used as criterion for significance. Dose-response relationships of mean fluid "intake volume" and mean "number of licks" on days 2 – 5 of acquisition for the low toxin level groups

(0.005 to 0.02 M LiCl) were examined with best fit polynomial curves and the associated formulae were then used to calculate the ID_{50} (infective dose). This is used to examine the dose of an infectious agent required to produce infection in 50 percent of the experimental subjects. For our purpose the ID_{50} is the molar dose of LiCl at which 50% reductions in the dependent variables were observed.

2.3 Results

The pattern of results was very similar for the "volume intake" data and the "number of licks" data. As well, large positive Pearson product moment correlations were obtained between number of licks and volume intake across all groups (correlation range of $r = 0.79$ to 0.87). Thus, only the "number of licks" data are presented in Fig. 1A and 1B.

2.3.1 Acquisition Phase

2.3.1.1 Intake Volume (mL). Inspection of group mean volume consumption of a solution containing sucrose and varying levels of LiCl indicates that the animals in the negative control group (sucrose plus NaCl only) drank consistently high levels of fluid across acquisition days, attesting to the high level of palatability for this solution (data not shown). The ANOVA revealed a significant Day x LiCl concentration interaction, $F(17,175) = 4.27$, $p < 0.001$ and post-hoc pairwise comparisons indicated that rats drinking solutions with a LiCl concentration greater than 0.01 M were drinking significantly less than animals in the NaCl only control group. As expected the positive control group (0.12M LiCl) consumed significantly less than all other groups, on all testing days. The 0.02 M LiCl group drank significantly less than NaCl control group on days 2-5 and rats drinking 0.015 M LiCl drank less than the NaCl control group on days 2-4. A main effect of LiCl concentration was also obtained, $F(5,52) = 61.75$, $p < 0.001$, but no main effect of Day, $F(4,175) < 1$.

The dose related effect of low LiCl concentrations on drinking behavior was examined using mean intake volumes on days $2 - 5$ (data not shown). A significant relationship between mean intake volume and LiCl concentration was found, $F(4,45) = 29.53$, $p < 0.001$, with the best fit polynomial curve ($Y = 13.19 - 110.95X - 785.71 X^2 - 633333.33 X^3$; $R^2 = 0.998$) for these dose-response data indicating that $ID_{50} = 0.0188$ M LiCl for the intake volume. Calculation of the mean daily amount of LiCl consumed on

days 2 – 5 revealed that the 0.12 M LiCl group consumed a mean of 3.815 mg of LiCl. Similarly, the 0.02, 0.015 and 0.01 M LiCl groups consumed a mean of 4.756, 5.818, and 4.866 mg of LiCl, respectively. Group 0.005 LiCl only consumed a mean of 2.645 mg/kg. Taking body weights into account, groups 0.02, 0.015 and 0.01 M LiCl were thus exposed to an overall mean dose of 16.90 mg/kg and group 0.005 M LiCl to a mean dose 8.55 mg/kg. Thus, these data suggest that rats drinking solutions containing low levels of LiCl regulated their mean intake levels to no more than 16.0 to 18.0 mg/kg per day.

2.3.1.2 Number of Licks. Analysis of the group mean number of licks is shown in Figure 2.1A. Inspection of this figure indicates that the animals in the negative control group (sucrose plus NaCl only) exhibited consistently high levels of licking across acquisition days, again attesting to the high level of palatability for this solution. A significant Day x LiCl concentration interaction was found, $F(17,173) = 3.63$, $p < 0.001$ and post-hoc pairwise analyses showed that rats drinking 0.02 M, and 0.015 M LiCl differed from NaCl on days 2-5, and 2 and 5, respectively. Also, rats drinking 0.02 M LiCl displayed lower levels of licking than rats drinking 0.015 M, 0.01 M and 0.005 M LiCl on days 2 – 5. As expected the positive control group drinking 0.12 M LiCl displayed significantly lower levels of licking than all the other groups on all days. A significant main effect of LiCl concentration was also obtained, $F(5,52) = 51.9$, $p < 0.001$, but no main effect of Day, $F(4,173) < 1$.

The dose related effect of low LiCl concentration on licking behavior was also examined using mean number of licks on days 2 – 5 (Figure 2.1B). A significant relationship between mean number of licks and LiCl concentration solution was found, $F(4,45) = 25.01$, $p < 0.001$, with the best fit polynomial curve (Y = 2119,93 – 73830.24 X + 7798928.57 X^2 – 352433333.33 X^3; $R^2 = 0.996$) for these dose-response data indicating that $ID_{50} = 0.0194$ M LiCl for the number of licks.

2.3.2 Extinction Phase

2.3.2.1 Intake volume (mL). Group mean volume intake during the extinction phase (all groups drinking sucrose plus NaCl only) was also investigated (data not shown). A significant Day x LiCl concentration interaction was found, $F(18,187) = 8.87$, $p < 0.001$. Post-hoc analyses revealed that on days 1-3 of extinction, the positive control group drinking 0.12 M LiCl in the acquisition phase had significantly lower intake than
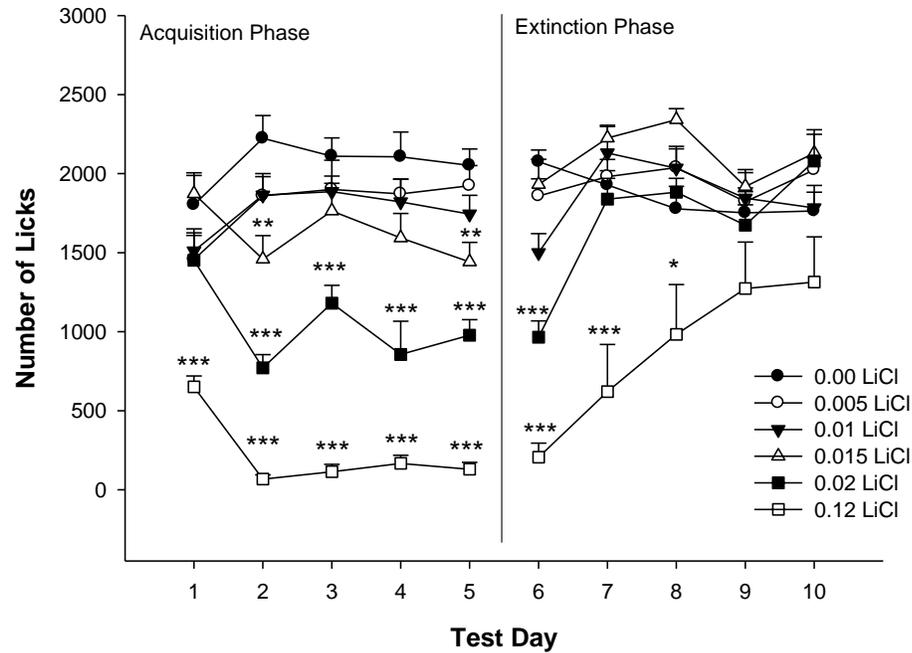
Figure 2.1 (**A**). Group mean number of licks when presented with sucrose plus salt solutions during both the acquisition phase (left panel) and the extinction phase (right panel). ANOVA of the acquisition data revealed a significant Day x LiCl concentration interaction, $p < 0.001$. Rats drinking 0.12 M LiCl and 0.02 M LiCl licked less than rats drinking NaCl. On days 2 and 5, rats drinking 0.015 M LiCl licked less than those drinking NaCl; ***$p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Rats drinking 0.02 M LiCl licked more than rats drinking 0.12 M LiCl, $p < 0.001$. ANOVA of the extinction data revealed a significant Day x LiCl concentration interaction, $p < 0.001$. On Days 1 -3, rats that consumed 0.12 M LiCl in the acquisition phase, licked less NaCl solution in phase 2, compared to rats drinking NaCl in both weeks. On Day 1, rats that consumed 0.02 M LiCl and 0.015 M LiCl in week 1, licked less NaCl solution in the extinction phase, compared to rats drinking NaCl in both phases; ***$p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Error bars represent S.E.M.
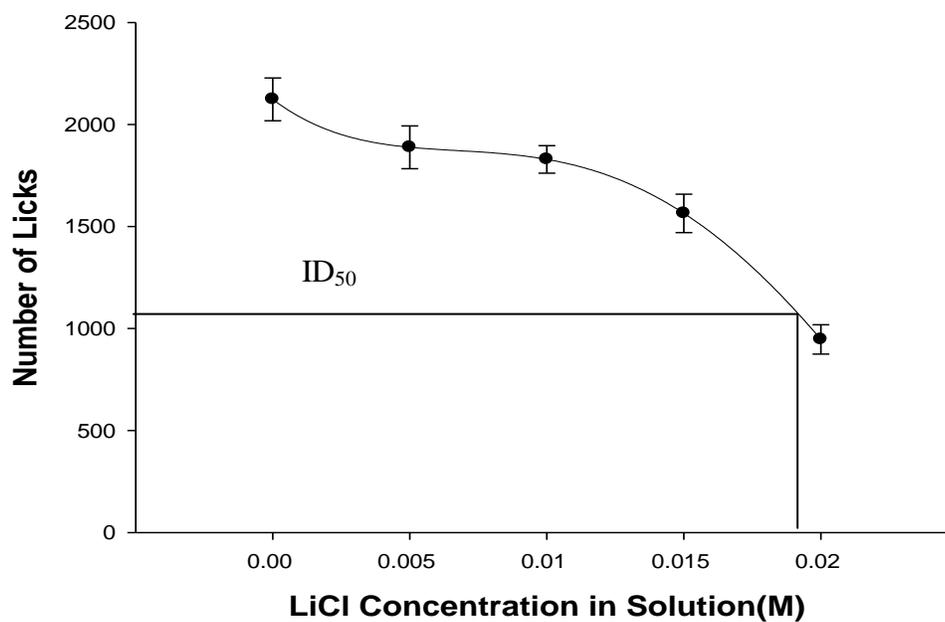
Figure 2.1(B) Dose response relationship for number of licks and LiCl concentration. Data points represent group mean number of licks for days $2 - 5$, for each solution. The best fit polynomial curve (see text) for this dose-response relationship reveals that $ID_{50} = 0.0194$ M LiCl for number of licks. Error bars represent S.E.M.

the NaCl group. Rats drinking 0.015 M and 0.02 M LiCl drank significantly less than those drinking NaCl on day 1 only. By day 3, all rats were drinking similar amounts of sucrose plus NaCl solution. A significant main effect of Day was also obtained, $F(4,187) = 30.58$, $p < 0.001$. The increase in fluid intake across days differed among groups (main effect of LiCl concentration), dependent on the concentration of LiCl encountered during the acquisition phase, $F(5,52) = 13.66$, $p < 0.001$.

     2.3.2.2 Number of Licks. Group mean number of licks during the extinction phase is depicted in Figure 2.1A (right hand panel). The ANOVA for these data revealed a significant Day x LiCl concnetration interaction, $F(17,177) = 6.05$, $p < 0.001$. Post-hoc analyses indicated that, in comparison to rats in the negative control group (sucrose plus NaCl in acquisition), rats drinking 0.12 M LiCl in acquisition exhibited significantly lower mean number of licks on days 1 – 3 of extinction. Those drinking 0.015 M and 0.02 M LiCl in the acquisition phase, licked significantly less frequently only on day 1 of extinction, in comparison to the NaCl only control group. Both a significant main effect of LiCl concentration, $F(5,52) = 12.61$, $p < 0.001$, and a significant main effect of Day were also obtained, $F(4,177) = 17.34$, $p < 0.001$.

2.4 Discussion

     The negative control group, drinking a 0.3 M sucrose plus 0.12 M NaCl solution, exhibited high and consistent levels of fluid intake and number of licks, both in the acquisition and extinction phases, indicating that this mixture had no obvious negative post-ingestive effects. In contrast, the positive control group, drinking a 0.3 M sucrose plus 0.12 M LiCl (toxin) solution during the acquisition phase, quickly reduced both fluid intake levels and number of licks to very low levels, indicative of a strong taste avoidance that already developed on the first acquisition day. The strong conditioned taste avoidance was further evidenced by the relatively slow return to baseline levels of both intake levels and lick number by this group during the extinction phase when drinking a toxin free solution. These findings are consistent with previous demonstrations of strong taste avoidance conditioning in rats drinking solutions with 0.12 M or higher levels of LiCl (Ladowsky & Ossenkopp, 1986; Loy & Hall, 2002; Cross-Mellor et al., 2004).

Of particular interest in the present study were the groups drinking low levels of LiCl, as these groups modeled feeding on foods with low levels of toxin. Dose dependent effects of LiCl on ingestion were evidenced by the dose related reductions in both volume intake and number of licks during the acquisition phase. During the extinction phase, all rats were presented with a solution of sucrose plus NaCl, allowing the animals, previously given LiCl, to learn that the sucrose/taste was no longer associated with toxic effects. During the extinction phase the low toxin level groups exhibited a rapid return to control values after the first day. These finding are consistent with the threshold hypothesis, which suggests that rats will regulate toxin intake, even at low levels (Glendinning, 2007). As the rats drinking the solution with 0.02 M LiCl drank significantly less than the NaCl negative control group on days 2 -5, this concentration of LiCl could represent a toxin threshold level to which the rats were regulating their behavior. Indeed, the ID_{50} values for both the intake volume and number of licks data are very close to this molarity of LiCl (0.0188 and 0.01935, respectively).

It is also interesting to note that the rats in the present experiment voluntarily regulated their intake of LiCl to no more than about 18 mg/kg per day. This value is comparable to the lowest dose (0.3 mEq/kg; 12.72 mg/kg) of LiCl which was shown to induce a significant conditioned avoidance in a more traditional taste avoidance learning paradigm, with rats first drinking a sucrose solution (10 min) followed by an injection (i.p.) with 0.15 M LiCl solution (Nachman and Ashe (1973). Clearly there are major differences in methodology between the present study and the Nachman and Ashe (1973) study; nevertheless, the levels of LiCl which the rats are willing to tolerate in the present experiment are similar to levels which have been shown to effectively induce conditioned avoidances of palatable tastes. The present rat model is thus useful for examining the regulated feeding mechanism, as it shows a clear toxin dose related, regulated feeding process. Quantification of consumption, by using orally presented solutions is simple, and it is easy to manipulate the toxin load in the drinking solutions.

The pattern of regulating feeding behavior observed in the present study is often seen in natural foraging conditions. Ruminants, such as sheep, have been found to alter their diet and limit intake of particular plants in order to minimize toxins and maximize nutrients (see those discussed in Pfister et al., 2010; Provenza, 1995; Provenza, 1996).

When presented with different plant species, each containing a different toxin, sheep will consume one plant until a threshold for the toxin in that plant is reach and then move on to the next plant (Pfister et al., 2010). This behavioral mechanism allows the animal to get the benefit of the food source, yet limit toxin effects.

Cross-Mellor et al. (2004) suggested that animals may have two different processes for regulating intake of toxic substances. If ingesting foods containing high toxin levels, animals develop a robust, long term rejection *and* avoidance of these foods. At high concentrations, LiCl and other toxins act on the chemosensitive area postrema (AP) in the brainstem to induce conditioned taste avoidances and aversions (Borison, 1989; Eckel & Ossenkopp, 1996; Ladowsky & Ossenkopp, 1986; Ossenkopp & Eckel, 1994, 1995; Ritter et al., 1980). As well, high doses of lithium have been shown to induce conditioned palatability shifts when paired with highly palatable tastes. These conditioned palatability shifts are likely mediated by the chemosensitive area postrema (Eckel & Ossenkopp, 1996; Ossenkopp & Eckel, 1995). By lesioning this structure in the brainstem conditioned taste and place avoidances can be blocked (Ladowsky & Ossenkopp, 1986; Ossenkopp et al., 1997; Ritter, McGlone, & Kelley, 1980). However, when animals are ingesting foods containing low toxin levels, they may be using a short-term transient mechanism to regulate feeding behavior within a meal and on a day to day basis. Evidence for this mechanism comes from rats learning to discriminate between an equimolar LiCl and NaCl solution, as these animals can develop a discrimination in the absence of an area postrema (Ossenkopp et al., 1997), suggesting that post-ingestive aversive cues from the gut are likely involved in the formation of this discrimination. It is likely that the behavioral regulatory process observed in the present study also depends on feedback of post-ingestive aversive cues from the gut. What the role of the area postrema chemosensor might be in such behavioral regulation remains to be determined.

In conclusion, it is evident that with oral ingestion of LiCl, rats will learn taste avoidances in a dose-dependent manner, and regulate their drinking behavior to correspond to a toxin threshold. The strength of the avoidance is a function of the concentration of the toxin in the food, and higher toxin concentrations result in faster acquisition and a longer extinction period for the avoidance.

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Chapter 3

EXAMINING POSSIBLE BEHAVIORAL TOXIN-NUTRIENT TRADE-OFFS IN RATS PRESENTED WITH LOW DOSE LITHIUM CHLORIDE

3.1 Introduction

Foraging animals are faced with the task of determining which foods in their environment are safe to consume, and which are harmful. In nature animals are often presented with multiple food sources that offer a variety of nutrients. However, these food sources may also be defended by a variety of toxins such as plant secondary metabolites (c.f. Forbey et al., 2009). When consumed in large amounts, these metabolites may cause a number of adverse side effects, such as gastrointestinal malaise (Pfister et al., 2010; Provenza, 1996). Animals can learn to use the integration of sensory input from the foods' characteristics and postingestive feedback to determine how much of one food can be ingested safely (e.g. Provenza, 1995; Provenza et al., 1999; Villalba & Provenza, 2007). An automatic and adaptive mechanism to help guide food choice or food preference is conditioned taste avoidance. When an animal ingests a novel food/flavour, and then becomes ill within minutes to hours later, it will learn to associate the sensory cues of that food with the illness and avoid it upon future encounters (Bures et al., 1998; Garcia, Kimeldorf, & Koelling, 1955; Garcia, Lasiter, & Bermudez-Rattoni, 1985).

Lithium chloride (LiCl) is a commonly used toxin, in laboratory investigations, to induce gastrointestinal malaise and mild nausea, and has been used frequently to investigate conditioned taste avoidances or aversions when paired with novel flavours (Garcia & Koelling, 1967, see Riley & Freeman, 2004). In rats, the effects of LiCl are manifested within 15-20 minutes, but the mildly adverse effects are short lived (Garcia et al., 1985; Loy & Hall, 2002; Provenza, 1996). Work by Nachman and Ashe (1973) showed that the route of administration does not significantly influence the toxic effects of LiCl. When presented orally, LiCl has been found to induce robust avoidances in a dose-dependent manner (Ladowsky & Ossenkopp, 1986; Loy & Hall, 2002), and even when tested at very low concentrations (Cross-Mellor et al., 2004; Good, et al., 2013). Many previous studies with LiCl used relatively high toxin concentrations (~95 – 128 mg/kg) to establish avoidances (c.f. Riley & Freeman, 2004; Nachman & Ashe, 1973). The mechanisms for avoidance learning at high LiCl concentrations may be different compared to low LiCl concentrations (Cross-Mellor et al., 2004). Typically, a palatable novel taste was paired with systemic LiCl injections and the rats subsequently reduced

intake of the conditioned food.

Foraging animals are generally exposed to very low concentrations of toxins in any one food source. When toxins are presented in food sources at low concentrations for oral consumption, animals are willing to voluntarily manipulate their intake to maximize nutrients, but minimize toxin intake (Good et al., 2013; Provenza, 1995; Provenza et al., 1999). Most species use physiological and behavioral adaptations to ensure a homeostatic balance between nutrient intake and toxin level (e.g. Barnett, Skelhorn, Bateson, & Rowe, 2011; Bevolsky, 1978; Chiwona-Karltun, et al., 2000; Dearing, Foley, & McLean, 2005; Duncan, Ginane, Elston, Kunaver, & Gordon, 2006; Glendinning, 2007). In a recent study, water-deprived rats were presented with a palatable sucrose solution containing various low concentrations of LiCl for oral consumption (Good et al., 2013), thus allowing rats to control toxin consumption levels. It was found that these rats used a threshold regulation strategy (suggested by Glendinning, 2007), in which they limited the intake of the toxic solution in a dose-dependent manner, and stopped consumption when post-ingestive feedback indicated that a certain toxin level had been reached, in order to minimize more serious toxic effects (Good et al., 2013). Duncan et al. (2006) showed that natural browsing ruminants show a similar dose-dependent decline in food preference when paired with a negative stimulus. They also suggested that the positive (i.e. nutrients) and negative effects (i.e. secondary metabolites) associated with a food seem to act independently, and it is the integration of both of these aspects that is responsible for the overall amount ingested.

Depending on the state of the animal, the positive post-ingestive effects from a food may include its caloric value. Rats' show distinct differential liking of sugars, and the hedonic value and palatability of a sugar changes with its concentration (Ackroff, Manza, & Sclafani, 1993; Richter & Campbell, 1940; Sclafani & Nissenbaum, 1987). Sclafani & Nissenbaum (1987) used a two-bottle preference test to determine a preference threshold for sucrose over water. The preference threshold was indicated at 0.0026M, but intake showed an inverted 'U' shape preference curve as concentration of sucrose was increased. Rats showed increased preference for sucrose at ~ 0.05 M - 0.10 M, and then preference leveled off until ~0.25 M sucrose, and decreased at 0.50 M sucrose (Sclafani & Nissenbaum, 1987). The various sucrose concentrations used in the

present study were based on this preference curve.

The difference in taste/palatability of sucrose solutions can be further analyzed by examining the licking behavior of the rat, especially when broken down into microstructural components. When ingesting a liquid food source, rats lick in rapidly occurring rhythmic tongue movements that occur in bursts (Davis, 1973). The intake and licking behavior system is under the control of a variety of influences, including sensory, gustatory, and post-ingestional variables (Davis & Perez, 1993). When a food source has an attractive smell and is accompanied by a "pleasant" taste, it is rated as having high palatability. Palatability and hedonic value of a solution can be inferred from both consumption and the microstructural analyses of licking patterns (Baird, St. John, & Nguyen, 2005; Dwyer, 2009; Weijnen, 1998). Microstructural analysis of licking patterns includes number of bursts, and number of clusters, and the size of bursts and clusters. Bursts and clusters of licks are defined by the pauses between lickings. By incorporating the toxin directly into a palatable solution, the microstructure of licking can offer information related to the animals' reaction to the palatability of the solution in ways that are not possible with other techniques such as taste-reactivity tests (Dwyer, 2012; Dwyer, Pincham, Thein, & Harris, 2009). Previous research has also shown that cluster size is independent of consumption and that the mean number of licks in a cluster shows a positive monotonic relationship with the concentration of a palatable fluid (Davis & Smith, 1992; Dwyer et al., 2009), such as a sucrose solution.

The purpose of the current study was to investigate the possible use of a toxin-nutrient trade-off strategy in rats. I was interested in examining changes in feeding behavior when a constant LiCl concentration is used, but the sucrose concentration is varied. Of particular interest was at what concentration the positive effects of sucrose would outweigh the negative effects of the LiCl. Trade-off strategy here refers to the willingness of the rat to increase its toxin load, by consuming a greater amount of solution in to maximize its caloric intake. Previous work from our laboratory suggested that rats regulate their intake to a possible LiCl threshold dose of 0.02 M (Good et al., 2013), a concentration that was used in the current study. By keeping the toxin concentration constant, it was possible to examine how much LiCl rats are willing to ingest as a function of available calories.

Previous demonstrations that the taste and smell of equimolar concentrations of LiCl and NaCl are indistinguishable to naive rats (Keifer, 1978; Loy & Hall, 2002; Nachman, 1963; Ossenkopp et al., 1997; Strom et al., 1970), allowed for the use of control solutions containing 0.02 M NaCl, in various sucrose solutions, as each comparable LiCl and NaCl group would initially be experiencing the same taste, until post-ingestive feedback relays the effects of LiCl. Rats that are presented with LiCl during the acquisition phase of avoidance learning, will also generalize their avoidance to NaCl when presented with a NaCl solution during the extinction phase. How long this generalization lasts is also an indication of the strength of the avoidance.

When water-deprived rats are presented with a toxic solution for oral consumption, with each group containing a different sucrose concentration, the amount consumed could be affected by both the palatability of the solution, and the available calories of each. It was hypothesized that when presented with a toxic sucrose solution, the amount consumed would vary according to the concentration of sucrose. That is, with a constant LiCl concentration, the benefits of sucrose would outweigh the toxic effects of LiCl only at higher sucrose concentrations, and rats would consume more of the test solution when compared to rats drinking solutions with low sucrose concentrations.

3.2 Methods

3.2.1 Animals

Fifty naïve adult male Long-Evans rats (Charles River, Quebec, Canada), weighing 175 - 200 g at the start of the experiment were used. Rats were individually housed in polypropylene cages, in a temperature controlled colony room maintained at 21 ± 1º C, with a 12-h light: 12-hr dark schedule (lights on at 07:00 h). Food (Prolab rat chow) and tap water were available ad libitum unless otherwise noted. The experimental methodology was carried out according to the Canadian Council on Animal Care guidelines and was approved by the Institutional Animal Care Committee.

3.2.2 Apparatus

An automated lickometer was used during testing (Contact 108 lick analysis system, Dilog Instruments, Tallahassee, Fl). Eight clear Plexiglas chambers (31cm X 31cm X 40 cm) with Plexiglas lids containing air holes were used. Each chamber had a 100 mL graduated drinking tube mounted and centered on the front, with the spout being

8 cm above the chamber floor. The drinking tube was accessible through an oval opening, in which only the rats tongue could make contact, and would not alter the tongue and jaw's natural movements. Each time the rat's tongue came into contact with the drinking spout, a non-detectable current (~60 nA) passed through the spout to complete an electric circuit, registering one lick. Signals were amplified before being stored on a desktop computer for compilation of licking measures (QLick, version 4.0).

3.2.3 Procedure

      3.2.3.1 Water Restriction and Habituation. Over a period of five days, a split water deprivation schedule was established. After 24h with no access to water, animals had access to water for 30 minutes, twice daily. For the first three days water access occurred in their home cages, first between 0900 h–1100 h, and then again between 1400 h–1600 h. Over the next two days, animals were habituated to the general testing procedure and lickometer testing chambers. For 20 minutes a day, access to water was given in these boxes (between 0900 h – 1100 h). After each test session, chambers were cleaned and deodorized using Alconox and baking soda. The habituation phase lasted until drinking levels were stable (approximately 7 mL/session) for all animals. Baseline drinking levels were recorded to ensure groups did not differ in their initial drinking levels. The additional 30 minutes of water access was given in the home cages between 14:00 – 16:00 h.

      3.2.3.2 Acquisition Phase. Once habituated, all animals were randomly assigned to one of eight different groups. For five days of acquisition training rats were presented with one of the following solutions for 20 minutes/day, in the lickometer chambers:

Salt	Sucrose	*n*	Salt	Sucrose	*n*
0.02 M LiCl	0.05 M	7	**0.02 M NaCl**	0.05 M	6
	0.10 M	6		0.10 M	6
	0.25 M	7		0.25 M	6
	0.50 M	6		0.50 M	6

Intake volume, number of licks, number of clusters, and size of clusters were the dependent variables. After each animal was tested, the chambers were cleaned and deodorized. An additional 30 minutes of water access was again given in the home cages between 14:00 – 16:00 h.

   3.2.3.3 Extinction Phase. Over the subsequent five days of testing all rats were given 20 minutes of access to the appropriate control solution (0.02 M NaCl + sucrose) in the lickometer chambers. Intake volume, number of licks, number of clusters, and size of clusters were again recorded. 30 minutes of water access was again provided at the usual time in the home cages.

3.2.4 Data Analysis

   The lickometer system recorded and stored number of licks. The QuickLick program was used to obtain number and size of clusters. Intake volume was recorded by noting the starting and finishing volumes in the graduated tubes, recorded to the nearest half mL. Microstructural lick variables are defined by pauses between licks, as shown in Figure 1 from Davis & Perez (1993).

The QuickLick program (Davis and Smith, 1992) identified a burst of licking as a run of licks before a pause of more than 250 msec. Bursts separated by pauses of >250 msec are grouped into clusters (of bursts). Time between clusters is >500 msec.

   Data were analyzed across each phase with a mixed design analysis of variance (ANOVA) procedure. The within-subjects factor was Day (at five levels; 1, 2…5; for each phase). For group data collapsed across days, a between-subjects factor ANOVA was used. In all analyses the between subjects factor was Salt (at two levels; NaCl or LiCl) and Sucrose Concentration (at four levels; 0.05 M, 0.10 M, 0.25 M, or 0.50 M). Significant interactions were subsequently investigated with Tukey's HSD test of

pairwise comparisons, and $\alpha = 0.05$ was used as criterion for significance.

3.3 Results

3.3.1 Acquisition Phase

3.3.1.1 Intake volume (mL). Group mean volume consumption of a solution containing 0.02 M LiCl or 0.02 M NaCl with varying levels of sucrose is depicted in Figure 3.1A/B. The mixed-design repeated measures ANOVA revealed a significant Day X Salt interaction, $F(4,133) = 10.28$, $p < .001$, and a main effect of Day, $F(4,133) = 4.01$, $p < .01$. Overall, rats drinking solutions containing 0.02 M LiCl, consumed less than rats drinking 0.02 M NaCl solutions. A significant Salt X Sucrose interaction was also found, $F(3,42) = 5.48$, $p < 0.01$. Post-hoc comparisons revealed a significant suppression of drinking for all LiCl + Sucrose groups, except the LiCl + 0.50 M Sucrose group. This group did not consistently differ from its control as it drank less than its control only on day 2, $p < 0.05$. There was also a main effect of Salt, $F(1,42) = 162.85$, $p < .001$, but no main effect of Sucrose, $F(3,42) <1$.

Collapsing across days, the total group mean volume consumption for the entire acquisition phase (five days) is presented in Figure 3.2A. All of the groups drinking LiCl, except LiCl + 0.50 M Sucrose, showed a significant suppression of drinking when compared to their NaCl controls, ps $< .001$.

3.3.1.2 Number of Licks. Group mean numbers of licks for a solution containing 0.02 M LiCl or 0.02 M NaCl with varying levels of sucrose are depicted in Figure 3.3A/B. The ANOVA revealed a significant Day X Drug interaction ($F(4,159) = 13.26$, $p < .001$), as well as a significant Drug X Sucrose interaction ($F(3,42) = 5.67$, $p < .01$). Post- hoc comparisons showed a significant decrease in the number of licks for all groups drinking LiCl + Sucrose, except the LiCl + 0.50 M Sucrose group, which had fewer licks than its control only on day 2. No main effect of Day or Sucrose was found; $F(4,160) <1$, $F(3,42) <1$, respectively.

Total group mean number of licks data for the entire acquisition phase are presented in Figure 3.4A. Overall, groups drinking a LiCl + Sucrose solution had signicantly fewer licks when compared to their corresponding NaCl + Sucrose control, ps$<.01$, except the LiCl + 0.50 M Sucrose group, which was similar to its NaCl control.
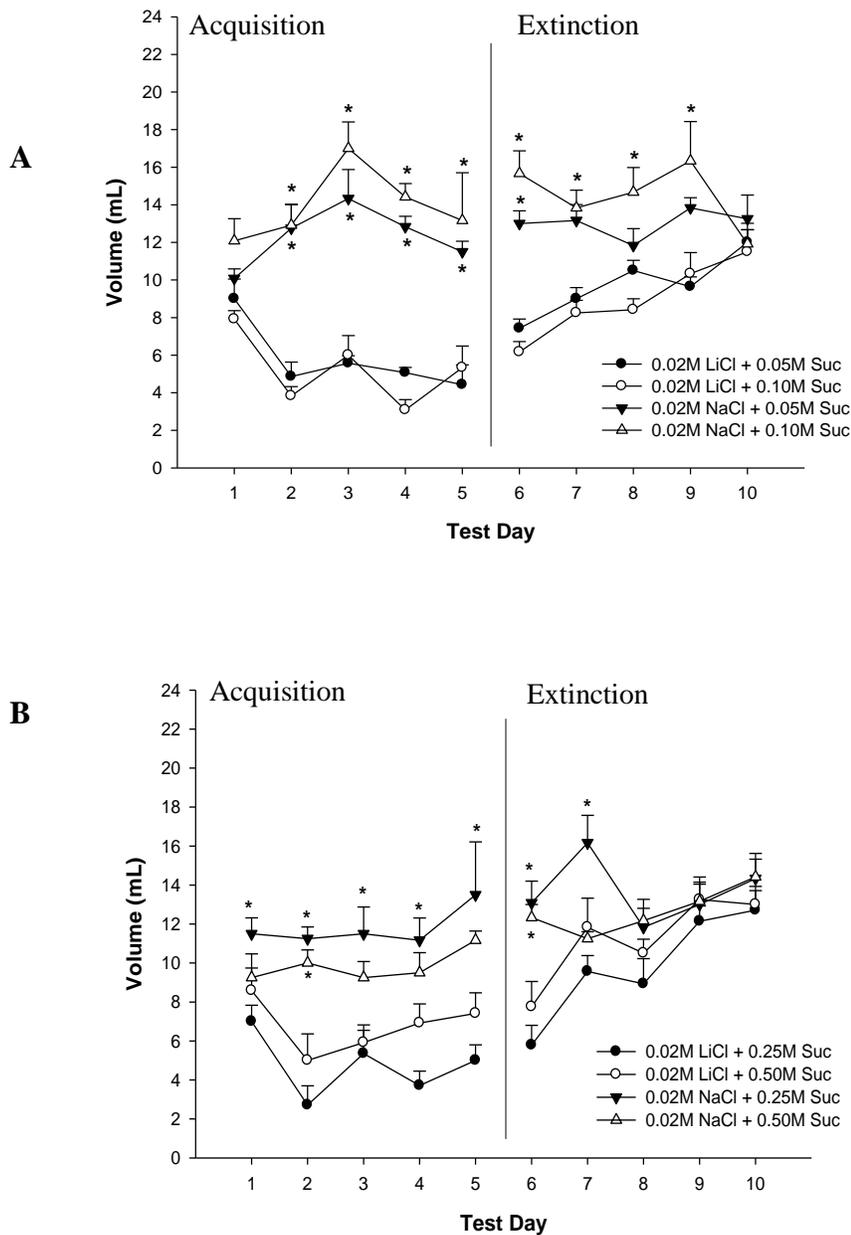
Figure 3.1. Volume intake (mL). Group mean volume intake (mL) + SEM across both the acquisition phase (days $1 - 5$) and extinction phase (days 6-10). **(A)** Groups drinking 0.05 M sucrose + LiCl (n=7) and 0.05 M Sucrose + NaCl (n=6), and groups drinking 0.10 M Sucrose with either NaCl or LiCl (n=6/group). **(B)** Groups drinking 0.25 M sucrose + LiCl (n=7) or NaCl (n=6) and 0.50 M sucrose solutions containing either LiCl (n=6/group) or NaCl (n=6/group). Significant differences indicated between corresponding LiCl and NaCl groups, $*p < .05$.
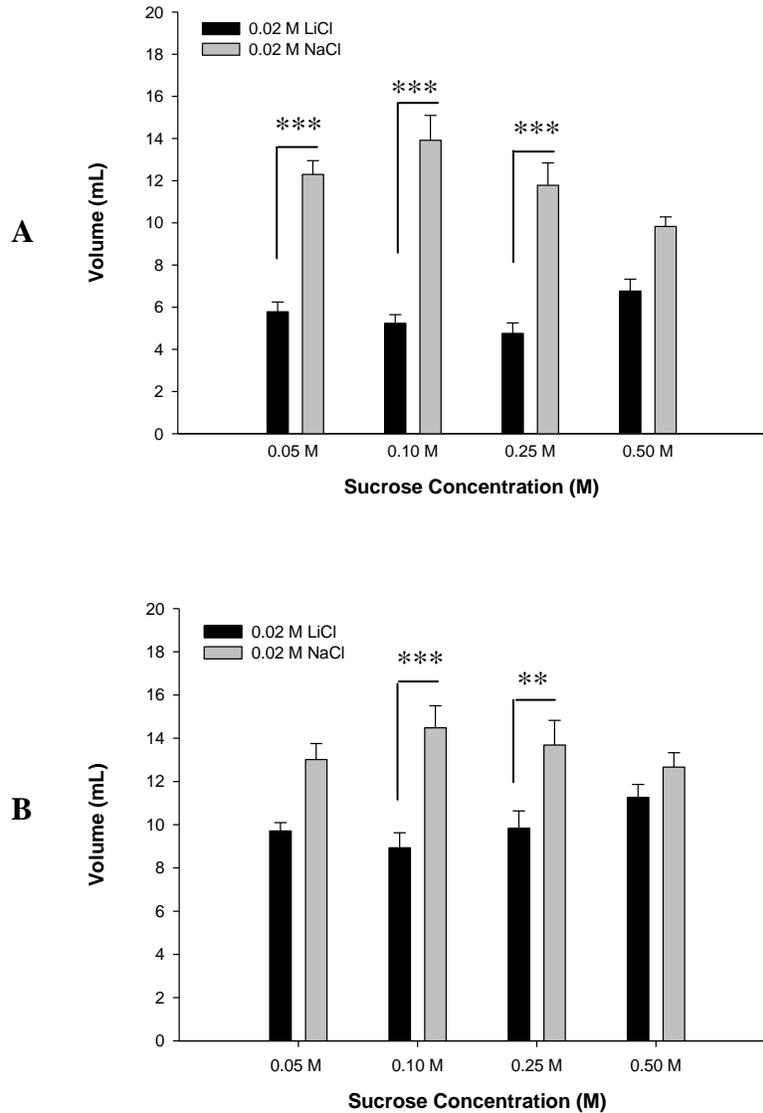
Figure 3.2. Total phase intake (mL). Total group intake (mL) + SEM for **(A)** acquisition phase and **(B)** extinction phase, of rats drinking a solution of 0.05 M sucrose + LiCl (n=7), 0.05 M sucrose + NaCl (n=6), 0.10 M sucrose + NaCl or LiCl (n=6/group), 0.25 M sucrose + LiCl (n=7), 0.25 M sucrose + NaCl (n=6) or 0.50 M sucrose + NaCl or LiCl (n=6/group). Significant differences between corresponding LiCl and NaCl groups are indicated, *$p < .05$, **$p < .10$, ***$p < .001$.
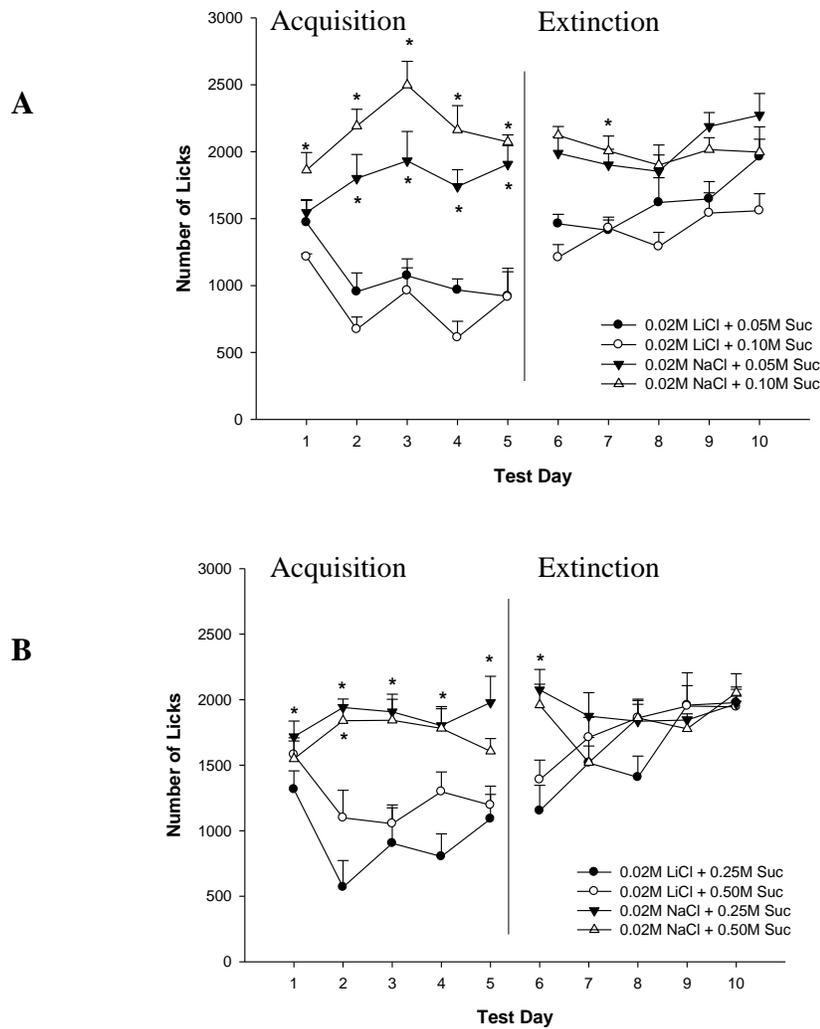
Figure 3.3. Number of licks. Group mean number of licks + SEM across both the acquisition phase (days 1 – 5) and extinction phase (days 6-10). **(A)** Groups drinking 0.05 M sucrose + LiCl (n=7) and 0.05 M Sucrose + NaCl (n=6), and groups drinking 0.10 M Sucrose with either NaCl or LiCl (n=6/group). **(B)** Groups drinking 0.25 M sucrose + LiCl (n=7) or NaCl (n=6) and 0.50 M sucrose solutions containing either LiCl (n=6/group) or NaCl (n=6/group). Significant differences indicated between corresponding LiCl and NaCl groups, *p* < .05.
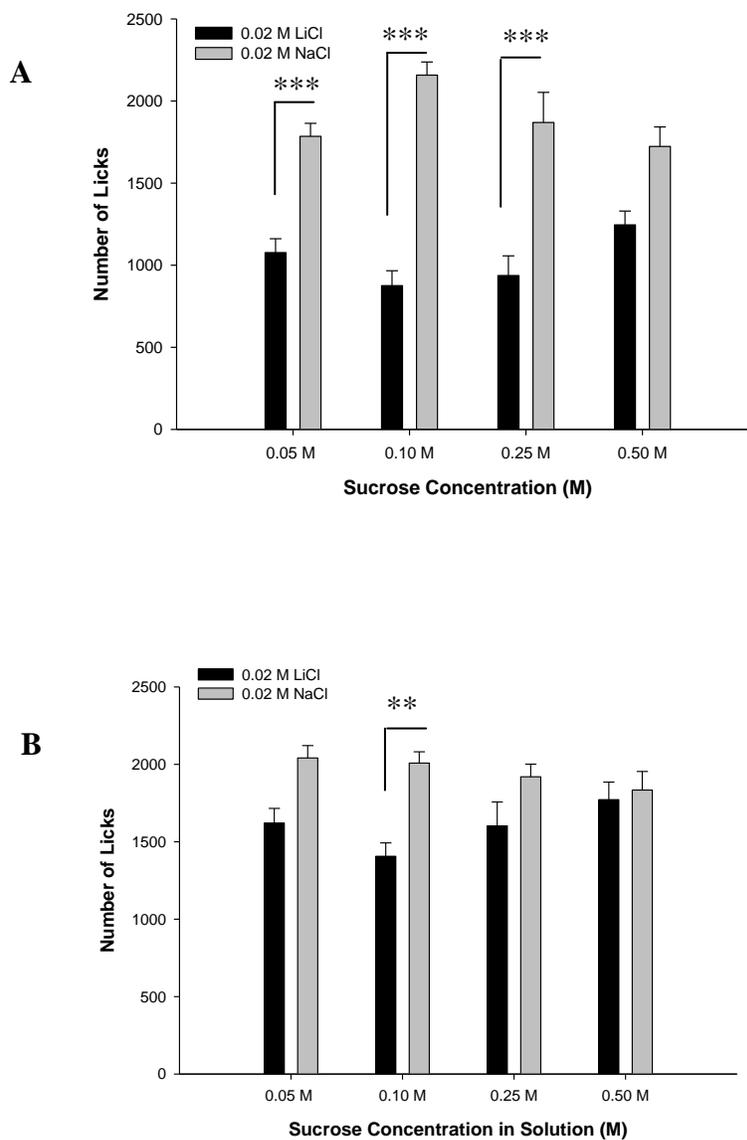
Figure 3.4. Total phase number of licks. Total mean number of licks + SEM for **(A)** acquisition phase and **(B)** extinction phase, of rats drinking a solution of 0.05 M sucrose + LiCl (n=7), 0.05 M sucrose + NaCl (n=6), 0.10 M sucrose + NaCl or LiCl (n=6/group), 0.25 M sucrose + LiCl (n=7), 0.25 M sucrose + NaCl (n=6) or 0.50 M sucrose + NaCl or LiCl (n=6/group). Significant differences between corresponding LiCl and NaCl groups are indicated, *$p < .05$, **$p < .10$, ***$p < .001$.

3.3.1.3 Number of Clusters. Group means for number of clusters, while drinking a solution containing 0.02 M LiCl or 0.02 M NaCl with varying levels of sucrose are depicted in Figure 3.5A/B. The ANOVA revealed only a main effect of Day, $F(4,120) = 6.69$, $p < .001$, as each group showed a different pattern of cluster number across days. No main effects of Salt or Sucrose were obtained, $F(1,42) < 1$, $F(3,42) < 1$, respectively.

Total group mean number of clusters for the entire acquisition phase (five days) is presented in Figure 3.6A. There were no significant differences between groups drinking LiCl and their corresponding NaCl controls.

3.3.1.4 Size of Clusters. Group mean cluster sizes while drinking a solution containing 0.02 M LiCl or 0.02 M NaCl with varying levels of sucrose are depicted in Figure 3.7A/B. The mixed-design repeated measures ANOVA revealed a significant interaction of Day X Sucrose ($F(10,136) = 2.07$, $p < .05$) and Day X Salt ($F(4,136) = 3.55$, $p < .05$). There was also a significant Salt X Sucrose interaction, $F(3,42) = 4.44$, $p < .01$. Post-hoc comparisons revealed a significant decrease in cluster size on at least two days for rats drinking LiCl, except the LiCl + 0.05 M Sucrose group, which never differed from its control group, ps $< .05$. A significant main effect of Day was also recorded, $F(4,136) = 8.56$, $p < .001$, but no main effect of Sucrose, $F(3,42) < 1$.

Total group mean data collapsed across days for cluster size for the entire acquisition phase (five days) are presented in Figure 3.8A. In total, the only group to not show significantly smaller cluster sizes compared to their NaCl controls was LiCl + 0.05 M Sucrose, ps $< .01$.

3.3.2 Extinction

3.3.2.1 Intake volume (mL). Group mean volume consumption of a solution containing 0.02 M NaCl and varying levels of sucrose, after drinking a sucrose solution of either 0.02 M LiCl or 0.02 M NaCl in the previous phase, are depicted in Figure 3.1A/B (days 6-10). The ANOVA revealed a significant three way interaction between Day, Salt, and Sucrose Concentration for volume consumed across days, $F(11,152) = 3.37$, $p < .001$. Post-hoc comparisons showed significantly less consumption compared to NaCl controls, on Days 6-9 for rats drinking LiCl + 0.10 M Sucrose in the acquisition phase, and less consumption for LiCl + 0.25 M Sucrose groups on Days 6-7, ps $< .05$. Additionally, both the Day X Salt interaction and the Day X Sucrose interaction were
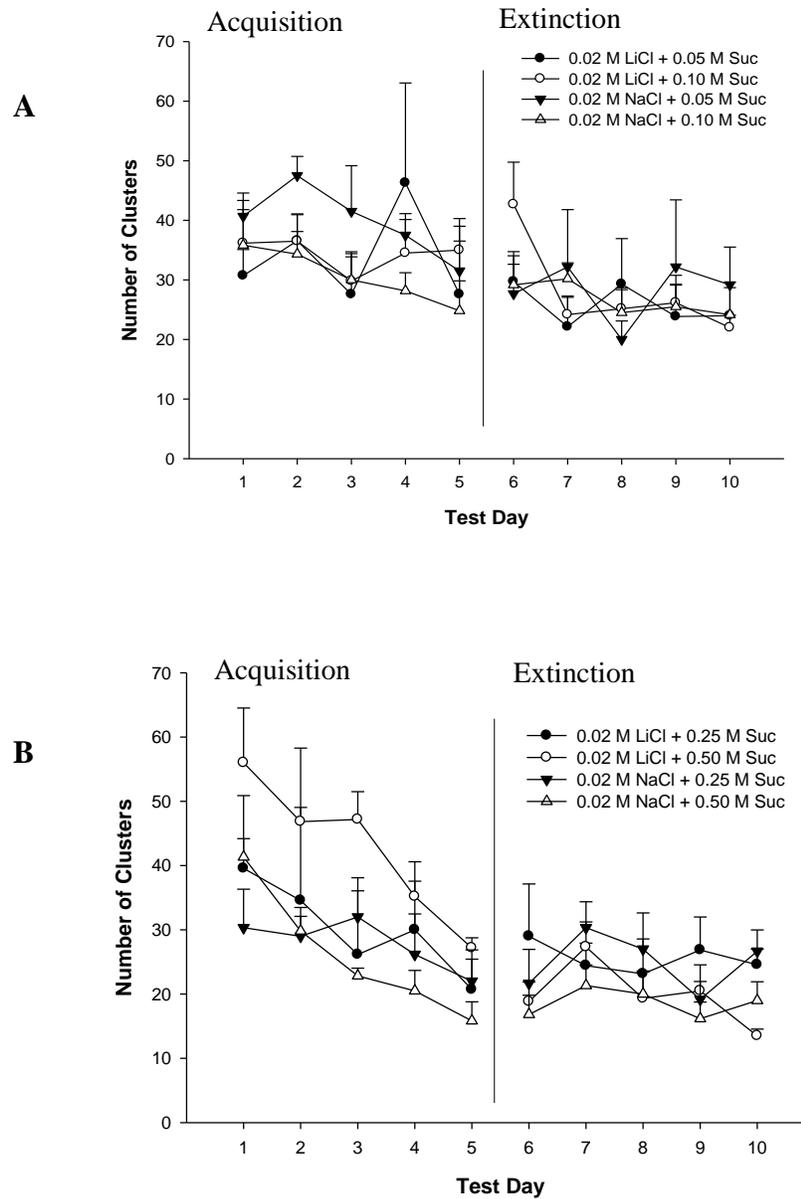
Figure 3.5. Number of clusters. Group mean number of clusters + SEM across both the acquisition phase (days 1 − 5) and extinction phase (days 6-10). **(A)** Groups drinking 0.05 M sucrose + LiCl (n=7) and 0.05 M Sucrose + NaCl (n=6), and groups drinking 0.10 M Sucrose with either NaCl or LiCl (n=6/group). **(B)** Groups drinking 0.25 M sucrose + LiCl (n=7) or NaCl (n=6) and 0.50 M sucrose solutions containing either LiCl (n=6/group) or NaCl (n=6/group). Significant differences indicated between corresponding LiCl and NaCl groups, *$p < .05$.
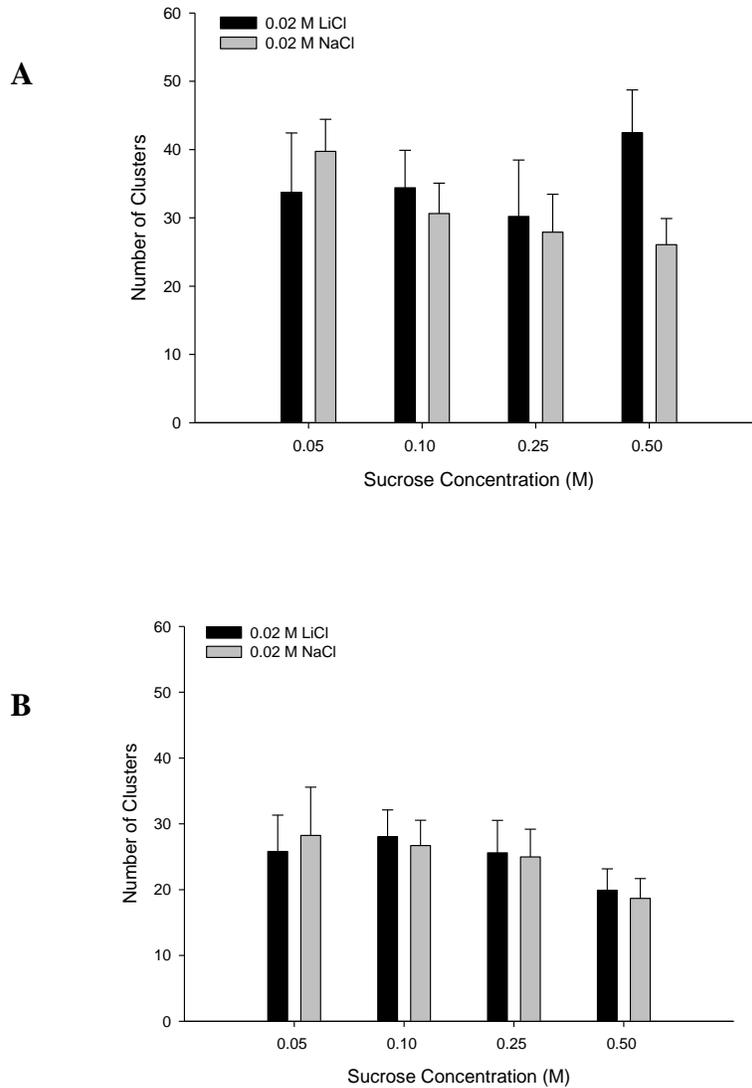
Figure 3.6. Total phase number of clusters. Total number of clusters + SEM for **(A)** acquisition phase and **(B)** extinction phase, of rats drinking a solution of 0.05 M sucrose + LiCl (n=7), 0.05 M sucrose + NaCl (n=6), 0.10 M sucrose + NaCl or LiCl (n=6/group), 0.25 M sucrose + LiCl (n=7), 0.25 M sucrose + NaCl (n=6) or 0.50 M sucrose + NaCl or LiCl (n=6/group). Significant differences between corresponding LiCl and NaCl groups are indicated, *$p < .05$, **$p < .10$, ***$p < .001$.
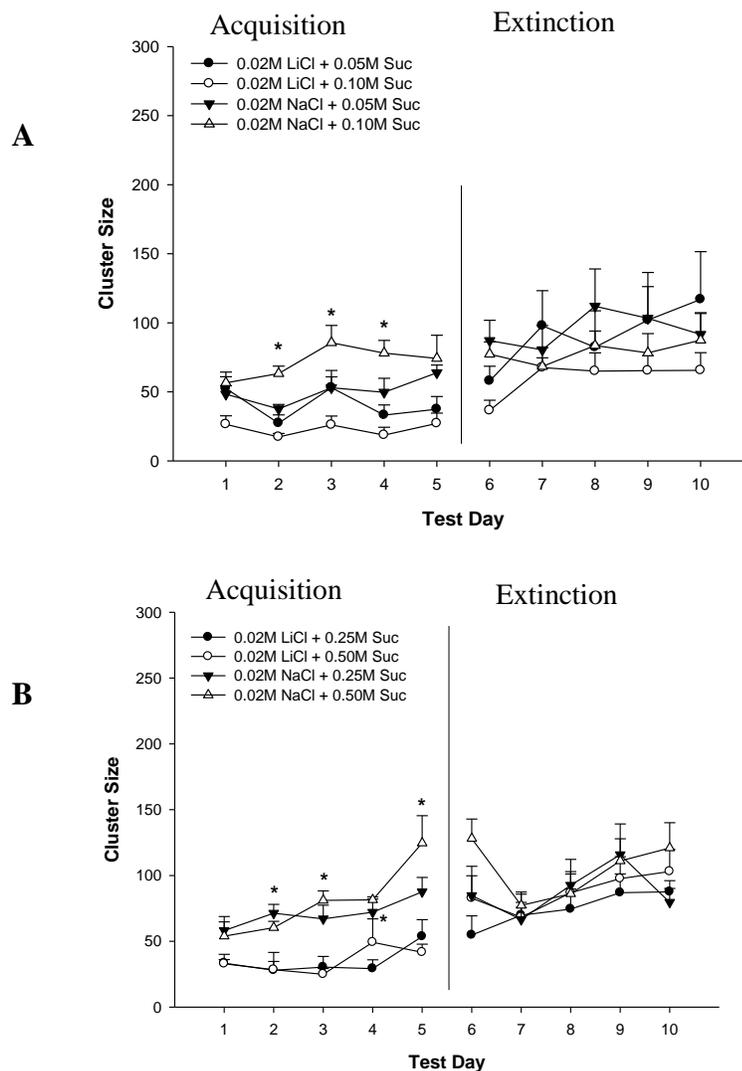
Figure 3.7. Cluster size. Group mean cluster size + SEM across both the acquisition phase (days 1 − 5) and extinction phase (days 6-10). **(A)** Groups drinking 0.05 M sucrose + LiCl (n=7) and 0.05 M Sucrose + NaCl (n=6), and groups drinking 0.10 M Sucrose with either NaCl or LiCl (n=6/group). **(B)** Groups drinking 0.25 M sucrose + LiCl (n=7) or NaCl (n=6) and 0.50 M sucrose solutions containing either LiCl (n=6/group) or NaCl (n=6/group). Significant differences indicated between corresponding LiCl and NaCl groups, *$p < .05$.
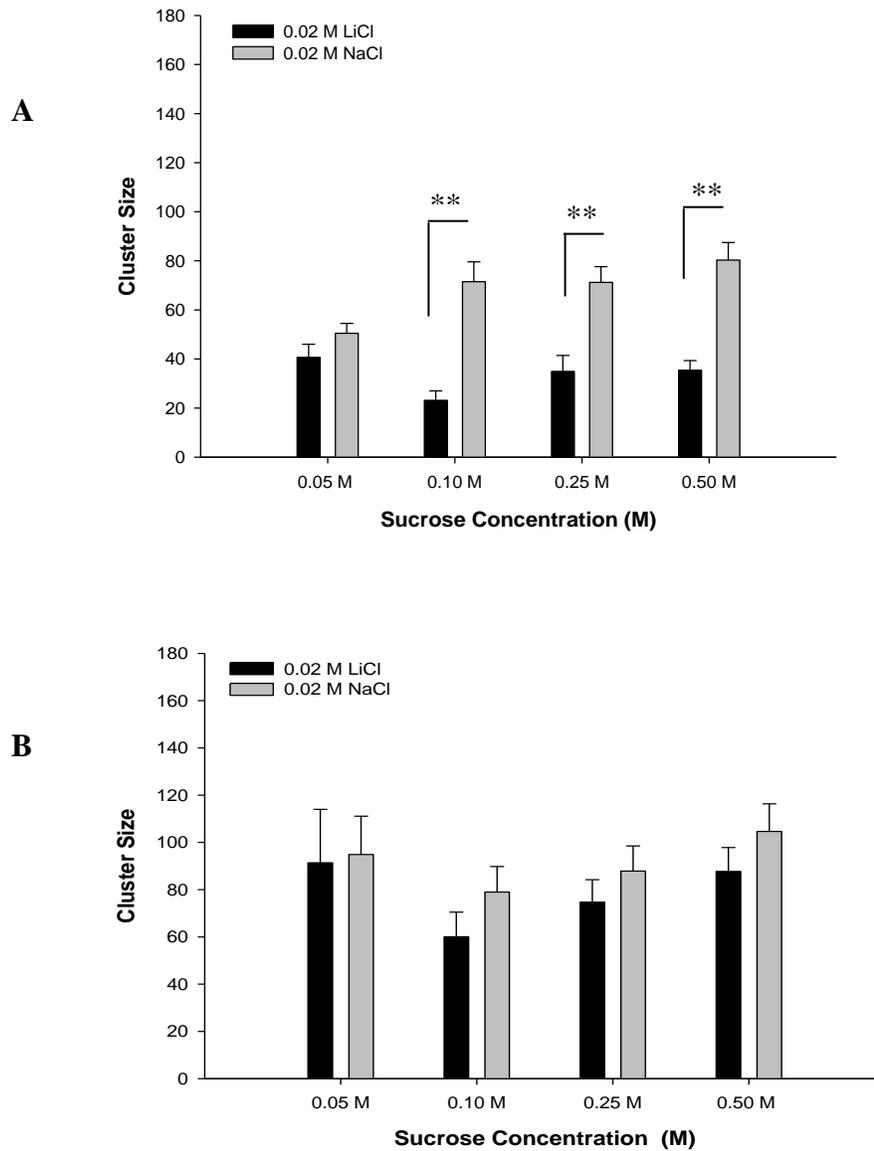
Figure 3.8. Total phase cluster size. Total cluster size + SEM for **(A)** acquisition phase and **(B)** extinction phase, of rats drinking a solution of 0.05 M sucrose + LiCl (n=7), 0.05 M sucrose + NaCl (n=6), 0.10 M sucrose + NaCl or LiCl (n=6/group), 0.25 M sucrose + LiCl (n=7), 0.25 M sucrose + NaCl (n=6) or 0.50 M sucrose + NaCl or LiCl (n=6/group). Significant differences between corresponding LiCl and NaCl groups are indicated, *$p <$ .05, **$p <$ .10, ***$p <$ .001.

found to be significant, ($F(4,152) = 16.85$, $p < .001$; $F(11,152) = 2.42$, $p < .001$). Both groups drinking LiCl + 0.05 M Sucrose and LiCl + 0.50 M Sucrose in the acquisition phase, consumed less NaCl solution compared to their controls, only on Day 6. A significant main effect of Day was also found, $F(4,152) = 20.77$, $p < .001$. Between groups a significant main effect of Salt was found, $F(1,42) = 40.98$, $p < .001$, but not Sucrose, $F(3,42) < 1$. All LiCl groups consumed significantly less compared to their controls on Day 6. The LiCl + 0.10 M Sucrose group showed an extended extinction period, drinking less compared to the NaCl control on days 6 through 9, $p < .05$.

Data for total group mean volume consumption for the entire extinction phase (five days) are presented in Figure 3.2B. Overall, the 0.10 M and 0.25 M Sucrose groups that were presented with LiCl + Sucrose in the acquisition phase, consumed less than their controls when drinking the NaCl + Sucrose solution in the extinction phase.

3.3.2.2 Number of Licks. Group mean numbers of licks for a solution containing 0.02 M NaCl and varying levels of sucrose, after drinking a sucrose solution of either 0.02M LiCl or 0.02 M NaCl in the previous phase, is depicted in Figure 3.3A/B (Days 6-10). The ANOVA revealed a significant three way interaction between Day, Salt, and Sucrose Concentration for number of licks across days, $F(12,160) = 1.94$, $p < .05$. Additionally, a significant Day X Salt interaction was found, $F(4,160) = 9.24$, $p < .001$. Post-hoc comparisons showed significantly fewer licks on Day 2 for the LiCl + 0.10 M Sucrose group and on Day 1 for the LiCl + 0.25 M Sucrose group, ps $< .05$. Significant main effects of Day, $F(4,160) = 13.84$, $p < .001$, and Salt, $F(1,42) = 21.69$, $p < .001$, were also found. Depending on the Salt consumed in the acquisition phase (LiCl vs NaCl), rats showed differing patterns in number of licks throughout the extinction phase.

Data for group mean numbers of licks for the entire extinction phase (five days) are presented in Figure 3.4B. Only the group that consumed LiCl + 0.10 M Sucrose in phase 1, showed significantly fewer number of licks compared to its NaCl control that received NaCl + 0.10 M Sucrose in both phases.

3.3.2.3 Number of Clusters. Group mean numbers of clusters while drinking a sucrose solution containing 0.02 M NaCl, after drinking a sucrose solution of either 0.02 M LiCl or 0.02 M NaCl in the previous phase, are depicted in Figure 3.5A/B (Days 6-10). The ANOVA revealed only a significant Day X Salt interaction, $F(4,154) = 2.91$, $p < .05$.

Across days, the number of clusters changed differently depending on the Salt consumed during the acquisition phase. No main effects of Day, Salt, or Sucrose were found, $F(4,154) <1$, $F(1,42) <1$, $F(3,42) <1$.

Data for group mean numbers of clusters for the entire extinction phase (five days) are presented in Figure 3.6B. Overall, groups did not differ in their number of clusters for extinction phase.

3.3.2.4 Size of Clusters. Analyses of the group mean cluster size, across days, while drinking a sucrose solution containing 0.02 M NaCl, after drinking a sucrose solution of either 0.02 M LiCl or 0.02 M NaCl in the previous phase, are depicted in Figure 3.7A/B (Days 6-10). The ANOVA revealed a significant Day X Salt interaction, $F(4,129) = 2.73$, $p < .01$ and a main effect of Day, $F(4,129) = 4.35$, $p < .01$. No main effects of Salt or Sucrose were found, $F(1,42) <1$, $F(3,42) <1$, respectively. Like number of clusters, the pattern for size of clusters depended on which Salt rats were presented with in the acquisition phase.

Data for total group mean cluster size for the entire extinction phase (five days) is presented in Fig 3.8B. Overall, groups did not differ in the size of clusters for extinction phase.

3.4 Discussion

I examined the intake and licking behavior of water-deprived rats voluntarily consuming various sucrose solutions, containing either LiCl or NaCl. As expected, the results showed that rats used a trade-off strategy for ingesting LiCl and sucrose, based on the caloric value of the sucrose. The interactions between Salt and Sucrose concentration also indicate that this trade-off is most pronounced when a very high concentration of sucrose is used (0.50 M). Sucrose concentration also interacted with the salt in solution and it was interesting to see that within each phase, NaCl control groups (but not LiCl groups) showed a preference for sucrose similar to the 'U' shaped preference function described in previous research (Sclafani & Nissenbaum, 1987). The groups drinking LiCl did not show this same preference curve, possibly due to the threshold level of toxin, or the aversive effects of the toxin, which are less notable as the sucrose concentration is increased.

Rats are using this trade-off mechanism to balance calories and toxic effects, and

the most robust effects are seen when drinking the 0.02 M LiCl + 0.50 Sucrose solutions, as it has the highest caloric value. In terms of drinking a toxin solution, it would be expected that groups drinking a less preferred concentration of sucrose would limit their intake, but that intake would be modulated further with the added negative effects of the toxin, and intake would be suppressed even more. As previously mentioned, all groups drinking LiCl suppressed their intake, number of licks, and cluster sizes during the acquisition phase. The group drinking 0.02 M + 0.50 M Sucrose had an intake value that was greater than would be expected with a less preferred sucrose solution containing toxins, which could indicate a trade-off for calories versus toxic effects. A similar result was found when 0.0009 M LiCl was injected (i.p.) into food-deprived rats that were subsequently presented with various sucrose solutions (Grigson & Gomez, 1999). When testing with 0.1, 0.3, and 0.5 M sucrose solutions, there was attenuation of LiCl suppressive effects only with rats drinking 0.5 M sucrose (Grigson & Gomez, 1999). In accordance with the current results, these studies show that rats will increase consumption of a taste associated with negative effects, but also that they are willing to do so on a voluntary level, when consuming a toxic solution.

To further assess the idea that these rats are using a trade-off system, and that it is based on the caloric value of the solution and not taste, the microstructural licking variables of cluster size was examined. Cluster size has been reported to show a monotonic increase with increased concentration of sugar solutions, such as sucrose (Davis & Smith, 1992). Examining cluster sizes for the 0.02M LiCl + 0.50 M Sucrose group, revealed that although they consumed approximately the same amount of solution as their NaCl controls, the cluster sizes were smaller for the LiCl group. This difference suggests that although both groups drank the same amount, it was not for reasons solely based on the taste of the solution. During the extinction phase, when all groups were presented with their corresponding NaCl + Sucrose solution, cluster sizes increased to control levels, indicating a shift in palatability, as the solutions were no longer associated with negative post-ingestive feedback effects. These results have also been shown using fructose as the palatable taste, where toxin induced reduction in palatability was also not permanent (Dwyer et al., 2009; Dwyer, 2012).

The pattern of trade-offs seen in the current study are often seen in nature with

various foraging animals. For example, both fruit eating birds and foraging ruminants will consume one food source containing various toxins to maximize the available nutrients, before moving on to another (Schaefer, Schmidt & Winkler, 2003; Duncan et al., 2006; Provenza, 1996). At low toxin concentrations, rats have been shown to utilize a threshold regulation as to maximize calories and minimize toxins (Glendinning, 2007, Good et al., 2013). Previous work in our laboratory has indicated that 0.02 M LiCl is a possible threshold that rats use when drinking a toxin + sucrose solution (Good et al., 2013). The post-ingestive feedback rats received while ingesting the 0.02 M LiCl, could have a range of succinct effects on gastrointestinal processing. If the toxic food had no other added benefits than hydration, for example, the rat may only consume the food until toxin load reaches the lower limit of this range. If there are added benefits, such as calories, the rat may be willing to use this trade-off system and increase consumption to the upper limit of its toxin load.

In conclusion, there is some evidence that rats will use a trade-off system between calories and toxin. Cross-Mellor et al. (2004) suggest there are two mechanisms for the regulation of toxin intake and avoidance learning; one that is used with high toxin concentrations and one that is used with low toxin concentrations, the strategy that seems to occur in the current study. It is probable that at these low toxin concentrations, the malaise-inducing and gut-related side effects of LiCl are regulating the behavior shown in these rats. Although some previous research has shown similar intake values based on low levels of LiCl, these studies also used forced exposure to the toxin (via i.p. injections). The current study shows that rats voluntarily trade-off between calories and toxins, and they are willing to ingest a larger amount of toxin to gain calories.

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Chapter 4

TOXIN-NUTRIENT TRADE-OFFS: EXAMINING THE EFFECTS OF CALORIC VALUE ON RATS' TOLERANCE OF LOW DOSE LICL

4.1 Introduction

Conditioned taste avoidance (CTA) is an innate learning mechanism, which allows organisms to make associations between novel tastes or flavors and the accompanying negative sensory and post-ingestive effects of that food. When a novel taste is paired with nausea-inducing effects, most species, such as rodents, ruminants, or humans, will avoid consuming this flavor upon further presentations (Bernstein, 1999; Bures, Bermúdez-Rattoni, & Yamamoto, 1998; Garcia, Kimeldorf, & Koelling, 1955; Garcia et al., 1985; Provenza, 1995). Classical avoidance behavior often produces complete avoidance of the conditioned food, but this occurs when high concentrations of toxins are used (Cross-Mellor, Clarke, & Ossenkopp, 2004; Good, Kavaliers, & Ossenkopp, 2013; Nachman & Ashe, 1973). Most of the previous research done using the CTA paradigm controls the amount of toxin introduced to the subject by using injection protocols (i.e. Bernstein, Chavez, Allen, & Taylor, 1992; Cross-Mellor, et al., 2004; Grigson & Gomez, 1999; Nachman & Ashe, 1973). However, low concentrations of toxins are much more likely to be encountered in nature, and foraging animals can voluntarily control the intake of the toxins they encounter. For foraging animals, diets are usually made up of a variety of food sources, each with varying types of toxins and toxin concentrations (Pfister et al., 2010; Provenza, 1996; Provenza et al., 1998). Non-emetic species, such as the omnivorous rat, have highly tuned sensory and post-ingestive defense mechanisms to avoid over loading on any one toxin-containing food. These conditions can be mimicked in the lab using a CTA paradigm with very low levels of toxins such as lithium chloride (LiCl).

Lithium chloride is a commonly used toxin in avoidance and aversion studies as it produces dose-dependent conditioned suppression of food intake, and has been shown to activate a multitude of central neural circuits that underlie avoidance learning and gastric emptying (Bernstein et al., 1992; Cross-Mellor et al., 2004; Good et al., 2013; McCann et al., 1989; Nachman & Ashe, 1973; Ossenkopp & Eckel, 1995; Rinaman & Dzmura, 2007). The dose-dependent intake suppression associated with LiCl suggests that its negative post-ingestive effects are concentration dependent. A study using food-deprived rats examined the suppressive effects of LiCl when it was paired with sucrose. Researchers found that attenuating effects on intake of a sucrose solution were dependent

on the caloric value offered from the conditioning stimulus (Gomez & Grigson, 1999). These researchers found that when rats were presented with a sucrose solution (0.1 M, 0.3 M or 0.5 M) and then injected (i.p.) with extremely low concentrations of LiCl (0.009 M), their resulting intake was dependent on the concentration of the sucrose solution. The suppressive effects of LiCl disappeared when the sucrose concentration was 0.5 M, as both test and control groups' intake was similar. It was suggested that this effect was due to the increased calories of the sucrose solution outweighing the negative effects of LiCl (Gomez & Grigson, 1999). Results such as these could be an indication of a behavioral mechanism producing a trade-off between toxins and nutrients when consuming a single toxic food source.

Intake is often based on the integration of nutritional and hedonic values, where nutritional values refer to keeping a balanced homeostatic state and hedonic values are associated with pleasurable sensory properties of a food. Various studies have shown that animals learn to associate the flavors/tastes with specific nutrients and caloric value, as indicated by post-ingestive feedback (Beeler et al., 2012; Glendinning et al., 2010; Lucas & Sclafani, 1989; Perez et al., 1999). Others have shown that intake is based on the motivational state of the animal (Scheggi et al., 2013). These studies use calorie-dense solutions, such as sucrose, and compare them to non-caloric sweeteners, such as saccharin. Further, Beeler et al. (2012) suggest that the nutritional and hedonic value of food can independently affect consumption, but when examined in concert, the positive effects of a solution are synergistically increased.

Parker (2003) proposed that nausea might be irrelevant to taste avoidance learning, but needed for the establishment of conditioned 'dislike' or rejection of a taste. Like or dislike of a taste is represented by the palatability and hedonic value of a solution, which can be inferred from both consumption and the microstructural analysis of licking patterns (Baird, St. John, & Nguyen, 2005; Dwyer, 2009; Weijnen, 1998). When ingesting a liquid food source, rats lick in rapidly occurring rhythmic tongue movements that occur in bursts (Davis, 1973). Microstructural analysis of licking patterns includes number of bursts, and number of clusters, and the size of bursts and clusters. Bursts and clusters of licks are defined by the types of pauses between lickings. By incorporating the toxin directly into a palatable solution, the microstructure of licking can

offer information related to the animals' reaction to the hedonic value of the solution in ways that are not possible with other techniques, such as taste-reactivity tests (Dwyer, Pincham, Thein, & Harris, 2009). Previous research has also shown that cluster size is independent of consumption and that the mean size of the cluster shows a positive monotonic relationship with the concentration of a palatable fluid (Davis & Smith, 1992; Dwyer et al., 2009). Palatability, or hedonic value of a solution, can be determined by comparing the cluster sizes between rats drinking a sucrose or saccharin solution containing LiCl.

In looking at this compounding effect of palatability with calories, I chose to investigate the effects of consuming a toxin containing solution with either dense calories or no calories. Sucrose preference in rats exhibits an inverted "U" shape, with most consumption occurring at intermediate concentrations (~0.10 - 0.25 M), with preference declining around 0.50 M (Sclafani & Nissenbaum, 1987). Sucrose is calorie dense and provides post-ingestive feedback as it is consumed. Consumption tends to decrease across time because caloric needs are being met (Smith, 2000). In my previous experiment a trade-off between toxin and sucrose at 0.50 M was observed, suggesting that caloric load has to be relatively high for it to outweigh the toxic effects of LiCl (Chapter 3). Saccharin on the other hand, is a calorie-free sweetener, which shows a slightly different licking pattern when ingested. When drinking a saccharin solution, rats lick at a sustained rate across time, whereas when drinking sucrose, rats lick faster at first, and the number of licks declines across time (Smith, et al., 1987; Smith, 2000). The differences in the pattern of intake could reflect differences in post-ingestive feedback from the two sweeteners.

Due to the nature of foraging, there are many behavioral strategies animals can adopt to avoid or reduce toxin. The present study examined the use of toxin threshold regulation, in which animals voluntarily tolerate a toxin load up to a certain level before stopping consumption (Glenndinning, 2007). Rats, ruminants, and humans, have all been shown to use this mechanism to some degree (Chiwona-Karltun, et al., 2000; Glenndinning, 2007; Jones, 1998; Pfister et al., 2010; Provenza, 1996). Since the basic idea of threshold regulation is to maximize calories/nutrients while minimizing toxin levels, it was of interest to determine to what extent animals will trade-off calories for

tolerating a higher toxin load. Trade-off strategy here refers to the willingness of the rat to increase its toxin load, by consuming a greater amount of solution in to maximize its caloric intake. Results of prior studies found that at low concentrations of LiCl (0.02 M), rats will trade-off the negative effects of LiCl and consume more of a toxic sucrose solution, only when the sucrose concentration is high enough (0.50 M) (Chapter 3). Although consumption was higher than controls, palatability indicators were lower. This suggests the trade-off was based on the caloric value of the solution, rather than taste (Chapter 3).

It was hypothesized that rats presented with a toxic saccharin solution would show less consumption and evidence of decreased palatability, compared to rats drinking a toxic sucrose solution. That is, rats drinking the calorie free saccharin would not exhibit trade-off behavior, because there is no added benefit of consuming an increased amount of the toxin. Also, rats drinking the toxic sucrose solution were expected to exhibit increased consumption, but still show a LiCl-induced conditioned decrease in behaviors indicative of the palatability of the solution.

4.2 Methods

4.2.1 Animals

Thirty-two naïve adult male Long-Evans rats (Charles River, Quebec, Canada), weighing 200 - 225 g at the start of the experiment were used. Rats were individually housed in polypropylene cages, in a temperature controlled colony room maintained at 21 ± 1º C, with a 12-h light: 12-hr dark schedule (lights on at 07:00 h). Food (Prolab rat chow) and water were available ad libitum unless otherwise noted. The experimental methodology was carried out according to the Canadian Council on Animal Care guidelines and was approved by the Institutional Animal Care Committee.

4.2.2 Apparatus

An automated lickometer was used during testing (Contact 108 lick analysis system, Dilog Instruments, Tallahassee, Fl). Eight clear Plexiglas chambers (31cm X 31cm X 40 cm) with Plexiglas lids containing air holes were used. Each chamber had a 100 mL graduated drinking tube mounted and centered on the front, with the spout being 8 cm above chamber floor. The drinking tube was accessible through an oval opening, in which only the rats tongue could make contact, and would not alter the tongue and jaws'

natural movements. Each time the rat's tongue came into contact with the drinking spout, a non-detectable current (~60 nA) passed through the spout to complete an electric circuit, registering one lick. Signals were amplified before being stored on a desktop computer for compilation of licking measures (QLick, version 4.0).

4.2.3 Procedure

4.2.3.1 Water Restriction and Habituation. Over a period of five days, a split water deprivation schedule was established. After 24h with no access to water, animals had access to water for 30 minutes, twice daily. For the first three days water access occurred in their home cages, first between 0900 h–1100 h, and then again between 1400 h–1600 h. Over the next two days, animals were habituated to the general testing procedure and lickometer testing chambers. For 20 minutes a day, access to water was given in these boxes (between 0900 h – 1100 h). After each test session chambers were cleaned and deodorized using Alconox and baking soda. The habituation phase lasted until drinking levels were stable (approximately 7 mL/session) for all animals. Baseline drinking levels were recorded to ensure groups did not differ in their initial drinking levels. The additional 30 minutes of water access was given in the home cages between 14:00 – 16:00 h.

4.2.3.2 Acquisition Phase. Once habituated, all animals were randomly assigned to one of four different groups. On five days of acquisition training rats were presented with one of the following solutions for 20 minutes/day in the lickometer chambers: 0.02 M LiCl + 0.04 M Saccharin (n = 8); 0.02 M LiCl + 0.58 M Sucrose (n = 8); 0.02 M NaCl + 0.04 M Saccharin (n = 8); 0.02 M NaCl + 0.58 M Sucrose (n = 8) Intake volume, number of licks, number of clusters, and size of clusters were the dependent variables. An additional 30 minutes of water access was again given in the home cages between 14:00 – 16:00 h. These two concentrations were chosen based on previous research that showed equivalent preference of sucrose and saccharin at these concentrations (Messier &White, 1983).

4.2.3.3 Extinction Phase. Over the subsequent five days of testing all rats were given 20 minutes of access to their corresponding control solution (0.02 M NaCl + 0.04 M Saccharin or 0.02 M NaCl + 0.58 M Sucrose) in the lickometer chambers. Intake

volume, number of licks, number of clusters, and size of clusters were again recorded. 30 minutes of water access was again provided at the usual time in the home cages.

4.2.3 Data Analysis

The lickometer system recorded and stored number of licks. The QuickLick program was used to obtain number and size of clusters. Intake volume was recorded by noting the starting and finishing volumes in the graduated tubes, recorded to the nearest half mL. Microstructural lick variables are defined by pauses between licks, as shown in Figure 1 from Davis & Perez (1993).

The QuickLick program (Davis and Smith, 1992) identified a burst of licking as a run of licks before a pause of more than 250 msec but less than 250 msec. Bursts separated by pauses of >250 msec are grouped into clusters (of bursts). Time between clusters is greater than 500 msec.

Data were analyzed across each phase with a mixed design analysis of variance (ANOVA). The within subjects factor was Day (at five levels; 1, 2…5; for each phase). For total acquisition group mean data, the within subjects factor was Familiarity (at 2 levels: Day 1(novel) vs Days 2-5 (familiar)). For total extinction group mean data, the within subjects factor was Time (at 2 levels: day 6(early) vs days 7-10 (late). In all analyses the between subjects factors were Drug (at 2 levels; NaCl or LiCl) and Sweetener (at 2 levels; 0.04 M Saccharin or 0.58 M Sucrose). Significant interactions between factors were subsequently investigated with Tukey's HSD test of pairwise comparisons, and $\alpha = 0.05$ was used as criterion for significance.   Within subject's pairwise comparisons were investigated using Dunnett Test, and $\alpha = 0.05$ was used as criterion for significance.

4.3 Results

4.3.1 Acquisition

4.3.1.1 Volume (mL). Group mean volume consumed for the sucrose or saccharin solutions, containing either LiCl or NaCl, are depicted in Figure 4.1. The mixed-design repeated measures ANOVA revealed a significant interaction between Day and Salt, $F(4,97) = 5.91$, $p < .01$, as well as Day and Sweetener, $F(4,97) = 2.93$, $p < .05$. A significant main effect of Day was also obtained, $F(4,97) = 5.32$, $p < .001$. Post-hoc analyses revealed significant differences between corresponding Saccharin and Sucrose groups only on Day 1; the Suc + LiCl group consumed more than the Sac + LiCl group, and the Suc + NaCl group consumed more compared to the Sac + NaCl group. From Days 1 – 5, both Saccharin and Sucrose groups drinking LiCl consumed less than their corresponding NaCl controls, ps $< .05$. Main effects of both Sweetener and Salt were observed, $F(1,28) = 5.78$, $p < .05$, and $F(1,28) = 96.41$, $p < .001$.

Comparison of Day 1 group (novel) means with the combined means of Days 2 -5 (familiar), (ANOVA) revealed a significant interaction between Familiarity and Sweetener ($F(1,28) = 16.25$, $p < .001$), as well as Familiarity and Salt ($F(1,28) = 30.74$, $p < .001$). As seen in Figure 4.2A, pairwise comparisons revealed that the Suc + LiCl group consumed significantly more on Day 1 than on Days 2-5. All other groups consumed *less* on Day 1 compared to Days 2-5. A significant main effect of Familiarity was also recorded, $F(1,28) = 29.26$, $p < .001$. As well as significant main effects of Sweetener ($F(1,28) = 16.13$, $p < .001$) and Salt ($F(1,28) = 66.88$, $p < .001$).

4.3.1.2 Number of Licks. Group mean number of licks, when drinking a sucrose or saccharin solution, containing either LiCl or NaCl, is depicted in Figure 4.3. The ANOVA revealed a significant Day X Salt and Day X Sweetener interaction, $F(4,104) = 6.75$, $p < .001$, and $F(4,104) = 7.43$, $p < .001$, respectively. A significant main effect of Day was also recorded, $F(4,104) = 5.31$, $p < .01$. Post-hoc analyses revealed significant differences between the Saccharin and Sucrose groups, independent of Salt, only on Day 1 (novel). Groups drinking a sucrose solution licked more when compared to their corresponding saccharin group. From Days 2 – 5, both Saccharin and Sucrose groups drinking LiCl licked fewer times than their corresponding NaCl controls, ps $< .05$. Between subjects, a main effect of Salt was observed, $F(1,28) = 86.66$, $p < .001$. No
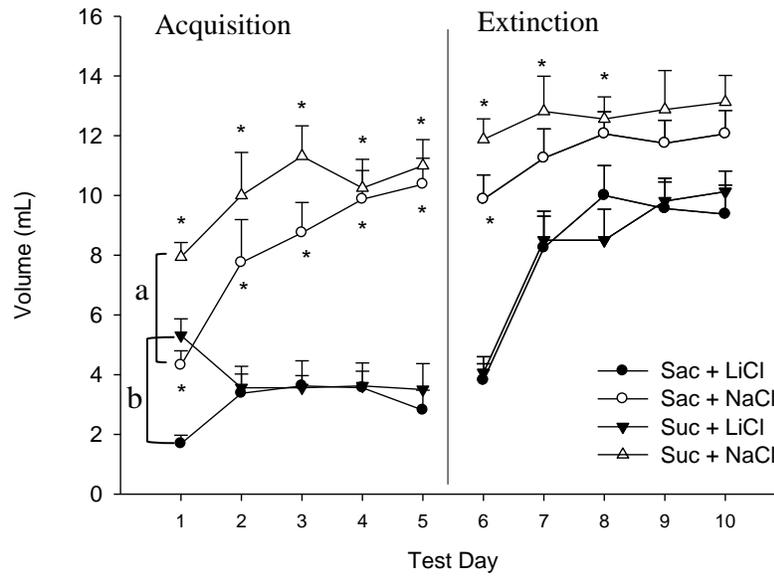
Figure 4.1. **Volume intake (mL).** Group mean volume intake (mL) + SEM across both the acquisition phase (days 1 – 5) and extinction phase (days 6-10). Groups drinking Sucrose or Saccharin solution containing LiCl (n=8/group), and groups drinking Sucrose or Saccharin solution containing NaCl (n=8/group). Significant differences between Sucrose + NaCl and Saccharin + NaCl are indicated by "a". Significant differences between Saccharin + LiCl and Sucrose + LiCl are indicated by "b". Significant differences indicated between corresponding LiCl and NaCl groups, $*p < .05$.
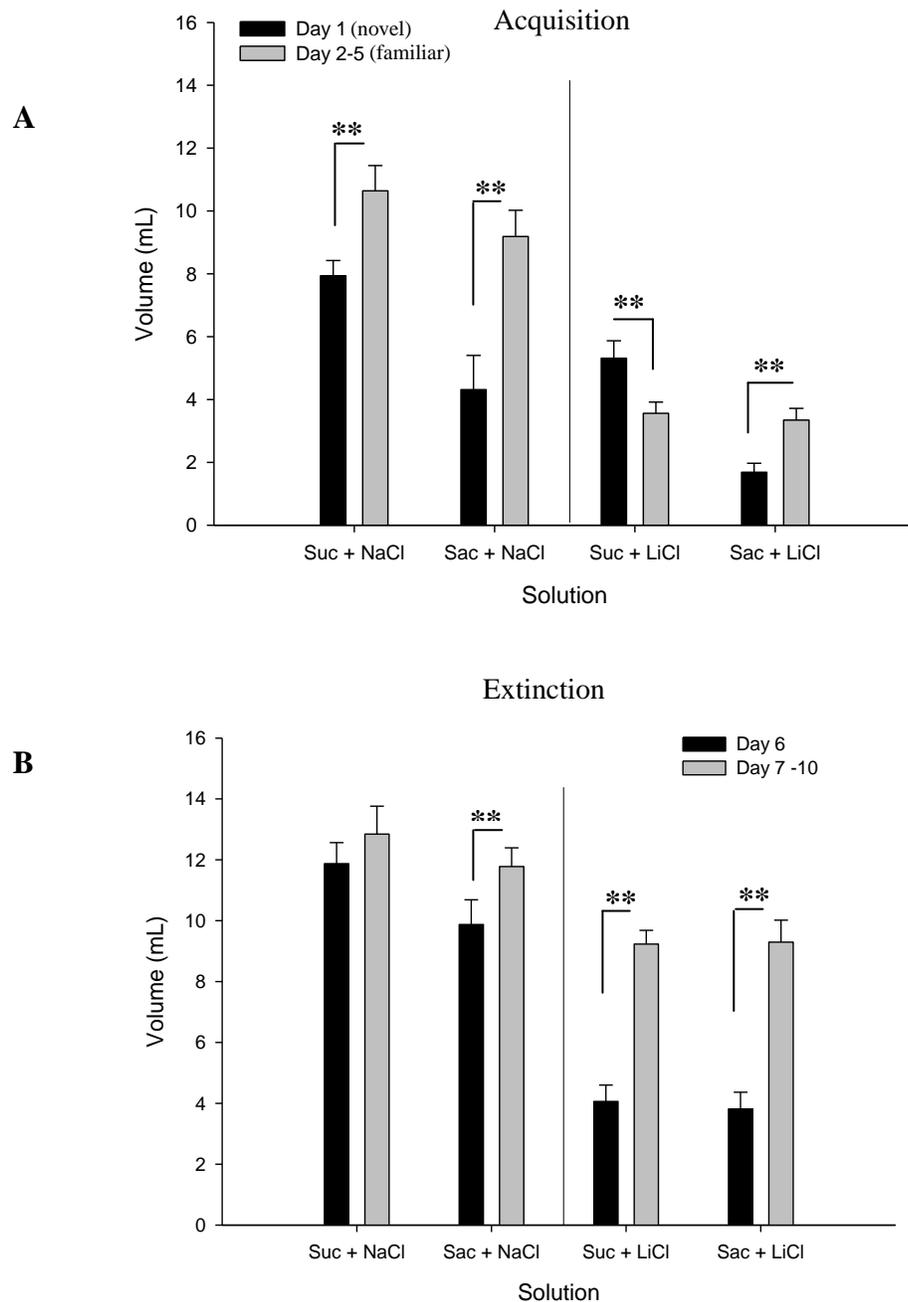
Figure 4.2. Phase mean volume intake (mL). (A) Day 1 mean volume intake (mL) + SEM compared to days 2-5 mean for the acquisition phase, with groups drinking Sucrose + NaCl, Saccharin + NaCl, Sucrose + LiCl, or Saccharin + LiCl (n=8/group). **(B)** Day 6 mean volume intake (mL) + SEM compared to days 7-10 mean for the extinction phase, of rats drinking Sucrose + NaCl or Saccharin + NaCl (n=8/group). Legend refers to solution consumed in acquisition phase. Significant differences between corresponding groups on days 1 vs days 2-5 are indicated, $*p < .05$, $**p < .10$, $***p < .001$.
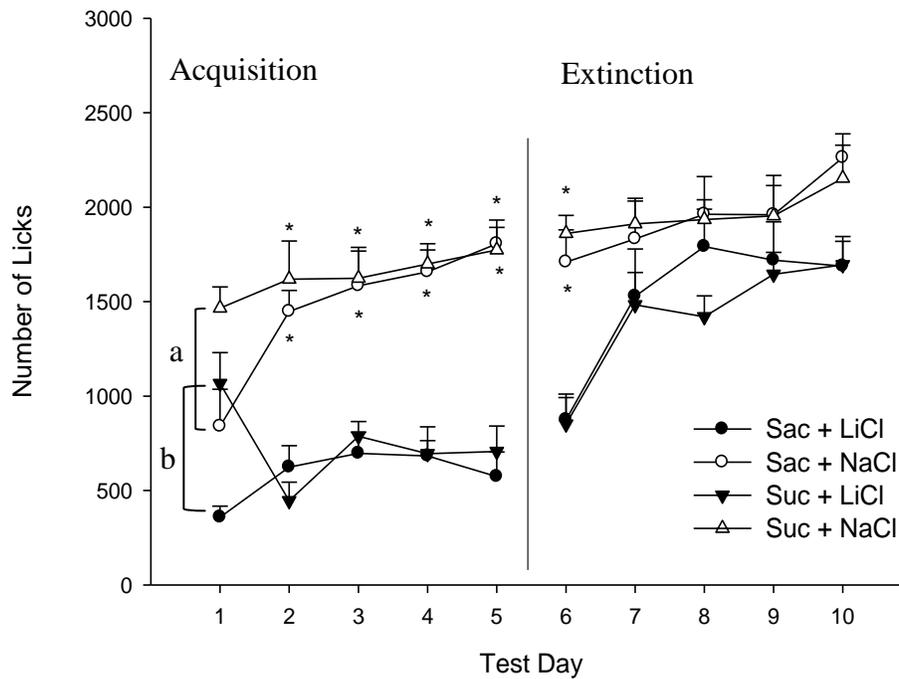
Figure 4.3. **Number of Licks.** Group mean number of licks + SEM across both the acquisition phase (days 1 – 5) and extinction phase (days 6-10). Groups drinking Sucrose or Saccharin solution containing LiCl (n=8/group), and groups drinking Sucrose or Saccharin solution containing NaCl (n=8/group). Significant differences between Sucrose + NaCl and Saccharin + NaCl are indicated by "a". Significant differences between Saccharin + LiCl and Sucrose + LiCl are indicated by "b". Significant differences are indicated between corresponding LiCl and NaCl groups, *$p < .05$.

effect of Sweetener was found, $F(1,28) <1$. Further, in comparing day 1 group (novel) means with the combined means of Days 2 -5 (familiar), the ANOVA revealed significant interactions between Familiarity and Sweetener ($F(1,28) = 22.69, p < .001$), as well as Familiarity and Salt ($F(1,28) = 17.82, p < .001$). As seen in Figure 4.4A, pairwise comparisons revealed that the Sac + NaCl and Sac + LiCl groups licked significant fewer times on Day 1 (novel), compared to Days 2-5 (familiar). Alternatively, the Suc + LiCl group licked *more* on day 1 compared to Days 2-5, ps < .05. No significant differences were recorded for the Suc + NaCl groups. A significant main effect of Familiarity was also recorded, $F(1,28) = 10.87$,

$p < .01$. Between groups, significant main effects of Sweetener ($F(1,28) = 11.52, p < .01$) and Salt ($F(1,28) = 48.61, p < .001$) were also obtained.

4.3.1.3 Number of Clusters. Group mean analyses of number of clusters when licking a sucrose or saccharin solution, containing either LiCl or NaCl, are depicted in Figure 4.5. The ANOVA revealed significant main effects of Day ($F(4,88) = 6.30$, $p < .01$) and Sweetener ($F(1,28) = 10.79, p < .01$). Post-hoc analyses revealed one significant difference between the Saccharin and Sucrose groups drinking LiCl on Day 2, $p < .05$. Unlike previously mentioned variables, number of clusters was affected only by Sweetener type, as no main effect of Salt was found, $F(1,28) <1$.

Comparing Day 1 (novel) group means with the combined means of Days 2 -5 (familiar), the ANOVA revealed a significant main effect of Familiarity ($F(1,28) = 12.76$, $p < .001$), and Sweetener ($F(1,28) = 8.44, p < .01$).   As seen in Figure 4.6A, pairwise comparisons revealed one significant difference between the Suc + LiCl group on Day 1 to Days 2-5, with a greater number of clusters on Day 1, ps < .05. Overall, the groups drinking a saccharin solution tended to exhibit a larger number of clusters. No main effect of Salt was found, $F(1,28) <1$.

4.3.1.4 Size of Clusters. Group mean analyses of the size of clusters, when licking a sucrose or saccharin solution, containing either LiCl or NaCl, are depicted in Figure 4.7. The ANOVA revealed a significant Day X Salt interaction, $F(4,75) = 2.92$, $p < .05$. Overall, groups drinking NaCl solutions had larger sized clusters. A main effect of Day was also found, $F(4,75) = 8.93, p < .001$. Between groups, main effects of Sweetener ($F(1,28) = 5.05, p < .05$) and Drug ($F(1,28) = 12.52, p < .01$) were found. On

Figure 4.4. Phase mean Number of Licks. (A) Day 1mean number of licks + SEM
compared to days 2-5 mean for the acquisition phase, with groups drinking Sucrose +
NaCl, Sucrose + LiCl, Saccharin + NaCl, Saccharin + LiCl (n=8/group). **(B)** Day 6 mean
number of licks + SEM compared to days 7-10 mean for the extinction phase, of rats
drinking Sucrose + NaCl or Saccharin + NaCl (n=8/group). Legend refers to solution
consumed in acquisition phase. Significant differences between corresponding groups on
days 1 vs days 2-5 are indicated, *p < .05, **p < .10, ***p < .001.

Figure 4.5. **Number of Clusters.** Group mean number of clusters + SEM across both the acquisition phase (days $1 - 5$) and extinction phase (days 6-10). Groups drinking Sucrose or Saccharin solution containing LiCl (n=8/group), and groups drinking Sucrose or Saccharin solution containing NaCl (n=8/group). Significant differences between Sucrose + NaCl and Saccharin + NaCl are indicated by "a". Significant differences between Saccharin + LiCl and Sucrose + LiCl are indicated by "b". Significant differences are indicated between corresponding LiCl and NaCl groups, *$p < .05$.
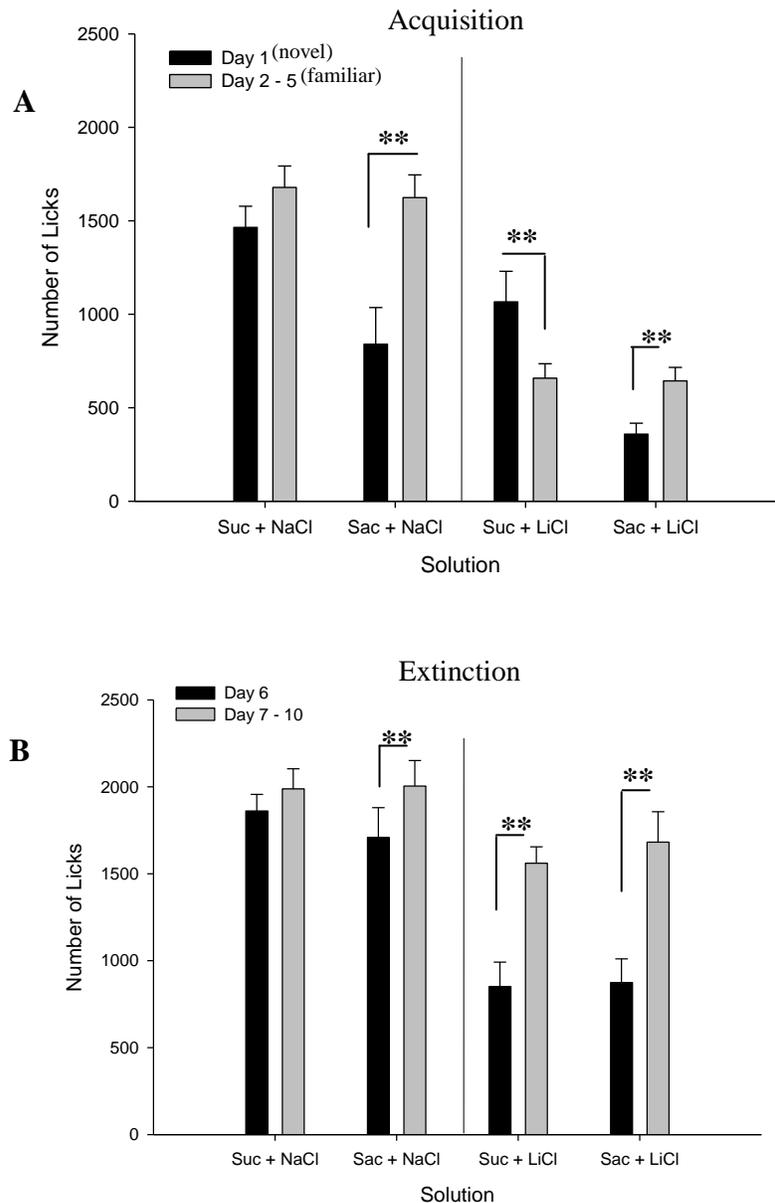
Figure 4.6. Phase mean number of clusters. **(A)** Day 1 mean number of clusters + SEM compared to days 2-5 mean for the acquisition phase, with groups drinking Sucrose + NaCl, Sucrose + LiCl, Saccharin + NaCl, Saccharin + LiCl (n=8/group). **(B)** Day 6 mean number of clusters + SEM compared to days 7-10 means for the extinction phase, of rats drinking Sucrose + NaCl or Saccharin + NaCl (n=8/group). Legend refers to solution consumed in acquisition phase. Significant differences between corresponding groups on days 1 vs days 2-5 are indicated, *$p < .05$, **$p < .01$, ***$p < .001$.
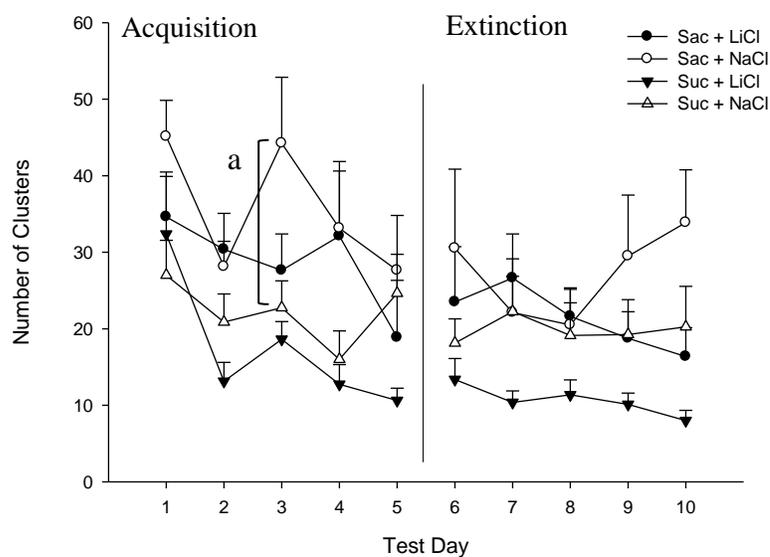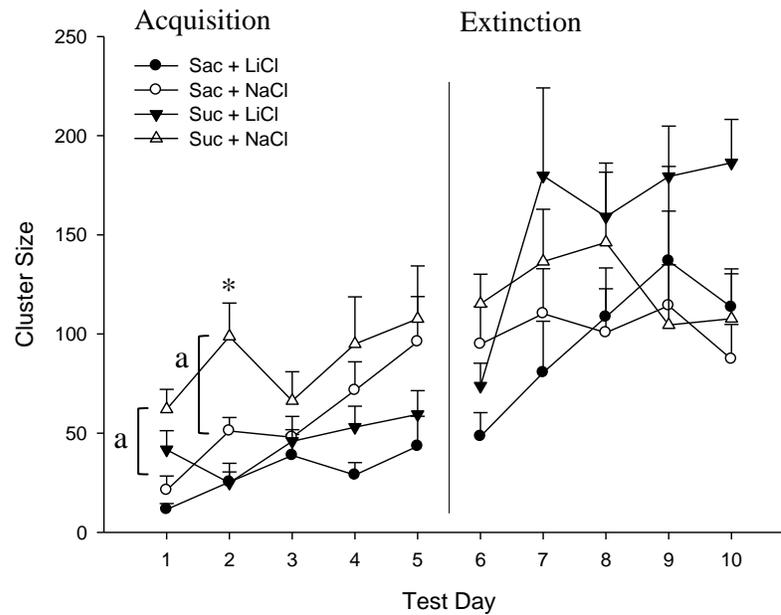
Figure 4.7. **Cluster Size.** Group mean cluster size + SEM across both the acquisition phase (days $1 - 5$) and extinction phase (days 6-10). Groups drinking Sucrose or Saccharin solution containing LiCl (n=8/group), and groups drinking Sucrose or Saccharin solution containing NaCl (n=8/group). Significant differences between Sucrose + NaCl and Saccharin + NaCl are indicated by "a". Significant differences between Saccharin + LiCl and Sucrose + LiCl are indicated by "b". Significant differences indicated between corresponding LiCl and NaCl groups, $*p < .05$.
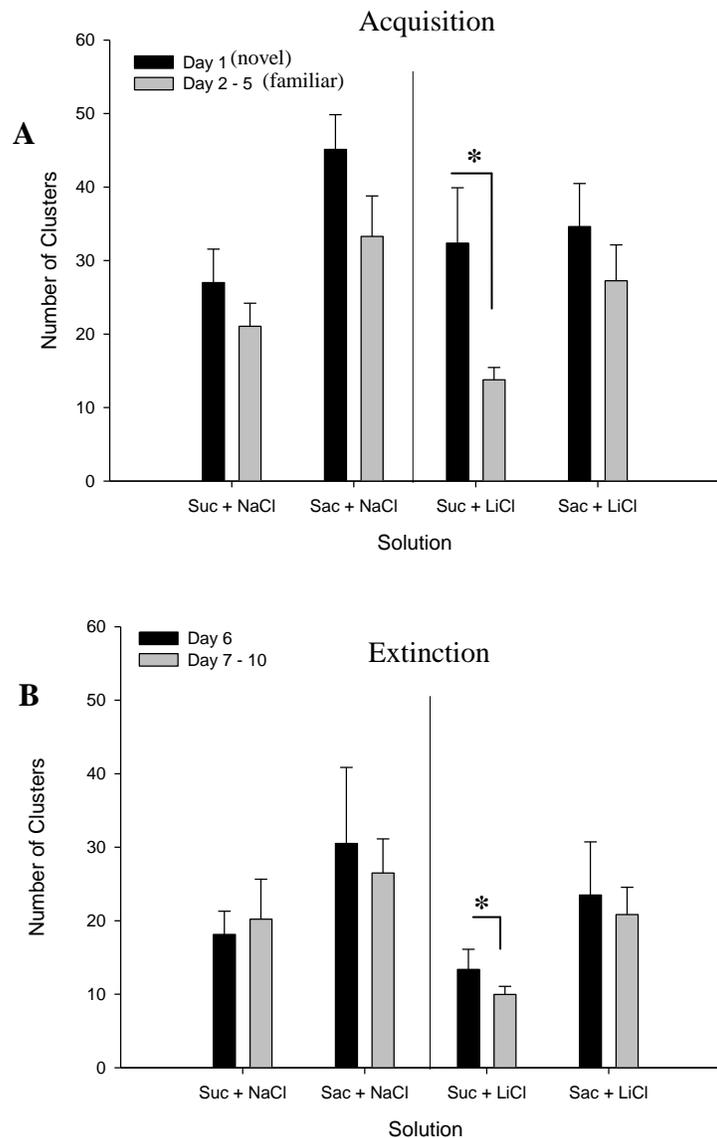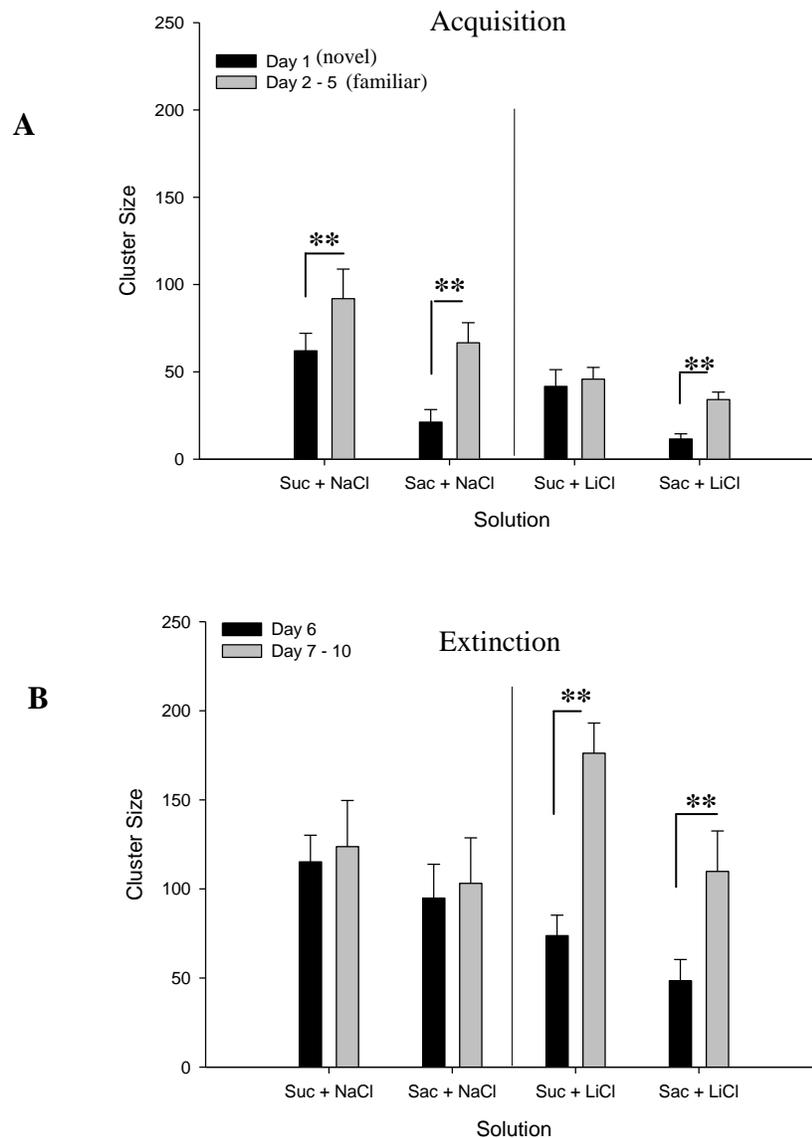
Figure 4.8. Phase mean Cluster Sizes. **(A)** Day 1 mean cluster sizes + SEM compared to days 2-5 mean for the acquisition phase, with groups drinking Sucrose + NaCl, Sucrose + LiCl, Saccharin + NaCl, Saccharin + LiCl (n=8/group). **(B)** Day 6 mean cluster sizes + SEM compared to days 7-10 mean for the extinction phase, of rats drinking Sucrose + NaCl or Saccharin + NaCl (n=8/group). Legend refers to solution consumed in acquisition phase. Significant differences between corresponding groups on days 1 vs days 2-5 are indicated, $*p < .05$, $**p < .10$, $***p < .001$.

Day 1, post-hoc analyses revealed a significant difference between the Saccharin and Sucrose groups drinking NaCl, $p < .05$. The Sac + NaCl group shows significantly smaller clusters. On day 2, the Suc + NaCl group showed significantly larger cluster sizes when compared to Suc + LiCl. No significant differences between the Sac + NaCl and Sac + LiCl were found. When comparing Day 1 (novel) group means with the combined means of Days 2 -5 (familiar), the ANOVA revealed a significant Familiarity X Salt interaction, $F(1,28) = 29.05$, $p < .001$. As seen in Figure 4.8A, groups tended to have smaller cluster sizes on Day 1, except those drinking Suc + LiCl, which had approximately the same cluster sizes both days, $ps < .05$. Main effects of Sweetener and Salt were also found, $F(1,28) = 10.50$, $p < .01$ and $F(1,28) = 10.63$, $p < .01$. Generally, saccharin groups had smaller cluster sizes compared to the sucrose groups.

4.3.2 Extinction

     4.3.2.1 Volume (mL). Group mean volume consumption of a Suc + NaCl or Sac + NaCl solution (after drinking a sucrose or saccharin solution containing LiCl or NaCl in the acquisition phase), are depicted in Figure 4.1(Days 6-10). The mixed-design repeated measures ANOVA revealed a significant Day X Salt interaction, $F(4,104) = 6.30$, $p < .001$. A significant main effect of Day was also obtained, $F(4,104) = 19.21$, $p < .001$. Post hoc analyses revealed that groups drinking Suc + LiCl in phase 1, continued to drink less than their Suc + NaCl control on Days 6-8. Comparatively, the Sac + LiCl group only consumed less than its Sac + NaCl control on Day 6, $ps < .05$. A main effect of Salt was observed between subjects, $F(1,28) = 37.26$, $p < .001$, but no effect of Sweetener, $F(1,28) < 1$.

     When comparing Day 6 (early) group means with the combined means of Days 7-10 (late), the ANOVA revealed a significant interaction between Time and Salt ($F(1,28) = 29.57$, $p < .001$), and a main effect of Time ($F(1,28) = 89.41$, $p < .001$). As seen in Figure 4.2B, pairwise comparisons revealed that all groups, except the Suc + NaCl group, consumed significantly less on Day 6 compared to Days 7-10, $ps < .05$. Both groups consuming LiCl in the acquisition phase increased their consumption after Day 6. Between groups, a significant main effect of Salt ($F(1,28) = 75.31$, $p < .001$), was observed, but no effect of Sweetener, $F(1,28) < .1$.

4.3.2.2 Number of Licks. Group mean number of licks, when drinking a Suc + NaCl or Sac + NaCl solution (after drinking a sucrose or saccharin solution containing LiCl or NaCl in acquisition phase), is depicted in Figure 4.3 (Days 6-10). The ANOVA revealed a significant Day X Salt interaction, $F(4,99) = 5.49$, $p < .01$. A significant main effect of Day, ($F(4,99) = 18.14$, $p < .001$) and Salt, ($F(1,28) = 13.66$, $p < .01$) were also obtained. Post-hoc comparisons showed that the group drinking Suc + LiCl in the acquisition phase still licked significantly fewer times on Day 6, compared to its Suc + NaCl control, ps $< .05$. On Days 6 and 10, the group drinking Sac + LiCl in the acquisition phase, licked significantly less compared to their Sac + NaCl control. No effect of Sweetener was found, $F(1,28) < 1$.

When comparing Day 6 (early) group means with the combined means of Days 7 – 10 (late), the ANOVA revealed a significant interaction between Time and Salt ($F(1,28) = 30.45$, $p < .001$), as well as a main effect of Time, $F(1,28) = 95.61$, $p < .001$. As seen in Figure 4.4B, pairwise comparisons revealed all groups, except those drinking Suc + NaCl, licked significantly fewer times on Day 6 compared to Days 7-10, ps $< .05$. Between groups, a significant main effect of Salt ($F(1,28) = 25.57$, $p < .001$), but not Sweetener ($F(1,28) < 1$), was found. Those groups drinking LiCl in the acquisition phase still licked less when drinking NaCl on Day 6.

4.3.2.3 Number of Clusters. Group mean numbers of clusters, when drinking a Suc + NaCl or Sac + NaCl solution (after drinking a sucrose or saccharin solution containing LiCl or NaCl in phase 1) are depicted in Figure 4.5 (Days 6-10). The ANOVA revealed only a main effect of Sweetener, $F(1,28) = 4.81$, $p < .05$. Overall, the groups drinking solutions containing Sucrose had fewer numbers of clusters. No main effects of Day or Salt were found, $Fs(1,28) < 1$.

Further, in comparing Day 6 (early) group means with the combined means of days 7-10 (late), the ANOVA revealed a significant main effect of Sweetener, $F(1,28) = 4.29$, $p < .05$. As shown in Figure 4.6B, groups drinking saccharin had fewer clusters than sucrose groups. No main effects of Time or Salt were found, $Fs(1,28) < 1$.

4.3.2.4 Size of Clusters. Group mean cluster sizes, when drinking a Suc + NaCl or Sac + NaCl solution (after drinking a sucrose or saccharin solution containing LiCl or NaCl in the acquisition phase), are depicted in Figure 4.7 (Days 6-10). The mixed-design

repeated measures ANOVA revealed a significant Day X Salt interaction, $F(4,75) = 2.65$, $p < .05$. A main effect of Day was also found, $F(4,75) = 2.65$, $p < .05$. Generally, the groups that drank LiCl in acquisition phase, showed a steeper increase in cluster size, across days. No main effects of Sweetener or Salt were found, $Fs(1,28) <1$.

Further, in comparing Day 6 (early) group means with the combined means of Days 7-10 (late), the ANOVA revealed a significant Time X Salt interaction, ($F(1,28) = 13.71$, $p < .001$), as well as a main effect of Time ($F(1,28) = 20.67$, $p < .001$). As seen in Figure 4.8B, both groups drinking LiCl in the acquisition phase, have significantly smaller clusters on Day 6, compared to Days 7-10 (of phase 2), ps $< .05$. No main effect of Sweetener or Salt was found, $F(1,28) <1$.

4.4 Discussion

The current study demonstrates that rats, when voluntarily consuming a toxic food source, will exhibit a trade-off between calories and toxicity of a solution. These findings are also consistent with previous demonstrations of dose-dependent consumption across the acquisition phase and behavioral regulations of a toxin containing solution (Good et al., 2013; Glenndinning, 2007). This calorie based trade-off is supported by the distinct dissociation seen in both intake and palatability measures between rats drinking a sucrose solution versus a saccharin solution. However, the trade-off effect was most prominent only Day 1 in rats drinking a calorie rich solution (0.58 M sucrose), when the taste was still novel.

Regardless of the sweetener type, LiCl (toxin) caused a suppression of intake and number of licks during the acquisition phase, when compared to the corresponding effects of NaCl. Where some LiCl suppressive effects were expected, intake and palatability differences between the two sweeteners were also of interest. As sucrose is calorie rich, and saccharin a non-caloric sweetener, one can compare the drinking and licking behavior of the two sweet solutions to infer behavioral changes based on calories. Previous research has also indicated that rats utilize caloric information when consuming a food, but that information is often modulated when the food has high palatability (Glendinning et al., 2010; Beeler et al., 2012; Scheggi et al., 2013). The control solutions containing NaCl and sucrose or saccharin showed high intake levels across both the acquisition and extinction phases. Since the two solutions were consumed in

approximately the same amounts after Day 1, this could indicate that both the sucrose plus NaCl and saccharin plus NaCl solutions were palatable, with no obvious negative post-ingestive effects. On Day 1 of acquisition both sucrose groups, regardless of salt, consumed more fluid when compared to their saccharin counterparts. Since the difference only occured on Day 1, this could be an indication of differences in neophobic behavior. The saccharin groups showed the expected neophobic reaction to a novel solution in both the intake and cluster size. When consuming a novel solution, rodents generally limit their intake and the palatability is shown to be low, until the solutions effects are realized and the rat can learn how to regulate this solution (Lin, Amodeo, Arthurs, & Reilly, 2012). Both intake and cluster size increase in the saccharin group after Day 1 indicating that they learned the associative negative effects of LiCl, but also how to regulate it to a point before toxicosis. Since the sucrose and LiCl groups showed an opposite patter, it may indicate that when a new taste/food is introduced, it is more likely to be consumed in greater amounts if it is accompanied by positive post-ingestive feedback, such as needed calories (Lin et al., 2012).

As noted in Chapter 3, rats drinking a LiCl (0.02 M) plus 0.50 M sucrose solution consumed more than would be expected according to the behavioral threshold regulation strategy. I suggested that this could represent a trade-off between calories and toxin level, in that the rat was willing to consume more of the toxic solution in order to maximize its calories ingested. Current results show a similar trade-off between calories and toxin load, in that the sucrose plus LiCl group drank more than the saccharin plus LiCl group on day 1 of the acquisition phase, compared to the other four days. Previous research using i.p. injections of low dose (0.0009 M) LiCl, showed a similar attenuation of LiCl suppression when food-deprived rats were presented with a 0.50 M sucrose solution (Grigson & Gomez, 1999). Rats in the current study were water deprived, which is generally accompanied by a voluntary restriction of food intake (Collier & Knarr, 1966). The attenuating effects of 0.50 M sucrose shown here, and in previous research, seem to be mediated by the caloric value of the solution being consumed, as the same pattern is not seen when saccharin is used (Bell et al., 1997; Grigson, 1997; Gomez & Grigson, 1999).

It can be inferred that this trade-off is based on the caloric value of the solution and not based on taste for two reasons: (A) the sucrose plus LiCl group consumed and licked more solution on Day 1, but the saccharin plus LiCl group consumed and licked more solution on Days 2-5; and (B) the cluster sizes of the sucrose plus LiCl group were approximately equal throughout acquisition, but the cluster sizes in the saccharin plus LiCl group increased significantly after Day 1 of acquisition. Once the toxin was removed from the solution during the extinction phase, both volume and cluster size increased after day 6 in the rats drinking LiCl. If taste was the primary factor in stimulating the use of this trade-off, it would be expected that rats would show large cluster sizes while consuming greater amounts of the solution. Previous research indicates that at the microstructural level, cluster size can be inferred to index palatability as it is a separate entity from total intake, and the cluster sizes tend to increase monotonically with increasingly palatable solutions (Davis, 1973; Davis & Smith, 1992; Glendinning, 2010).

Although previous research has used LiCl injections to monitor its suppressive effects, the current study demonstrates that rats will voluntarily regulate their intake of a toxic solution to fulfil their calorie needs. At a LiCl concentration of 0.02 M, (one that is more likely to be seen in a natural foraging environment), rats appear to be willing to consume this level of toxin and are regulating their behavior and intake levels to this concentration. Rats will consume the solution until the post-ingestive feedback reaches a certain degree of malaise, and then they will stop. With a greater calorie reward, they may push their toxin load to its maximum level before stopping consumption. After the initial day of drinking, the consumption of the LiCl solutions remained relatively constant for the rest of the acquisition phase.

The large difference in intake seen between the sucrose and saccharin solutions on Day 1 compared to Days 2-5 may also be related to a difference in central dopamine release. Beeler and colleagues (2012) showed that dopamine released from the nucleus accumbens is released preferentially when a sweet taste is nutritional relative to a sweet taste alone (i.e. sucrose compared to saccharin). Although rats showed an increase in dopamine release when presented with saccharin, the response was attenuated, likely due to the lack of positive post-ingestive feedback. Further research, also showed that the

dopaminergic response to a sweet taste shows an habituation effect, with diminished responding after the first encounter (Scheggi et al., 2013). This could explain why a calorie-toxin trade-off was seen only on day 1 for consumption the sucrose plus LiCl solution.

In conclusion, when presented with a calorie rich toxic solution, rats will voluntarily trade-off calories and toxic effects. These rats were more willing to engage in trade-off behavior for increased calories, where palatability played a secondary role. When the amount of available calories is high enough, rats will consume more toxic solution and deal with greater negative side effects to maximize the caloric intake. This same pattern is not seen when the sweet taste is a non-caloric sweetener, which indicates that this trade-off pattern is based on nutrition, and not palatability. This feeding behavior may have a greater role in natural foraging conditions, where animals may be limited on their food choices and utilize feeding strategies that will maximize their nutrient intake.

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Chapter 5

GENERAL DISCUSSION

5.1 General Discussion

The current thesis developed a possible model of foraging behavior, using a conditioned taste avoidance paradigm and based on voluntary intake and regulation of a toxin-containing food. Previous research has typically utilized a paradigm of 'forced exposure' to a toxin, with little focus on voluntary and oral consumption of toxin-containing foods. Across the three current experiments, the toxin LiCl was incorporated into sugar solutions to more closely mimic naturally foraging conditions. It was observed that rats learned to associate toxin-containing foods with the negative post-ingestive feedback, in a dose-dependent manner. Rats voluntarily consumed a toxic food in an attempt to maximize their nutrient intake but limit the intake of the toxin, up to a certain threshold level of toxicity.

In Chapter 2, rats drinking low levels of LiCl in a sucrose solution during an acquisition phase limited their intake and number of licks in a dose-dependent manner. As the group drinking 0.02 M LiCl drank significantly less than the NaCl controls on most days, and the ID_{50} values were very close to this molarity, it is likely that 0.02 M LiCl represents a toxin threshold level to which rats were regulating their feeding behavior. These findings are consistent with the threshold hypothesis, which suggests that rats will tolerate a certain amount of toxin, and regulate intake even at low levels (Glendinning, 2007). Herbivores have also been shown to reduce their meal size or increase intervals between meals to minimize toxin exposure (Sorensen et al., 2005; Torregrossa & Dearing, 2009; Wiggins et al., 2003). In Chapter 2, the positive control group, drinking a 0.3 M sucrose plus 0.12 M LiCl (toxin) solution during the acquisition phase, showed a strong taste avoidance on the first day of acquisition, an observation which is consistent with previous demonstrations of strong taste avoidance conditioning in rats drinking solutions with 0.12 M or higher levels of LiCl (Cross-Mellor et al., 2004; Ladowsky & Ossenkopp, 1986; Loy & Hall, 2002; Nachman & Ashe, 1973).

Cross-Mellor et al. (2004) suggested that animals may have two different mechanisms for regulating intake of toxic substances. If ingesting foods containing high toxin levels, animals develop a robust, long term avoidance *and* rejection of these foods. At high concentrations, LiCl and other toxins act on the chemosensitive area postrema (AP) in the brainstem to induce conditioned taste avoidances and aversions (Borison,

1989; Eckel & Ossenkopp, 1996; Ladowsky & Ossenkopp, 1986; Ossenkopp & Eckel, 1994, 1995; Ritter et al., 1980). The area postrema has a reduced blood brain barrier and activates the appropriate toxin-defense feedback loops to modify behavioral reactions to tastes associated with the effects of toxins such as LiCl (Ossenkopp & Eckel, 1995). When animals ingest LiCl, central neural circuits underlying hypophagia and adaptive associative learning are also activated (Rinaman & Dzmura, 2007). However, there is a dissociation between the hypophagic effects and the conditioning of taste aversions (Ossenkopp & Eckel, 1994, 1995; Rinaman and Dzmura, 2007). If the area postrema is lesioned, rats are unable to form conditioned taste avoidances/aversions when novel tastes are paired with LiCl. However, the anorexic effects of LiCl are still obtained, as rats continue to decrease eating and lose weight (Borison, 1989; Eckel & Ossenkopp, 1996; Ladowsky & Ossenkopp, 1986; Ossenkopp & Eckel, 1994, 1995; Ritter et al., 1980). Rinaman and Dzmura (2007) provided further evidence for the dissociation of LiCl induced hypophagia and conditioned taste avoidance. When noradrenergic neurons in the area postrema and nucleus of the solitary tract were lesioned, rats no longer exhibited LiCl-induced hypophagia (Rinaman & Dzurma, 2007).

When animals are ingesting foods containing low toxin levels, they may be using a short-term transient mechanism to regulate feeding behavior within a meal and on a day to day basis. Evidence for this mechanism comes from rats learning to discriminate between equimolar LiCl and NaCl solutions, as these animals develop a discrimination in the absence of an area postrema (Ossenkopp et al., 1997), suggesting that post-ingestive aversive cues from the gut are likely involved in the formation of this discrimination. Post-ingestive feedback lets animals associate a foods' taste with its homeostatic utility and create automatic changes in palatability for that food (Garcia, et al., 1985; Provenza, 1995). An increase in preference or hedonic value will occur for foods with appropriate nutrients, whereas decrease in preference will occur for foods with excess toxins or inadequate nutrients, and these changes can occur within a meal or across meals (Garcia et al., 1985; Provenza, 1995, 1996). It remains unclear what role the area postrema plays with low levels of LiCl, or what other processes become activated.

In Chapter 3, the threshold toxin concentration identified in Chapter 2 was used to investigate rats' use of a "trade-off" strategy for maximizing calories and minimizing

toxins. It was found that when presented with toxin-containing foods, rats will use this trade-off mechanism, and it is most pronounced when the benefits greatly outweigh the costs; for example, when the sucrose concentration is high (great caloric value). These results agree with a previous study that showed a lack of drinking attenuation when sucrose was combined with LiCl (Grigson & Gomez, 1999). Although the manner of LiCl exposure was different from the current study (injection), and the dose was much smaller, a similar effect on drinking behavior was seen. Rats increased their sucrose consumption relative to that of NaCl control levels when the sucrose concentration was above 0.3 M (Grigson & Gomez, 1999). Duncan, Ginane, Elston, Kunaver, and Gordon, (2006) examined "trade-off" effects in a toxin containing food consumed by goats. They presented goats with different conifers followed by a positive stimulus, cornstarch, or negative stimulus, LiCl. Each stimulus had a low, medium, and high option (Duncan et al., 2006). Researchers concluded that browsing herbivores also use a trade-off strategy, which results from the integration of the dose-dependent positive and negative effects of the food in question (Duncan et al., 2006). This is similar to the findings of the current study that when the positive effects of a toxin containing food outweigh the negative effects, the animal will consume more.

To search for further evidence that the trade-off strategy was based on caloric value rather than taste, Chapter 4 tested the calorie free sweetener saccharin versus calorie rich sucrose. On Day 1 of testing, each solution was novel and results showed that the nutritional effects on consumption are most pronounced on day one. The saccharin groups tended to consume less compared to their sucrose counterparts, whether drinking NaCl or LiCl, which indicated that rats were getting more positive post-ingestive feedback from sucrose compared to saccharin.

Again I found that rats were willing and able to voluntarily consume a toxin-containing solution, and regulate their fluid intake to a toxin threshold level. In Chapter 4, rats drinking the sucrose and LiCl solution showed intake levels matching their NaCl controls. However, the palatability indicator of cluster size, showed significantly smaller sizes for the LiCl group, when compared to the controls. These microstructural variable effects support the idea that taste or palatability was not likely the reason for increased consumption. These findings were also similar to the 0.50 M Sucrose + LiCl group in

Chapter 3 that exhibited increased intake levels but smaller cluster sizes, compared to their NaCl controls.

In conclusion, rats are able to voluntarily control the amount of toxin they consume. When the nutrient or calorie benefits greatly outweighed the negative effects of the toxin, rats tolerated a greater amount of the toxin. The results presented here add to the expanding field of research on foraging behaviors and strategies. Foraging animals are likely to come into contact with many different food sources containing different low levels of toxins, such as plant secondary metabolites. Research has shown that these animals are able to determine which foods, and how much of those foods, will maximize their nutritional needs but minimize their toxin intake (Good et al., 2013; Forbey et al., 2009; Provenza, 1995;1996).

Avoidance learning and feeding regulation result from the integration of gustatory and visceral information. Visceral afferents may interact with gustatory and olfactory afferents to facilitate or inhibit food ingestion (Forbey et al., 2009; Provenza, 1995). Affective processes integrate the taste of food and its post-ingestive consequences, whether positive or negative (Provenza, 1995). Foraging animals use this integration of information to find strategies that will minimize effects of toxins. Due to the wide variety of plant secondary metabolites, complete avoidance of some foods would not be a successful strategy (Forbey, et al., 2009).

The area of threshold regulation feeding behavior would benefit from future research investigating the potential pathways of these short-term and transient mechanisms that are seen within meals for many foraging animals. The area postrema has been implicated as a necessity for forming conditioned avoidances, but most research has used high concentrations of LiCl (Eckel & Ossenkopp, 1996; Ladowsky & Ossenkopp, 1986; Ossenkopp & Eckel, 1994). Examining the effects of area postrema lesions on conditioned avoidance using low doses of LiCl, similar to the threshold level presented in the current thesis, would give insight to possible pathways.

As the current results showed, nutritional value overrides the palatability of a food, but it has been suggested that the nutritional value of food also plays a role in establishing its hedonic value. Monitoring palatability indicators, such as cluster size, are useful in understanding the resulting drinking behavior. To study the palatability changes

more in depth, it could be useful to employ a taste-reactivity measure. Since avoidances can be conditioned within a meal, it is possible to use the taste-reactivity approach (TRT) later in the same day. After rats consume a toxin containing solution in the Lickometer, testing them with the same solution through TRT hours later can give insight into rejection behaviors. Further, the solutions used in the current study were seemingly palatable, until post-ingestive feedback signaled the negative effects of LiCl. Another possible study design could include adding a bitter taste, such as quinine, to a sucrose + salt solution. The bitter taste would upset the negative versus positive factors in solution, and rats may not be willing to tolerate as much solution with an aversive taste.

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