Determinants of Conditioned Prey Avoidance in the Fire Bellied Toad, *Bombina, Orientalis*

by

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ABSTRACT

DETERMINANTS OF CONDITIONED PREY AVOIDANCE IN THE FIRE BELLIED TOAD, *BOMBINA ORIENTALIS*

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Conditioned prey avoidance (CPA) is a behaviour that prevents the consumption of harmful prey. It occurs when a novel prey is paired with an aversive event. Previous behavioural studies in amphibians have presented conflicting evidence surrounding the ability of amphibians to conduct CPA. This study evaluated the ability of the fire-bellied toad (*Bombina orientalis*) to display a CPA and investigated three competing hypotheses (avoidance intensity, sex and body size) to determine what factors could predict CPA. During this study, toads learned to avoid mealworms a week after pairing this novel prey with the emetic substance copper sulfate. This behavioural response was specific to mealworms since usual cricket prey was consumed readily at testing time. The results show that avoidance behaviour intensity was an important predictor of CPA, whereas sex and body size had no influence. Therefore, fire-bellied toads possess similar capacities for CPA as demonstrated in other vertebrates.

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The original goal of my project involved qPCR and *in situ* hybridization methods to investigate brain responses associated with learning and memory. However, numerous setbacks with these methods have forced me to adopt a behavioural perspective for the final thesis. I would like to express my gratitude for my advisors continued support and concern with my research endeavors.

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Background

Conditioned food avoidance

Avoidance behaviour is defined as an organism's tendency to reduce exposure towards a stimulus that has been deemed harmful or potentially dangerous. Freezing, fleeing and fighting are three broad categories of defensive behaviours that animals use to avoid harmful stimuli or situations (Eilam 2005). The ability to display these defensive behaviours is innate and the choice of which behaviour to express depends on the type of danger present in a specific situation. On the other hand, acquired avoidance behaviours are learned, whereby an animal associates a novel situation with an aversive stimulus and avoids the situation after this experience. Researchers have developed various metrics to study acquired food avoidance behaviour, such as condition taste avoidance (CTA), conditioned taste aversion (CTAv) and conditioned prey avoidance (CPA). CTA and CTAv are typically used interchangeably in the scientific literature; however, there is evidence to support that these two responses could represent different learning processes. CTA and CTAv tests involve the pairing of a novel taste with an aversive event followed by a subsequent consumption test to assess behavioural responses. CTA is measured by the amount of food consumed during a consumption test. On the other hand, CTAv uses a taste reactivity test to measure the reaction towards a previously paired flavor stimulus (Parker 2003). CPA is similar to CTA except that CPA measures consumption of prey items without manipulating sensory stimuli such as taste, smell and vision which could influence avoidance learning. CTA in humans has been demonstrated from pre-clinical trial data showing aversion to foods and beverages after a single pairing with a chemotherapeutic agent (Schwartz et al. 1996). Mammals such as rats and shrews can be tested with a CTA paradigm and display avoidance learning when a novel flavor/taste is paired with a toxic substance (Parker

2006). Unlike acquired food avoidance, clay or pica consumption tests are used as an index of general discomfort or illness. Clay consumption measures whether an animal will consume a non-nutritious substance like dirt in response to being given a toxic stimulus. The consumption of a non-nutritious substance could be an adaptive response to nausea as it may dilute the nauseating effects of the toxin. For example, rats consume kaolin clay when injected with a chemotherapeutic agent like cisplatin (Yamamoto et al. 2002). These examples showcase the variety of methods used to study food avoidance behaviour in animals. CPA is often mediated by aversive stimuli that cause illness. Extensive research has investigated the role of signals originating from the gut in inhibition of goal-directed behaviour (Maniscalo and Rinaman 2008). Therefore, it has been argued that illness symptoms due to interoceptive discomfort promote avoidance learning.

Illness and Emesis

In general, illness is defined as the subjective experience of disease symptoms or an extended period of sickness that alters the mind or body. Researchers have investigated illness by studying various symptoms that are a by-product of disease. Nausea is a symptom of illness that has been well studied in humans (Sanger and Horn 2006; Horn 2014). Nausea is caused by a variety of stimuli in humans, including cancer therapeutic agents, radiotherapy, opioid and general analgesics, and gastrointestinal diseases. Many of these treatments and diseases can induce nausea in organisms by altering the gastrointestinal tract (Sanger and Andrews 2006). However, the ability to study nausea and illness accurately in other organisms is an ongoing problem for researchers. This problem stems from the fact that nausea is a self-reported symptom and thus is difficult to quantify in non-human animals. General changes in behavioural activity

have been used to study illness and nausea in animals. For example, the lack of motor movement and reduced food intake are common side effects of nausea and illness in animals (Horn 2014).

Emesis is another aspect of illness that describes the set of events that lead to vomiting. Emesis is easier to study and interpret than illness because it does not rely on self-reported symptoms and can be observed in a laboratory setting. Lithium chloride (LiCl) is a commonly used emetic agent that is known to cause illness and vomiting when injected in emetic animals (Parker and Kemp 2001; Rabin and Hunt 1992). However, research on the vomiting reflex has illustrated important species differences (Andrew and Horn 2006). The vomiting reflex is not required for survival but can be useful for preventing the consumption of harmful substances. For example, rats are incapable of vomiting but display overt disgust reactions when injected with LiCl. Moreover, there is a lack of consistency when measuring sensitivity to different emetic agents between species. For example, the rank order of decreasing sensitivity to apomorphine exposure is dog>human>ferret>cat with shrews and monkeys being unresponsive. While X-ray radiation exposure has a rank order of ferret>dog>human=monkey>cat (Andrews et al. 1990). The efficacy of emetic substances is an important factor for eliciting avoidance behaviour in animals.

Investigating avoidance learning in vertebrates

Important differences in emotion and learning capacities in vertebrates are hypothesized to have emerged after the divergence between amniotes and non-amniotes. For example, heart rate, an index of emotional state, is accelerated in mammals, birds and reptiles following gentle handling. However, this response is reportedly absent in amphibians (Cabanac 1999; Cabanac and Aizawa 2000; Cabanac and Cabanac 2000). CPA is a behaviour that prevents the consumption of harmful prey items. It is demonstrated experimentally, by providing a novel prey

item and pairing it with a subsequent aversive event, like illness. An organism is thus able to associate the aversive event with the novel taste or other aspects of the prey, and then learn to avoid it. CPA represents a behavioural response whose origins are openly debated. This behavioural response could have evolved in amniote vertebrates (mammals, reptiles and birds) or could have evolved in non-amniote animals such as amphibians, fish or invertebrates (Mackay et al. 1974; Mikulka et al. 1981; Paradis and Cabanac 2004). General prey avoidance behaviour has been documented in amphibians in response to certain aversive stimuli like bee stings (Brower and Brower, 1962), unpalatable mealworms (Sternthal 1974), and consuming toxic prey items (Greenlees et al., 2010); however, there is still an ongoing debate about whether amphibians share the ability to demonstrate CPA with amniote vertebrates (Mikulka et al. 1981; Paradis and Cabanac 2004; To and Laberge 2014).

A previous study reported that amphibians were not able to demonstrate CPA when a novel food item was paired with an intraperitoneal injection of LiCl (Paradis and Cabanac 2004). More specifically, the study showed that four species of reptiles could avoid food items paired with LiCl injection but two species of amphibians did not. Therefore, the study concluded that CPA had not evolved in amphibians. However, these finding conflicts with an earlier study by Mikulka et al. (1981) which showed that *Bufo americanus* (American toad) can avoid a novel prey item that was previously paired with LiCl. It is important to note that both studies of amphibians exposed to LiCl did not report overt illness symptoms such as vomiting or retching. Therefore, it is unknown whether LiCl is a suitable emetic substance for amphibians because it is well known that amphibians can display overt illness reactions when exposed to emetic substances other than LiCl (Naitoh et al. 1991).

Another study investigated avoidance behaviour in amphibians using copper sulfate ($CuSO_4$) as an emetic agent to induce CPA (To and Laberge 2014). Hydrochloric acid (HCl) and water (H_2O) were used as control substances that were known not to cause illness symptoms. The results of the study showed that consumption of mealworms dipped in $CuSO_4$, but not HCl or water, induced prey avoidance in fire-bellied toads. This prey avoidance behaviour was specific to novel prey (mealworms), since typical prey (crickets) was readily consumed. The study demonstrated that fire-bellied toads can show CPA. Therefore, it is highly likely that CPA is shared between amphibians and amniotes. Emesis was an important factor for facilitating prey avoidance, since $CuSO_4$ caused illness and CPA in toads, while HCl only induced immediate aversion without CPA. Interestingly, an equal proportion of toads exposed to $CuSO_4$ either completely avoided mealworms or consumed them readily. Toads in the $CuSO_4$ group followed a bimodal distribution where only half of the toads displayed CPA upon re-exposure to mealworms during consumption tests. In general, there is limited research pertaining to avoidance learning in basal vertebrates (Thompson and Boice 1975). Therefore, it would be beneficial to explore amphibian learning behaviour to confirm the validity of the observation of CPA behaviour and understand the factors that may explain the 50% learning success in the To and Laberge (2014) study.

The present study follows the work of To and Laberge (2014) by investigating three potential factors that may explain why only half of the toads expressed a CPA response. The factors are: 1) avoidance intensity 2) sex, and 3) body size, which are presented as competing hypotheses to explain variation in CPA in the fire-bellied toad. However, it is important to note that these three hypotheses are not mutually exclusive, meaning that a variable such as avoidance intensity may be influenced by body size and sex because the response to an emetic agent may

be due to these factors. Overall, by comparing toads that successfully learn to avoid mealworms and toads that continuously attack mealworms, I will test if sex, body size and avoidance intensity are factors that determine CPA learning in the fire-bellied toad.

The prey avoidance intensity hypothesis postulates that prey avoidance learning is dependent on the severity of the avoidance behaviour displayed during the initial pairing between novel prey and emetic agent. It is expected that toads have individual differences in their reactivity to the emetic substance $CuSO_4$ and that these differences should impact their avoidance behaviour upon exposure and subsequent learning. I predict that toads that avoid or display little interest in prey after consuming mealworms dipped in $CuSO_4$ will readily avoid mealworms upon subsequent re-exposure, while toads that show active interest in prey after consuming mealworms after dipped in $CuSO_4$ will not avoid consuming mealworms in subsequent exposure.

The sex difference hypothesis proposes that CPA learning in fire-bellied toads depends on sex. Because To and Laberge (2014) found a 50% CPA learning rate but did not identify the sex of the toads during their study, I predict a very large difference between males and females if the difference in CPA learning is due to sex. I also predict that females should be more resistant to novel prey avoidance learning than males. This is because there is evidence that females in certain species generally perform poorly on avoidance tasks (Marin et al. 1994; Pryce et al. 1999) and female amphibians invest a large amount of energy in egg production (Shine 1979), so they are more likely to continue consuming a noxious prey item despite the negative consequences of consuming such prey due to higher energetic needs.

Research involving sex and size dimorphism in amphibians has revealed that females are the larger sex in 90% of anurans species (including *Bombina orientalis*). This fact has led researches to believe that most anuran species display sexual size dimorphism due to the fecundity hypothesis (Shine 1979). This hypothesis states that females allocate more resources into reproduction and therefore would be larger in size to accommodate their larger gametes. Furthermore, it is generally known that females of certain species will often compete for food resources to enhance their growth. For example, *Lissotriton boscai* (Bosca's newt) females increase their foraging habits when in the presence of conspecifics, while males do not (Aragon 2009). This type of foraging behaviour has also been shown in female guppies (*Poecilia reticulata*) which show more feeding on dangerous food patches compared to males, since the possible nutritious benefits of gaining a meal outweighs potential predation (Abraham and Dill 1989).

The effect of sex on avoidance learning in animals has been studied in a variety of learning paradigms. For instance, fear conditioning tasks provide an unconditioned stimulus cue (sound) which is paired with a foot shock treatment. Fear avoidance is measured by the duration of freezing behaviour when the cue is reintroduced. It has been demonstrated that female rats exhibit less fear conditioning than males (Maren et al. 1994; Pryce et al. 1999). Females are also more likely to extinguish taste aversion learning sooner than males, since female rats will consume a novel solution in less time and greater quantity during repeated consumption tests (Randall-Thompson et al. 2003). Thus, female fire-bellied toads could be more inclined to continue eating mealworms despite the negative consequences due to copper sulfate that followed the first consumption of this particular prey.

The body size hypothesis emphasizes that the size of toads will affect prey avoidance. I predict that larger toads will be more resistant to a toxic substance due to a dilution effect. A larger toad will have a lower dose to body weight ratio and thereby show a reduced illness reaction and subsequently a lower prey avoidance response. Numerous studies have documented how body size can affect the toxicity of certain compounds. For example, environmental studies have demonstrated how intraspecific body size variation predicts resistance to heavy metal toxicity in freshwater organisms. *Daphnia magna*, a common freshwater copepod, shows increasing resistance to zinc toxicity with increasing body size (Sarka & Jacobus 2006). Similarly, yellow catfish that are larger survive exposure to higher concentrations of ammonia (Zhang et al. 2011). Thus, larger toads will be less impacted from $CuSO_4$-mealworm pairings and display lower prey avoidance learning.

Research objectives

The current study investigated whether fire-bellied toads can conduct a CPA response and tested three hypotheses: avoidance intensity, sex and body size to determine the factors that are involved in predicting prey avoidance behaviour. The avoidance intensity hypothesis postulates that prey avoidance learning is dependent on the severity of the avoidance reaction during the initial pairing between novel prey and emetic agent. The sex difference hypothesis proposes that CPA learning in fire-bellied toads depends on sex. The body size hypothesis emphasizes that the size of toads will impact their susceptibility to $CuSO_4$-induced toxicity and thus influence their prey avoidance behaviour.

Furthermore, I attempted to supplement the behavioural observations conducted during this study by investigating the brain pathways involved in CPA using molecular methods such as qPCR and *in situ* hybridization (see Appendix). These methods investigate temporal and spatial

patterns of gene expression and could elucidate the molecular mechanisms surrounding CPA in amphibians. This work could provide a better sense of how amniote brains have evolved in terms of memory and learning, as amphibian brains are simpler compared to the more derived brains of amniote vertebrates.

Methods

Animals

The experiment used fire-bellied toads, which were purchased as adults from a local supplier (National Reptile Supply, Mississauga, ON). Fire-bellied toads were held at a temperature of 21°C with 12 hours of daylight and 12 hours of nighttime. Toads were kept in plastic cages (18 cm x 18cm x 18cm) with gravel substrate, broken clay pots and stones for cover. Before experimentation, the toads were deprived of food for one week and given free access to water at all times. This experiment was approved by the Animal Care Committee at the University of Guelph (AUP# 3590).

Effect of acute exposure to copper sulfate on avoidance behaviour

The first phase of this study involved behavioural observation of fire-bellied toads exposed to mealworms dipped in $CuSO_4$ (treatment) and mealworms dipped in H_2O (control). The initial phase was done to characterize the avoidance response of toads exposed to $CuSO_4$. Note that some of the toads used to study the acute effects of $CuSO_4$ were sacrificed shortly after behavioural observations to investigate brain gene expression (7 $CuSO_4$ toads and 4 H_2O toads in summer 2017, and 8 $CuSO_4$ toads and 4 H_2O in summer 2014). Note that the body size and number of snaps was not measured in 2014. A subset of 20 additional toads exposed to $CuSO_4$ was not sacrificed at that time. For theses toads the initial pairing of $CuSO_4$ and mealworms was an “acquisition trial” and is meant to establish the association between aversive event and novel

prey (mealworms) that would produce conditioned prey avoidance (see below). Fire-bellied toads were placed into an experimental context (See Fig 1) with a transparent glass dish covering four live mealworms. Once the toads had detected the mealworms and begun displaying predatory behaviour such as approaching, staring and snapping, there was a 2-min wait period before toads were fed a mealworm dipped in a solution of 3% $CuSO_4$ (n=15) or H_2O (n=8). A 3% $CuSO_4$ concentration was chosen because it is known to induce illness in toads without being lethal (E. To and F. Laberge unpublished observations). After being paired with either $CuSO_4$ or H_2O coated mealworms, toads were kept in the experimental context for an hour where their behaviour was observed. A stop-watch and a tally counter was used to measure time spent interested (TSI) in mealworms and the number of snaps attempted. TSI in mealworms consisted of toads staring, approaching and snapping at mealworms. Time spent looking away or trying to escape the context was not included when measuring TSI in mealworms. Whether toads vomited during the 24 hr period following mealworm consumption was also documented. The toads were weighted and then anesthetized by immersion in 1% buffered MS-222 before being sacrificed to determine sex and extract their brains for qPCR and *in situ* hybridization (see Appendix on molecular studies attempted).

Conditioned prey avoidance response one week later

In order to assess prey avoidance learning, the second phase of this study was conduced one week later in order to assess prey avoidance learning. This second phase tested whether avoidance intensity upon initial pairing, sex, and body size were important factors that determined CPA in fire-bellied toads. The 20 toads used in this phase underwent one $CuSO_4$-mealworm pairing followed by the consumption test in the same experimental context one week later. the toads were not sacrificed after initial exposure to $CuSO_4$ but instead returned to their

holding tanks after an observation period of 60 min inside the context. This observation period allowed for assessment of individual variation in illness behaviour. They were not fed during the week that preceded the consumption.

The consumption test consisted in a series of prey exposure events. First, toads were placed into the experimental context with four crickets trapped under a Petri dish and observed for any predatory or prey-avoidance behaviours (TSI in mealworms) for two min. This initial step with crickets was used to determine whether toads had retained normal prey-catching behaviour and motivation to feed after exposure to $CuSO_4$. The hindlimb of crickets were removed to prevent jumping outside the Petri dish. The second step involved swapping the Petri dish with crickets for another dish covering four mealworms. Toad behaviour toward the mealworms was then observed for two min. Thereafter, for 17 of the 20 toads tested, the Petri dish was overturned, mealworms were placed inside the dish and toads were given free access to attack mealworms for an additional two min. This additional manipulation was performed to see if olfactory cues or unconstrained access to the prey would influence behaviour. Lastly, the toads were given the option to consume four crickets from an open Petri dish to again verify if prey avoidance was specific to the mealworms. The maximum latency to attack prey was 120 sec and if animals did not attack prey within this time interval, they were given the maximum 120 sec value. A stop-watch and a tally counter were used to measure the latency to attack prey, TSI in prey and the number of snaps attempted. Whether mealworms or crickets were consumed was also noted. If toads avoid mealworms but not crickets during this consumption test, it would demonstrate the specificity of prey avoidance toward mealworms. Also, if toads avoided consuming mealworms they would be denoted as learners, while toads that actively consumed mealworms would be non-learners.

Statistics

When comparing differences in continuous variables between two independent groups, the data never met the assumption of normality so the non-parametric Mann-Whitney test was used. When comparing the proportion of responders between sexes, the Fischer exact test was used. When analyzing the association between two continuous variables, the Pearson correlation was used when the data met the assumption of normality and the Spearman rank correlation was used when the assumption was not met. Finally, when analyzing responses between events during the consumption test, the Wilcoxon matched pairs test was used. All statistical tests were computed using Prism version 5 (GraphPad Software, La Jolla, CA, USA). Power calculations were done with SPSS Sample Power 3 (IBM Corporation).

Results

Only 18 out of 45 toads tested in the present study attacked mealworms dipped in $CuSO_4$ but did not consume them. These toads were rejected from the analysis. Twenty-one out of 27 toads that accepted mealworms dipped in $CuSO_4$ displayed illness symptoms, including open mouth gape, face wiping, retching and vomiting during the observation period. Toads fed mealworms dipped in H_2O showed no such signs of illness. One individual was discarded due to severe illness that debilitated its response during the consumption test.

Acute effect of copper sulfate on avoidance behaviour

Toads that had consumed a mealworm coated with $CuSO_4$ demonstrated less interest (mean: 7.2 ± SD: 7.4 min) in mealworms under the Petri dish compared to toads that had consumed mealworms coated in H_2O (39 ± 5.6 min) during the 60 min observation period (Figure 2A). A Mann-Whitney test showed that the treatment toads spent significantly less time

interested in mealworms compared to the control toads (U = 4, n = 35 for $CuS0_4$, n= 8 for H_2O, P <0.01). Another Mann-Whitney test showed that the treatment toads snapped significantly less at mealworms than the control toads (Figure 2B; U = 0, n = 27 for $CuSO_4$, n = 4 for H_2O, P <0.01). Both groups displayed interest in mealworms prior to consumption of either H_2O or $CuSO_4$-coated mealworms. In general, $CuSO_4$-treated individuals avoided mealworms, while control toads continued showing an active interest in mealworms following prey consumption. Thus, this variable was used as an indirect approach to quantify avoidance behaviour in toads, with the lesser time spent interested in mealworms corresponding to more avoidance.

The effect of sex on avoidance behaviour was investigated during acquisition trials. A Mann-Whitney test showed that males and females did not differ significantly in TSI in mealworms after consuming mealworms dipped in $CuSO_4$ (Figure 3A; U = 90.5, P = 0.10, n = 23 for males, n = 12 for females, P = 0.10). Additionally, males and females did not differ significantly in their vomiting response after consuming mealworms dipped in $CuSO_4$ (Figure 3B; n = 23 for males, n = 12 for females, Fischer's exact P = 0.69).

The effect of body size on avoidance behaviour was analyzed using correlational analysis. The analysis showed that body size and TSI in mealworms after consuming mealworms dipped in $CuSO_4$ were not significantly correlated (Figure 4; r = -0.16, n = 27, p = 0.42).

Factors that influence conditioned prey avoidance

During consumption tests a week after the initial pairing of novel prey and $CuSO_4$, a clear difference in response toward mealworms and crickets was observed. The latency to attack prey was much higher for mealworms compared to crickets whether they were covered by the Petri dish or not (Figure 5). Wilcoxon matched pairs tests showed significant differences in latency to

attack mealworms (novel) and crickets (usual) when toads could only see prey items under the Petri dish (Figure 5A; $W = -210$, $n = 20$, $P < 0.01$) or when prey items were accessible to toads (Figure 5B; $W = -111$, $n = 17$, $P < 0.01$). More specifically, 19 of 20 toads did not attack mealworms under the Petri dish and 14 out of 17 toads did not consume mealworms when given free access during the consumption test. The results of the consumption test demonstrated that toads specifically avoided mealworms while retaining their normal predatory behaviour towards crickets.

The overall relationship between sex and CPA was analyzed by examining the proportion of male and female toads that avoided mealworms completely during the consumption test. During this test, 5 out of 16 males and 2 out of 4 females completely avoided mealworms. A Fischer exact test showed that there was not a clear difference in the proportion of toads that avoided mealworms (Fischer's exact $P = 0.59$, $n = 16$ for males, $n = 4$ for females). Therefore, sex did not clearly explain the 50% CPA learning ratio seen in To and Laberge (2014). It is important to note that extensive sampling only yielded 4 female toads for the CPA experiment, which limits the statistical power to accept the null hypothesis that there is no difference in avoidance between sexes. Under the assumption that 90% of males and 10% of females would be avoiders the statistical power for this test was 93.4%, but power was reduced dramatically under a hypothetical effect size of 80% male and 20% female avoiders. Thus, this test and the sample sized used here were only able to detect a putative strong effect of sex on CPA, not a more subtle effect.

The relationship between body size and CPA was analyzed using a Spearman correlation of the response observed during the consumption test. The relationship between body size and

TSI in mealworms during the consumption test was not significantly correlated (Figure 7; $r = 0.06$, $n = 20$, $P = 0.81$). Therefore, body size did not determine CPA.

The relationship between TSI in mealworms during acquisition trials and TSI in mealworms during the consumption test was analysed to test the avoidance intensity hypothesis. A Spearman correlation test indicated that the relationship between TSI in mealworms during acquisition trials and consumption tests was significantly and positively correlated (Figure 8, $r_s = 0.7$, $n = 20$, $P < 0.01$). Toads that spent less time attending to mealworms during an acquisition trial were more likely to avoid mealworms during the consumption test a week later. This result supports the hypothesis that avoidance intensity determined the strength of CPA.

Discussion

The objective of this study was to investigate whether fire-bellied toads could produce a CPA response and elucidate potential factors that are involved in CPA. It was demonstrated that fire-bellied toads can demonstrate a CPA response when mealworms were paired with $CuSO_4$. More specifically, a majority of toads rejected mealworms a week after receiving a $CuSO_4$-mealworm pairing. This is in stark contrast to the To and Laberge (2014) study where only half of the toads fed mealworms dipped in $CuSO_4$ showed a CPA response. The present study suggests that sex and body size do not explain differences in CPA in this species. However, the low statistical power of the test used to assess the sex difference hypothesis did not allow for the detection of potential subtle differences in CPA between sexes. Clearly, the 50% proportion of CPA learning observed by To and Laberge (2014) was not due to a sex difference. The present results, on the other hand, showed that the intensity of avoidance experienced during the acquisition phase of CPA correlated positively with avoidance learning. Despite the latter, it is unclear why the toads differed in avoidance intensity.

This study originally aimed to use molecular methods to complement behavioural observations and investigate the relationship between brain gene expression and avoidance learning. Quantitative polymerase chain reaction (qPCR) was used to investigate temporal gene expression, while *in situ* hybridization explored spatial gene expression in the brain. Unfortunately, the findings were mostly inconclusive due to varying gene expression in individual toads during qPCR experiments and inconsistent staining with *in situ* hybridization. Details of the attempts to implement molecular methods to study brain gene expression in fire-bellied toads are provided in appendix form.

CPA in the fire-bellied toad

This study demonstrated that CPA is a prominent behavioural adaptation that *Bombina orientalis* shares with many other vertebrates. However, this finding differs from a previous study by To and Laberge (2014) which showed that only 50% of toads fed mealworms dipped in $CuSO_4$ displayed a CPA response. To and Laberge (2014) concluded that this bimodal distribution may be the result of two distinct learning types for nausea-induced CPA in the fire-bellied toad. A potential explanation for the discrepancy between the two studies could be due to differences in the copper sulfate content of prey items. Both studies used 3% $CuSO_4$ solutions, but mealworm exposure to 3% $CuSO_4$ was not standardized in either study. Furthermore, the amount of $CuSO_4$ that adhered to mealworms was dependent on mealworm size and was not controlled. Intraperitoneal injections were discussed as a possible solution to control for the volume of $CuSO_4$ administered, however such an injection could cause undue stress and interfere with learning. Also, injecting $CuSO_4$ directly into mealworms was not a successful strategy for controlling dosage because preliminary observations showed that the $CuSO_4$ solution was not retained inside mealworms after piercing through their cuticle. Thus, both studies opted to induce

aversion by dipping mealworms in a $CuSO_4$ solution. Because it is clearly not possible to control the exact dosage of the emetic agent using this method of administration, it is possible that a systematic bias in dosage happened across studies. The mealworms might not have been dipped as thoroughly in $CuSO_4$ before presentation to the toads in To and Laberge (2014) compared to the present study. If this occurred, then toads used by To and Laberge (2014) would have received on average lower doses of $CuSO_4$, reducing avoidance intensity, which in turn would have reduced the chance that they display mealworm avoidance during the consumption test. Unfortunately, the quantity of $CuSO_4$ administered could not be quantified or standardized thoroughly across experimenters in the two studies. Another possible explanation could be genetic differences between different batches of toads used in the two studies. Unfortunately, no information is available on the genetic background of the toads used in these studies. Another possibility, since these animals were purchased as adults, the animals used in the two different studies could have had a different history of exposure to mealworms prior to arriving in the lab. Again, this could not be controlled for, as the type of food used before purchasing the toads was unknown.

Time spent interested in prey as an indirect measure of prey avoidance

TSI in prey was considered a measure of prey interest and used as an indirect measure of avoidance intensity. TSI in mealworms clearly varied between toads fed mealworms dipped in $CuSO_4$ and toads fed mealworms dipped in water. Therefore, TSI was used as an indirect method to measure prey aversion intensity where less time spent interested in prey equated to more avoidance. However, it should be noted that other factors could influence TSI in prey, such as context exploration behaviour or random distractions. Even though these factors could impact TSI, the strong difference in TSI shown between control and treatment toads is unlikely to be

due to increased distraction or a stimulatory effect of the emetic agent on exploration. Because there was no possible way to measure pure avoidance in this study, a lesser TSI in prey was used as an indirect approximation of prey avoidance.

The role of emesis in facilitating learning

The role of emetic agents in facilitating avoidance behaviour has been documented in a variety of vertebrates, however some vertebrates lack an emetic response. Rats are a non-emetic species that can display CTAv via disgust reactions that involve gaping, chin rubbing and paw treading when an intra-oral injection of lithium chloride is paired with a novel solution (Rabin and Hunt 1992; Parker and Kemp 2001; Parker 2006). Furthermore, rats only display disgust reactions when a flavor is paired with a substance that induces emesis in other animals (Parker 2003). However, an emetic agent that induces nausea is insufficient for producing a taste avoidance response in rats, since pre-treatment of an anti-emetic agent like odansetron blocks disgust reactions but not CTA. Therefore, it seems that nausea is necessary for the induction of conditioned taste aversion but not conditioned taste avoidance in rats (Rabin and Hunt 1992; Parker and Kemp 2001). Since rats are incapable of vomiting, it is speculated that rats associate the taste of LiCl as a danger signal rather than an illness signal and this facilitates their CTA response. Thus, it has been proposed that CTA in rats is dependent on fear rather than nausea (Parker 2006; Parker 2008).

Shrews are an emetic species that demonstrate CTA and, unlike in rats, this response can be attenuated by pre-treatment with odansetron (Kwiatkowska and Parker 2005). Therefore, emetic agents are important for facilitating CTA in emetic species like shrews. Paradis and Cabanac (2004) used LiCl as an aversive stimulus and concluded that amphibians did not possess the ability to perform a CPA response. However, LiCl is not an emetic agent in amphibians

(Mikulka et al. 1981; Naitoh et al. 1991). This current study and the study by To and Laberge (2014) demonstrated that fire-bellied toads that are treated with a suitable emetic agent ($CuSO_4$) can produce a CPA response. These examples demonstrate how animals of different species and taxa could have adopted different strategies or mechanisms for conducting an avoidance response based on their ability to respond to specific aversive stimuli. Overall, I propose that unconditioned stimuli that specifically cause illness and emesis are key for facilitating conditioned prey or taste avoidance behaviour in animals.

Variation in sensitivity to emesis

In general, variation in sensitivity to an emetic substance is a major factor in determining conditioned avoidance in animals. Nevertheless, a species may have a specific adaptation for eliminating toxins to minimize the impact of emetic substances on overall health and decrease the need for a CPA response. For instance, various fish species utilize metallothionein, a metal binding protein, which binds physiological metals for transport and detoxification (Philippe et al. 2017). Furthermore, knockout mice have shown that metallothionein genes (MTI and MTII) can protect against zinc deficiency and zinc toxicity (Kelly et al. 1996). It is possible that species or individuals have different capacities for regulation of copper levels and thus vary in sensitivity to $CuSO_4$-induced emesis. Furthermore, different species have different sensitivity to certain emetic substances. For example, dogs and cats differ drastically in their sensitivity to apomorphine. Small doses of apomorphine (ug/kg range) cause violent vomiting in dogs while the same illness reaction in cats requires a much larger dose (mg/kg range) (Andrews et al. 1990). Therefore, not only are unconditioned stimuli that induce emesis key for developing a CPA/CTA response, but the individual and species-specific sensitivity to emetic agents play an important role in

determining the strength of conditioned avoidance displayed by animals, as observed in this present study.

Another possible reason behind variation in sensitivity to emetic substances is the multiple sensory modalities that are involved in the association between novel prey and avoidance. Some species are better adapted to using certain sensory stimuli to encode information. For example, zebrafish are better able to associate a chemical stimulus with aversion than a visual stimulus (Morin et al. 2013). As stated earlier, rats lack the vomiting reflex but it has been speculated that rats possess highly specialized olfactory sensors that serve as a defensive mechanism against consumption of harmful foods (Davis et al. 1986). During this study toads were given the option of attacking mealworms during restricted access (Petri-dish covered) or free access (inverted Petri-dish). Even though both situations produced similar results, it could have been predicted that toads would display higher avoidance during free access compared to restricted access if olfactory cues were important for CPA in toads. Overall, sensory cues used to associate novel situations with an aversive condition could be important factors for determining conditioned avoidance behaviour in animals. It is plausible that emetic and non-emetic species differ in their learning and memory abilities based on their use of different sensory stimuli.

Phylogeny of conditioned prey avoidance

Paradis and Cabanac (2004) proposed that conditioned prey/taste avoidance behaviour originated in amniote vertebrates (reptiles, birds and mammals) and is absent in non-amniote vertebrates (fish and amphibians). However, studies conducted in fish have demonstrated their ability to avoid a novel prey or taste that has been paired with an aversive stimulus (Mackay 1974; Little 1977; Manteifel and Karelina 1996). Furthermore, To & Laberge (2014) have

demonstrated CPA in amphibians, since 50% of fire-bellied toads in their study avoided consuming mealworms that were previously paired with $CuSO_4$. The present study confirmed the finding of CPA in the fire-bellied toad and highlighted the importance of avoidance intensity and emesis in determining prey avoidance behaviour. Therefore, it can be concluded that some non-amniote vertebrates like fish and amphibians clearly share conditioned prey avoidance behaviour with reptiles, birds and mammals (Burghardt et al. 1973; Brett et al. 1976; Parker 2006). The fire-bellied toad is especially well positioned in anuran phylogeny to help support this conclusion, as it is a member of a basal anuran group (Pyron and Wiens 2011).

Nevertheless, the phylogeny of CPA in animals could depend on the stimuli that mediate CPA learning. It could be speculated that CPA learning based on different types of stimuli could have evolved at different times in a variety of animal lineages. I propose a distinction between CPA-mediated learning responses that are displayed by animals. Emesis-facilitated CPA learning, as demonstrated during this study, emphasizes how an animal associates the novel prey/taste with an emetic reaction which promotes avoidance behaviour in subsequent exposure to prey items or tastes. On the other hand, toxicosis-facilitated CPA learning is less sensitive and animals associate the novel prey/taste with negative changes in their physiological state that promotes avoidance behaviour in subsequent exposure to prey items or tastes. I propose that emetic animals typically display emesis-facilitated prey avoidance when given the correct emetic agent at an appropriate dose, while toxicosis-mediated CPA is a response that depends on the toxic effects of a substance without the benefit of an adaptive emetic response.

Emesis-facilitated CPA learning is based on an organism ability to associate a novel stimulus with emesis or illness. For example, the catfish *Ictalurus punctatus* displays an emetic response toward LiCl, which facilitates taste avoidance responses towards specific amino acid

flavors (Little 1977). Shrews (*Suncus murinus*) are another emetic species that display conditioned taste avoidance when LiCl is paired with a novel taste and demonstrate overt illness reactions such as vomiting during the initial chemical-food pairing (Kwiatkowska and Parker 2005; Parker 2006). Emesis-facilitated learning is more sensitive by initiating illness behaviour before the onset of toxic effects, which allows the animal to specifically associate the novel stimulus with illness.

Toxicosis-mediated CPA learning is mediated by an association with a negative change in physiological state such as declining bodily functions that produce an impaired behavioural response. This type of impairment might produce fear due to a loss of behavioural control. Mikulka et al. (1981) demonstrated that high doses of LiCl induced general avoidance behaviour in American toads (*Bufo americanus*). Toads would avoid a novel mealworm equally as much as familiar prey (crickets), suggesting toxic effects of LiCl at high doses. This type of behaviour denotes that toads were impaired in their general ability or motivation to consume prey. This generalized response was not associated with emesis because the toads exposed to LiCl showed no overt signs of illness at high or low doses. Even if the toads could show some specificity of prey avoidance after pairing with a low dose of LiCl, it is likely that the toxic effects of this substance induced a danger signal distinct from emesis behaviour to allow the association with novel food. Since an increased dosage resulted in non-specific prey avoidance, and even death a few weeks after a single injection, it suggests that toxicosis-mediated CPA is not as sensitive as emesis-mediated CPA. A similar example is seen in snails where CTA is accomplished by pairing a novel sucrose solution with an aversive stimulus like KCl. A subset of snails will associate KCl as a danger signal and retract into their shell in subsequent consumption tests. Studies have speculated that snails experience a state of heightened arousal similar to fear when

sucrose is re-introduced during consumption tests, and it has been proposed that CTA in snails may be facilitated by fear (Sugai et al. 2007; Kita et al. 2011). As explained earlier, conditioned taste avoidance has been speculated to develop from a change in physiological state that signals danger and includes fear avoidance in rats, which would provide evidence for a toxicosis-facilitated learning response mediated by fear (Parker 2006; Parker et al. 2008). Because rats are non-emetic, I suspect that emesis-facilitated CPA learning was possibly lost somewhere in the rodent lineage while maintained in insectivores like shrews. Overall, it has been clearly demonstrated that amphibians and fish possess capabilities of performing CPA/CTA responses, but it is possible that emesis-facilitated and toxicosis-facilitated prey avoidance learning have evolved at different times, and emesis-facilitated CPA appears to have been lost at least once in the lineage leading to rats. More research on the nature of prey avoidance responses in different groups of animals will be needed to refine our knowledge of the phylogeny of CPA.

Figures

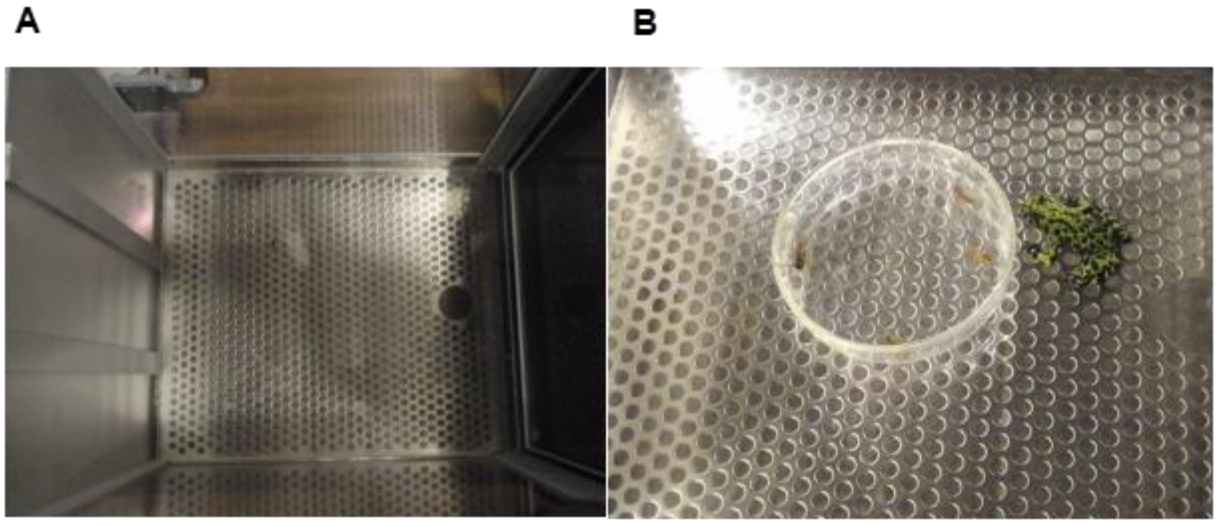

Figure 1. Experimental context. Fig 1A shows a top-down view of the empty context. Fig 1B shows a fire-bellied toad staring at crickets in a Petri dish during a consumption test.

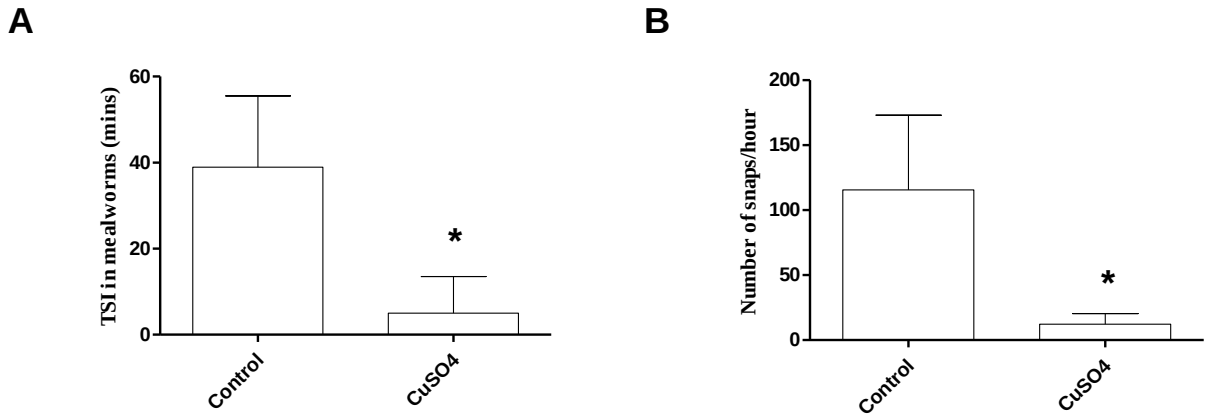

Figure 2. The effect of $CuSO_4$ on predatory behaviour. Fig. 2A presents the median amount of time spent interested (TSI) in mealworms for the two groups of toads during the 1 hr observation period in acquisition trials. Toads in the copper sulfate group spent significantly less time attending to mealworms underneath the Petri dish compared to control toads ($P < 0.01$); n = 8 for control, n = 35 for $CuS0_4$). Fig. 2B presents the median number of snaps attempted by toads. Toads in the copper sulfate group snapped significantly less at mealworms underneath the Petri dish than toads in the water control group ($P < 0.01$, n = 4 for control, n = 27 for $CuS0_4$). Vertical bars represent the upper quartile values.

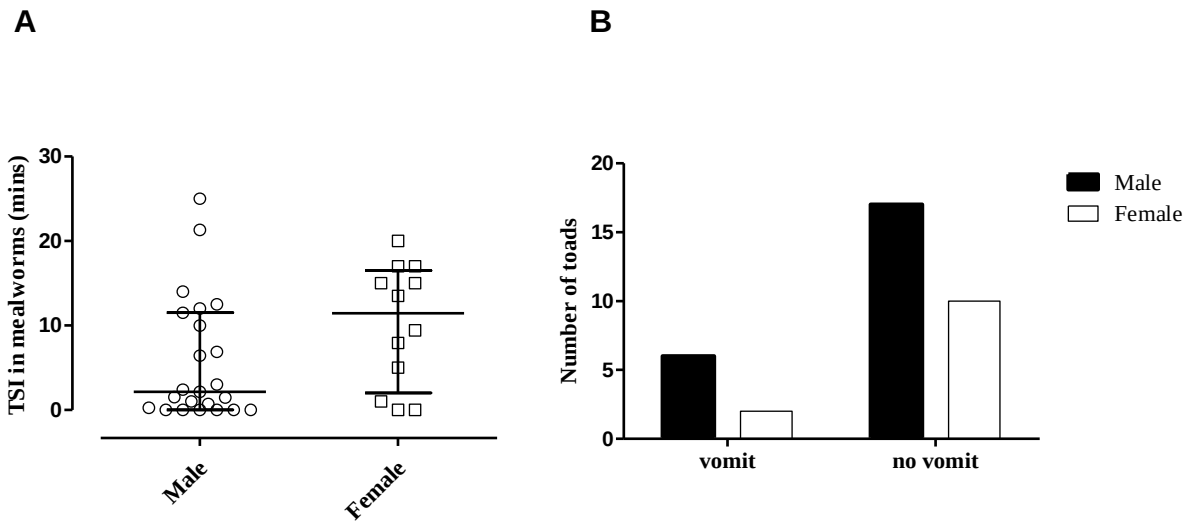

Figure 3. Avoidance and illness responses between sexes. Fig. 3A presents the median amount of time spent interested (TSI) in mealworms between male and female toads in the hour following consumption of a mealworm coated with copper sulfate. Males and females did not differ significantly in their TSI in mealworms after consuming copper sulfate treated prey (P = 0.10, n = 23 for males, n = 12 for females). Horizontal bars represent median ± interquartile range. Fig. 3B presents the number of toads that vomited in the 24-hour period that followed mealworm consumption. The proportion of males and females that vomited in reaction to mealworms dipped in copper sulfate did not differ significantly (P = 0.69, n = 23 for males, n = 12 for females).

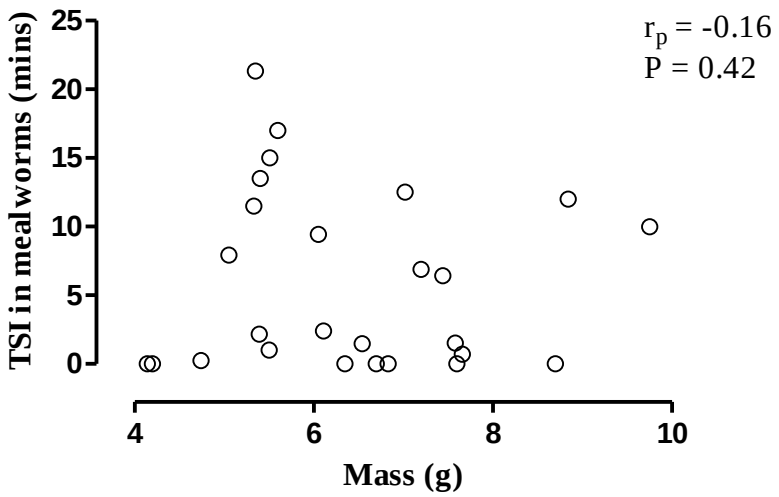

Figure 4. Body size and avoidance response. Relationship between body mass and time spent interested (TSI) in mealworms during acquisition trials. The relationship was not statistically significant (n = 27).

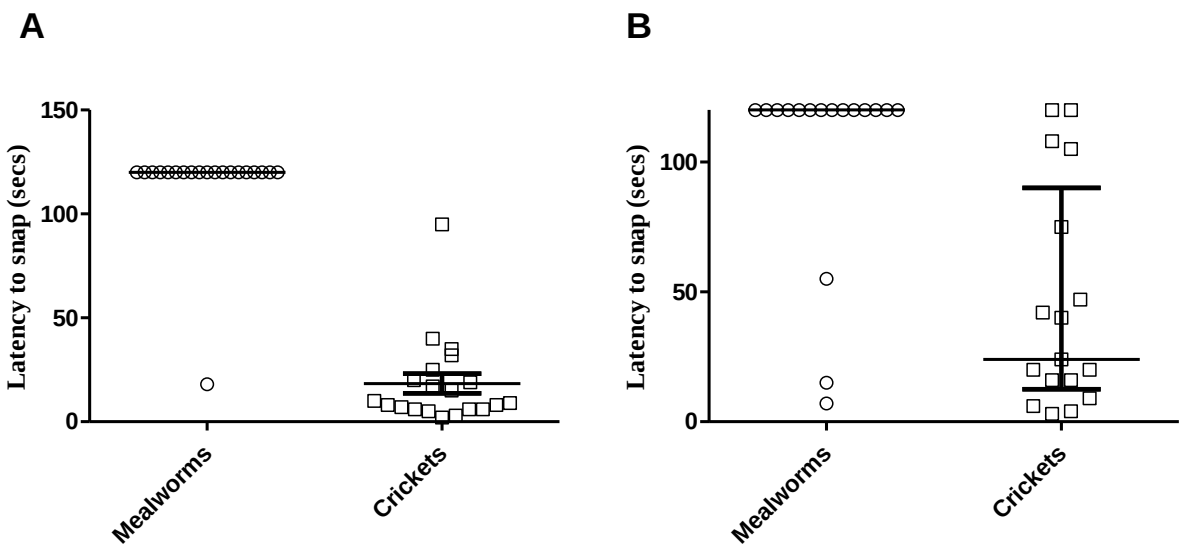

Figure 5. Specificity of prey avoidance. Fig. 5A presents the median latency to strike toward mealworms and crickets during restricted access in consumption tests. A Wilcoxon matched pairs test showed a significant difference ($P < 0.01$, $n = 20$) in latency to attack mealworms (novel) and crickets (usual) when toads were exposed to prey items trapped underneath a Petri dish. Fig. 5B presents the median latency to strike mealworms during unrestricted access in consumption tests. A Wilcoxon matched paired test showed a significant difference ($P < 0.01$, $n = 17$) in latency to attack mealworms (novel) and crickets (usual) when they were given free access. Horizontal bars represent median ± interquartile range. Individual values are also shown to illustrate inter-individual variation.

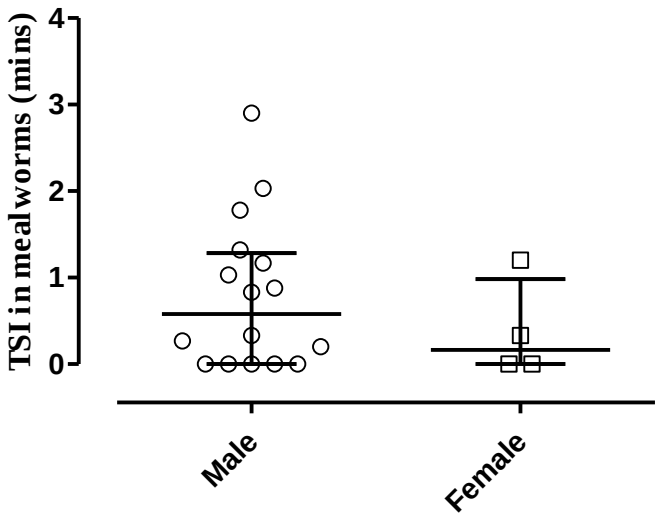

Figure 6. Sex and conditioned prey avoidance. Sex did not have a significant effect on time spent interested (TSI) in mealworms in consumption tests (P = 0.50, n = 16 for males, n = 4 for females). Horizontal bars represent median ± interquartile range.

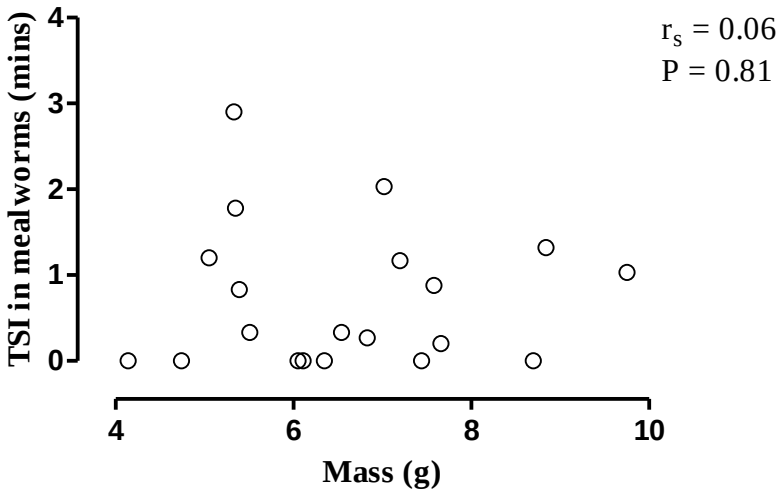

Figure 7. Body size and conditioned prey avoidance. The relationship between body size and time spent interested (TSI) in mealworms during consumption tests was not significant (n = 20).

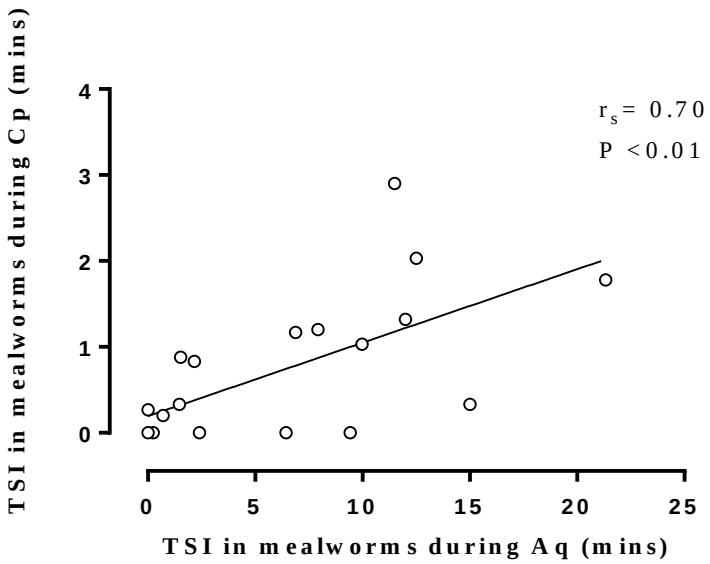

Figure 8. Avoidance intensity and conditioned prey avoidance. The relationship between time spent interested (TSI) in mealworms (MW) during the acquisition (Aq) and consumption phases (Cp) were significantly and positively correlated (n = 20).

General Conclusion

In this research project, sex, body size and avoidance intensity were investigated as potential factors involved in determining conditioned prey avoidance in fire-bellied toads. It was demonstrated that sex and size did not determine CPA in fire-bellied toads but that the strength of avoidance learning in toads is dependent on their initial reaction to copper sulfate coated prey. These findings are somewhat in line with the prior study conducted by To and Laberge (2014), where only half the toads could produce a CPA response in a similar situation compared to the majority of the toads showing CPA in the present study. Potential explanations for these discrepancies include differences in presentation of prey items or genetic differences between toads used in both studies. Regardless of the extent of prey avoidance displayed it is evident that fire-bellied toads can exhibit CPA. Overall, conditioned prey avoidance was correlated with initial behaviour towards mealworms during acquisition pairing such that avoidance intensity was the prevailing factor determining the strength of conditioned avoidance displayed by toads. It is likely that initial differences in prey avoidance displayed by toads are due to biological differences between individual toads that affect their susceptibility to $CuSO_4$. Therefore, future studies should pinpoint the biological mechanism involved in determining sensitivity to emetic substances.

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Appendix

Importance of molecular mechanisms in understanding learning processes

This study presents evidence of conditioned prey avoidance in the fire-bellied toad and highlights the importance of avoidance intensity in shaping prey avoidance behaviour. To supplement this behavioural observation, I attempted to study the brain pathways involved in CPA. Investigating the brain pathways involved in CPA in amphibians could clarify the mechanisms mediating the CPA response. Similar molecular studies have been successful in investigating cognitive mechanisms in fish and rats (Hoke et al. 2005; Lin et al., 2012).

The amphibian brain has many regions homologous to those of mammalian vertebrates. For example, the amygdala of amphibians is similar to the amygdala complex found in mammals (Laberge et al., 2006) and the telencephalon of amphibians is comparable to the cerebral hemispheres of amniotes (Laberge and Roth 2007). These regions are likely important in learning and associated limbic functions in amphibians. Most of the studies investigating conditioned avoidance have been done in rats, and have identified important brain regions involved in this phenomenon, such as the amygdala, gustatory thalamus and insular cortex (Koh et al., 2003; Dardou, 2006; Lin et al., 2012). To effectively study brain and behaviour relationships in animals it is necessary to use an appropriate tool for measuring brain activity.

Immediate-early genes (IEG) were first described as rapidly transcribed viral genes that utilize pre-existing transcription factors of the host cell and function in the absence of *de novo* protein synthesis (Morgan et al., 1987). In healthy cells, IEG are rapidly and transiently expressed proteins that can function in two distinct ways; they can act as an effector protein which directly impacts cellular function or they can act as transcription factors and regulate gene expression. IEGs are vital in triggering the genomic response of activated neurons, since they are

transcription regulators and DNA binding proteins which interact with response elements of a myriad of downstream effector genes to regulate overall gene expression (Veyrac et al., 2014). IEGs have been useful to localize brain activation in various contexts such as predatory behaviour, learning and long-term memory. For example, the general feeding behaviour displayed by rats was demonstrated to involve the visceral and gustatory cortex, since after feeding these specific brain regions exhibited an increase in IEG expression (Comoli et al., 2005). Therefore, IEG are a useful tool for studying brain responses.

Similarly, late expressed genes (LEG) are useful tools for studying brain responses in animals. LEG are synthesized in response to a variety of external stimuli such as secondary messengers and IEG (Clayton 2000). For example, calcium/calmodulin-dependent protein kinase II (CAMKII) is a calcium activated enzyme that directly influences voltage-gated ion channels, like NMDA and AMPA receptors, and therefore is involved in modifying electrochemical signalling of neurons (Lisman 2002). Furthermore, CAMKII is known to be involved in facilitating long-term potentiation (LTP) and enhancing the efficacy of synaptic transmission (Lisman 2002). The investigation of CaMKII autophosphorylation and dephosphorylation activation has led to the notion that this protein could serve as a molecular switch that allows for long-term memory storage (Lisman 2012).

In general, there is limited research pertaining to the brain pathways involved in learning in basal vertebrates. Therefore, it would be beneficial to explore amphibian brain function to understand how their relatively simple brains control behaviour. Additionally, such work may help provide a better understanding of how amniote brains have evolved since amphibian brains seem to represent a basic prototype of the more derived brains of amniote vertebrates. Using molecular approaches like qPCR and *in situ* hybridization allow for the temporal and spatial

quantification of gene expression in brain areas of interest. These molecular approaches were conducted to investigate mechanisms involved in learning and memory by using IEG and LEG as markers for brain activity and isolating specific brain regions involved in CPA. qPCR was conducted to investigate when brain expression of IEG and LEG reached peak expression in response to CPA. *In situ* hybridization would provide further clarity by studying the spatial resolution of IEG and LEG gene expression in specific brain regions that are speculated to be involved in illness and learning.

RNA extraction and cDNA synthesis

To prepare fire-bellied toad brains for RNA extraction, the brains were dissected under RNAse and DNase free conditions, the brains were separated into three regions (telencephalon, diencephalon and medulla/spinal cord) and stored in ethanol on ice. The extraction began by replacing ethanol with 1 ml of Trizol solution (Ambion Life Technology, Van alley way, CA). Briefly, each brain sample was homogenized using plastic pestles. The homogenized samples are then centrifuged at 12,000g for 10 minutes at 4°C. The tissue was incubated at room temperature for 5 minutes to allow for complete dissociation of nucleoprotein complexes. 250 ul of chloroform (Fischer Chemicals, Geel, BE) was added and sample tubes are shaken thoroughly for 15 seconds and incubated at room temperature for 15 minutes. Samples are then centrifuged at 12,000g for 15 minutes at 4°C. Thereafter, the mixture will have separated into an aqueous upper phase, an interphase, and a lower red phenol-chloroform phase. The upper aqueous phase was transferred into a fresh tube without disturbing the interphase. The next step involves precipitating the RNA out using isopropyl alcohol, glycogen and sodium acetate (reagents listed above are provided by Fischer Scientific, Lithuania, EU). The samples are then incubated overnight at -20°C.

The next day the samples are centrifuged for 10 minutes at 12,000g at 4°C before the supernatant was removed completely and the RNA pellet was washed with 75% ethanol. The samples are then mixed and centrifuged at 7.5g for 5 minutes at 4°C. The previous wash step was repeated once again and all leftover ethanol is removed. The RNA pellet was then air-dried for approximately 5-10 minutes. The purity of the RNA was assessed using the Nanodrop 8000 Spectrophotometer.

The next step after RNA extraction was cDNA synthesis. For one sample, a master mix involving the following components was created: 2.0µl of 10x Reverse Transcription Buffer, 0.8µl of 25x dinucleotide triphosphate (dNTP), 2.0µl of 10x Reverse Transcribe Random Primers, 1.0µl of Reverse Transcriptase and 4.2µl of Nuclease-free water (reagents supplied by Applied Biosystems, Foster City, CA). The next step involves using the S1000 Thermal Cycler machine and adjusting the properties to optimize cDNA synthesis. Step one involves incubation of RNA sample at 20°C for 10 minutes, while step two entails incubation at 37°C for 120 minutes, and lastly step three was run at 85°C for 5 minutes. The resulting cDNA can be used for qRT-PCR experiments.

qPCR protocol

qRT-PCR experiments were used to quantify the amount of target gene that is amplified in real time, this technique allows for highly accurate quantification of starting material. Internal control genes such as *18s*, *40s*, and *elfa* were used to compare against IEG and LEG expression. Time course experiments were conducted to investigate a proper time point for maximum gene expression. In preparation for qRT-PCR, primers are diluted 1:10 and primer mixes are prepared by adding reverse and forward primers with water. Sample master mixes are prepared by diluting stock samples 1:10 and by adding the appropriate amount of SYBR Green. SYBER Green is a

fluorescent dye that binds to double stranded DNA and emits green light. It was used to detect the amplification of target sequences during qRT-PCR. A 96 well plate was prepared by adding sample and primer mixes into each well. The 96 well plate was covered under a membrane sheet, spun in a centrifuge at 2600 rpms for 10 seconds and finally placed into the Applied Biosystem One-Step Plus qRT-PCR machine.

Preliminary qPCR experiments

Before conducting the qPCR experiments, various IEG and LEG were researched in relation to learning and memory. IEG and LEG primers were designed by using a whole-body transcriptome created by the lab of Dr. Jinzhong Fu, which contains an index of genes from a variety of amphibian species. Common genes were found in amphibian species closely related to and including the fire-bellied toad, and later validated through multiple NCBI blast searches. Further confirmation analysis involved using GenBank and NCBI to confirm the correct identity of the gene procured from the transcriptome (See Table Ap1 below). Thereafter, utilizing the CLC main bench program, each gene sequence was translated and the first open reading frame was taken and placed into the GenBank primer design feature.

Table Ap1: qPCR Primer Design. Several IEG and LEG sequences of interest were retrieved from the transcriptome.

Gene	Sequence (5'→3')	GenBank
bdnf (brain dervided neutrophic factor)	Fwd: CAGAGGACAGAGTGGCTTGG Rev: GGGTAGTTCGGCACTGTGAA	NM_001085482
c-fos (proto-oncogene)	Fwd: CTCTACTTGCCCTGACCTGC Rev: AGGTGTCAAAGAGGTTCCA	EF566821
CAMKIIA (calcium/calmodulin-dependent protein kinase type II alpha)	Fwd: TTTGGACTGGCCATTGAGGT Rev: TCGCCAGCATTGTGGTTAGG	NM_001017741.2
Egr-1 (early growth response protein)	Fwd: AACCTGGTCCCAATAGCAGC Rev: GGGGAGGTAGCAACTGACAC	NM_001097361.1
Fra-2 (fos-like antigen)	Fwd: TGGAAGCAGCAGTTCTCCAG Rev: ACAAAGCCAGAAGTGTGGGG	NM_001100929
GLUR1 (glutamate receptor 1)	Fwd: ACATGCGCTCAGCAGAACCT Rev: TGACCACTCTGCCATTCTCAC	NM_031608
Homer1	Fwd: GGGGGAGCAACCCATCTTTA Rev:TCCATCCAGGATCTCTAAAAGTGT	NM_001095583

Homer 1 is a protein induced by activity in neurons playing a role in synaptic plasticity (Foa et al., 2005). BDNF is a neurotrophic factor facilitating growth and differentiation of neurons and synapses in the central and peripheral nervous systems (Jenks et al., 2012). Egr-1 is a nuclear protein that functions as a transcriptional regulator implicated in long-term synaptic potentiation and memory consolidation (Morin et al. 2015). c-Fos and Fra-2 belong to the Fos family of leucine zipper proteins, which can dimerize with other IEGs to form transcription factor complexes and regulate gene expression (Kovács, 2008).

Late expressed genes were also retrieved from the transcriptome. CAMKIIA, a subunit of CAMKII holoenzyme, is involved in facilitating long-term potentiation (Lisman 2002). GLUR1 is a glutamate receptor subunit involved in memory and learning. More specifically, both proteins work in tandem to allow for the localization of AMPA receptor to the membrane surface. AMPA receptors induce the influx of calcium and sodium ions into the cell which influences action potential in neurons (Lisman 2012).

qRT-PCR time course experiments were conducted to determine the peak expression of IEG compared to housekeeping genes (*18S, 40S & elfa*). After toads were exposed to $CuSO_4$ as described in the Methods, they were sacrificed at varying intervals (30 mins, 60 mins, 90 mins, 120 mins and 180 mins). Whole brain samples were processed for RNA extraction and cDNA synthesis. Once a proper timeframe for mRNA expression was determined, qRT-PCR experiments were conducted on dissected brain regions between toads in the control (H_2O) and treatment groups ($CuSO_4$) to see if broad differences could be seen in regional IEG expression.

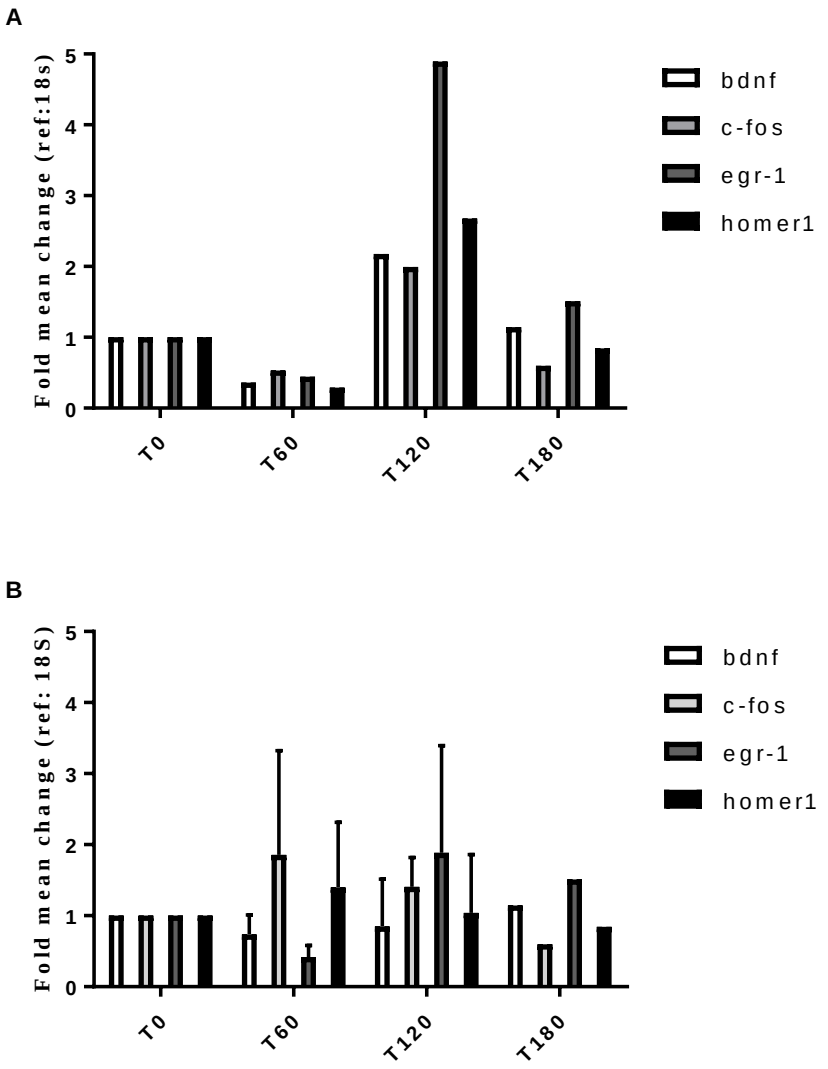

Figure Ap1. Time course of expression patterns of various IEGs at specific time points after toads had consumed a mealworm dipped in $CuSO_4$. Initial qPCR experiment results (Ap1A). Pooled sample of multiple time course experiments (Ap1B; n = 3 at T60 and T120 time points, n = 1 at T180). All time course experiments had one untreated animal (T0) that served as an internal control for other time points.

Initial qPCR time course experiments yielded inconsistent results across time points. Increased gene expression in select genes such as *egr-1* and *homer1* but minimal expression of other IEG made inferring patterns difficult (Figure Ap1A). Afterwards, a pooled sample of multiple qPCR runs was created to determine the general pattern of expression among IEG. Unfortunately, most IEG had minimal changes throughout the time course. Overall, there was no consistent pattern displayed when the data was pooled (Figure Ap1B). Observed differences in IEG expression between sexes led to subsequent experiments revolving around sex-specific time courses.

Unlike in previous qPCR experiments, egr-1 and homer1 showed no increase in expression 120 minutes after $CuSO_4$ exposure in both sexes (Figure Ap2). Unfortunately, the general trends of IEG expression in both sexes were stochastic and unpredictable. It is possible that there is a high degree of individual variation in IEG expression, which would contribute to the stochastic nature of gene expression displayed at various time points. The inability to produce conclusive results with IEG led us to switch towards the study of LEG.

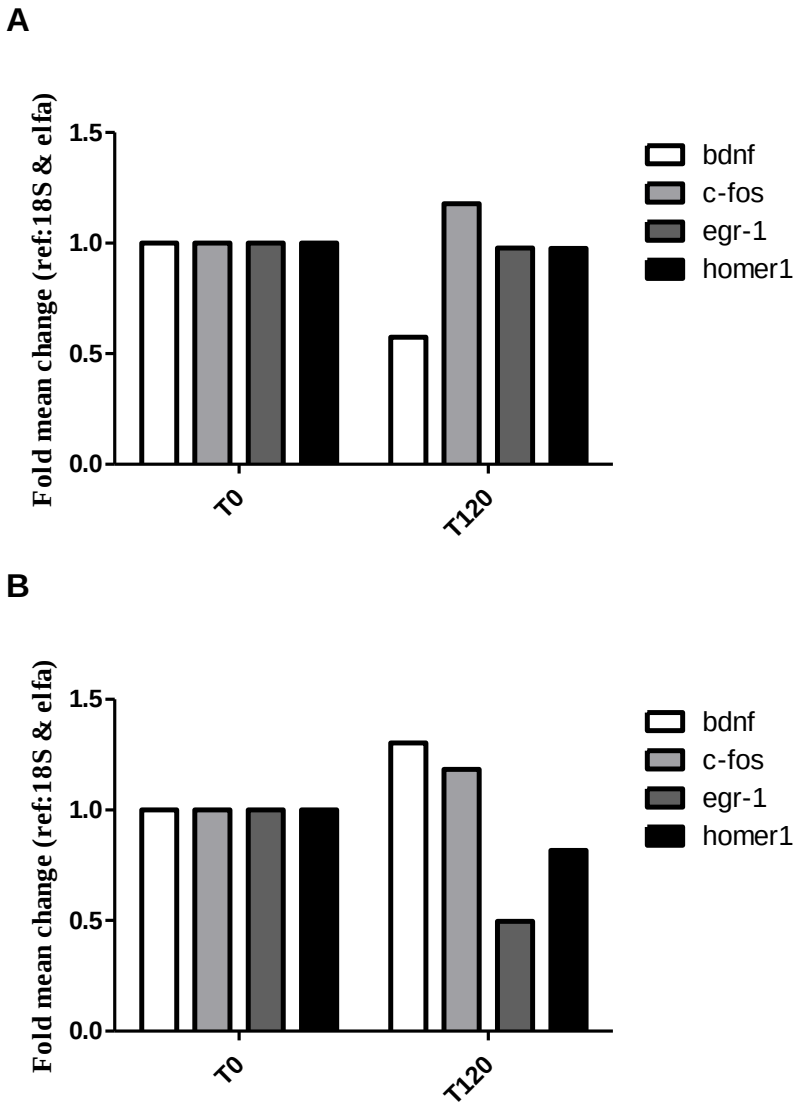

Figure Ap2. Sex differences in immediate-early gene (IEG) expression at 120 minutes after consumption of a mealworm coated with copper sulfate. The 120 min time point was chosen based on prior experiments showing upregulation of *egr-1 and homer1* at that time point in both sexes. IEG expression in females (A) and males (B) is the mean fold change of expression in relation to the mean of the reference genes *18s* and *elfa*. All time points were tested with one individual.

A time course of the late expressed gene *camkiia* yielded very favourable results by demonstrating high levels of gene expression 4-24 hours after toads were trained on a prey catching task (Figure Ap3). During this task, toads underwent 2 prey catching training sessions of 6 trials where they were fed a cricket reward after 5 snaps towards a cricket video stimulus with each session being separated by 15 minutes and each trial separated by 3 minutes. Toad snapping frequency increased with experience and was used as a measure of learning (not shown). Using the results from this experiment, 4 hrs was determined to be the standard sacrifice time point for experiments investigating LEG expression. However, qPCR tests with toads exposed to $CuSO_4$ produced no amplification of LEG. A second attempt using LEG with toads trained on the prey avoidance task showed lower mean-fold changes (~4-7) compared to the first attempt (~300-700), thereby showcasing the unreliability of testing these genes. Ultimately, the qPCR approach was abandoned due to inconclusive results.

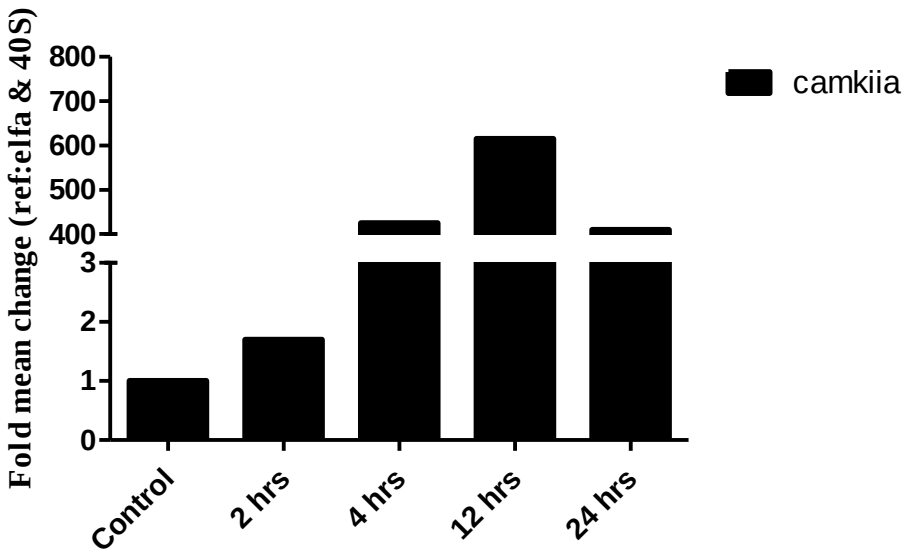

Fig Ap3. Time course of a late expressed gene after toads underwent prey catching conditioning. A time course of the late expressed gene *camkiia* yielded very favourable results by demonstrating high levels of gene expression at 4-24 hours after toads were trained on a prey catching task. Expression is the mean fold change of *camkiia* expression in relation to the mean of the reference genes *18s* and *elfa.* Note the broken y axis due to very high *camkiia* expression levels at late time points.

RNA probe synthesis

In-situ hybridization is a useful molecular tool for identifying spatial patterns of gene expression and can be used to indicate what brain areas are potentially involved in CPA in conjunction with IEG and LEG markers. Designing a proper RNA probe is important for ensuring specificity of *in situ* assays. RNA probe synthesis is required for conducting *in situ* hybridization experiments. Specific primer pairs are used to synthesize a DNA sequence with a T7 promoter region (TAATACGACTCACTATAGGG) which is used to facilitate RNA synthesis using T7 RNA polymerase.

Regular PCR was performed by creating a master mix reaction using 10 ul of HF Buffer, 1 ul of 10mM dNTP 2.5 ul of template cDNA (see RNA extraction and cDNA synthesis), 31 ul of RNAse free water, 0.5 ul of DNA polymerase and 2.5 ul of forward and reverse primers for the specific gene of interest (All reagent listed above are provided by Bio-Rad Laboratories, Hercules, CA). This master mix was placed into the S1000 Thermal Cycler machine. The machine underwent denaturation, annealing and extension steps to promote DNA synthesis. The machine denatures the cDNA template at 98°C for 40 seconds. Next the temperature was lowered to 52°C for 30 seconds which allowed for primer annealing. Then the temperature was raised to 72°C to initiate DNA extension. The denaturing, annealing and extension steps were repeated for 35 cycles. Lastly, the machine was run at 72°C for 5 minutes. Afterwards, the DNA product is tested on a gel to confirm the correct product was made. Lastly, the PCR product underwent DNA purification before being used for RNA synthesis.

The first step in RNA synthesis was making a master mix solution using a DNA template with a T7 tag (see above), 1 ul 10X RNA polymerase buffer, 1 ul DIG labelling mix, 1 ul T7 RNA Polymerase and 1 ul RNAse Inhibitor (reagents obtained from Roche, Fairlawn, NJ). Next

the master mix solution was incubated at 37°C for 3.5 hrs. Afterwards, 1 ul of DNAse1 (Ambion Life Technology, Van alley way, CA) was added to the master mix and then incubated at 37°C for 15 mins.

Then, 1.5 ul of a 7.5M $LiCl_2$ (Ambion Life Technology, Van alley way, CA) and 50mM EDTA (Fischer Bio Reagents, Fairlawn, NJ) solution was added to the master mix solution. 38 ul of 100% ethanol was gently mixed and the resulting solution was transferred into a 1.5 ml tube. This solution was kept overnight at -20°C. The next day this solution is spun at 12,000g for 20 mins at 4°C. The supernatant was removed while carefully avoiding the RNA pellet. 70% ethanol was then added to the 1.5 ml tube and spun for 5 mins at 7,500g. The supernatant was removed and the RNA pellet is dried for 2-5 mins. Lastly, the RNA pellet was re-dissolved in 20 ul of water and frozen (-20°C) until later use. The RNA probe contains the DIG antibody that is required for the *in situ* hybridization color reaction and visualization of mRNA expression.

In situ **hybridization protocol**

In-situ hybridization allows for the cellular localization of gene expression in tissues by utilizing either RNA or DNA probes to hybridize a known target sequence within a sample. The general procedure in this experiment utilized digoxigenin labelled RNA probes, anti-digoxigenin antibodies conjugated with alkaline phosphatase, and nitro blue tetrazolium chloride or 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as an alkaline phosphate substrate to localize mRNA probes. Visualization is accomplished by dephosphorylation of the alkaline phosphatase substrate forming a purple coloured precipitate (Jezzini et al., 2005). In preparation for in-situ hybridization *Bombina* brains were fixed with 4% (wt/vol) paraformaldehyde (PFA) at 4 °C for 2 d. The general procedure followed the Jezzini et al. (2005) protocol.

Over a three-day period *Bombina* brains were progressively acclimated in OCT (optimal cutting temperature compound, VWR, Radnor, PA) prior to sectioning. On the first day, brains were kept in a 30% sucrose solution in 0.1 M phosphate buffer overnight. The second day, the brains are moved into a half 30% sucrose and half OCT solution. The last day, the brains are immersed in a 100% OCT solution. The next day the brain tissue was frozen in a small basket in preparation for cryo-sectioning. The brain was imbedded within a frozen block and placed into a cryostat (Leica CM 3050S) for sectioning. Toad brains were sectioned in multiple series at 12 µm thickness. This produced multiple complements of sections representing the complete rostrocaudal extent of the brain for each individual toad. This method allows for different treatments to be applied on tissues obtained from the same individual. The *in situ* hybridization protocol is a 3-day procedure of progressive washes to prepare the sections for hybridization, antibody incubation and colour reaction.

Day One

Slides undergo air drying at room temperature to acclimate the tissues for washing procedures. They are subsequently fixed in 4% paraformaldehyde (PFA) before being thoroughly washed to remove excess PFA. The slides are washed in phosphate buffer saline (PBS) solution 3 times for 3-minute intervals at room temperature before being moved into a phosphate buffer saline-tween (PBST) solution and washed 3 times for 15-minute intervals at 70°C. In preparation for probe hybridization the slides are slowly acclimated to a hybridization buffer (HB: 25% Formamide, 12% Saline-sodium citrate buffer (SSC) x20 and 0.3% Tween 20) mix through progressive exposure via PBST and HB mixed solutions. Thereafter, the slides are immersed in full HB solution for an hour at 70°C before being exposed to the RNA probe/HB mix overnight

at 70℃. All reagents involved in making the HB are provided by Fischer Bio Reagents (Fairlawn, NJ).

Day Two

The next day the slides are removed from the RNA probe/HB mix and washed in 2X SSC 3 times for 30-minute intervals at 70℃. The slides are then washed in 0.2X SSC 2 times for 30-minute intervals at 70℃. A third wash in 0.2X SSC is done at room temperature for 30 minutes before the slides are placed into a tris-buffered saline-tween (TBST) solution and washed 3 times for 15-minutes intervals. The slides are then incubated in the block solution (bovine serum albumin and PBST) for 1 hr at room temperature. Thereafter, the slides are immersed in the Anti-DIG (anti-digoxigenin conjugated w/ alkaline phosphatase) solution at 4℃ overnight.

Day Three

After the overnight antibody incubation, the slides are washed in TBST 5 times for 30-minute intervals at room temperature. The slides undergo AP (alkaline phosphatase) buffer washes 3 times for 5-minute intervals at room temperature, followed by a colour reaction incubation (NBT/BCIP fragments) at room temperature in which the RNA labelled probe with the attached anti-dig antibody will give a purple coloured precipitate (the Anti-DIG antibody and colour reaction used reagents provided by Roche, Mannheim, Germany). The development time for this reaction varied depending on which gene was used. Typically, highly expressed genes like 18s took 30 minutes, while IEGs and LEG which are moderately expressed took longer (~1-3 days). Microscope slides containing brain sections are subjected to air drying and permanent mounting medium procedure to create long lasting cover-slipped slides for microscope viewing.

In situ hybridization preliminary experiments

Table Ap2: *In situ* Probe Design. Before conducting *in situ* hybridization experiments, it was important to design RNA probes for specific genes of interest. As described earlier, IEG and LEG probes were designed using the transcriptome provided by Dr. Jingzhong Fu. A T7 Promoter region is tagged to the 5' end of reverse primers to promote RNA synthesis.

Gene	Sequence (5'→3')	GenBank
c-fos	Fwd: AGCCTGACCTACTACCCGTC Rev:(TAATACGACTCACTATAGGG)AGGTGTGCAAAGAGGTTCCA	EF566821
CAMKIIA	Fwd: AAGAAGGGCACCATTACCTGG Rev:(TAATACGACTCACTATAGGG)ATGGCCCCCTTTAGCTTTCT	NM_001017741.2
egr-1	Fwd AACCTGGTCCCAATAGCAGC Rev:(TAATACGACTCACTATAGGG)GGGGAGGTAGCAACTGACAC	NM_001097361.1
GLUR1	Fwd: CCAAGTCATCTGCGCTCTCT Rev:(TAATACGACTCACTATAGGG)AAACTGATCCCCCTGGCAAC	NM_031608
homer1	Fwd: ACCAGCAAACATGCCGTTAC Rev:(TAATACGACTCACTATAGGG)AGGCGTTGTTCTAAGTCGCT	NM_001095583

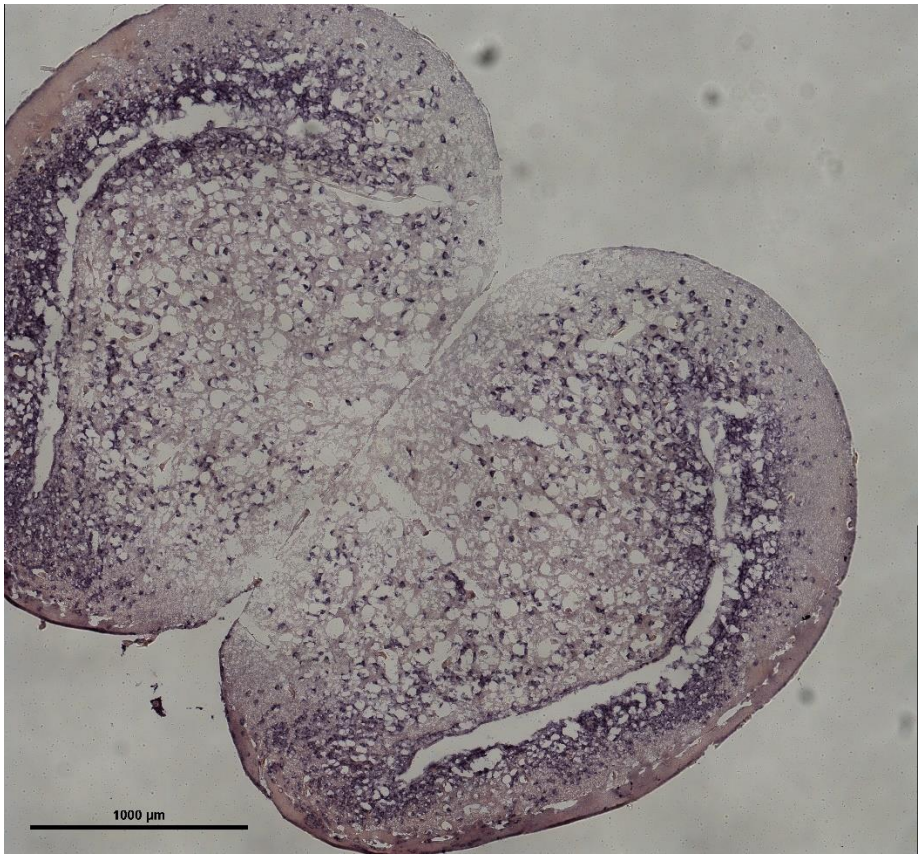

Fig Ap4. Spatial expression of 18s in a section of the fire-bellied toad telencaphon. An 18s antisense probe was used to test the *in situ* hybridization protocol on toad brain tissue. This individual was treated with a $CuSO_4$ coated mealworm and sacrificed after 4 hours. Abundant dark purple cytoplasmic labelling can be seen throughout the telencephalon, likely representing neurons with a high protein synthesis activity.

The preliminary *in situ*-hybridization experiments yielded positive results, as protocol testing was very successful with the standard control probe 18s (Figure Ap4). However, when the assay was tested for IEG and LEG the results were not successful. In general, there was inconclusive staining throughout the brain and on occasion there was unspecified nuclear staining which did not fit the profile of the genes being tested. It was suspected that the inconclusive staining was due to the generally low expression of IEG. Troubleshooting the *in situ* protocol might remedy the lack of consistent staining seen when testing LEG probes. The foundation for spatial mapping of gene expression in fire-bellied toads requires further refinement of the *in situ* hybridization protocol to properly investigate spatial gene expression with low abundance transcripts.

General Findings

Overall, initial qPCR experiments with IEG demonstrated that these genes were not well suited for studying brain activity in toads due to low and variable amplification displayed during preliminary trials. A potential explanation for this outcome could be naturally high variance in IEG expression among individual toads. Using LEG proved more fruitful since initial qPCR experiments demonstrated how a LEG like CAMKIIA was upregulated between 4 and 24 hrs after prey catching training. Unfortunately, continued testing with qPCR led to inconclusive results, likely due to technical problems. Possible troubleshooting steps for increasing gene amplification would involve tweaking the cDNA synthesis protocol to increase quantity and quality of cDNA for qPCR experiments.

The *in situ* hybridization method was developed from toad brain tissue and provided a reliable way to visualize spatial patterns of gene expression. Preliminary trials with 18S showed the promise of this method, unfortunately more effort is needed to develop a suitable protocol for

target genes expressed at low levels. For example, increasing the concentration of RNA probe or modifying the colour reaction components could be potential troubleshooting options for increasing resolution of LEG expression. Overall, there is potential merit for continued investigation of spatial and temporal gene expression in amphibian brains associated with learning and memory. The framework of such investigations has been established and can be modified to answer interesting questions about brain-behaviour relationships in basal animals such as amphibians.

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