METHODOLOGICAL APPROACHES FOR SOUND TRAINING IN UNDEREPRESENTED LEARNERS: A CASE STUDY WITH AMERICAN TOADS (*ANAXYRUS AMERICANUS*)

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

METHODOLOGICAL APPROACHES FOR SOUND TRAINING IN
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(*ANAXYRUS AMERICANUS*)

presented by Deise Cruz Santos,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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This is dedicated

to my family

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ABSTRACT

The efforts to better understand the minds of animals have been flourishing, with methodological breakthroughs and a remarkable increase in the number of publications dealing with a wide variety of non-model species. The growing interest in species that are distantly related to humans in the field of comparative physiology and cognition was confirmed with the general reviewed performed in this dissertation. Yet, the progress is unbalanced among the ectothermic vertebrates (fish, reptiles, and amphibians), with almost no research on amphibians. Many animals remain unstudied, even though they may possess unique and powerful adaptations to respond to environmental stimuli that can be useful for learning and cognition research. Inspired by the efforts to increase species representation in studies of learning and cognition, this dissertation also explored two methods of spatial learning to train American toads to respond to tone burst cues in order to find the reward. As frogs and toads have been able to acquire maze task associated to visual cues and mating calls, I predicted that a protocol based on these previously successful methods could be reliable in testing toads to associate and discriminate tone bursts of different frequencies (HZ). None of the methods were effective in demonstrate learning abilities in American toads, but the results pointed to important challenges to calibrate methods for future studies. Aspects to consider such as sex effects on side bias and can be used to reflect behavioral plasticity as a metric for the process of learning, such as time latency (longer it takes a toad to succeed, the more likely they will be successful) and the behavior displayed during the task as an

indication of behavioral flexibility for decision making. Besides these aspects of the procedure, there are physiological and evolutionary aspects that might make toads unable to interact with non-mating sounds. These aspects and the level of hearing constraints that can affect learning assessment in toads are critical to answer broad questions on anuran auditory role beyond mating purposes.

CHP 1 – INTRODUCTION

Our understanding of the learning and cognition abilities of animals is expanding, and often we discover shared cognitive traits following the evolutionary perspective of Darwin in the evolution of minds. Abilities that heretofore were attributed just to humans (e.g., mirror recognition, tool-use and cultural transmission) are manifested in some form in other primates, elephants, birds, fish and social insects (De Waal 2016; Bshary and Brown 2014; Giurfa 2015). The efforts to better understand animal's minds are flourishing because of methodological breakthroughs and a remarkable increase in the number of publications dealing with a wide variety of non-model species in the last 15-20 years (Healy 2019; Vonk 2016; Blaser and Bellizzi, 2014; Shettleworth 2009). Many animals and large taxa nevertheless remain unstudied even though they possess unique and powerful adaptations to respond to environmental stimuli. As researchers and humans, the main challenge to building a robust database of the cognitive abilities of animals lies in setting reasonable circumstances and environments for the animal to display their cognitive skills (Bräuer et al. 2020; de Waal, 2016). The experimental protocol and test environment ideally matches the ecological relevance and specialization of the sensory modality being investigated. For instance, assays that require animal learning based on vision are inappropriate when environmental adaptations are driven by other domains, such as smell, sounds, taste, and magnetic field cues. We are therefore more challenged by developing a proper experimental setup and anthropocentric interpretation rather than the lack of cognitive skills in the tested animal.

The relevance of expanding knowledge about learning and cognition of underrepresented taxa has also become a growing demand in human studies (Fshbein et al. 2019; Fitch 2020). While the evolutionary analysis of human language has depended on large amounts of data from birds and other mammals, there is a glaring gap in an early tetrapod group: the Anurans, an Order that includes frogs and toads, whose vocal communication has been well studied (Gerhardt and Huber 2002; Ryan 2001). The vast majority of anurans frequently communicate using acoustic signals in the context of mate identification, attraction, territorial defense, and other forms of male-male aggressive interactions. There is also a vast literature dealing with the neurophysiology and sensory physiology of the auditory system of anurans. Many of these studies document hormonal regulation of the auditory system and a hearing capability for sounds with different physical properties than their natural mating sounds (Capranica and Moffat 1983; Gerhardt and Schwartz 2001; Simmons et al. 2007; Walkowiak et al. 1981; Wilczynski and Lynch 2011; Wilczynski and Burmeister 2016). Still only a handful of studies have applied learning paradigms from experimental psychology and behavioral ecology to explore the auditory capabilities of anurans in natural and laboratory situations (Bee and Gerhardt 2001, 2002; Elepfandt et al. 2000; Sotelo et al. 2019). Even fish have been studied more extensively than anurans in the context of auditory conditioning (Bshary and Brown 2014; Brown 2015).

An overall skepticism about the learning abilities of amphibians is prevalent regardless of the sensory modality. Unlike birds, most anurans do not seem to be able to learn sounds even though they generally display avoidance to unfamiliar sounds, which suggests some mechanism of the auditory process to unwanted sound stimuli. Therefore, in this dissertation, I explore methodological approaches and discuss essential parameters to consider when developing training methods for terrestrial anurans in sound learning and discrimination tasks. The work on frogs and the entire amphibian class requires a major effort to claim even a modicum of visibility relative to other animal groups including other ectothermic vertebrates.

1.1 Anuran hearing behavior

Hearing is a crucial sensory modality for reproductive success and species recognition in anurans, playing a central role in making decisions by gravid females and male vocal competitors (Gerhardt and Huber 2002; Rand 1985; Ryan 2009; Wells 2007). The auditory system of anurans has effectively evolved to identify, choose, and locate conspecific calls (Ryan 2009, Gerhardt and Bee 2007; Gerhardt and Huber 2002) in environments that are frequently densely vegetated, and dark — conditions in which vision might be unreliable. The hearing adaptations and the reproductive dependence on vocal signals have placed anurans as traditional models for studies of sexual selection and the evolution of sound communication and behavior (Bee 2015; Ryan 2009; Gerhardt and Huber 2002). Much of the research on the anuran hearing system has evaluated responses to properties of sounds in a breeding context rather than assessing auditory sensory behavior in general. Some of these data will be reviewed below (see *Neurophysiological Studies)*. Frogs have evolved a powerful hearing system, especially sensitive to the reproductive repertory of the conspecific advertisement call (matched filter hypothesis [Capranica and Moffat 1983]), although not limited to it (Jaeger 1976; Wilczynski and Burmeister 2016). While descriptions of their neurophysiology capability demonstrate that anurans can detect alternative sounds, the behavioral responses towards non-breeding stimuli remain poorly known.

1.2 Non-mating function of the auditory perception

Some insights into hearing behavior in non-breeding contexts have been gained by studying anuran responses to sounds related to predation and escape behavior. Large cane toads were observed orienting to the túngara frogs advertisement call and consequently feeding on them (Jaeger 1976). In phonotaxis assays, common toads (Taylor 2001) and green treefrogs (Höbel et al. 2014) failed to demonstrate the auditory role in foraging behavior towards orthopteran signals. Albeit significant, avoidance of green treefrogs in the direction of cricket's sound suggests the stimulus was perceived, but not interpreted as a feeding opportunity (Höbel *et al*. 2014). In a field playback experiment to assess escape behavior (Grafe et al. 2002; Llusia et al. 2010), common toads showed a weak response when presented to predator acoustics cues, thus indicating a deficit in the recognition of predation risks (Llusia et al. 2010). In a most surprising study, Grafe et al. (2002) showed that African savannah reed frogs fled the source of the sound of a fire towards fire-resistant sites and vegetation. This behavior however did not occur when these frogs were tested during the rainy season when fires were rare or non-existent. This result reflects seasonal effects of hormones on the auditory behavior of vertebrates (Arch and Narins 2009; Maney and Pinaud 2011; Sisneros et al. 2004; Watts 2020; Wilczynski and Burmeister 2016). Indeed, this is a critical factor to consider when studying the hearing abilities of frogs and toads, mainly the plasticity of mate choice in different reproductive states (Hillery 1984; Kelley 1980; Lynch *et al*. 2006; Wilczynski and Lynch 2011). In fact, non-gravid or post-reproductive female anurans seldom show phonotactic responses to conspecific calls in the field or in test arenas (Klump 1995) and require injections of hormones to stimulate female phonotaxis in either situation (Chakraborty and Burmeister 2009; Gordon and Gerhardt 2009; Ward et al. 2015). Moreover, modulation in hormone levels, seasonality, reproductive state, auditory sensitivity can affect a variety of physiological responses to sounds. For instance, post-mating females, although behaviorally unresponsive at low steroid levels, had low thresholds and high hearing sensitivity to tone and conspecific call frequencies in treefrogs (Baugh *et al.* 2019; Gall *et al.* 2019). When castrated, green treefrogs show no difference in auditory sensitivity compared to reproductive state individuals (Penna *et al.* 1992). In addition, non-breeding and immature froglets show positive phonotaxis (Baugh and Ryan 2010; Lea et al. 2002), suggesting that anuran auditory systems do not always promote only sexual behaviors.

1.3 Psychophysical contributions

Behavioral approaches to questions about general hearing abilities require the use of psychophysical methods. This requires the use of experimental stimuli that do not necessarily correspond to signals in their natural repertoire and individuals that are not primed naturally or artificially to be responsive to sound playbacks. Besides relying on behavioral responses that express how animals perceive a certain property of a stimulus, psychophysical studies also require conditioning experiments for better control of motivation and an eventual bias for previous experience (Dent 2017; Dooling et al. 2000; Fay 1989). In anurans, a major challenge is to find relevant tasks to employ operant conditioning species subjects, mainly accessing the hearing domain. Earlier attempts to condition anurans with terrestrial habits failed (Brzoska and Schneider 1978; McGill 1960; Yerkes 1903), and ever since, experiments to test frog association to non-breeding sound cues have proved to be difficult, long-term, and inconsistent. Studies performed of aquatic clawed frogs claimed to have demonstrated sound learning and training subjects to determine frequency discrimination of tone burst stimuli is possible in anurans (Elepfandt et al. 2000). These experiments were however flawed by the fact that vibrational cues were not excluded during aquatic sound presentations and the punishment (hitting the frog) was both uncontrolled, unquantified, and could not be replicated because of changes in animal care and use protocols. Using classical conditioning, such as reflex modification and respiration movement responses, frogs and treefrogs were subjects of psychophysical approaches to determine hearing sensitivity as alternatives to traditional methods in electrophysiology (Kleerekoper and Sibabin 1959; Megela-Simmons et al. 1985). Some of these studied produced could threshold curves similar to those generated by neural measurements taken at the midbrain level (see Fig. 1).

Using habituation (a primary type of learning), Bee and Gerhardt (2001, 2002) evaluated aggressive responses of bullfrogs (*Lithobates catesbeianus*) to determine differential threshold in territorial call parameters. Applying synthetic advertisement calls they showed that sound frequency was a key property used to distinguish between the calls of established neighboring rivals and unfamiliar territorial rivals. The minimum change in the frequency of the low-frequency spectral peak in these stimuli that elicited aggressive behavior was about 5%, which is well above estimates of the frequency resolution ("just noticeable difference") estimated from neurophysiological studies of hearing in this species and other anurans (details below in the next two sections). A limitation of this approach was the lack of a way to control the motivation of the frog via a reward or punishment by the researcher. Indeed, aggressive responses to other males or playbacks were limited to the breeding season of this species. In general, the number of studies that have used classical conditioning approaches to quantify sound discrimination in airborne-hearing frogs pales in comparison to the extensive studies of other vocal vertebrates such as fish, birds, and mammals (Dooling and Saunders 1975; Fay and Wilber 1989; Heffner et al. 1971; Jacobs and Tavolga 1968; Langemann and Klump 1992; Marvit and Crawford 2000; Park et al. 1985; Walker et al. 2009). This is almost certainly a major reason for the paucity of studies of learning and conditioning in amphibians in general and frogs in particular (see Chapter 2).

1.4 Neurophysiological studies of anuran hearing

Most information about the hearing ability of frogs has focused on measuring sensitivity, in terms audible frequencies (Hz) and thresholds (dB), commonly depicted in audiogram curves. The absolute hearing threshold is simply the lowest sound intensity in which a certain frequency can be detected, and generally, the estimations vary over species, sex, the stimulus, and the method applied (Lauridsen et al. 2020; Taylor et al. 2019; Buerkle et al. 2014; Werner et al. 2009; Megela-Simmons et al. 1985). Anuran audiograms have been accessed through a wide range of techniques and approaches, such as auditory brainstem responses (ABR), multiunit electrophysiological responses, and behavioral responses (Taylor et al. 2019). These authors applied an unconditioned stimulus to trigger phonotaxis responses as opposed to the psychophysical approaches described above (Elepfandt et al. 2000; Megela-Simmons et al. 1985).

Overall, anuran audiograms show a wide range of auditory sensitivity, reinforcing the idea that anurans hear more than their stereotyped mating calls. For example, in one study a bullfrog was estimated to hear 100 – 3000Hz at a standard intensity of 60dB SPL (see fig. 1A, Heffner and Heffner 2007). Another study of this species that compared their behavioral and neural audiograms found these frogs were behaviorally more sensitive, with a threshold of only 20dB in a range of 400 – 1600Hz (see fig. 1B, Megela-Simmons et al. 1985). Within this hearing range, two specialized organs of the peripheral auditory operate with distinct tuning mechanisms (Capranica and Moffat 1983). The low-frequency tuned amphibian papilla (AP), with tonotopically organized hair cells has a greater number of cells than the basilar papilla (BP) which detects higher frequency and an overlapping range (1000-1200 Hz) to which the AP is also sensitive (Wilczynski and Burmeister 2016; Ryan 2009; Simmons et al. 2007; Gerhardt and Schwartz 2001).

1.5 Behavioral approaches to estimating JNDs

To fully understand hearing in anurans across entire audible range, it is essential to determine if a failure to respond or discriminate is due to a failure in motivation or in auditory processing. Considering hearing definition as the behavioral response to sound involving the whole, awake organism, a key psychophysical metric is the just noticeable difference (JND) (Akre and Johnsen 2016, Dooling et al. 2000, Fay 1988). The single study in anurans that provided such a metric was the flawed study by Elephandt et al. (2000) described above; they estimated the finest differential hearing perception JND of 2% in the purely aquatic frog (*Xenopus laevis*). To my knowledge no JND has been estimated for terrestrial anurans through conditioning methods. The lowest estimation of 5% frequency discrimination demonstrated in bullfrogs, performed through habituation assays (Bee 2016), is more conservatively characterized as a JMD (Just Meaningful Difference) than a JND (Nelson and Marler 1990).

Other protocols that have attempted to estimate hearing discrimination thresholds, and possibly JMDs, of terrestrial frogs have used untrained animals in traditional playback and phonotaxis experiments in mate choice context. For instance, the smallest difference in frequency (Hz) that triggered female preference to one conspecific call over the other in treefrogs was reported around 10 – 15% (Hobel 2016; Gerhardt 1991). In other words, females show no preference between acoustic signals with smaller percentage differences. Generally, a male toad can emit calls with 5% intraindividual variation in dominant frequency, and chorus males 7% inter-individual variation (Howard and Young 1998; Sullivan 1992). Such levels of discrimination have not been reported to be discriminated by females in phonotaxis tests. This means that the signal variation among males of 5-7% may be biological irrelevant to the female decision, under the risk of predation and waste of energy to act towards small increments of mates. Moreover, the female choosing randomly does not reflect incapability of hearing (e.g., JND < 7%), but it could represent an estimate of the JMD as a limitation of phonotaxis metrics. Therefore, methodologies focused on trained subjects with controlled motivation are recommended to indicate whether the lack of response to signals reflects a failure on the hearing ability (sensory resolution) or lack of motivation (ecological relevance and psychological experience).

1.6 Learning skills of anurans

While the skepticism about frog learning abilities, mainly associated to sound cues, stems from early and unsuccessful attempts to pure tone association in the green frog (Yerkes, 1903) and European toad (Brzoska & Schneider 1978), the recent studies that applied habituation (Bee and Gerhardt

2001, 2002; Davis 1987) and conditioning paradigms (Elepfandt et al. 2000) reinforce the possibilities of investigating the auditory cognition and behavior constraints in anurans. More recently, Argentine toads (*Rhinella arenarum*), a species with an airborne ear system, successfully performed in a maze task following conspecific advertisement calls (Sotelo et al. 2019). Males acquired the association of conspecific sound with hydration reward faster than when trained to associate visual and geometric cues (Sotelo et al. 2015, 2017, 2019; Daneri et al. 2011, 2015). However, numerous studies describe the learning skills of anurans in spatial tasks associated with visual cues (Brattstrom 1990; Daneri et al. 2011, 2015; Liu et al. 2016, 2020; Sotelo et al. 2015, 2017, 2019; Ventura et al. 2019). Poison dart frogs have also demonstrated cognitive capacity in maze tasks though (Liu et al. 2016, 2020), motivated with distinct rewards and procedures than those developed using the Argentine toad model. Overall, these results, even though most promising for the visual modality, represent some progress in assessing anuran sensory capabilities. If classical conditioning protocols can eventually delimit the full function of the frog auditory system, this knowledge will also complement and contribute to testing hypotheses about sexual selection and signal evolution in airborne systems (Akre and Johnsen 2014; Bee 2016). Specifically, whether a slight difference between two signals is relevant to express mating decision and whether discrimination constraints of the listener limit competition among signalers. These determinants of auditory behavior might impair signal elaboration and instead drive new and multimodal mating attributes.

1.7 Conclusion

As an important model for acoustics behavior and communication studies, the complete understanding of the hearing abilities of anurans should be extended beyond the breeding context. In Chapter 2, I review – over several decades - the status of studies of ectothermic vertebrates (fish, amphibians and reptiles) to evaluate the progress and efforts to understand the mental skills of those animals. In Chapters 3 and 4, I then used two different conditioning approaches to evaluate the potential to assess the auditory sensitivity of American toads (*Anaxyrus americanus*) by attempting to condition them to respond to tone bursts and to distinguish between tones of different frequency. I used two different procedures and rewards to compare the performance of in maze tasks. In Chapter 3, I tested a method based on food reinforcement, following procedural settings used to condition poison frogs to escape a two-arm maze associated with visual cues (Liu et al. 2016). Chapter 4, I replicated other methods based on partial-dehydration and water reward associated to mating sounds cues (Sotelo et al. 2019). Moreover, I modified the goal of these studies to evaluate a suitable protocol for conditioning toads based on sound frequency discrimination. I predicted that American toads would respond best to water reward conditioning, which was successfully applied to studies of Argentine toads (Sotelo et al. 2019). My attempts to condition American toads were unsuccessful, and I discuss some possible reasons for this (e.g., low of motivation and seasonality) and unexpected complications such as sex-specific side biases. My results should nevertheless help to guide future research that can advance our understanding of the finer-scale sound discriminating abilities of anurans.

1.8 Figures

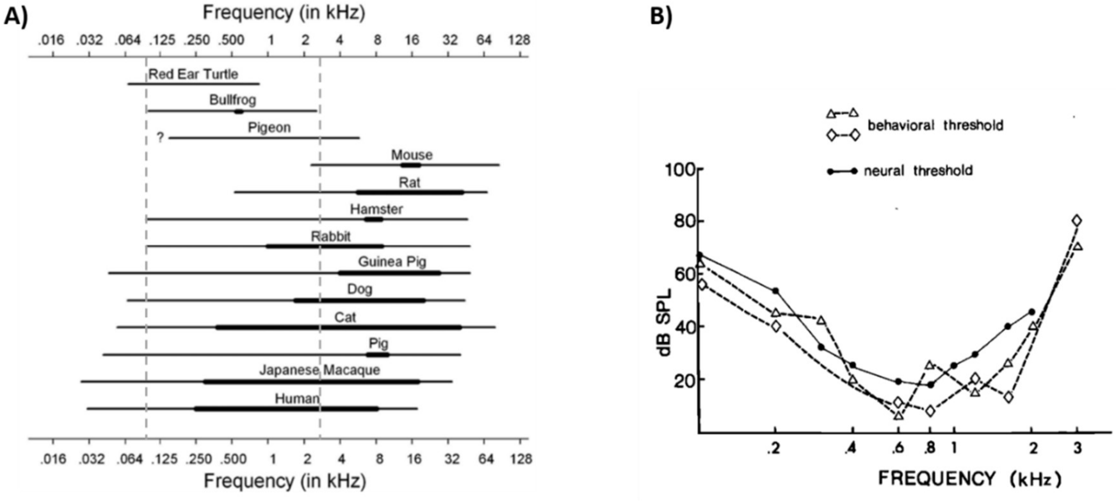

Figure 1. Hearing range of laboratory animals and bullfrog audiograms. Left - Bullfrog frequency range by Heffner and Heffner (2007) overlapping with other animals (within dash lines). Right - Audiogram of two bullfrogs by Megela-Simmons et al. (1985) comparing hearing curves of neural and psychophysical measurements.

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CHP 2 – THE NEED TO INCREASE THE DIVERSITY OF APPROACHES TO ANIMAL COGNITION IN NON-TRADITIONAL SPECIES

Deise Cruz

2.1 Challenges

Since Pavlov, Thorndike, and Skinner presented techniques to demonstrate learning in dogs, cats, and rats, respectively, a large number of species have been successfully conditioned (Wynne and Udell 2013). Conditioned animals are capable of demonstrating associative learning, the most assessed type of learning in animals (Blaser and Bellizzi 2014; Lambert et al. 2021). Associative learning is based on the causal relationship among stimuli and actions built through training and reinforcements (Breed and Moore 2015; Laland et al. 2020), but this form of learning is just one piece of the vast spectrum of possibilities in animal cognitive skills. The endless interest of scientists to reveal learning and cognition in non-human animals has resulted in the documentation of impressive complex cognitive abilities in birds and primates such as memory, imitation, face recognition, and problem-solving; skills that were once only attributed to humans (De Waal 2016). These amazing discoveries do not imply that some animals are “smarter” than others but instead indicate that there are other minds than humans to be understood for a complete comprehension of mental abilities. Reaching such research status requires, however, a concerted effort to develop techniques and experimental procedures with meaningful circumstances to the animal. Moreover, there are still large taxonomic groups of vertebrates (e.g., reptiles and amphibians) for which even basic aspects of learning capacity are still relatively unexplored (Matsubara et al. 2017; Vonk 2016; Burghert 2013).

A gross imbalance also exists with regard to species diversity in the fields dedicated to understanding animal mental skills, which cover learning and other mechanisms of cognition. The goal of increasing the range of taxa aligns with methodological, conceptual, and structural debates that point to a series of challenges and considerations within comparative cognition and related fields. Many of the challenges can be related to the distribution of studies within the broad field of cognition – "ways in which animals take in information about the world through the senses, process, retain and decide to act on it through mechanisms of learning, perception, memory, and decision" (Shettleworth 2001). Such studies are often reported in a variety of contexts (e.g., spatial, social, numerical cognition) and overlap through multidisciplinary perspectives, such as comparative psychology, cognitive ethology, behavioral ecology, sensory ecology, neurobiology, and evolutionary psychology (Shettleworth 2009; Wasserman 1997). This extensive scope supports recommendations to strengthen cross-disciplinary collaborations (e.g., field of psychology, evolution biology, or statistics [Beran et al. 2014; Krasheninnikova et al. 2020]).

Cognitive researchers have accumulated vast amounts of information on a limited number of non-human animals (primates, rats, pigeons), while information on the majority of the animal kingdom is lacking (Beran et al. 2014; Fragaszy 2018; Healy 2019; Vonk 2016). Recently, this quantitative disparity in species coverage in animal cognition studies has become noticeable and has resulted in a considerable shift of the range studied in the last decade (Bräuer et al. 2020; Shettleworth 2009). Under the umbrella of animal cognition and comparative psychology, numerous reviews have surveyed and discussed progress and pointed out gaps in the cognitive literature of non-human species (c.f. Beran et al. 2014; Blaser and Bellizzi 2014; Bräuer et al. 2020; Domjan and Krause 2002; Healy 2019; Krasheninnikova *et al*. 2020; Papini 2002; Shettleworth 2010 and 2009; Stevens 2017; Vonk 2016). The discussions in these reviews are extensive and debates about potential solutions focus on three main aspects: 1) Inclusion of new taxa and species as subjected to cognition studies; 2) diversification on topics, questions, and problems involving animal cognitive capability; 3) broad integration among fields of biology and psychology with robust multidisciplinary collaborations. If successful, these solutions will provide data bearing on broader evolutionary cognition hypotheses, a goal based on the mental continuity in comparative psychology that follows the Darwinian frame – animals and humans also share elements of mind (Papini 2002; Shettleworth 2001 and 2009; Wasserman 1997; Vonk 2016). Some progress is in the form of quantitative surveys reporting the increasing number of publications on animal cognition (Beran et al. 2014; Shettleworth 2001) and also a rising interest in species and taxa that are not traditionally studied (Beran et al. 2014; Healy 2019; Vonk 2016). Nevertheless, most achievements of cognitive evolution are still limited to humans, other primates, and the traditional species of mammals and birds (MacLean et al*.* 2012; Bräuer et al. 2020).

One of the main obstacles to diversifying the species studied is the tradeoff between standard methods and possible negative consequences of modifying those methods to accommodate studies of “untraditional” species. Critics see comparative cognition as an open and uncertain field, challenging its structure and scientific rigor (Boyle 2021; Farrar and Ostojić, 2019). They also point to these inconsistent parameters for validation and the lack of a study’s reliability through replication as an example (Brecht et al. 2021; Beran 2020; Farrar et al. 2020; Halina 2021, Stevens 2017). To deal with these concerns it will be necessary to establish consistent methodological patterns for multispecies comparison and evolutionary approaches, as well as to be attentive to appropriate protocol adjustments depending on the species. This is a major challenge for establishing an across species standard that controls for species-specific factors (Krasheninnikova et al. 2020; Shaw et al. 2021). When reproducibility of studies is not feasible, Shaw et al. (2021) suggest a transparent and exploratory phase that illustrates the use of various tools and approaches to assure robust methodologies in untested species. While standards are crucial, there are also discussions about revising approaches to data collection and interpretations (Beran et al. 2014; Krasheninnikova et al. 2020; Vonk 2016). For instance, using continuous metrics instead of the typical binary data (yes/no) that focus on frequencies of assertive response or the number of scores and errors to criterion. Traditional approaches like forced-choice tests could result in the loss of valuable and informative data, such as response latency or the turning movement of an animal in expressing a moment of decision (Krasheninnikova et al. 2020). Other considerations include motivation control, warm-up trials, and intertrial interval. Evaluations of animal learning techniques have supported the relevance of both aspects (Bräuer et al. 2020; Domjan 2000; Papini 2002): the biocentric/ecological view, focusing on learning traits as adaptive processes; and the general process view that can target stable learning characteristics shared across animals.

2.2 Prospects for Progress

Non-traditional models like arthropods (e.g., bees, wasps, and ants) have become increasingly popular, and there are now numerous studies demonstrating their several forms of cognition (spatial, social, and collective) and unique cognitive flexibility (Chittka and Niven 2009; Perry et al. 2017; Pfeffer and Wolf 2020; Simons and Tibbetts 2019). The successful performance studies of some arthropod species have impacted the cognitive literature, generated methodological breakthroughs, and discovered their unprecedented learning capabilities. Some of these findings have even found notoriety in the public domain, such as the “the soccer players bumblebees” (Loukola et al. 2017). Experiments have documented a high level of behavioral flexibility in bees, including the ability to use tools for a food reward, the capacity of learning from a conspecific demonstrator, and even improving on the performance of the demonstrator. While demonstrating a shift toward studies of animal cognition in “unconventional” species, these studies still represent a small fraction of the total devoted to non-human animals (Beran et al. 2014; Healy 2019, Vonk 2016).

Among ectothermic vertebrates, remarkable abilities are being discovered, such as tool-use in fish (Brown 2015; Kuba 2010) and counting skills in fish and reptiles (Bshary and Brown 2014; Pouca and Brown 2017; Salena et al. 2021; Sneddon and Brown 2020; Szabo et al. 2021), with possible evidence in amphibians (Stancher et al. 2014). Cognitive research on fish, for instance, is now considered well-developed, as it has quickly reached an accumulated level of knowledge and discoveries primarily dominated by studies on mammals and birds (Miller 2017; Pouca and Brown 2017).

The proportional progress on taxonomic patterns still remains unbalanced, not only among the invertebrates but also with regard to the representation of large groups of vertebrates, with most studies focused on mammals and birds. Questions remain about the status of the other animals, such as the understudied ectothermic vertebrates – fish, amphibians, and non-avian reptiles? What progress has been made in reporting learning and cognitive performance in these groups may well help us understand the mental diversity on earth? Assessing these groups can contribute to the scientific literature by identifying unique capabilities and patterns in understudied groups of animals, whose behavior was for long interpreted as merely stimulus-response effect (Burghardt 2013; Luchiari et al. 2021).

2.3 The Structure and Goals of this Review

Rather than updating the progress in the number of publications in all species and topics covered in the comparative cognition demands, my aim is to summarize the status of learning and cognition in ectothermic vertebrates and the publication trends of journal articles about their learning assessments during the last 6 decades. Learning has been a major part of the cognition processes studied over the years and is considered the central mechanism to be explored in comparative research (Blaser and Bellizzi 2014; Lambert et al. 2021). Learning is generally defined by behaviorists as changes in behavior due to experience (Shettleworth 2010). Among the learning paradigms from 1994-2013 associative learning (classical and operant conditioning) corresponded to 90% of the publications, with an increasing number of publications using non-human subjects (Blaser and Bellizzi 2014; Domjan and Krause 2002). Such research activity also reflects the important applicability of animal learning beyond task ability descriptions, serving as methodological support for psychophysics, neuroscience, biomedicine, and conservation biology investigations.

This current review also aims to provide a synopsis of learning publications in underrepresented vertebrates (fish, amphibians, and reptiles). I compile data at ten-year intervals instead of performing an exhaustive survey, which is available in the reptile and fish literature (c.f. Brown 2015; Bshary and Brown 2014; Szabo et al. 2021). I present this review in two parts. Part I summarizes the perspectives discussed in the latest literature reviews of learning and cognition, highlighting the status of studies on fish, amphibians, and reptiles. Then, in Part II, I present a quantitative report compiling studies that used learning assays on these animal groups of vertebrates in the last sixty years at ten-year intervals. The learning studies collected referred to all papers that described learning experiments, including studies that used learning as a tool to investigate other mechanisms (memory, perception, and problem-solving, drug and substance effects, psychophysics processes, etc.). I also summarize the number of journals and species used within each taxon to discuss the level of publishers' interest and the diversity of species-subject.

In order to update the current status on learning and cognition of the ectothermic vertebrates – fish, amphibians, and reptiles (Part I), I searched articles through three Platforms: Google Scholar, Pubmed, Web of Science. In addition, I explored the "citations by" tool to find articles through backward and forward searches from the leading publications. To compose the survey on Part II, I accessed these search Platforms collecting publications that described experiments of learning processes in these three animal groups, sampled every 10 years: 1970, 1980, 1990, 2000, 2010, and 2019. The choice of sampling 2019 instead of 2020 was necessitated by the global pandemic that happened in 2020, which could have affected many research laboratory experiments, scientific routines, and publications. The search was performed by selecting three parameters: year, key-words referring to the target animals (fish, teleost, shark, amphibian, salamander, newt, tadpole, frog, treefrog, toad, reptile, no-avian-reptile, lizard, snake, serpent, tortoise, turtle, crocodile, alligator, and tuatara) and the study elements (learning, task, training, reward, conditioning, cognition, reinforcement, discrimination).

Each journal I surveyed was assigned to one of two categories to better frame the field efforts and scope in the past 60 years. The publishers were listed as Traditional or Alternative. The Traditional journals are the commonly mentioned journals of several literature reviews in animal learning and cognition and are considered leading comparative learning and cognition journals (Beran et al. 2014; Blaser and Bellizzi 2014; Domjan and Krause 2002; Shettleworth 2009). Here, I listed 12 traditional journals: Animal Cognition (AC); International Journal of Comparative Psychology (IJCP); Journal of Comparative Psychology (JCP); Journal of Comparative and Physiological Psychology (JCPP); Journal of Experimental Psychology: Animal Behavior Processes (JEP: ABP); Journal of Experimental Psychology (JEP); Journal of the Experimental Analysis of Behavior (JEAB); Learning and Behavior (L&B); Learning and Memory (L&M); Learning and Motivation (L&M); Animal Learning & Behavior (AL&B); and Quarterly Journal of Experimental Psychology (QJEP). The Alternative category is all the others, which approach animal behavior and behavioral experiments integrated into their main focus. For instance, these are journals that focus on studies of specific taxa or groups (e.g., Copeia, Herpetologica), cover psychology, physiology, and ethology themes (e.g., Physiology & behavior, Ethology) and general science topics (e.g., Science, Proceedings of the National Academy of Sciences).

2.4 Part I.

2.4.1 Current state of learning and cognition literature in fish

Studies of fish cognition are numerous and have grown substantially in the last decades (see fig.1 [Pouca and Brown 2017; Miller 2017; Salena *et al*. 2021]).

To date, a great portion of the knowledge of fish cognition literature covered skills tested in goldfish, zebrafish, guppies, and sticklebacks (Salena *et al*. 2021). The concentration of studies on these four model species has improved our understanding of their various cognitive abilities, but these species only represent a small fraction of fish diversity which includes more than 30,000 species (Fernö et al. 2020a; Newport 2021; Salena et al. 2021). This is certainly a noticeable imbalance that reinforces demands on the field to increase the diversity of fish subjects to better understand cognitive features across the phylogeny and their ecological diversity (Bshary and Brown 2014).

The advancements documented in fish cognition have been remarkable and have broken ground into exploring the cognitive capabilities that before were only intensively explored in mammals and birds. Besides the most common assessments of spatial cognition and associative learning (see fig.1 [Salena et al. 2021]), the cognitive repertoire of fish has expanded to consider complex skills. The cognitive literature currently includes data on tool-use like behavior, abstract concepts of learning, individual and self-recognition, conflict resolution, collective decision-making, reciprocity, and cooperation (Brown 2012; Bshary and Brown 2014; Luchiari et al. 2021; Miller 2017; Newport 2021; Pouca and Brown 2017; Salena et al. 2021; Sneddon and Brown 2020). Over recent decades, the studies have diversified to explore their numerical capabilities and even sentience, such as pain and consciousness linked to cognitive parameters (Brown 2015; Bshary and Brown 2014; Pouca and Brown 2017; Sneddon and Brown 2020). Fish can discriminate absolute and discrete quantities, and studies have even developed methods to investigate the multisensory roles of numerical stimuli (Fernö et al. 2020b; Pouca and Brown 2017; Salena et al. 2021). Using sets of numbers, features, and objects in complex discrimination tasks researchers have tested abstract concepts of learning to provide conclusive results bearing on the question of whether fish are capable of transferring previous experiences based on concepts and not just the procedure acquisition of a specific situation (Newport 2021).

Fish have also been subjects of studies of sentience: awareness of experiences and emotions, feelings, and its own life (Brown 2012; Learmonth 2020; Pouca and Brown 2017; Sneddon and Brown 2020). The interpretation of pain feeling is based on the existence of cellular mechanisms and receptors in the peripheral nervous system of fish that are homologous to those in other vertebrates as well as notable observations of behavioral changes in response to painful stimuli and their modulation through analgesic treatment (Bshary and Brown 2014; Sneddon and Brown 2020). Evidence of sentience that involves complex cognitive domains has been debated in reviews to support improvements of management and welfare protocols of fish and reptiles (Brown 2015, Burghardt 2013; Fernö et al. 2020b; Learmonth 2020; Pouca and Brown 2017; Sneddon and Brown 2020). According to Miller (2017) “fish has been quickly catching up to those traditional model taxa” (e.g., primates, mammals, and birds) and push forward the debate about whether they and other “lower” vertebrates may possess cognitive elements that before now were only attributed to humans and their closest relatives.

Although the developments in fish cognition are growing impressively and have gained a greater place in the comparative cognition field of the new century, there are still many challenges to using them as models to improve our comprehension of animal minds. Even on large studies topics likes spatial learning, memory, and social capabilities, there are still many unexplored avenues to advance fish cognition studies, especially those related to mechanisms underlying quantity discrimination, brain regions, neural plasticity, gene expression, and individual variation and personality; the physiological factors underlying their cognitive performance is also a relatively new research direction (Babkiewicz et al. 2021; Bshary and Brown 2014, Pouca and Brown 2017). In the realm of ultimate causation, Salena et al. (2021) have advocated a more ecological approach to learning and fish cognition. They suggest that studies need to shift efforts from standard laboratory models to contextualize the function of cognition in their natural habitat. Changes to the status of fish cognition research contribute to narrow gaps in the unbalanced diversity of data within the field of comparative cognition, placing the status of fish cognition ahead of many other terrestrial vertebrate species, such as amphibians and reptiles.

2.4.2 Current state of learning and cognition literature in reptiles

The most recent update of the status of learning and cognition status in reptiles has also demonstrated robust growth in the number of studies over the last decades (Fig. 2 [Szabo et al. 2021]). The existing reptiles, known as non-avian reptiles (turtles, the tuatara, crocodylians, and squamates [lizards, snakes, and amphisbaenids)]) form a diverse taxonomic group of about 11,570 species (as of May 2021; Uetz, Freed and Hošek, 2021). The studies exploring the cognitive abilities of these animals have been mostly centered on a few species of turtles (*e.g*., genus *Chrysemys* and *Chelonoidis*) and lizards (*e.g*., genus *Anolis* and *Varanus*), with very few studies of snakes and crocodiles (Burghardt 2013, Szabo et al. 2021). Large efforts are needed to broaden the range of species sampled (Matsubara et al*.* 2017; Wilkinson and Huber 2012).

With regard to new discoveries, recent research has demonstrated that the reptile cognitive processes are complex, presenting social learning, problem-solving skills, and evidence of play behavior (Burghardt 2020; Burghardt 2013; Learmonth 2020; Roth et al*.* 2019; Wilkinson and Huber 2012). Non-avian reptiles have demonstrated broad behavioral flexibility in experiments concerned with associative, discrimination, a version, reversal, and detour learning, as well as spatial learning and memory (Burghardt 1977; Szabo et al. 2021). As suggested by Szabo et al. (2021) this may well be the time for the expansion of the field and a shift away from just focusing on descriptive studies towards more comparative approaches of psychology and cognition. Development of future questions in this realm could include topics such as variation in sexual dimorphic cognition, fitness relevance, behavioral changes in invasive species (Pettit et al. 2021), social relationships (Kis et al. 2015), decision-making processes, and problem-solving abilities (Storks and Leal 2020; Szabo et al. 2018). Although not fully tested, there is evidence in reptiles for cognitive elements of play behavior and sentience that suggests a newer type of investigation about the state of the reptilian mind (Burghardt 2013; Learmonth 2020). Sentience in reptiles is based on evidence of emotional fever and increases in body temperature under stress or handling (Burghardt 2013).

According to Roth et al. (2019), there are numerous bodies of evidence on reptilian complex spatial cognition processes consistent with those in mammals and birds. Moving forward, reptilian studies may discover even more sophisticated abilities that will certainly contradict the view of reptile behavior as merely sedentary and instinctive (Burghardt 2020). These more recent studies celebrate the popularity of non-avian reptiles as a model system for animal cognition questions and how non-avian reptiles are an important territory to expand our understanding of non-human cognitive mechanisms and to contribute to the increasing diversity of species studied in the field.

2.4.3 Overall perspective on learning and cognition in amphibians

Compared to the accumulated knowledge on learning and cognition studies among all vertebrates, our knowledge of learning and cognition in amphibia is extremely limited both in terms of the number of species and topics studied. The Amphibia are an important group at the base of the tetrapod evolution that links vertebrate adaptations from aquatic to terrestrial life (Duelmman and Trueb 1986). It is currently composed of about 8,352 species (as of July 2021; AmphibiaWeb 2021) of three living orders: Anura (toad, frog, and treefrog), Caudata (salamander and newt), and Gymnophioba (caecilian). The paucity of quantitative learning and cognitive literature on amphibians stands out in our current overview and comparative evaluation of research progress across vertebrates. In an earlier literature review, Suboski (1992) addressed the learning skills of amphibians and reptiles as being able to define the releaser-induced recognition category of learning based on innately organized released responses in classical conditioning. More recently, Burghardt (2013) discussed the complex behaviors in amphibians that are required in cognition mechanisms and their performance from studies on habituation (simplest form of learning) to classical and operant conditioning. Berry (2019) reviewed potential evidence for numerical discrimination in salamanders (Krusche et al. 2010; Uller et al. 2003), and numerical skills reported through quantity preference assays have also been demonstrated in toads (Stancher et al. 2014). Nevertheless, there is still a gaping deficiency even in the broad behavior repertoire and sensory adaptations to be explored in cognitive approaches.

An updated and systematic review on amphibian learning is badly needed. Such a review could provide a complete picture of the current research status in the group, setting the stage for a summary of what is necessary to expand on some recent studies of habituation, avoidance learning, discrimination learning, plasticity on learning extinction, reverse learning, spatial orientation, maze learning, some extent of play behavior in tadpoles, and sexual conditioning (Bee and Gerhardt 2001, 2002; Burghardt 2013; Brattstrom 1990; Elepfandt 1985; Ferrari et al. 2009; Kundey et al. 2018; Liu et al. 2016; Muzio and Puddington 2018; Muzio et al. 2018). Potentially breakthrough contributions have been made in studies of a terrestrial toad (*Rhinella arenarum*) using a training protocol based on water rehydration motivation to test appetitive, aversive, and spatial learning

(Daneri and Muzio 2013; Muzio 1999; Muzio et al. 1992; Puddington and Muzio 2013; Sotelo and Muzio 2015; Schmajuk et al. 1980, 1981; Schmajuk and Segura 1982). These studies have found that partially dehydrated subjects of *R. arenarum* have successfully learned to associate water reward and avoid saline solution reward in tasks involving cues as body turn, geometric, visual, and conspecific sounds in distinct training structures such as mazes, arenas, shuttle box and runways (Muzio and Puddington 2018; Muzio et al. 2018). As in fish and reptiles, several abilities demonstrated by these toads raise amphibians to the baselines dominated by mammals and birds, allowing identification of similar characteristics through several mechanisms of learning.

In addition to the learning skills, these experiments have explored and found areas of the brain related to learning behavior. The medial pallium area of the amphibian brain is homologous to the hippocampus of mammals. These areas are related to spatial learning and extinction responses, while the striatum of amphibians is homologous to the amygdala of mammals and is active during aversive learning (Daneri and Muzio 2013; Muzio 1999; Puddington and Muzio 2013; Sotelo and Muzio 2015). Such evidence supports the use of trained amphibians as a simple brain model to understand the conservative basis of the evolution of learning mechanisms across vertebrates. Liu et al. (2020) found that differences in gene expression in the medial pallium could explain a better maze performance of poison frogs (*Dendrobates auratus*) than túngara frogs (*Engystomops pustulosus*). These outcomes are important discoveries for the status of amphibians learning and supporting vast possibilities for exploring learning and other cognitive abilities among the vast number of species in a wide variety of ecological domains.

2.5 Part II.

The quantitative survey that compared the number of studies covering learning among the ectothermic vertebrates (fish, amphibians, and reptiles) confirms the highly unbalanced numbers of studies of learning and cognition in terms of taxonomy. Among lower vertebrates, for example, a survey of 173 publications showed that 74% analyzed fish, 15% reptiles, and 11% amphibians. Fish were the only group showing an increasing trend in the number of papers whereas the frequency of studies in the amphibians and reptiles remained much lower and showed no consistent trends (Fig. 3). Fish learning studies were still 50% of the total published since 1960 (Salena *et al*. 2021). In reptiles, the number of studies doubled in 2019 compared to the previous decades; nevertheless, in the same year studies of fish learning increased fourfold. The increase in reptile studies is a positive development in the taxonomic diversification of the field (see Szabo *et al*. 2021; Fig. 2) but highlights the existing paucity of studies of amphibians even more in the present data.

Broad literature reviews surveying learning and cognition generally focus on the number of studies of a given topic in a limited number of model species of mammals and birds; other animal groups are placed into large categories (e.g., “others”), combined (e.g., “amphibians/reptiles", “fish/marine animal”) or not even considered because the number of studies is critically low (Beran et al. 2014; Blaser and Bellizzi 2014; Bräuer et al. 2020; Bueno-Guerra 2021; Domjan and

Krause 2002; Healy 2019; Krasheninnikova et al. 2020; Papini 2002; Rosenthal et al. 2017; Shettleworth 2010 and 2009; Stevens 2017; Vonk 2016). This generalized approach reinforces the minimal representation of the majority of the animal kingdom and ignores the progress, however limited, in studying groups of animals with distinctive life histories. Moreover, large-scale literature compilations usually address several research topics or a particular topic that overlaps with multiple fields. Animal learning, for example, is usually a short section of literature reviews of fields such as ethology, animal behavior, comparative cognition, and psychology (Beran et al. 2014; Bueno-Guerra 2021; Rosenthal et al. 2017; Shettleworth 2009). Animal learning paradigms can also be found in several resources that are not even examined by the traditional psychology and cognition literature (see discussion and data below about “Alternative Journals”). Therefore, emphasizing systematic approaches, as the recent review on learning of non-avian reptiles (Szabo et al*.* 2021), can clarify research progress, and redirect alternatives targeting quantitative and qualitative data of historically underestimated animals.

In 1990 only 12 papers were found that explored the learning performance of ectothermic vertebrates (Fig. 3), presumably reflecting a time when many scholars expressed concerns about the survival of research involving experiments in animal learning and cognition (King and Viney 1992; Viney et al. 1990). During the late 70s to the 90’s, productivity in the field of comparative psychology was mostly reported as stable or declining (Blaser and Bellizzi 2014; Domjan and Krause 2002; Szabo et al. 2021; Viney et al. 1990). This period even saw a decline in the number of studies on traditional animal models such as great apes, cats, rats, and pigeons (Viney et al. 1990). Domjan and Krause (2002) pointed out several potential causes for this decline: (1) the increase in animal rights activism that imposed strict regulations on experiments with animals in the laboratory (Dewsbury 1990; King and Viney 1992; Viney et al. 1990; Wasserman 1997); (2) the costs and challenges to operate animal facilities (Gallup and Eddy 1990); and (3) the peripheral role of nonhuman research in psychology that influenced a decline in job interest, training, and fundings (Dewsbury 1992; King and Viney 1992). There were also numerous critiques focused on the universal applicability of the fundamental principles of learning and even the use of conditioning designs as cognitive metrics (Barrett 2016; Domjan 1983, 2000; Heyes 2015; Shettleworth 2009), perhaps a revival of the debates in the 1970's about the limitations found in classical and instrumental conditioning that contrasted with the General Process Learning Theory – accounts all learning as a universal set of principles (Shettleworth 2001). An animal's failure to acquire a task in a standard associative protocol using a sound cue for example might not reflect its learning ability, but rather the time during its growth and development or even the season of the year. Rather than a generalized view, subjects' specialization could thus be considered – "constraints on learning" – considering factors under natural species-specific circumstances and evolutionary adaptations (Domjan 1983, 2000; Shettleworth 1972). These factors potentially account for the lowest number of learning studies in the ectothermic vertebrates registered in 1990 even in alternative journals (Fig. 4).

Similarly, Szabo et al. (2021) reported an abrupt reduction in studies of reptile learning during 1981-2000, compared to higher productivity from the previous two decades (1960 – 1980; see Fig. 2).

After these periods of crisis, studies on mechanisms of animal learning cognition started to grow again in this century. Here, this recovering period is visible through the frequency of publication in the past two decades in alternative journals, predominantly reflecting the number of studies in fish (Fig. 3) and in their advanced research status compared to the reptile and especially amphibia (revisit Part I). As the coverage of non-human cognitive mechanisms has flourished, demands have also grown for research with more interdisciplinary collaboration, diversification of species studied, and methodological rigor (Boyle 2021; Beran et al. 2014; Domjan and Krause 2002; Farrar and Ostojic 2019; Healy 2019; Krasheninnikova et al. 2020; Vonk 2016).

In this survey, I found a small number of species and a consequent low rate of species diversification of learning studies within fish, amphibians, and reptiles. Fifty-six species of fish were involved in learning assays, followed by 25 species of reptiles, and 15 species of amphibians. This is a modest coverage relative to the total number of species that currently compose each group: fish (0.19%), non-avian reptiles (0.22%), and amphibians (0.18%) (See Table 1 for the complete list of species coverage). By comparison, the number of bird species whose cognitive abilities were tested in the last five years was 1.41%, while the primate order was 15% (Lambert et al. 2021, Many Primates 2019). These comparisons emphasize the necessity to extend the taxonomic database on studies of cognitive mechanisms on a wider range of non-human animals to make feasible a meaningful review of comparative and evolutionary approaches.

A major reason for the lack of species diversity even within a group is the large number of studies conducted with a limited number of model species. In my summary, for example, the fish species most studied were the well-established subject models, such as zebrafish (*Danio rerio*, 20.0%) and goldfish (*Carassius auratus,* 18.0%), corroborating other recent fish cognition surveys (Salena et al. 2021). Interestingly, they showed a different temporal progression on the number of studies performed. Studies on goldfish started earlier, at least, since 1970, while zebrafish became part of learning experiments later in 1990. Zebrafish then prevailed with the highest frequency of publications in 2010 and 2019 (92%, n = 28), while goldfish decreased (24%, n = 25) in those same years. Widely used in embryology, genetics, toxicology, and pathology, zebrafish has also been considered a newcomer in comparative psychology, demonstrating consistent performances in experiments on learning, memory, and cognition (Gerlai 2020; Luchiari et al. 2021; Sison and Gerlai 2010). Within the reptiles, most of the subjects used in learning studies were species of lizards (71%), but no particular species was largely predominant among the studies. Twenty percent of the studies used chelonids, partially using red-footed tortoises (*Chelonoidis carbonarius*) and painted turtles (*Chrysemys picta picta*). A similar pattern was reported by Szabo et al. (2021), showing species representation in the studies were dispersed among the numerous species of lizard, as well the less studied reptiles (snakes, crocodilians, and tuataras). Among the few amphibian species represented in the learning publications during the years sampled here, the tiger salamanders (*Ambystoma tigrinum*, 13.6%) and the Argentine common toad (*Rhinella arenarum*, 13.6%) were the species most frequently studied, followed by frogs of the genus *Lithobates*, 22.7%). However, the lack of a complete and updated literature review of amphibian learning prevents inferences as to whether these species could potentially serve as models within their order Caudata and Anura, respectively. Besides, the total number of studies on these species is well below model references like zebrafish and goldfish (Table1).

Studies of ectothermic vertebrates were found much more frequently in alternative journals than in traditional ones (148 studies versus 25). Although there was a large difference between the number of traditional (n = 12) and alternative (n = 76) journals surveyed, the rate of publication per journal was about the same: 2.1 and 1.9 respectively. In the traditional journals, the periods with the highest focus on fish, amphibians, and reptiles learning were in 1970 and 2019 (Fig. 4), between a declined period from 1980 to 2010. The publications in the alternative journals were similar to the traditional journals in 1970 but then increased over the last three decades (Fig. 5). This growth of articles published in alternative journals is expected, as the category increases as new journals are created. For instance, there were only seven alternative journals that published at least one article describing learning in fish, amphibians, or reptiles in 1970; this number grew to 30 in 2019. Furthermore, the alternative category is composed of journals focusing on different topics and disciplines that also use learning as a method to integrate other biological processes. This expands collaborative possibilities and encourages researchers to explore new avenues and methods for our understanding of learning in a wider array of organisms.

Publications in the traditional journal category were found most frequently in the *Journal Comparative Psychology* (JCP, 32%), followed by *Animal Cognition* (AC, 28%). Most of the studies published by JCP were counted in 1970 (75%, n = 8), while AC published more frequently in 2019 (71%, n = 7). The JCP has been issued since 1921 and is one of the oldest journals reported here, with 75% of publications focused on fish, and 13% focused on both amphibians and reptiles. In a most recent JCP Editorial, Fragaszy (2018) reported an increase in the proportion of studies on amphibians and a more balanced number of studies done among all three ectothermic vertebrates from 2000 onwards. Such taxonomic equilibrium reflects a positive shift to diversify the organisms studied. However, this general view prevents a conclusive impact on the learning studies as the journal covers other broad areas of cognition, behavior, and psychology. As the second most active traditional journal, *Animal Cognition* is a recent journal, first issued in 1998, and has published studies on learning in reptiles (43%) and fish (57%). Although no article on amphibians learning was reported in the years sampled here, there are studies published after 2011. (c.f. Ferrari and Chivers 2011; Kundey et al. 2016; Mesich et al. 2021; Puddington et al. 2011; Sotelo et al. 2015, 2020).

Of the alternative journals, *Animal Behaviour* (AB, 9.5%) and *Behavioural Brain Research (*BBR, 6.7%) published most of the articles covering learning in the ectothermic vertebrates, especially in the last three decades. Overall, *Animal Behaviour* published 7% of the studies on reptiles, 14% on amphibians and 79% on fish. Most of these studies were found in 2000, 2010, and 2019 (71%, n = 14), even though AB has been issued since 1953. One would expect more publications AB, given its age and its broad focus. However, there is not a high demand for studies on learning and least of all studies focused on learning in fish, amphibians, and reptiles. As stated, AB comprises a broad spectrum of behavioral topics besides learning, such as communication, parental care, mating, foraging, and predation. Until up to 2015, AB had an intense over-representation of mammals and birds making up 50% of the publications, and only 7% of articles were on reptiles and amphibians combined (Rosenthal et al. 2017). Such minute coverage only reinforces the lack of studies focused on these two groups of animals (Burghardt 2013; Roth et al. 2019) and indicates that taxonomic bias is common in many other areas of behavioral and biological research (Bueno-Guerra 2021, Troudet et al. 2017; Zuk et al. 2014). Reviewing this issue in the *Journal of Animal Behaviour*, Rosenthal et al. (2017) also pointed out strong concentration in the type of studies carried out on a particular taxon and behavior. For instance, 68% of the publications on frog behavior focus on mating and/or communication. The second most active alternative journal reported here was the *Behavioural Brain Research*, which was first issued in 1980. Most of the articles on learning in ectothermic vertebrates were published in 2010 and 2019 (90%, n = 10), covering only experiments done in two fish species: zebrafish and goldfish. The studies from BBR generally apply methods of learning as a tool to underline neural mechanisms of cognitive behavior This approach takes advantage of the accumulated knowledge and consistency of well-established model organisms, characteristics suitable for zebrafish and goldfish, but remain undeveloped in many other species of fish, amphibians, and reptiles.

2.6 Conclusion and Future Directions

The number of studies of learning and the progress in cognitive research status in historically understudied vertebrates has greatly increased in recent decades. However, these parameters expanded unevenly among the three taxa I have focused on in this review. Studies in fish were the most prevalent especially in the past three decades. The publication trends found on reptiles and amphibians were reduced, with publications on reptiles only increasing over the last decade, which suggests an optimistic growth of future studies. Moving forward, the field of learning and cognition should develop strategies that accompany the growth of studies done in neglected study organisms among the already underrepresented groups of animals. This can be specifically addressed by 1) Selection of study systems to diversify the number of organisms and species within these understudied taxa; 2) Create open-science collaborations to discuss, revise and develop adapted learning and cognition methods; 3) Support studies through publications and funding of less-common species; 4) Encourage the publication of negative-results and exploratory studies in methods development.

Among the three ectothermic vertebrates, amphibian subjects were the least documented: fewer publications on learning; the lowest within species representation; and outdated status of reported cognition surveys. An updated amphibian review is needed for comparison among the reviews of the cognitive abilities in fish and reptiles. Reports comparable to a systematic compilation recently published for reptiles (Szabo et al., 2021) can provide an overview of learning topics described in amphibians and their common experimental elements (e.g., type of task, type of reward, and learning criteria). Fish, although the most studied taxa among the amphibians and reptiles, tended to concentrate publications on a limited number of species (e.g., zebrafish, goldfish). In fact, the relative diversification coverage within fish was comparable to that seen in amphibians, with studies only spanning 0.19% of the taxa. In contrast to fish, reptiles have a broader within-representation of species in learning research, with most studies focusing on species of lizards. These findings emphasize the need for strategies to investigate a wider range of taxa, starting with what species to select as experimental subjects beyond the model species. Although model species have provided valuable insight in the learning and cognition literature, they do not improve our knowledge regarding the evolution of learning and cognitive skills across a broader range of species. In that line, it is essential to expand the focus of science beyond species that are lab reliable and affordable (e.g., zebrafish). Therefore, some approaches to improve species diversity would be to comb through the comparative cognition databases, find species that have been well studied, and then focus on lesser-studied but closely related taxa.

Such a multi-level technique would increase the number of species studied and could expand the methods that have been used on similar species (Krasheninnikova et al. 2020). Finally, expanding on the number of species studied in cognitive abilities can support the welfare and enrichment of exotic animals in captivity, like many fish (Brown 2005), amphibians, and reptiles (Burghardt 2020).

As the number of studies on species distantly related to humans increases, concerns about methods standards will be constantly raised, given the species-specific learning styles of animals ("species-fair"; Krasheninnikova et al. 2020) and their differential motivations and ecological relevance (biocentric approach, Bräuer et al. 2020). The problem is that a lack of standards might lead to inconsistent comparative approaches and amplify a current replication crisis such as the inconsistent results that arose from repeated studies among traditional subjects (Beran 2020, 2018; Beran et al. 2014; Brecht et al. 2021). Solutions to increase transparency and interdisciplinary collaboration can possibly mitigate this issue while maintaining the diversification efforts following the scientific rigor in a certain range of methodological plasticity. Beran et al. (2014) and Krasheninnikova et al. (2020) suggest strength "team-science" produces reliable species-representative data. These collaborations can be initiated and facilitated at any stage of research, but ideally during the first attempts to discuss the implementation of a learning approach to a novel species (Shaw et al. 2021). In addition, recent open-source science networks are providing transparent digital channels that are useful for collaborative animal behavioral research, such as the ManyPrimates, ManyDogs, and ManyBirds projects, and Open Science Collaboration (Lambert et al. 2021; Many Primates et al. 2019). Those tools could be the next step for scholars to produce robust work on new endeavors within the behavior, learning, and cognition field of ectothermic vertebrates.

Lastly, future alternative and traditional journal publications should reserve more space for studies that describe results of newly tested subject species in learning and cognition experiments. These actions would require a shift in the culture of science that tends to focus mostly on positive outcomes and charismatic animals, which also attract greater research funding support (Lambert et al. 2021; Rosenthal et al. 2017). Instead, peer-reviewed sources need to be more accepting of negative outcomes when it comes to the learning process and observations that show a more robust methodological approach of animal performance, rather than focusing on straight metrics of yes/no questions designed for humans and more-traditionally studied animal models. Additionally, journals should be open to creating a space for related descriptions of observations from natural history and exploratory science (Bräuer et al. 2020) in order o to frame relevant experimental designs on newer learning and cognitive assessment species. These changes will promote more studies to explore a wider range of species and explore questions involving ecological, comparative, and evolutionary variations of cognition and learning.

2.7 Figures

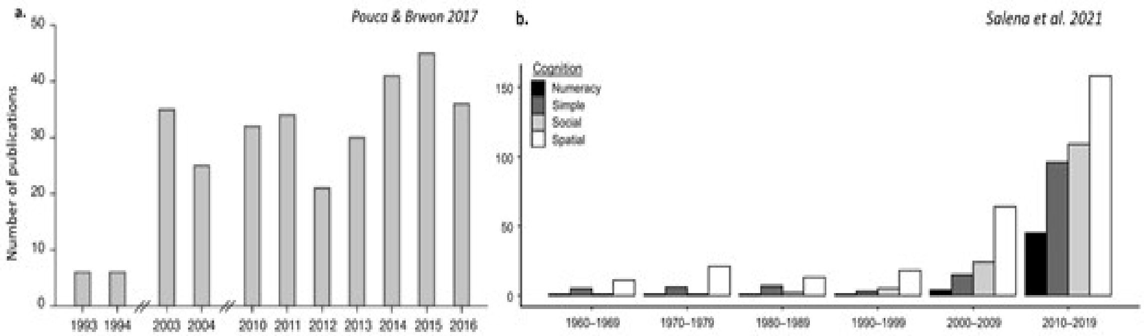

Figure 1. Number of articles published on fish learning and cognition in 1993-2016 reviewed by Pouca and Brown, 2017 (a); and the number of publications on fish cognition (numerical, simples, social and spatial) in 1960 - 2019 reviewed by Salena et al. 2021 (b).

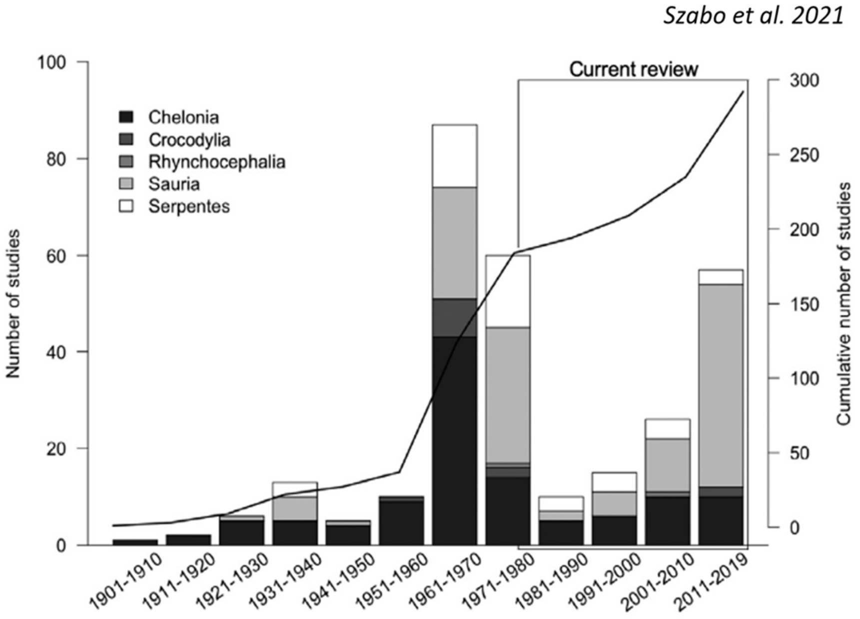

Figure 2. The number of articles published on reptile cognition in 1901-2019. Publications prior to 1977 were reviewed by Burghardt (1977). The following years were added in Szabo et al. (2021).

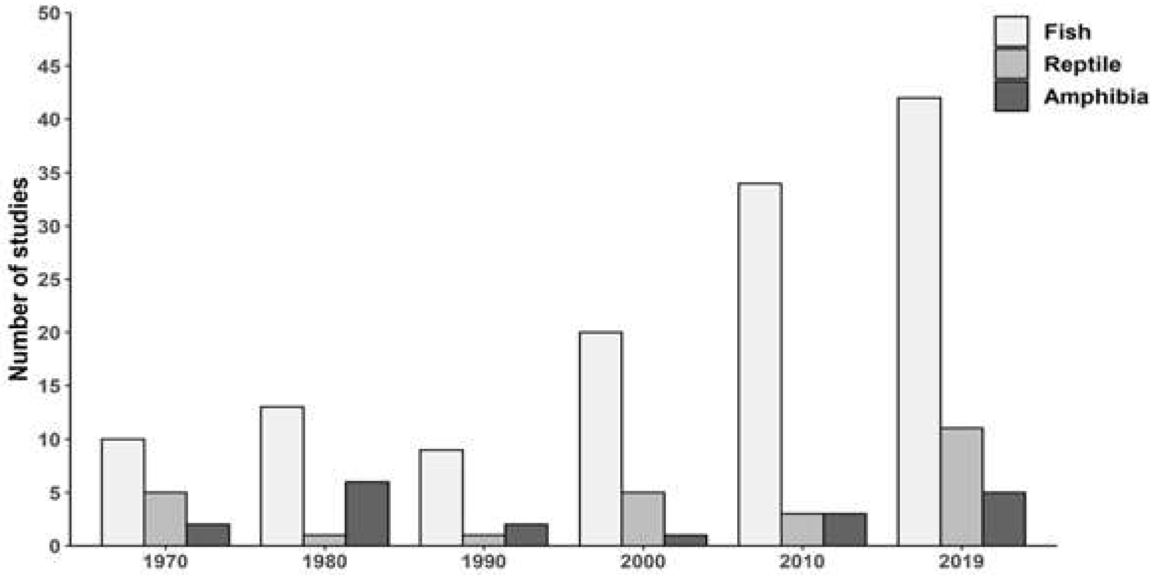

Figure 3. Number of publications reporting learning paradigms in the three groups of vertebrates (fish, reptile, and amphibia) across the latest every 10 years interval.

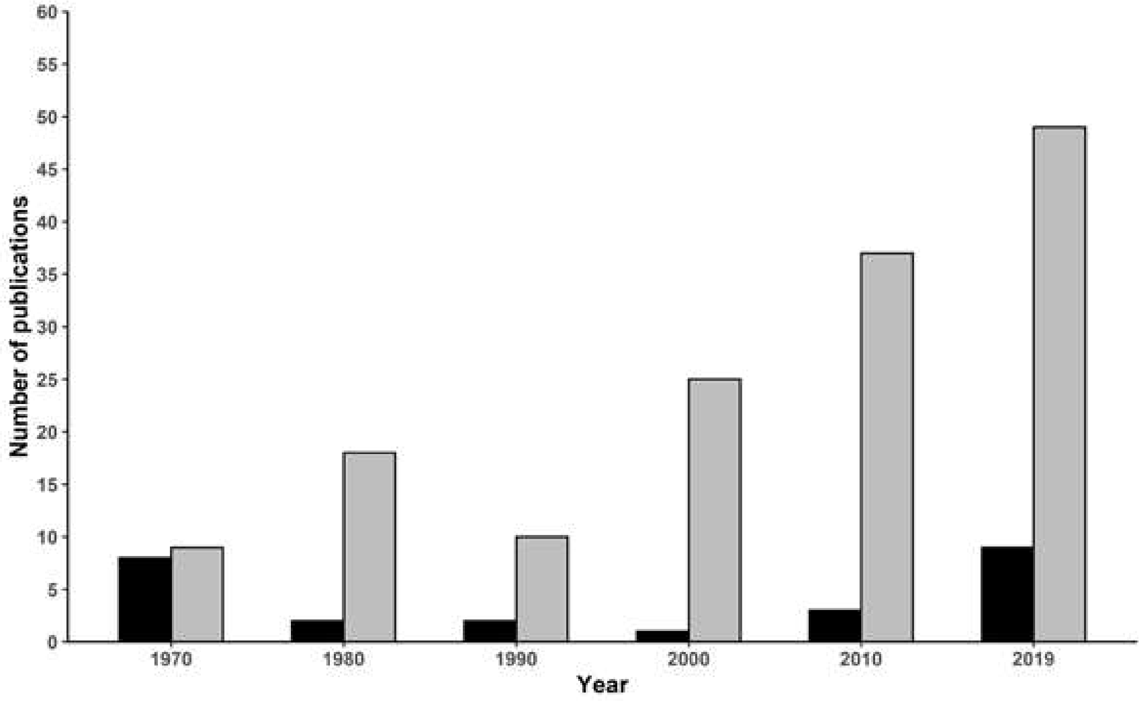

Figure 4. The proportion of studies published by journals considered Traditional (black) and Alternative (grey).

2.8 Tables

Table 1. The summary table shows the list of species of the three groups of vertebrates (fish, amphibians, and reptiles) assessed on learning studies published every ten years over the last 60 years.

FISH			
#	**Species**	**Journal**	**Source**
1	brown bullhead (*Ameiurus nebulosus*)	Journal of comparative physiology	Johnsen & Teeter, 1980
		Chemical Senses	Valentincic, 2000
		Journal of Experimental Biology	Valentincic et al., 2000
2	electric fish (*Apteronotus leptorhynchus*)	Journal of Comparative Neurology	Harvey-Girard et al., 2010
3	cavefish (*Astyanax jordani)*	Animal Behaviour	Popper, 1970
		Journal of comparative physiology	Weiss & Martini, 1970
4	river fish (Astyanax mexicanus)	Animal Behaviour	Popper, 1970
5	betta (*Betta splendens*)	Journal of comparative physiology	Baenninger, 1970
		Data in brief	Frank et al., 2019b
		Journal of comparative physiology	Hogan et al., 1970
		Animal Behaviour	Hogan & Bols, 1980
		Behavioural processes	Hurtado-Parrado et al., 2019a
6	cichlid fish (*Callochromis pleurospilus*)	PLoS biology	Kotrschal & Taborsky, 2010
7	goldfish (*Carassius auratus*)	Brazilian Journal of Medical and Biological Research	Abreu et al., 2019
		Journal of Experimental Psychology: Animal Behavior Processes	Amiro & Bitterman, 1980
		Journal of comparative physiology	Bowman & Sutherland, 1970
		Science	Braud, 1970
		The Psychological Record	Breuning et al. 1980
		Behavioural brain research	Broglio, et al., 2010

7	goldfish (*Carassius auratus*)	Journal of Comparative Physiology A	Dörr & Neumeyer, 2000
		Zeitschrift für Tierpsychologie	Dücker at al., 1980
		Behavioural Brain Research	Durán et al., 2010
		Journal of theoretical biology	Eijkman, 1970
		Journal of the experimental analysis of behavior	Fay, 1970a
		Journal of comparative physiology	Fay, 1970b
		Neuroscience	Gómez et al., 2010
		Experimental Neurology	Greif & Scott, 1980
		Behavioral Neuroscience	Laming & McKinney, 1990
		Psychological Reports	Miskovsky et al., 2010
		Behavioral Neuroscience	López et al., 2000a
		Behavioural Brain Research	López et al., 2000b
		Pharmacology Biochemistry and Behavior	Satake, 1980
		Brain Research	Vargas et al., 2000
		Behavioral and Brain Functions	Yoshida & Hirano, 2010
		The Psychological Record	Zella & Wolach, 1980
		Animal Learning & Behavior	Zerbolio & Golden, 1990
		Animal learning & behavior	Zerbolio & Wickstra, 1980
		Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences	Zippel, 2000
8	crucians (*Carassius carassius*)	Zeitschrift für Tierpsychologie	Dücker at al., 1980
9	red sea bream (*Chrysophrys major*)	Journal of fish biology	Kaneko et al., 2019
10	lumpfish (*Cyclopterus lumpus*)	Frontiers in veterinary science	Staven et al., 2019
11	common carp (*Cyprinus carpio*)	Environmental Biology of Fishes	Bajer et al., 2010
		Behavioural processes	Zion et al., 2010
12	zebrafish (*Danio rerio*)	Animal Behaviour	Ayoub et al., 2019
		Scientific reports	Baker & Wong, 2019
		Behavioural brain research	Bloch et al., 2019
		Behavioural brain research	Daggett, et al., 2019

12	zebrafish (*Danio rerio*)	International Journal of Comparative Psychology	Fort et al., 2019
		Nature neuroscience	Frank et al., 2019a
		Behavioural brain research	Gómez-Laplaza & Gerlai, 2010
		Neuroscience Research	Kim et al., 2010
		Journal of the experimental analysis of behavior	Kuroda et al., 2019
		Current biology	Lee et al., 2010
		Animal Behaviour	Lindeyer & Reader, 2010
		Behavioural brain research	Maximino et al., 2010
		Symmetry	Miletto Petrazzini et al., 2019
		Scientific reports	Potrich et al., 2019
		Journal of Neurobiology	Pradel et al., 2000
		Current Biology	Randlett et al., 2019
		Journal of Comparative Physiology A	Suboski et al., 1990
		Behavioural Brain Research	Wong et al., 2010
		PloS one	Roberts et al., 2019
		Scientific reports	Roy et al., 2019
		Learning & behavior	Ruiz-Oliveira et al., 2019
		Behavioural brain research	Sison et al., 2010
		Neurotoxicology and teratology	Smith et al., 2010
		Zebrafish	Stefanello et al., 2019
		The American Biology Teacher	Weber et al., 2019
		Frontiers in behavioral neuroscience	Yang et al., 2019
		Frontiers in Robotics and AI	Yang et al., 2019a
		Bioinspiration, biomimetics, and bioreplication IX	Yang et al., 2019b
13	Caribbean cleaning goby (*Elacatinus prochilos*)	Animal Cognition	Mazzei et al., 2019
14	Atlantic cod (*Gadus morhua*)	Aquaculture	Nilsson & Torgersen, 2010
15	mosquitofish (*Gambusia holbrooki*)	PloS one	Agrillo et al., 2010

16	three-spined stickleback (*Gasterosteus aculeatus*)	Behaviour	Jenkins & Rowland, 2000
		Current zoology	Keagy et al., 2019
		Ethology	Peeke & Dark, 1990
16	three-spined stickleback (*Gasterosteus aculeatus*)	Animal Behaviour	Rowland, 2000
17	African mormyrid (*Gnathonemus petersii*)	Frontiers in behavioral neuroscience	Von Der Emde, 2010
18	Atlantic halibut (*Hippoglossus hippoglossus*)	Behavioural processes	Nilsson et al., 2010
19	Port Jackson sharks (*Heterodontus portusjacksoni*)	Behavioral Ecology and Sociobiology	Vila Pouca et al., 2019
20	bluegill (*Lepomis macrochirus*)	Journal of the Faculty of Applied Biological Science-Hiroshima University	Yamate et al. 1990
21	paradisefish (*Macropodus opercularis*)	The Psychological Record	Melvin & Anson, 1970
22	rock-dwelling cichlid (*Metriaclima benetos*)	Journal of Experimental Biology	Escobar-Camacho et al., 2019
23	largemouth bass (*Micropterus salmoides*)	Behavioral Ecology and Sociobiology	Louison et al., 2019a
		Fisheries Management and Ecology	Louison et al., 2019b
24	electric Fish (*Mormyrus rume proboscirostris*)	Ethology	Walton & Moller, 2010
25	lemon sharks (*Negaprion brevirostris*)	Marine Biology Research	Spaet et al., 2010
		Fish Physiology and Biochemistry	Watsky & Gruber, 1990
26	golden shiner (*Notemigonus crysoleucas*)	Animal Behaviour	Reebs, 2000
27	rainbow trout (*Oncorhynchus mykiss*)	Journal of Comparative Physiology A	Hawryshyn et al., 1990
		Journal of Comparative Physiology A	Hawryshyn & Bolger, 1990
		Transactions of the American Fisheries Society	Munson at al., 1980
		Animal cognition	Nordgreen et al., 2010
28	Chinook salmon (*Oncorhynchus tshawytscha*)	Journal of fish biology	Cogliati et al., 2019
29	Nile tilapia (*Oreochromis niloticus*)	Aquacultural Engineering	Zion et al., 2000
		Scientific reports	Brandão at al., 2019

30	African cichlid fish (*Pelvicachromis taeniatus*)	Behavioural processes	Hesse et al., 2019
31	blind cave cyprinid (*Phreatichthys andruzzii*)	Ethology Ecology & Evolution	Sguanci et al., 2010
32	fathead minnow (*Pimephales promelas*)	Animal Behaviour	Ferrari et al., 2010a
33	sailfin mollies (*Poecilia latipinna*)	Animal Behaviour	Gibelli et al., 2019
		Current zoology	Fuss & Witte, 2019
34	Atlantic mollies (*Poecilia mexicana*);	Current zoology	Fuss & Witte, 2019
35	guppy (*Poecilia reticulata*)	Current zoology	Fuss & Witte, 2019
		Animal Cognition	Lucon-Xiccato et al., 2019a
35	guppy (*Poecilia reticulata*)	Ethology	Lucon-Xiccato et al., 2019b
		Behavioural processes	Lucon-Xiccato & Bertolucci, 2019
		Animal Behaviour	Reader & Laland, 2000
		Vision research	Sibeaux et al., 2019
		Japanese Psychological Research	Sugita, 1980
		Animal Behaviour	Swain et al., 2019
36	electric fish (*Pollimyrus adspersus*)	The Journal of the Acoustical Society of America	Marvit & Crawford, 2000a
		The Journal of the Acoustical Society of America	Marvit & Crawford, 2000b
37	Pacific threadfin (*Polydactylus sexfilis*)	Journal of Fish Biology	Masuda & Ziemann, 2000
38	coral reef damselfish (*Pomacentrus amboinensis*)	Behavioural Processes	Holmes & McCormick, 2010
		Behavioral Ecology	Simpson et al., 2010
39	coral reef damselfish (*Pomacentrus brachialis*)	Behavioral Ecology	Simpson et al., 2010
40	coral reef damselfish (*Pomacentrus moluccensis*)	Behavioral Ecology	Simpson et al., 2010
41	coral reef damselfish (*Pomacentrus nagasakiensis*)	Behavioral Ecology	Simpson et al., 2010
42	freshwater stingrays (*Potamotrygon falkneri*)	Animal cognition	Kuba et al., 2010

43	striped jack (*Pseudocaranx dentex*)	Aquaculture	Kuwada et al., 2000
44	ninespine stickleback (*Pungitius pungitius*)	Behavioral Ecology	Pike, 2010
45	triggerfish (*Rhinecanthus aculeatus*)	Journal of Experimental Biology	Cheney et al., 2019
46	Atlantic salmon (*Salmo salar*)	Aquaculture International	Bratland et al., 2010
		Aquaculture	Folkedal et al., 2010
		Journal of Comparative Psychology	Morin et al., 1990
		Frontiers in veterinary science	Staven et al., 2019
47	brown trout (*Salmo trutta*)	The Progressive Fish-Culturist	Willis & Flickinger, 1980
48	brook trout (*Salvelinus fontinalis*)	The Progressive Fish-Culturist	Willis & Flickinger, 1980
		Canadian Journal of Zoology	Mirza & Chivers, 2000
49	lake trout (*Salvelinus namaycush*)	The Progressive Fish-Culturist	Willis & Flickinger, 1980
50	rudd (*Scardinius erythrophthalmus*)	Journal of Thermal Biology	Rahmann et al., 1980
51	mandarin fish (*Siniperca chuatsi*)	International journal of molecular sciences	Peng et al., 2019
52	fifteen-spined stickleback (*Spinachia spinachia*)	Behaviour	Girvan & Braithwaite, 2000
		Animal Behaviour	Hughes & Blight 2000
53	damselfish (*Stegastes fuscus*)	Journal of fish biology	Silveira et al., 2019
54	corkwing wrasse (*Symphodus melops*)	Animal Behaviour	Hughes & Blight 2000
55	jack mackerel (*Trachurus japonicus*)	Journal of fish biology	Takahashi et al., 2010
56	redtail splitfin (*Xenotoca eiseni*)	Behavioural Brain Research	Truppa et al., 2010
AMPHIBIA			
#	**Species**	**Journal name**	**Source**
1	tiger salamander (*Ambystoma tigrinum*)	Behavioural Processes	Kundey & Phillips, 2019
		Chemical Senses	Mason et al., 1980
		Animal Behaviour	Ray, 1970
2	larval spotted salamander (*Ambystoma punctatum*)	Brain Research Bulletin	Pietsch & Schneider, 1990
3	toad (*Anaxyrus terrestris*)	Behavioral and neural biology	Mikulka et al., 1980

4	woodhouse's toad (*Anaxyrus woodhousii*)	Journal of Comparative Psychology	Boice, 1970
5	fire-bellied toad (*Bombina orientalis*)	Journal of Herpetology	Brattstrom, 1990
		Learning and Behavior	Jenkin and Laberge, 2010
6	poison frog (*Dendrobates auratus*)	Journal of Experimental Biology	Liu et al., 2019
7	túngara frogs (*Engystomops pustulosus*)	Current zoology	Ventura et al., 2019
8	green frog (*Lithobates clamitans*)	Journal of Comparative Psychology	Boice, 1970
		Animal Behaviour	Adler, 1980
9	leopard frog (*Lithobates pipiens*)	Journal of Comparative Psychology	Boice, 1970
10	wood frog tadpoles (*Lithobates sylvaticus*)	Biology letters	Crane et al., 2019
		Biology Letters	Ferrari et al., 2010b
11	Iberian greenfrog tadpoles (*Pelophylax perezi*)	Behavioral Ecology and Sociobiology	Gonzalo et al., 2010
12	toad (*Rhinella arenarum*)	Behavioral and neural biology	Schmajuk et al., 1980
		Acta physiologica latino americana	Schmajuk & Segura, 1980
		Brain, behavior and evolution	Sotelo at al., 2019
13	cane toad (*Rhinella marina*)	Perceptual and Motor Skills	Janes & Falkenberg, 1980
14	spadefoot toad (*Spea hammondii*)	Journal of Comparative Psychology	Boice, 1970
15	clawed frog (*Xenopus laevis*)	Journal of Experimental Biology	Elepfandt et al., 2000
REPTILE			
#	**Species**	**Learning/Task**	**Source**
1	spotted pythons (*Antaresia maculosa*)	Journal of Herpetology	Stone at al., 2000
2	alligator (*Caiman sp.*)	Perceptual and Motor Skills	Williams & Robertson, 1970
3	oriental garden lizard (*Calotes versicolor*)	Amphibia-Reptilia	Shanbhag et al., 2010
4	red-footed tortoise (*Chelonoidis carbonarius*)	Amphibia-Reptilia	Wilkinson et al., 2010
		Animal Cognition	Bridgeman & Tattersall, 2019
		Journal of Comparative Psychology	Santacà et al., 2019

5	painted turtle (*Chrysemys picta picta*)	Learning and Motivation	Pert & Bitterman, 1970
		Brain research	Reine & Powers, 1980
		Physiology & Behavior	Grisham & Powers, 1990
6	collared lizard (*Crotaphytus collaris*)	Physiology & behavior	Davidson & Richardson, 1970
7	Laurent's whiptail (*Cnemidophorus murinus*)	Herpetologica	Schall, 2000
8	Jamaican Iguana (*Cycluracollei collei*)	Copeia	Cooper et al., 2019
9	indigo snakes (*Drymarchon couperi*)	Psychonomic Science	Kleinginna, 1970
10	leopard gecko (*Eublepharis macularius*)	Animal Cognition	Simpson & O’Hara, 2019
11	eastern water skink (*Eulamprus quoyii*)	Animal Cognition	Szabo et al., 2019b
12	five-lined skink (*Eumeces Inexpectatus*)	Psychonomic Science	Powell & Peck, 1970
13	Guatemalan Beaded Lizards (*Heloderma charlesbogerti*)	Copeia	Cooper et al., 2019
14	reeves’ pond turtles (*Mauremys reevesii*)	BioSystems	Nomura & Gunji, 2000
15	Catalan wall lizard (*Podarcis liolepis*)	Behavioural processes	Font, 2019
16	bearded dragon (*Pogona vitticeps*)	Journal of Comparative Psychology	Santacà et al., 2019
17	south Indian rock agama (*Psammophilus dorsalis*)	Biology Letters	Batabyal & Thaker, 2019
18	eastern fence lizard (*Sceloporus undulatus*)	American Naturalist	Thaker et al., 2010
		Biological Invasions	Venable et al.,2019
19	sleepy lizard (*Tiliqua rugosa*)	Canadian Journal of Zoology	Zuri & Bull, 2000
20	astern blue-tongue skink (*Tiliqua scincoides*)	Animal Behaviour	Szabo et al., 2019a
21	red-eared slider turtle (*Trachemys scripta elegans*)	Learning and Behavior	López et al., 2000c
		Journal of Molecular Neuroscience	Zheng et al., 2019
22	collared spiny lizard (*Tropidurus catalanensis*)	Journal of Zoology	Rodrigues & Kohlsdorf, 2019

23	Mertens' Water Monitors (*Varanus mertensi*)	Copeia	Cooper et al., 2019
24	emerald tree Monitors (*Varanus prasinus*)	Copeia	Cooper et al., 2019
25	roughneck Monitor (*Varanus rudicollis*)	Copeia	Cooper et al., 2019

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CHP 3 – EXPLORING CONDITIONING METHODS FOR ASSOCIATION AND DISCRIMINATION OF SOUND FREQUENCY IN AMERICAN TOADS (*ANAXYRUS AMERICANUS*)

Deise Cruz

3.1 Introduction

A key to understanding the evolution of acoustic communication is the ability to discriminate differences in sound signals in the natural environment. In the vocal communication systems of most anurans (frogs and toads), reproductive success relies on the auditory system. Males call to attract females, requiring females to discriminate among the sounds of different males of their own and other species, which are essential processes underlying sexual selection and pre-zygotic isolating mechanisms (Gerhardt and Huber 2002; Ryan 2001). The calls produced by many anurans have a relatively simple structure with relatively small variation within one or a few sound properties, which can be considered conservative species-specific traits (Wells 2007; Duellman and Trueb 1986). Other properties differ significantly between males of the same species and are considered targets of sexual selection (Gerhardt and Huber 2002; Gerhardt 1991). In their natural breeding habitat, when a female frog chooses between two calling males the preference might be a matter of only a small change in one or more physical properties of the calls. An advantage of studying the acoustics of frogs is that calling males often respond to field playbacks, and gravid females show phonotactic approaches to speakers playing back appropriate signals (Gerhardt 1995; Klump 1995). By varying the values of different properties of synthetic-call models of natural calls researchers are able to estimate the minimum differences in properties that males use to distinguish between different rival neighbors (Bee 2016; Bee and Gerhardt 2002) and females use to distinguish between prospective mates (Howard and Young 1998; Howard and Palmer 1995; Gerhardt 1991; Morris and Yoon 1989; Sullivan 1982). These discrimination thresholds that are tested through habituation paradigms and mate preferences of two-stimuli (forced-choice playbacks) are considered to be just-meaningful differences (JMDs; Nelson and Marler 1990).

Like birds and mammals, anurans have been the subjects of extensive literature in the fields of acoustic behavior and auditory physiology, but compared to other vertebrates the literature concerned with cognition and auditory conditioning in anurans pales in comparison to that devoted other vertebrates. For instance, birds and mice are constant subjects of instrumental learning assays and have been shown to be a great model in responding to sounds and detecting acoustic variations that affect decision making (Anand and Nealen 2019; Dent et al. 2000; Hienz et al. 1980; Kobrina et al. 2018; Screven and Dent 2016). Estimates of the true resolving power of the auditory system require psychophysical approaches with conditioned individuals, and the metric estimated is termed the just-noticeable difference (JND). In contrast, the JMD is typically much larger than the JND because resolution of subtle differences in the natural world is seldom necessary and can even be disadvantageous depending on the within- and between-individual differences in key acoustic properties (Bee 2016; Bee and Gerhardt 2001, 2002).

Studies that aim to understand an animal's decision criteria require methods that quantify the relationship between a stimulus's physical magnitude and its perception ability (Akre et al. 2011; Akre and Johnsen 2016; Dent 2017). For this purpose, conditioning methods that have been used on birds and mice are considered reliable techniques because subjects are trained to make a prompting response upon detection of a target stimulus to obtain the reward (Heffner and Heffner, 2001; Niemiec and Moody 1995; Wever 1985). Moreover, experiments based on operant conditioning allow better control for motivation to respond to acoustic differences and be used to estimate an animal 'audiogram' without non-verbal yes/no responses to slight variants of the conditioned signal. JNDs are readily obtained from animals that can be conditioned to respond to sounds (many mammals, birds, and even fish; Fay 1988; Klump 1995; Manabe and Dooling 2020). But it is odd that such estimates for frogs, for which estimates of JMDs are common, are only available for one kind of aquatic frog (Elepfandt et al. 2000), given how dependent on auditory signals frogs are in the wild. Elepfandt et al. (2000) trained *Xenopus leavis* to learn and discriminate tones, reporting 2.4% JND for detectable difference in sound frequency. Results from this study need to be taken with caution because of possible interference from the frequency of water waves detected by the lateral line system, as well as the uncalibrated intensity of punishment.

Frogs are potentially important models to explore auditory JNDs, but psychophysical estimates of spectral and temporal resolution of their acoustics system are generally lacking (Bee 2004, 2016). The smallest estimation of sound discrimination in frequency documented in terrestrial anurans was 5% (Bee 2016, almost certainly representing the JMD) based on phonotaxis assays in untrained animals. Two-choice phonotaxis tests have registered smallest discrimination values for sound frequency of 14 and 15% differences in American toads and gray treefrogs, respectively (Gerhardt 1991; Howard and Palmer 1995; Sullivan 1982). Although toad males can emit calls with 5% differential variation in dominant frequency, and chorus males 7% inter-individual variation (Howard and Young 1998), such levels of discrimination have not been reported to be discriminated by females. In other words, methods designed to test whether females are able to discriminate signals with frequency differences smaller than 7% (e.g., bullfrogs 5% JMD; Bee 2016) or even 2% (*e.g.*, *X. leavis*; 2.4% JND; Elepfandt et al. 2000) can potentially indicate whether the lack of response to signals reflects a failure on the hearing ability (sensory resolution) or lack of motivation (ecological relevance and psychological experience).

The skepticism about the ability of amphibians to be conditioned to sounds stems from earlier unsuccessful attempts to train frogs to associate the reward with tone bursts (Brzoska and Schneider 1978; Yerk 1903). Historically, conditioning frogs is considered a very labor- and time-intensive task, and often considered impossible (for review, Megela-Simmons and Moss 1995, 1985; Strother 1962). Much of this skepticism to conditioning anurans is because of the idea that the behavior of animals like amphibians, fish, and reptiles are considered merely instinctive (Burghardt 2013; Luchiari et al. 2021). However, more recent studies of some anurans have had extensive success in instrumental and spatial learning tasks related to visual and position cues (Brattstrom 1990; Daneri et al. 2011, 2015; Liu et al. 2016; 2020; Muzio et al. 1992, 1994, 1999; Papini et al. 1995; Puddington et al. 2018; Sotelo et al. 2015, 2017, 2019).

In Argentinian toads, the conspecific mating call was used as a conditioned stimulus in a partial dehydration protocol that was used to guide the toad in a plus-maze task (Sotelo et al. 2019). The study found that the acquisition based on mating sound was faster than toads that learned spatial navigation through visual cues (Daneri et al. 2011, 2015). But poison dart frogs (*Dendrobates auratus*) performed well in maze tasks displaying visual cues for associative and reverse learning (Liu et al. 2016, 2020). Multi-species replication of the spatial training method found that poison frogs acquired the discrimination task faster following visual stimuli, like symbols, while túngara frogs (*Engystomops pustulosus*) used beacons (door color) for acquisition. Exploring the success of these recent methods for conditioning anurans can help increase our understanding their learning skills and open a new perspective to test their sound learning and discrimination behavior of their auditory interaction with the environment. The possibility of an accurate estimate of their auditory discrimination (JNDs) will indicate the impacts of auditory limitations and behavioral decisions on the sexual selection of anurans.

The goal of this study was to apply a sound training approach to explore the possibility of a territorial anuran to associate a novel tone burst with food and escape reward. First, the experimental settings and procedures were based on the spatial learning study done on poison frogs (Liu et al. 2016; 2020), although there were modifications applied in order to test the most suitable methods for American toads (*Anaxyrus americanus*). Second, subjects that passed the training criterion were trained to distinguish the target-tone that was acquired previously from broad and narrow levels of difference in frequency magnitude (Hz). Retention of choices across the training phases and performance parameters were considered for potential elements to develop methods to explore sound learning, discrimination, and psychoacoustic tests to determine JNDs in anurans.

3.2 Methods

Subjects. Adult American toads (*Anaxyrus americanus*) were collected in Boone County, Missouri, from 2017 to 2019. They were housed in the anuran amphibian facility at the University of Missouri, Columbia, MO. Toads were treated with antibiotics (Kanamycin) to prevent infection before being transferred to home-cages. The cages contained coarse gravel mixed with charcoal, logs for hiding, and a water bowl. Toads were fed every three days with a single live cricket covered in a vitamin supplement. The facility was maintained at 21°C temperature, 50-80% humidity and 12/12 light/dark cycle. These facilities and procedures were approved by the Animal Care and Use Committee of the University of Missouri (Protocol ACUC 9531).

Acoustic stimuli. Toads were presented with a pair of tone-burst with identical spectral and temporal characteristics, generated with Audacity 2.3.2 as a sine wave, 0.6 dB amplitude, sample rate 44.1 kHz, and 32-bit stereo. The tones were set as s-curves envelope, 300 ms duration, 50 ms rise/fall time, and 500 ms inter-tone interval. The stimulus was repeated every second during the trials and broadcasted using two loudspeakers connected to a stereo amplifier (Alesis RA150) and computer system output. Stimuli presented during the discrimination test phase (Fig. 1) were identical in the aspects described but differed in frequency (Hz). The stimuli were adjusted to 63 dB SPL in the central area of the maze apparatus using a Larson-Davis 800B level meter and a LD826B microphone that controlled the stimulus output. White noise with an intensity of ± 36 dB SPL was constantly emitted as background from the top of the apparatus using a single channel Dell (Rev A00) speaker connected to a Laptop.

Apparatus. The task apparatus was a T-maze constructed with ½ cm wire mesh (Fig. 2). The dimensions were the same as the maze of Experiment 1, but it was closed from the floor to the top at 15 cm height. The top of the central area was covered with a plexiglass plate lid which allowed the experimenter to remove animals in failed trials. The maze was bright and dry, and the temperature was maintained at 25°C ± 2. Brightness was set with four LED light bulbs of 60W each, providing a total brightness of 200 – 220 Fc. Such light intensity is higher than the average of 0.47 Fc measured in frog’s natural reproductive sites during the night (Höbel et al. 2014). The humidity in the maze ranged from 20 – 35% and was maintained with a mini-dehumidifier (Eva-Dry-E-500 Renewable) and a layer of Drierite crystals. The crystals were arranged under the maze floor, covered with absorbent paper to prevent the animal from touching the crystals. The maze was surrounded by white cardboard panels (43cm height) that also covered the doors. The doors operated as guillotines, separating the maze from the shelter. The shelter area was 20L x 43H x 29W cm. The shelter floor was humid (covered with wet cloth) and surrounded by a dark wall (0.2-0.4 Fc), lowering brightness to 0.2-0.4 Fc. A petri dish with mealworms (food reward) was available in the shelter between the door and the loudspeaker (15cm behind the door). White fabric covered the entire apparatus, from the ceiling to the floor, to eliminate potential visual cues. This experiment also took place in a semi-anechoic chamber, and behavior was monitored through a camera from outside. The experimenter manually controlled ropes to open the door and release the toad at the starting area of the maze.

General Procedure. The experiment was divided into two conditioning stages: single-tone and discrimination training. The goal was to evaluate the toads conditioning performance on single-stimulus association as simple associative learning. In the first training, toads listened to a tone on one side of the maze while the other side of the maze played no sound. The second stage aimed to evaluate toad performance in a more complex cues perception task. This discrimination training aimed to evaluate toad performance in recognizing and choosing a specific stimulus (previously associated) against a similar but not equal alternative stimulus. Specifically, toads were trained to discriminate the previously associated target-tone (S+) against an alternative-tone (S-) released at the opposing side of the maze. I anticipated that reliable performances of toads in this experiment could serve as a suitable conditioning protocol, potential method, and a relevant reward–motivation tool for future psychoacoustic tests on sound frequency discrimination of anurans.

In addition, my experiments compared more suitable elements of the protocol regarding subject responses and experiment durability. Therefore, the protocol was adjusted to consider the number of trials a toad was subjected to in a day, facilitated phases, and type of sound cues. To test these aspects, toads were trained in two distinct treatments: Naïve–1000-Hz and Pre-training–1500-Hz. These treatments differed in the number of daily trials, facilitating experiment phase, and the conditioned stimulus (S+). In the discrimination training stage, the treatments also differentiated regarding the tone combinations presented in alternation from both sides of the maze. In the Naïve–1000-Hz treatment the subject always had to discriminate the target tone against a higher frequency alternative tone (plus), as 1000Hz vs. 2000Hz. For the Pre-training–1500-Hz, an alternative tone (minus) that was below the frequency magnitude of the target-tone (S+) was added, and the trials alternated between S+ vs. minus or plus (e.g., 1500Hz vs. 1800hz; or vs. 1200Hz). These procedure for stimulus presentation can indicate whether toads recognize a specific stimulus frequency or are instead responding to confounds of the highest/lowest magnitude.

Behavioral Procedures. Subjects were randomly assigned into two treatments (Naïve–1000-Hz and Pre-training–1500-Hz). Five naïve–1000-Hz toads were trained from June – August 2017. Eighth pre-training–1500-Hz toads were trained from May – July 2018. At the single-tone training stage, the naïve–1000-Hz toads started the experiment without the pre-training phase, received only two trials a day, and were conditioned to tones of 1000 Hz. While the pre-training–1500-Hz toads received an additional experimental phase of pre-training trials, three trials per day, and were conditioned to tones of 1500Hz. The subjects were maintained in standard housing conditions when rested in the homecages throughout the experiment period, except for the feeding schedule. The feeding schedule was restricted to a single cricket every four days, on rest days (no experiment was performed). During this experiment, a toad was given 2-3 mealworms (~ 0.17g total) only on correct trials. The sex and bodyweight of the toads were not determined in either of the treatments.

Acclimation. The experiment started with a single acclimation trial of 10 min, in which no sound stimulus was presented, and the doors were opened. Acclimation lasted two days. The naïve naïve–1000-Hz toads started these trials on the starting dish in the initial aisle of the maze. The pre-training–1500-Hz toads were released in the central area of the maze instead of in the initial aisle. In this case, access to the initial aisle was blocked and they had a view of only the two arms and the exit doors.

Pre-training. After acclimation, only the pre-training–1500-Hz toads received facilitating trials. This was designed to result in faster task acquisition than the toads that did not receive pre-training (naïve–1000-Hz). The pre-training phase lasted for three days of a single trial. In the first two days, the subject was released at the central area of the maze, initially with doors closed and without sound. The door opened after the animal turned its body toward one side when it reached the center area of the maze. Simultaneously, the tone signal was started on the chosen side. On day 3, the toad was then released at the starting dish in the initial aisle. Once the toad reached the central area and turned towards a door, it opened, and the tone signal started.

Single-tone Training. Toads were trained to perform the task of exiting the T-maze through the door behind which the conditioned tone was being emitted. Similar to experiment 1, the task stimulus was a single-tone choice (e.g., tones vs. silence). The target stimuli were 1000-Hz and 1500-Hz tones for toads assigned in the naïve–1000-Hz and pre-training–1500-Hz treatments, respectively. The training was performed at 14:00 – 19:00 h, and animals had a rest day after every four consecutive days of training. The naïve–1000-Hz received two trials per day while the pre-training–1500-Hz toads had three trials daily, both at ~ 60 min inter-trial intervals. A trial started when the experimenter released the subject from the starting dish, 30 seconds after the sound stimulus onset. The trial ended when the toad reached the decision point at one of the arms. The decision point was located 10 cm in front of the door and intended to minimize retreating behavior, given that toads might perceive the door movement as a threat. Only the door associated with the target tone (S+) was opened on correct trials, allowing the subject to enter the shelter and access the food reward. After entering the shelter, the door was closed, and toads were allowed to spend 5 min in the reward area before being removed and transferred back to the resting cage until the next trial. In trials resulting in an incorrect choice, the door did not open, and the subject was removed from the maze without accessing the reward, being placed back in the resting cage. If the toad did not leave the releasing area within 8 min, the trial was considered null (non-response). The testing order of subjects was randomized every day, and the apparatus was cleaned between trials to avoid olfactory cues. The side of the correct door was pseudo-randomized (avoiding the same side more than three consecutive times). The experiment was monitored through the camera. From outside the chamber, the experimenter manually operated doors and the releasing box attached to transparent ropes. Each toad was trained until it reached the individual criterion of equal or above 80% correct choices in the last consecutive days. In order to reach the criterion, the maximum number of trails received by the naïve–1000Hz toads was 70 while the pre-training–1500Hz was 25. The criteria based on a sliding window (e.g., seven correct responses on eight consecutive trials) are commonly used in learning experiments to evaluate reaching a given criterion. However, caution should be taken when interpreting this approach, as the robustness is highly dependable on the number of trials given to an individual. Furthermore, there is a risk of acquisition criteria representing merely chance, as the higher the number of trials, the higher the likelihood of reaching criteria by chance, which can also happen at the first eight trials.

Discrimination training. Toads that reached the criterion were trained in discrimination trials to determine if they could recognize the target-tone (S+), acquired during the previous training stage, against alternative tones (S-). The alternative tones were similar in sound properties to the target-tone, varying only in the frequency magnitude. The procedure used was the same as the previous training stage, except that both sides of the maze emitted alternating sound stimuli during the tests. For instance, a toad that acquired the 1500-Hz tones was then trained on discrimination trials that emitted 1500-Hz (S+) at one door versus 1800-Hz alternative tones (S-) at the other door (Fig 1). Toads had access to the reward only if they chose the door associated with the target tone (S+).

Discrimination training was divided into differential levels. The levels correspond to the percentage differences in tone frequency (Hz) between the target and alternative tones. Toads were trained from high to low differential levels: 100, 20, 10, and 5%. A criterion performance of equal or larger than 75% correct choice in consecutive trials was established to determine whether an individual advanced to the next level (Table 1). These sets of discrimination acquisition were intended to reinforce animal association ability for recognizing and discriminating the acquired tone from similar tones based only on frequency variation. The training through the discrimination levels stopped when the toad failed to reach the criterion within the average number of trials it has taken to pass through the experiment phases.

Treatments received two different combinations of stimulus-pairs: plus and plus/minus during the discrimination stage (Table 1). The naïve–1000-Hz toads at the discrimination training stage were presented to the 1000 Hz (S+) versus alternative plus-tones (S-) with frequency at a percentage of magnitude higher (e.g., 1000-Hz vs. 1200-Hz). They were only tested on the plus combination through the differential levels 100, 20, and 10%. While the pre-training–1500-Hz toads listened to the target-tone (S+) against alternative plus or minus. In alternated trials, the target-tone (S+) was presented against a tone whose frequency magnitude differed above (plus) in one trial and below (minus) in another trial (Table 1). The combinations were randomized among trials, with only trials with plus stimulus-pair or trials only with the minus stimulus-pair, never both stimuli together at the same trial. Thus, the target-tone (S+) was always presented in every trial. In that way, toads from pre-training–1500-Hz treatment could indicate recognition of the target-tone frequency (S+) regardless of whether the alternative was higher or lower frequency through the differential levels 20, 10, and 5%. The 100% differential level was only applied to the naïve–1000-Hz but not to the pre-training–1500-Hz treatment.

Data Analysis. The task performance was evaluated individually by counting the number of trials a toad required to reach the criterion. The latency was computed as the time in seconds it took animals to move from the start dish to the moment they reached the decision point in front of the door. To compare whether including a pre-training phase improved the performance of toads, I used the first six trials of the single-tone training phase as a baseline evaluation. The baseline parameters were the individual score of the first six trails (GLMMs [binomial]) as the dependent variable and toad as a random effect. Treatments were placed as fixed effects with other experimental parameters: latency (s), total trials; order of trials of the day (tr1, tr2, tr3), side choice (right or left door). The *glmer* model was formulated as: score ~ treatment + total trials + side_choice + trial_order + latency + (1 | Toad). The latency was then modeled (*lmer*) as a dependent variable with similar fixed and random effects. To test the significance of factors from linear mixed effect models (lmer), I performed the Likelihood Ration test (LRT) and model comparison through ANOVA. Multilevel models for baseline analyses were performed in lme4 1.1- 23 package (Bates et al. 2015) in R 4.0.2 software (R Development Core Team 2021). The overall data were explored and summarized, but due to reduced sample sizes and the unfixed number of trials fitting models and testing for significance was challenging. In the discrimination training stage, I explored the number of trials to criterion and proportion of errors of toads in each discrimination level.

3.3 Results

Seven out of 13 toads completed the single-tone stage training, reaching the criterion (Fig. 3). Three toads from the pre-training–1500-Hz group reached the criterion within a fewer number of trials (trial range: 9 – 10) compared to the naïve–1000-Hz toads (trial range: 30 – 56).

Baseline analysis demonstrated that the score [binomial] of the first six trials were not affected by the treatments (Pre-Training-1500-Hz: estimate = 0.2734 ± 0.68, z = 0.40, p = 0.68) or by other fixed parameters, such as latency, the number of trials a day, or by counting toads as the random element (Table 1). The baseline of latency time of the Pre-Training-1500-Hz was 118.5 sec ± 23.2 (sd) faster, obtained from Likelihood Ratio Test ($\chi 2(1)$ =14.8, p = 0.0001 [Fig. 4, left]). This speed to respond to trials suggests that the Pre-Training-1500-Hz treatment gained more familiarity with the task at the beginning of the initial trials. Considering that the treatments differed in three aspects (naive vs pretraining phases; two vs three trials per day; 1000 Hz vs 1500 Hz) it is hard to predict whether a single factor or a combination of factors improved the speed of toads during the trials.

Throughout the whole single training stage, toads that reached the criterion spent more time (average latency) to complete the task than those that failed within their groups (Fig. 4, right). The long latency time seems a frequent characteristic among those toads which accomplished this association stage in both treatments (Table 2). However, future studies need to establish a balanced sample size of trials per subjects which would properly test the overall relation between trial speed (latency) and successful acquisition.

Discrimination Training. Six toads responded to discrimination training in both treatments (Table 3). Only one toad (naïve–1000-Hz) that passed the criterion stopped responding to the experiment during the discrimination training. In this treatment, the performance varied among the level of discrimination training. Only a single toad presented a proportion of error below 0.25 at the first discrimination training (100% tone frequency difference [(Table 3)]). The other toads of the naïve–1000Hz treatment performed poorly with the highest proportion of error (>0.40) and a varied number of trials to criterion (Table 3).

The three toads from the pre-training–1500Hz treatment showed more consistency during all the levels of the discrimination training than the naïve-1000-Hz treatment. Their proportion of error of pre-training–1500-Hz toads was lower (avg. 0.33), and they reached the criteria faster (range: 10 - 14): only 2.5 trials above the average to succeed in the single-tone training stage. The individual toad that completed more levels of the discrimination stage was the Hf12 (Table 3); it reached the criterion performance over three differential levels (20, 10, and 5%) hearing alternating pair-stimulus of plus and minus frequencies (alternative tones with frequencies above or below the target tone frequency-Hz). This descriptive comparison suggests that the pre-training–1500-Hz treatment was somewhat effective, with subjects reaching criteria through narrow difference levels of discrimination in addition to the minus/plus combinations. In summary toads trained in the pre-training–1500Hz protocol performed the whole experiment faster, reaching criteria within fewer trials and committing lesser errors at discrimination.

3.4 Discussion

To better test the learning capabilities of amphibians, it is vital that we improve our understanding of their behaviors regarding various elements of protocols used in learning studied. Based on food reward and escaping behavior, 50% of the toads trained reached the individual criterion of a single-tone association. The subjects that succeeded in this phase tended to display long latency time to complete their trails than the those that failed to reach the criterion. This is common observation of successful learners in other animals (Jones et al. 2020; Mazza et al. 2018; Reinagel and Clark 2012). There was however a lack of robust discrimination performances from most of the subjects in subsequent phases (discrimination training) that indicated a weak acquisition of learning or inability to discriminate the tones. Overall, comparative examination between the two treatments that toads were submitted indicates important aspects to be considered when designing methods to evaluate sound learning for psychophysics paradigms in terrestrial anurans. The long training (naïve-1000-Hz group) increased chances of reaching the criterion, but there was a potential negative effect from overtraining which could have resulted in a decline in motivation in subsequent phases. Interestingly, the toad that acquired association the fastest at 1500 Hz showed more stable performance even with adjustments to the protocol to plus/minus frequencies. Control of sound intensity effects and loudness sensations are common procedures in psychoacoustics methods to describe differential hearing threshold approaches (Dooling and Saunders 1975; Henning 1966). The results from the fast-learning toads suggest that the frequency value (Hz) of the tone assigned and the properties interdependency (e.g., frequency-loudness) are essential parameters to calibrate in hearing discrimination studies of American toads.

The choice of distinguishing treatments settings regarding the number of daily trials, shaping phase, and frequency of the target-tone resulted in a tradeoff between training period and the number of toads that reached my criterion. Rather than testing which of those three elements affected the training performance, the primary intention was to obtain as many toads as possible that could rapidly reach the criterion because overtraining impacts in American toads are unknown. During this time I was careful to avoid early exhaustion of the subjects and loss of their motivation due to food satiation because I wanted these subjects to also respond in the discrimination trials. Methodological flexibility, such as the one applied for this research, is commonly applied in animal learning assay projects. For instance, in conditioning that includes several reversal phases it is common to establish a weak learning criterion to avoid initial overtraining of individuals that show robust performance on subsequent phases (Brucks and Bayern 2020; Fuss et al. 2019; Lucon-Xiccato and Bisazza 2014; Warren 1960). In my study, the pre-training–1500-Hz treatment that was not overtrained received a maximum of 25 trails to reach the criterion. This resulted in only 37% of the individuals reaching the criterion in comparison to the 80% of individuals who reached the criterion in the naïve–1000 Hz treatment (receiving one less trail per day and lack of pre-training). However, even though a larger proportion of the naïve-1000-Hz toads reaching the criterion compared to the pre-training-1500-Hz toads, they were less consistent (number of trials and errors) at the discrimination tests. The pre-training–1500-Hz animals were faster during the discrimination phases and were more consistent. Such results revealed a methodological conflict of prioritizing more samples (large accumulation trials per subject) and more chances to obtain subjects reaching criterion, under the risk of compromising additional experimental phases due to exhaustion or loss of motivation. Considering that amphibians and reptiles are ectotherms that only intake a moderate amount of food (Burghardt 2013), future studies exploring learning with food as a motivator should consider documenting feeding behavior and satiation level of their subjects adjusted to a training schedule.

The procedural differences between the treatments had no effect on the number of correct choices. This contrasted with the prediction that toads that received pre-training would be more successful in training and correct choices. But those toads with pre-training were faster at navigating the maze and solving the task, although not always correctly, compared to the naïve toads who were less familiar with the procedure. The fast latency is an important parameter to measure the familiarity of the subjects with the experimental routine, but rapid responses can potentially reduce the chance of that toads will reach the criterion within the allotted treatments. Still both the naïve and pre-training groups demonstrated that individuals that reached the learning criterion showed a longer latency time. The difference in latency with regard to successful and unsuccessful subjects is commonly found in multiple choice tasks, resulting in a trade-off between speed and accuracy, which is different compared to studies that use runway and go/no go tasks in which fast speeds improve success (Jones et al. 2020; Mazza et al. 2018; Reinagel and Clark 2012). Such latency and accuracy relationships support my ad hoc explanation for why some toads reached criterion compared to other toads in their own group. This relationship has been associated with inter-individual variation during learning tasks applied in birds and honeybees (Moiron et al. 2016; Tait and Naug 2020), but to date this has not been explored or understood in anurans. Moreover, the observation that individual differences in speed affected success in American toads still requires more data and robust statistical confirmation.

The better discrimination performance of the toads from the pre-training–1500-Hz treatment was surprising given they had an additional pair stimulus that differed from the target tone with either a frequency above or below. Toads from this group had to discriminate among three types of tone frequencies and were expected to successfully recognize the tone with the intermediate frequency. The goal of this study design was to see if toads can recognize a specific frequency magnitude instead of responding to the tone that was always a lower frequency, which is what the naïve–1000-Hz treatment toads were expected to do. Understanding the anuran auditory system might help to explain why the pre-training-1500-Hz toads appeared more successful at discriminating tones compared to the naïve-1000-Hz. The inner ear of anurans is composed of two distinct organs to process sound frequency (Hz). The Amphibian papilla (AP) is tuned to a low frequency, generally below 1000 Hz and the Basilar Papilla is sensitive to higher ranges that are above 1000 Hz (Wilczynski and Capranica 1984). This range limit of each papilla varies among species, and generally both can be activated by sounds of 1000 Hz (Capranica and Moffat 1983; Wilczynski and Burmeister 2016; Ryan 2009; Simmons et al. 2007; Gerhardt and Schwartz 2001). The carrier frequency of the advertisement call of American toads ranges between 1400 and 1900 Hz; thus the 1500-Hz stimulus is well within the mating-call frequency range (Howard and Palmer 1995, Howard and Young 1998, Sullivan 1992). Training frogs to tone burst whose frequency is processed by either the BP or AP and overlaps with the frequency of the advertisement calls might increase the chance of perception and discrimination. One example was the successful acquisition of Argentine toads in spatial leaning task associated to the mating call (Sotelo et al. 2019).

Another consideration that affects the auditory perception and discrimination is the fact that the auditory system is non-linear (Dooling and Saunders 1975; Henning 1966). According to an audiogram based on electrophysiology measurements, American toads are more sensitive to 1000 Hz than to 2000 Hz and would be expected to perceive the lower frequency to be more intense (Walkowiak et al. 1981). Thus, there is always the possibility that a difference in intensity rather than frequency could be responsible for correct choices. This can be tested by systematically varying the intensity of the tones while holding frequency constant. (c.f. Marvit and Crawford 2000; Screven and Dent 2016; Talwar and Gerstein 1998; Toal et al. 2016).

The results of this study can provide some hints and caveats for future attempts to conditioning anurans to sounds and to estimate JNDs. Although it was expected that a pre-training phase, in combination with 3 daily trials and 1500 Hz tone, would improve success in reaching criterion, this did not occur; however, the pre-training did improve maze familiarity and task associations. Another consideration is that rather than setting the number of trials to criterion, the latency time difference between the successful and unsuccessful individuals might be a better sign of successful association and auditory discrimination. It will also be important to systematically vary the intensity of stimuli of different frequency to confirm the basis for their discrimination.

Although sound learning was not achieved in American toads in my study, this does not mean that they or some other anurans cannot be conditioned. The success or failure may depend on finding effective rewards or punishments that have not yet been tried or which might be effective in other species, such as desert toads of Argentina discussed in Chapter 4. Alternatively, the failure to condition anurans to sounds may simply reflect the fact that they have no opportunity to learn sounds or discriminate among them and that they have to respond appropriately in the one or few breeding opportunities they are likely to have in their lives.

3.5 Figures

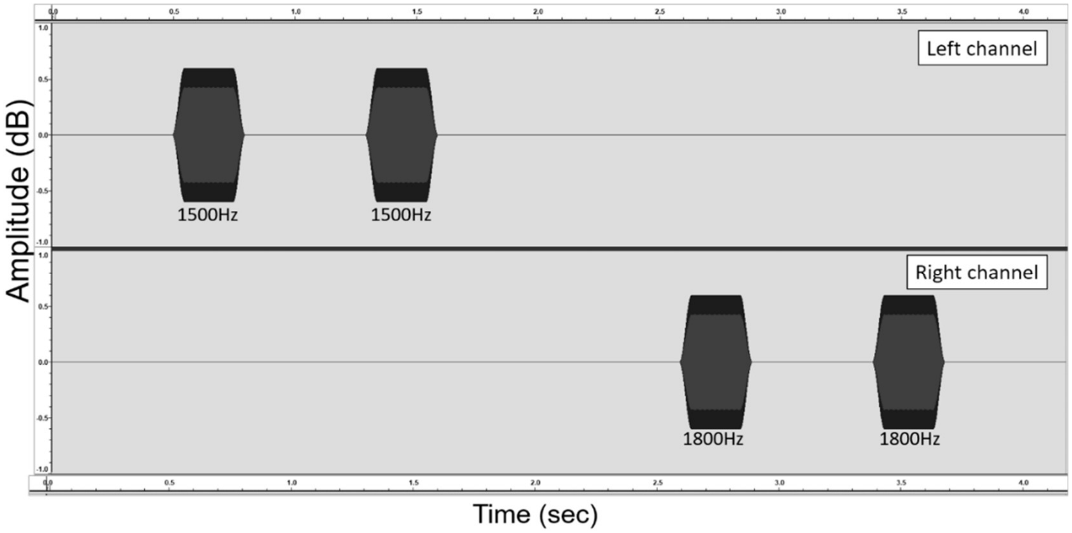

Figure 1. Diagram of the sound stimuli used in the discrimination training phase. Tones from the left (top) and the right (bottom) channels only differ in 20% frequency (Hz). During the single-tone training phase, only a single channel (left or right) emitted the target stimulus, while the other channel was silent (no tone).

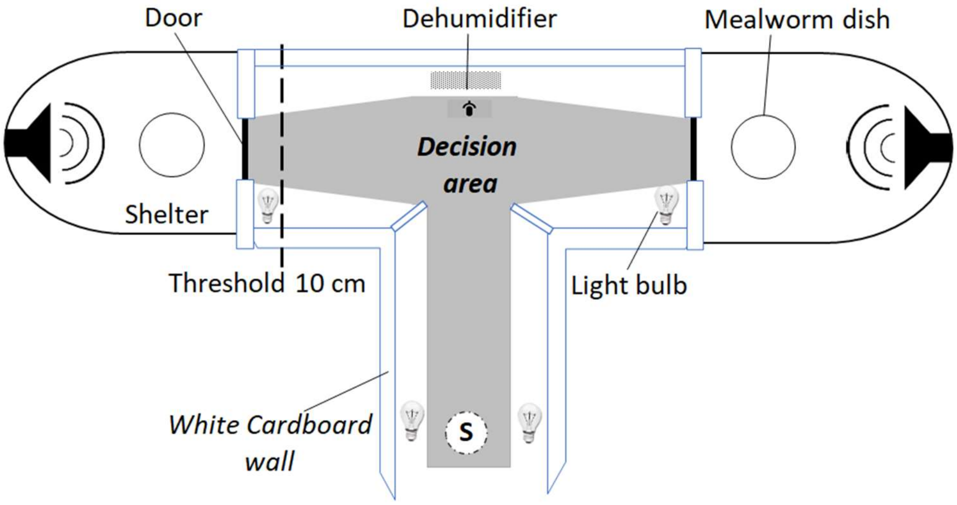

Figure 2. Experiment apparatus: T-maze dimensions – initial aisle 30L x 15H x 9W cm; arms 54L x 15H x 14W cm. The arms' endings narrow to 4 cm wide to form a funnel-shaped exit. Shelters (40L x 42H x 39W cm) limited by a wall built with wire mesh and dark fabric. Loudspeakers placed 32 cm behind the doors and camera 58 cm above the maze.

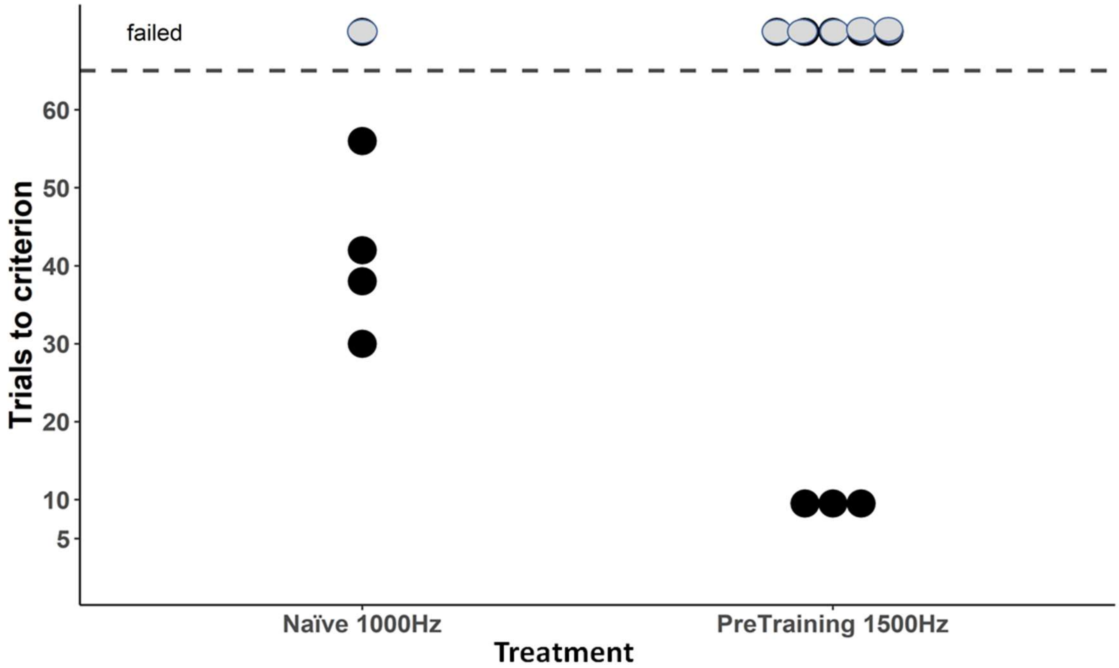

Figure 3. The number of trials to reach the criterion of the single-tone training stage. The plot shows toads (dots) from naïve–1000-Hz (N=5) and pre-training–1500-Hz treatments (N=8). The black dots represented all toads that reached the criterion. Toads that failed to reach the criteria are represented in gray color above the dashed line.

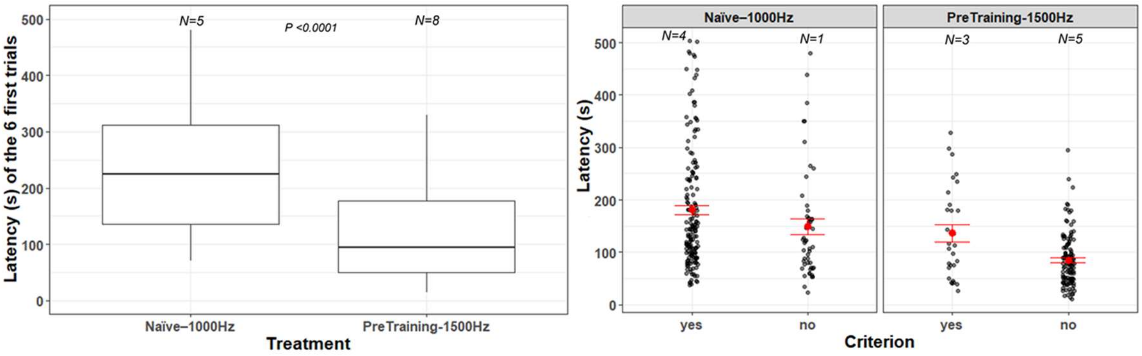

Figure. 4 Left: Baseline difference of latency time between the naïve–1000-Hz and Pre-Training-1500-Hz treatments during the six first trials of the single-tone training stage. Right: The latency throughout the entire duration of the single-tone training. Observations from naïve–1000-Hz (left) and Pre-Training-1500-Hz (right) based on the ability of toads to reach the criterion. Number of toads (N). Mean and standard error (red).

3.6 Tables

Table 1: Baseline (performance on the six first trials) effects and significance associated with the successful response. GLMMs estimates, expressed in logits. Toads (N= 13) and observations (N=78)

Fixed Effects	Estimate	Std. Error	Z value	Pr(>|z|)
(Intercept)	-0.5721	1.0068	-0.568	0.570
Pre-Training-1500-Hz	0.2734	0.6771	0.404	0.686
Total trials	-0.0218	0.1472	-0.149	0.882
Side choice (Right)	-0.2741	0.5270	-0.520	0.603
Second trial (tr2)	0.2265	0.5599	0.405	0.686
Third trial (tr3)	0.3249	0.7406	0.439	0.661
Latency (sec)	0.0028	0.0028	0.992	0.992
Random Effect		Variance	Std. Error	
Toad ID	(Intercept)	0.2438	0.4938	

Table 2: Toads performance by treatments during the single-tone training stage. The number of toads (N) and whether they reached the criterion (Criterion). The minimum and the maximum number of trials (trail range) and (Mean ± sd) of the total latency and baseline latency (six first trials of the training).

Treatment	N	Criterion	Trial range	Total Latency (s)	Baseline latency(s) of 6 trials
Naïve -1000-Hz	4	yes	30 – 56	180.6± 114.5	238.1±119.0
Naïve-1000-Hz	1	no	50	149.5± 105.9	216.6±107.7
Pre-Training -1500-Hz	3	yes	9 – 10	136.8± 88.8	155.5±93.9
Pre-Training -1500-Hz	5	no	21 – 31	85.1± 49.9	93.4±56.2

Table 3: The proportion of errors and number of trials: error rate (trials) during the discrimination training. Errors represented the incorrect choices (S-), as opposed to the target-tone (S+) of 1000Hz and 1500Hz for the Naïve and Pre-training treatments, respectively. The toads on the Naïve-1000-Hz treatment heard the tone frequency of S- always a percentage above 1000Hz (S+). The pre-training–1500-Hz toads were presented to alternated trials with S- differing the tone frequency at the percentage of magnitude above (plus) or below (minus) 1500Hz (S+). The discrimination training level advanced as the toad reached criteria above 80% or 75%*.

Naïve –1000-Hz: only plus alternative tones					Toad error rate (trials)	
Experimental phase	Level (%)	Difference (Hz)	stimuli (Hz)	Sal	Vit	Eva
Training	n/a	Single-tone	1000 vs silence	0.47 (30)	0.51 (56)	0.34 (38)
Test	100	+1000	1000 vs 2000	0.20 (10)	0.41 (41)*	0.42 (14)
	20	+200	1000 vs 1200	0.44 (18)		
	10	+100	1000 vs 1100			

Pre-Training –1500-Hz: plus and minus alternative tones					Toad error rate (trials)	
Experimental phase	Leve (%)	Difference (Hz)	stimuli (Hz)	Hf8	Hf12	Hf14
Training	n/a	Single tone	1500 vs silence	0.20 (10)	0.11 (9)	0.30 (10)
Test	20	±300	1500 vs 1800 1200	0.30 (10)*	0.33 (12)*	0.38 (13)
	10	±150	1500 vs 1650 1350		0.36 (14)	0.27 (11)
	5	±75	1500 vs 1575 1425		0.33 (12)	

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CHP 4 –TRAINING AMERICAN TOADS (*ANAXYRUS AMERICANUS*) TO LEARN NON-NATURAL SOUNDS WITH A WATER REWARD

Deise Cruz

4.1 Introduction

The partial-dehydration protocol is a promising method to train anurans and test their learning abilities and has been well suited for such species like *Rhinella arenarum*, the Argentine toad. This technique has been successfully implemented since the 1980s and has resulted in successful subject responses with the desired tasks (Schumajuk et al. 1981; Schumajuk and Segura 1982; Muzio 1999). The repetition of the method, using *Rhinella arenarum* as a model, produced a series of findings on learning paradigms, instrumental, spatial cognition, memory, and associations with brain activity (Daneri et al. 2011, 2015; Muzio et al. 1992, 1994, 1999; Papini et al. 1995; Puddington et al. 2016, 2018; Sotelo et al. 2015, 2017, 2019). More recently, Argentine toads (*R. arenarum*) showed an association of mating call signals to hydration reward in a maze setting within six days of training (Sotelo et al., 2019). This was a remarkably short time of acquisition compared to previous studies that used egocentric cues (e.g., turn left or right), visual and geometric methods (Daneri et al. 2011, 2015; Sotelo et al. 2015; 2016; Schumajuk et al. 1980). This kind of study represents a great opportunity to discover and use novel conditioning stimuli, apply replication for multispecies comparison, and increase the use of "non-traditional" species that have discovered new cognitive abilities in fish and reptiles (see Chapter 2).

The partial body dehydration protocol aims to increase the motivation of toads to seek water rewards during the tasks. Prior to the trials, *R. arenarum* subjects are partially dehydrated to ~80% of their standard body weight (SBW [Muzio et al. 2011]). The SBW is defined as the animal weight with the urinary bladder empty (Ruibal 1962). The urinary bladder of anurans is adapted to store water as diluted urine up to almost 30% of their total body mass, which works as a reliable source of water reabsorption in dehydration conditions, especially in anurans of territorial and arid habits (Bentley 1966; Ruibal 1962), like *R. arenarum* that are adapted to live in the arid areas of Argentina (Sanabria et al. 2007). In response to dehydration, amphibians display the water behavior by sitting on wet substrates allowing a faster absorption mechanism throughout their highly vascularized ventral skin in their pelvic area (Hillyard et al. 1998, 1999; Bentley 1966). In addition to the dehydration period, feeding is also suspended during the training to control for water loss due to excretions. These food regimes and schedule of hydration and dehydration in *R. arenarum* are far from the weight loss death risk of 40-45% for terrestrial anurans (Hillman et al. 2009; Hillyard 1999; Krakauer 1970; McNab 2002). Therefore, the controlled partial dehydration condition and natural water behavior are useful mechanisms to assess water uptake motivation to conditioning toads.

Other studies that have applied the partial-dehydration method in maze tasks and water reinforcement showed variation among populations and protocols. Brattstrom (1990) conditioned fire-bellied toads (*Bombina orientalis*) to navigate in a T-maze and a complex maze with multiple dichotomic turns. In this study, fire-bellied toads acquired the task in 3-4 days. They could follow egocentric cues, had more trials per day, were partially less dehydrated (< 10% of the body weight), and were provided only 30 sec to reabsorb water from the reward. More recently, Ouellet et al. (2020), reproduced the experiment with another population, but they did not find the faster learning ability of fire-bellied toads as was described in the study performed by Brattstrom (1990). Yet the spatial learning ability was confirmed after modifications on the dehydration level, reinforcement schedule, extended training period, control of turn preferences and analytical approaches (Ouellet et al. 2020).

These results, although confounded by critical components of experimental variation and replication, suggest a great possibility to investigate learning abilities in other amphibian species associating diversity of sensory cues with water motivation. Therefore, I tested whether the partial-dehydration method is suitable to conditioning American toads (*Anaxyrus americanus*) in maze tasks, based on association with a non–mating repertory stimuli (tone-bursts). I predict that American toads will respond to water reward conditioning, which was successfully applied to studies on Argentine toads and fire-bellied toads (Brattstrom 1990; Ouellet et al. 2020; Sotelo et al. 2019). More specifically, this study replicates the procedure performed with Argentinian toads (Sotelo et al. 2019), evaluating the method to conditioning other species of toads and their performance to learn non-mating acoustic stimuli.

4.2 Methods

Subjects. Adult American toads (*Anaxyrus americanus*) were collected in Boone County, Missouri, from 2017 to 2019. They were housed in the anuran amphibian facility at the University of Missouri, Columbia, MO. Toads were treated with antibiotics (Kanamycin) to prevent infection before being transferred to home-cages. The cages contained coarse gravel mixed with charcoal, logs for hiding, and a water bowl. Toads were fed every three days with a single live cricket covered in a vitamin supplement. The facility was maintained at 21°C temperature, 50-80% humidity and 12/12 light/dark cycle. These facilities and procedures were approved by the Animal Care and Use Committee of the University of Missouri (Protocol ACUC 9531).

Acoustic stimuli. Toads were presented with a pair of tone-burst with identical spectral and temporal characteristics, generated with Audacity 2.3.2 as a sine wave, 0.6 dB amplitude, sample rate 44.1 kHz, and 32-bit stereo. The tones were set as s-curves envelope, 300 ms duration, 50 ms rise/fall time, and 500 ms inter-tone interval. The stimulus was repeated every second during the trials and broadcasted using two loudspeakers connected to a stereo amplifier (Alesis RA150) and computer system output. Stimuli presented during the discrimination test phase (Fig. 1) were identical in the aspects described but differed in frequency (Hz). The stimuli were adjusted to 63 dB SPL in the central area of the maze apparatus using a Larson-Davis 800B level meter and a LD826B microphone that controlled the stimulus output. White noise with an intensity of ± 36 dB SPL was constantly emitted as background from the top of the apparatus using a single channel Dell (Rev A00) speaker connected to a Laptop.

Apparatus. The apparatus was a plus-maze built with acoustic foam wedges (Fig. 1). The wedge walls, which were not fixed to the floor, could be rotated each trial to prevent the animal from relying on visual or turn cues. The arm's dimensions were 55L x 15W x 43H cm and the central area of the maze was 15 cm wide. The top of the maze was uncovered and 1m above it there was a web camera and a 20W lamp providing 3.2Fc illumination (measured at the maze floor). Only three arms were used on each trial, as a T–shape, while the inactive arm was blocked with a foam wedge. The microphone was set across the blocked wall to calibrate sound intensity in the central area of the maze. The positive reinforcement was placed at the end of the choice arm in a dark container with 100 ml of water and covered with a wire mesh lid. The lid at the correct choice was loosely closed so it would sink once a toad sat on top, allowing the belly to touch the water for the rehydration reward. At the wrong choice, the lid was fixed to prevent animal contact with the water (settings adapted from Sotelo et al. 2019, 2015). On each side of the T-maze a loudspeaker was placed 15 cm above the water container, facing the center of the maze. Dark fabrics enclosed the entire maze structure to eliminate external visual cues. The entire apparatus was installed inside of a semi-anechoic chamber. The temperature and humidity were maintained at 21 – 24°C and 50%, respectively.

Water Deprivation Procedure. Two days before the experiment started, toads were placed in a container with water for 60 min to reach their full hydrated body weight. Subjects were then weighed to determine their standard body weight (SBW). To get the SBW the experimenter gently inserted a glass capillary in the animal cloaca and pressed the belly to empty the urinary bladder (Hillyard et al. 1998; Reboreda et al. 1991; Ruibal 1962). Toads were placed on an open, dry surface until the body weight dropped to 80% of their SBW. Then, they were transferred to individual dry cages to rest for the experiment the following day. Toads were expected to increase body weight during the daily trials when they reach the water reward. An hour after trials, toads were weighed to correct the ~ 80% SBW by dropping water into the plastic container of the ones that did not absorb enough water; and dehydrating the ones that were too hydrated. Feeding was suspended seven days before the experiment started to control body hydration and weight changes.

Behavioral Procedure. Toads were trained for three consecutive trials per day to accomplish the task of choosing the reward (accessible water container) at the maze arms associated with the target stimulus. They were assigned into three groups: female-tone (n=5) and male-tone (n=10) groups were trained to respond to the tone signal (1650 Hz); and a control group, male-silence (n=5), was trained to respond to the silent side. The experiment was conducted from May to July 2020.

Acclimation. The first two days of the experiment were composed of single trials, in which drops of water were placed on the floor to guide the toad towards one of the water containers that were accessible on both sides. Toads that did not reach the container within 10 minutes were gently guided by the experimenter towards the closest water reward. No tone stimulus was presented during this phase.

Training. The training phase followed the last day of acclimation. Each toad was trained every day with three consecutive trials per day at 14:00 – 19:00 h. Subjects were transferred to the maze using an upside-down starting box. The box was attached to a rope, which the experimenter could pull from outside of the chamber to release the animal. A trial started when the experimenter released the subject 30 seconds after the acoustic stimulus onset. In a trial, one side emitted the sound stimulus (tones) while the opposing side did not emit a sound (silence). It was only considered a correct choice when the toad sat on the top of the water container with all four legs on the tone side of the maze. The toad was then allowed to rehydrate on the container for 2 min before starting the next trial. When a toad chose the incorrect side, the subject was left sitting on the inaccessible container for 1 min as punishment, and then it was guided to the correct container for 2 min of rehydration. The experimenter guided toads to the water container if they did not move from the starting area after 3 min. In this case, the trial was repeated. Left and right sides were pseudo-randomized, avoiding repetition of the correct choices on the same side more than twice a day. The testing order of subjects was randomized every day, and the apparatus was cleaned after trials to avoid olfactory cues. The experimenter only approached the apparatus before and after a trial, as the experimenter could observe the behavior through the web camera from outside of the chamber. Toads trained for ten days to reach the learning criterion established as a group average of 75% correct choice on the last three consecutive days (Sotelo et al. 2019, Daneri et al. 2011).

Data Analysis. I evaluated five variables of toad performance in training trials: success rate, latency, water uptake, maze behavior and side choice. Success rate was computed as the overall group average of correct choices across the training days. Latency was the time an animal moved from the starting area until it stopped with all four of legs on top of the water container. Water uptake was measured through body weight variation and calculated as the difference between the weight before and after the daily trials, divided by the standard body weight (SBW) multiplied by 100 (Papini et al. 1995). Weight variability across training days has been used as an important parameter to infer toad motivation in partial dehydration protocols (Muzio et al. 1992). To check if toads assigned into the groups significantly differed in size (SVL [mm]) and weight (SBW[g]), I performed non-parametric analysis Kruskal-Wallis and pairwise Dunn's Test to compare body size. The SBW was compared using linear regression and randomization testing based on 10,000 resampling and pairwise permutation. This verification is important to understand weight variation related to the outcomes of water uptake.

The maze behavior parameter was composed of three types of behavior toads displayed during a trial before making a decision. Trials were classified as Regular, Escaping, or Indecisive. In Regular trials, toads walked straight to one of the sides and chose the water container. In Indecisive trials, toads spent most of the time in the center of the maze going back and forth between the two sides before making a decision. In the Escaping trials, toads were observed trying to escape from the maze by climbing the maze wall before making a decision.

Finally, analyses of a side bias were computed using a bias index (Petrazzini et al. 2020; Szabo et al. 2019). The bias index (BI) corresponds to the number of turns to the right and left: (R-L)/(R+L). I examined the BI to identify whether bias was pre-existent or developed during the training. For that, BI was used as the response variable (LMMs[continuous]), day was a fixed effect and added to a random intercept for each toad and random slope for day. Model was formulated as: BI ~ Day + (1+Day|toad). To examine effect of sex and side preference during the experiment, I examine side choice (GLMMs[binomial]) as the following model: side_choice ~ sex +total trials + trail order + tone-group + latency + behavior + (1|toad).

Generalized linear mixed models (GLMMs) were performed examined score (yes/no) and side choice as a dependent variable (*glmer* [binominal]) and toad identity of the toad as a random effect. Fixed effects included maze behavior and other training parameters: total trials, order trial of the day, side choice (left/right), sex (male/female), group (tone/silence) and latency (s). Linear mixed models (LMMs [*lmer* function]) and Likelihood Ratio Test (LRT) comparison model factors through ANOVA were used to investigate the predicted controls over water uptake and latency with same controls and random-level effect. All data were analyzed in R version 4.1.2 (R Core Team,

2021) and Multilevel mixed models performed with lme4 1.1-27.1 (Bates et al. 2015)

4.3 Results

Nineteen toads completed the training phase in three groups: female-tone (N=5), male-tone (N=9), and male-silence (N=5). See Appendix Table 1. Only one toad from the male-tone group was unresponsive and did not complete the training and was excluded from the analyses. All groups performed below the acquisition criterion (i.e., results were not significantly different from chance) (Fig. 2a), demonstrating absence of sound association with the water reward in a maze task.

Although toads failed to reach the learning criterion, toads were more likely to respond correctly in trials in which they behaved indecisively, going back and forth between the two sides of the maze before making a decision (estimate = 0.85 ± 0.16, z = 3.46, p = 0.015, Appendix Table 2). This suggests that the behavior displayed by the toad prior to making a decision can predict task outcomes and work as a control to calibrate animal and procedural constraints.

The water uptake pattern was inconsistent across days (Fig. 2b). As the number of trials advanced, the proportion of water uptake declined to -0.03 ± 0.006 (sd) (LRTs: $\chi2(1) = 30.5$, $p < 0.001$). The negative relationship confirms the lack of motivation. The expectation was that would increase the level of rehydration, thus indicating more motivation and consequently greater possibility of learning. Sex also acted as a significant predictor for water reabsorption, with the proportion of water-uptake by males being 1.64 g/100g ± 0.64 (sd) larger than females (LRTs: $\chi2(1) = 30.51$, $p < 0.0001$; Fig. 4). The difference in water uptake between females and the groups with males probably reflected the larger female body size and consequently smaller surface area to volume ratio rather than an indifferent reaction to the task and reward. Females body length (SVL) was larger than the males ($\chi2$ (2) = 10.59, $p = 0.005$; pairwise female vs. males $p < 0.0099$; Appendix Fig.1 and Table 1). Similar pattern was evident regarding body weight (SBW), with females on average weighing 50.8g compared to males 26.2g (SWB, randomization test [p=0.0001], pairwise permutation; female vs. males, $p < 0.0042$).

Toads responded to the task rapidly, averaging 44.0 seconds (Appendix Table 1). Latency significantly changed and was affected by the number of trials, order of trials, decision behavior, and side choice. As the number of trials increased, toads finished the trials more rapidly (LRTs s: $\chi2(1) = 3.96$, $p < 0.04645$). The time during first trials of the day (tr1) was longer than the time during the second (tr2) and third trials (tr3) of the day (LRTs: $\chi2(2) = 13.66$ $p = 0.0011$). Compared to the first trial/day (tr1), tr2 was 9.2 s ± 3.31 (sd), and tr3 was 11.8 s ± 3.34 (sd) faster (Fig. 5, Appendix Table 3). The reduction in latency response time among the trials and across the number of trials might have indicated a gain in familiarity rather than being related to the proportion of correct choice and a consequently learning skill. (Latency, GLMMs: estimate = -0.004 ± 0.002, $z = 1.74$, $p = 0.08$).

In addition to the number of trials and order, regression of latency also indicated a relationship between toads displaying indecisive maze behavior and side choice (turning right or left). Latency was positively related to the indecisive behavior (LRTs: $\chi2(1) = 90.46$, $p < 2.2e-16$), with indecisive trials being the longest (mean± sd: 52.3 s ± 3.34). Although a display of indecision generally resulted in a correct response, the GLMMs model for the proportion of correct choice, counting an interaction of latency and behavior as the fixed effect, was not statistically significant (estimate = -0.012 ± 0.007, z = 1.58, p = 0.113). The fourth parameter to account for variation in latency during the experiment was the side choice (LRTs: $\chi2(1) = 8.62$ $p < 0.003$). Responses to the right side of the maze were 8.4s ± 3.34 (sd) longer than a trial in which toads were decided for the left side.

Side choice was controlled by trial order, latency time and sex. In general, toads usually chose the left side at the first trial of the day (tr1), with an increasing probability to shift to the left in the following trials (GLMMs; tr2: estimate = 0.499 ± 0.22, z = 2.22, p = 0.026 [Appendix Table 2]). This trend demonstrates that toads used the left side as default with more flexibility to alternate sides in the second trial (Fig. 6). Supporting the findings of latency LMMs analyses, toads took longer to finish a trial when they chose right side of the maze (latency significantly increased with the proportion of choice to the right (GLMM; Latency: estimate = 0.008 ± 0.002, z = 2.79, p = 0.0052). Males chose the left side more often than females (GLMMs; male: estimate = -0.75 ± 0.31, z =

- 2.40, p = 0.016, Fig. 6). This suggests the performance of toads were strongly influenced by a preferred side.

The side choices investigated through BI (bias index) also demonstrated lateralization components influencing the performance of toads. The average bias index (BI) of the groups was far from zero, indicating bias to the right in females and the left side in males regardless of their groups [Fig. 7a]). Animals tended to show a side bias from day one of the experiment (Fig. 7b) instead of developing side bias over time (GLMMs; Day: estimate = -0.01 ± 0.01, p = 0.318). Although both sexes diverged regarding the preferred turning side at the beginning of the training, females indicated more plasticity to revert to this behavior after days of training.

4.4 Discussion

American toads (*A. americanus*) were unable to acquire the task of associating tone bursts (non-reproductive sound) with water as a reward. Unlike the toads *R. arenarum* and *B. orientalis*, dehydrated American toads did not uptake water (increasing their body weight) in the course of the experiment, and hence water did not function as a reinforcing reward. However, replicating this protocol (Sotelo et al. 2019) in different species and novel sound cues revealed important aspects to consider for future conditioning studies on anurans. In my experiments, side bias and sex differences strongly influenced the performance of American toads in a maze task based on binary responses. Observation of the behavior by the toads in the maze prior to a decision also emerged as a potential methodological parameter to evaluate the learning process of naive species.

Although the accessibility to the water container appeared irrelevant to American toads, such methodology proved to be the strongest reinforcement for learning method in spatial conditioning for the species *R. arenarum* and *B. orientalis* (Brattstrom 1990; Daneri et al. 2011; Ouellet et al. 2020; Sotelo et al. 2019). The amount of water toads reabsorbed and the weight gained across this protocol were considered the most critical feature for successful learning (Muzio et al. 1992; Schmajuk et al. 1980; Ouellet et al. 2020). The motivation to reabsorb water triggers anticipatory physiological responses (more irrigation at the ventral skin), prompting the toad to seek water and increasing chances of correct choices (Schmajuk et al. 1980; Schmajuk and Segura 1982). There are several implications related to the use of water reinforcement in dehydrated anurans. The tolerance to dehydration varies widely among terrestrial anurans, some are able to withstand up to 30%–50% decrease of their own body mass (Bentley 1966; Hillman et al. 2009; Hillyard 1999; Krakauer 1970; McNab 2002), with some species of amphibians being as tolerant to water loss as species of mice and lizards (Hillyard 1999; Thorson and Svihla 1943). Therefore, some level of dehydration might not represent a significant problem for one species as it is to others, and this varies with body weight (observed between male and female of American toad trained here), the habitat, temperature, permeability of the skin, and body condition of the individual animal (Bentley 1966; Roznik et al. 2018).

American toads are most often found away from bodies of water except during breeding season. Their habitat ranges from the forest, flat grassland, and even open fields where they will find moist soil for burrowing and hiding places (Bergstrom 2010). Argentine toads by contrast are commonly subjected to hydric stress because they inhabit a wide range of xeric habitats (Bionda et al. 2011; Sanabria et al. 2007). It is possible that American toads adapted to dehydration level were not stimulated to enter the water container because their typical water-balance behavior is to burrow in moist soil or to sit on a wet substrate (Rittenhouse et al. 2008; Stille 1958). Conversely, being in or seeking out standing water is a normal behavior for Argentine toads, given that they have a much longer breeding season (Mid-August to April) that requires more contact with ponds in contrast to the short breeding season of American toads (April-May). I also emphasize that American toads ignored the water rewards was not caused by weakness in motor movement caused by their dehydrated condition. Toads were for the most part active in the maze with very few null trials (unresponsive trials). Moreover, increased familiarity with the maze occurred as measured by the decrease in latency time as the experiment progressed. Gatten (1987) found that American toads were less sensitive to dehydration (reduced 30% of the body mass) when submitted to exercise and showed better aerobic capacity than the Northern leopard frog (*Lithobates pipiens*): 46% vs. 80% of the capacity affected, respectively. A series of investigations on dehydrated Argentine toads, prior to the establishment of the conditioning protocol, documented that at dehydration levels from 10 to 40%, toads more rapidly approached a water container in a runway apparatus. This shows that in this particular species there was a strong motivation to search for water and hence a potentially effective reward that could be used to reinforce or condition responses to other stimuli. Another contributor to the decrease in water uptake could be associated with animal stress from the experiment. Ouellet et al. (2020) reported this phenomenon when trained hydrated and dehydrated groups of fire-bellied toads in a plus-maze based on position cues. They found the control treatment that was trained at hydration condition to find the water reward and had constant access to water in the home cage ended up losing weight during the experiment period. They suggested this unexplained weight loss could be due to training stress. Future studies that explore the relevance of water reward for training need to consider the level of water motivation and the stress it could cause on American toads and other species before submitting them to this partial-dehydration protocol.

Side biases have also been reported in spatial training of fish and reptiles, indicating lateralization impacts on variations of learning outcomes at individual and population levels (Bridgeman and Tattersall 2019; Csermely et al. 2010; Liu et al. 2017; Pouca and Brown 2018; Pouca et al. 2019; Roche et al. 2020; Szabo et al. 2019). Red-footed tortoises (*Chelonoidis carbonaria*), for instance, exhibited individual biases during visual discrimination training in a y-maze, a tendency that was absent at the pilot and pre-training phases of the experiment (Bridgeman and Tattersall 2019). In contrast, eastern water-skink (*Eulamprus quoyii*), mixed sexes group, started visual discrimination training with positive BI (right side bias) and no significant training effect in developing it (Szabo et al. 2019). This tendency to follow egocentric cues (remember last turn direction) is a common strategy adopted by toads in spatial learning tasks. Daneri et al. (2011) tested this with Argentine toads (*R. arenarum*) and found they relied more upon turn cues in a maze task that combined side and visual cues. This side confound was also acting in spatial tasks that used anuran species, such as the fire-bellied toad (Ouelle et al. 2020) and túngara frogs (Liu et al. 2017; 2020). But this is not the case with all anurans, as poison frogs were able to navigate in maze tasks following visual cues without a reporting of any side constraints (Liu et al. 2016; 2020). This evidence showed side bias as a complex factor in conditioning paradigms, given that its effects vary among studies, methods, species, and individuals.

In my study, American toads trained through dehydration demonstrated a pre-existing side bias, with BI (bias or lateralization index) average far from zero since the beginning of the experiment. More surprising was the clear sex differences in preferred sides in the population of American toads trained here. The bias did not even develop during the experiment; females were able to inhibit their preference to the right side throughout the experiment while males persisted in choosing the left side preference. It is possible that these females could improve their performance with more days of training, given they were more able to approximate the BI closer to zero after 10 days of training. Liu et al. (2017) reported sex differences in spatial cognition of túngara frogs, in which both sexes preferred to turn to the left, but females tended to switch sides throughout the training. A later replication of the study, implementing side bias control (randomization of sides the cue was placed in the maze), found a reduction in learning performance and no differences between sex side preference in túngara frogs (Ventura et al. 2019). Data comparing sex-related side bias are lacking in successful anuran systems such as poison frogs, Argentine toads, and fire-bellied toads, although experiments are performed with mixed sexes (except Argentine toads which have mostly been tested in males). Ouelle et al. (2020) used both sexes and found a mixed bias to the right and left sides, which could reflect a sex difference, but this was not explored. Gender differences in learning and cognition have been reported in other animals, with males generally performing better than females in spatial tasks, although the contrary and lack of difference are also documented (Jozet-Alves et al. 2008; Keagy et al. 2019; Kriengwatana et al. 2016; Szabo et al. 2019b). Therefore, more than simple determination, the performance between sexes requires a better understanding of natural history, neural, physiological, sensory detection, and even personality and experience coping with novelty (e.g., neophobia).

In this study, the use of a side strategy by the American toads appeared to be an easier strategy as the latency became shorter as the toads became more familiar with the task. Studies on other animals have encountered this relation of familiarity working as a factor to strengthen side bias (Bridgeman and Tattersall, 2019; Lehman, 1980). For American toads, turning left appeared to be the default. The left choices were the faster and the first option in the first trial of the day, suggesting that toads used side as the solution to deal with the task. The turn strategy can change throughout the training or be maintained when the task is difficult to understand, or the reinforcements are irrelevant to the subject (Bridgeman and Tattersall 2019; Ouelle et al. 2020; Szabo et al. 2019). Fire-fire-bellied toads succeeded in learning after methodological adjustments in the reinforcement schedule, increased training period, and changing of the apparatus position (Ouelle et al. 2020). In contrast, the learning performance of túngara frogs decreased when the side of the cue was randomized across trials in a two-arm maze (Ventura and Burmeister 2019). Discrimination tasks become complex when association sides are randomized because it adds the navigational component on top of the stimulus cues, such as visual, sounds (Bridgeman and Tattersall 2019; Smith 2012). There are methods of adjustment to deal with side preferences, as increasing the training to provide time to the animals to overcome the bias; and repeatedly presenting the reward on the unbiased side during the training, excluding the corrections from the data.

Another consideration about the sound stimulus to which an individual is to be conditioned is the seasonality effect, which also interacts with auditory processing in the lateralized frog brain. The successful protocol that was done to train spatial learning in Argentine toads using the conspecific calls was done during their reproductive season (Sotelo et al. 2019). The resulting effect was a positive and fast learning outcome. This was potentially favored by the period when physiological regulation concentrates auditory functionality for mating signals (Arch and Narins 2009; Hillery 1984; Kelley 1980; Lynch et al. 2006; Maney and Pinaud 2011; Sisneros et al. 2004; Watts 2020; Wilczynski and

Burmeister 2011, 2016). In contrast, the American toads were subjected to the same training protocol but with tone association (novel sound) outside of the mating period or during the transition time (end of May through summer). Additional analyses from this experiment could reveal results for American toads trained after reproductive state, from August-October, when toads are commonly seen catching insects in residential areas before entering hibernation in the winter, when they may be more alert for environmental sounds than mates. Studies have used electrophysiological approaches to test lateralization functions in frogs' auditory systems in responses to conspecifics and white noise stimuli during reproductive and nonreproductive periods (Xue et al. 2016). They described strong responses to conspecific calls during the reproductive season but encountered sex variation at non-reproductive stages, in which females responded to white noise while the male response to it was not significant. The white noise is not a natural stimulus and animals could perceive it as a novel and dangerous signal. It is possible that trained American toads could overcome this interpretation of novel sounds with risk events and start to associate the tone (novel sound) with a relevant reward, perhaps food, when trained between before or right after winter. One example is a study of the African savannah reed frogs (*Hyperolius nitidulus*) that fled from the sound of fire towards fire-resistant sites and vegetation during the dry season but failed to respond to the sounds in the rainy season (Grafe et al. 2012).

The significant occurrence of correct responses when American toads displayed indecisive behavior before choosing the side of the maze represents a relevant behavior and methodological factor. This type of maze behavior was classified as position error (in a two-arm maze training Liu et al. 2016, 2020), where the frog first walks halfway to the incorrect arm before turning and deciding for the correct arm. Here, as a consequence of the displayed behavior of the American toads in the plus maze, I added classification of trials as regular and escaping, besides indecisive (position error). In a comparison between túngara and poison frogs performance on visual discrimination tasks, Liu et al. (2020) associated better learners to the fewer number of position errors that poison frogs presented. The túngara frog also reached the learning criterion but accumulating more trials and position errors. These comparisons support the influence of turn strategy used by túngara frog on previous training: a general bias to the left side and frequent indecisive behavior of females, resulting in better suppression of the left bias across the training (Liu et al. 2017). These behavioral variations indicate that we need to carefully analyze the learning processes regarding distinct species, the biological and methodological constraints. The observations of maze behavior of toads are important components to assess regarding the potential for the animal to learn given a specific methodological approach. For example, in the regular trials, American toads (current study) scored at the chance level, but this was also when they were the fastest going through the maze, indicating that toads understood the task of going to a side to get the reward and/or ending the procedure. Whereas the trails that toads tried to escape suggests either an inability to understand the desired task or a strong lack of interest, perhaps aversion, by the toad to find the water reward, given the lowest number of correct responses when they displayed the escape behavior (*vs*. regular and indecisive). These in-maze behaviors, both positive (fast latency to get to a side) and negative (escape), are important considerations for improving studies that wish to examine learning in anurans and other species in spatial learning.

In conclusion, the replication of a well-established protocol for spatial learning in anurans was not suitable to determine American toads (*A. americanus*) ability for sound association in a maze task. However, the training process revealed strong aspects that affected the possibility of a successful acquisition in this population. This study showed that water uptake was an irrelevant and ignored type of reinforcement. Two factors to consider in future investigations are the (1) natural mechanism of the species to hydrate (soil moisture absorption rather than a pond); and (2) the stress factor that might reduce the reabsorption mechanism. In addition, prior to initiating the training with American toads or other anuran species, we should integrate natural history, behavioral and physiological responses to understand the relevance of the reward to the animal (Schmajuk et al. 1980; Schmajuk and Segura 1982). The effect of turn strategy reported in other conditioning studies in frogs (Daneri et al. 2011; Liu et al. 2017; 2020; Ouellet et al. 2020) largely impacted American toad performance, with diverged directions between males and females. Studies need to turn attention to this confound and implement steps to control for side-bias, with turn preference pre-test and ongoing training bias corrections. Moreover, the clearly delimited sex difference in side preference and turning plasticity added crucial factors to explore in cognition and sexual selection studies, helping to understand how selective forces might act on mating decisions in anurans.

The sounds stimulus used (tone) might have added an extra level of difficulty to the toads. Although studies have shown that amphibians are able to hear a wide range of sounds not related to mating repertory and many times avoid them (Hobel et al. 2014; Hobel and Christie 2016; Klump 1995; Knight K. 2015; Xue et al. 2015, 2016), the failure to succeed in the acquisition phase precluded additional tests to confirm that toads were hearing the sound. Nevertheless, the number of correct responses registered when toads behaved indecisively and its higher average of water uptake, although not significant, expressed a potential of the toads to be attentive to the task and perhaps overcome side bias. Understanding the elements of experiments and their methodological effects is as crucial as the repeated successful performance of popular model species established in learning and cognition studies. It is essential to discuss analytical considerations of experimental effects throughout learning as a process, mainly to collaborate with the diversification of the comparative cognition database. Furthermore, we must embrace challenges and increase efforts to include underestimated species, like amphibians, whose cognition skills are undescribed and not suited to the traditional methods of study.

4.5 Figures

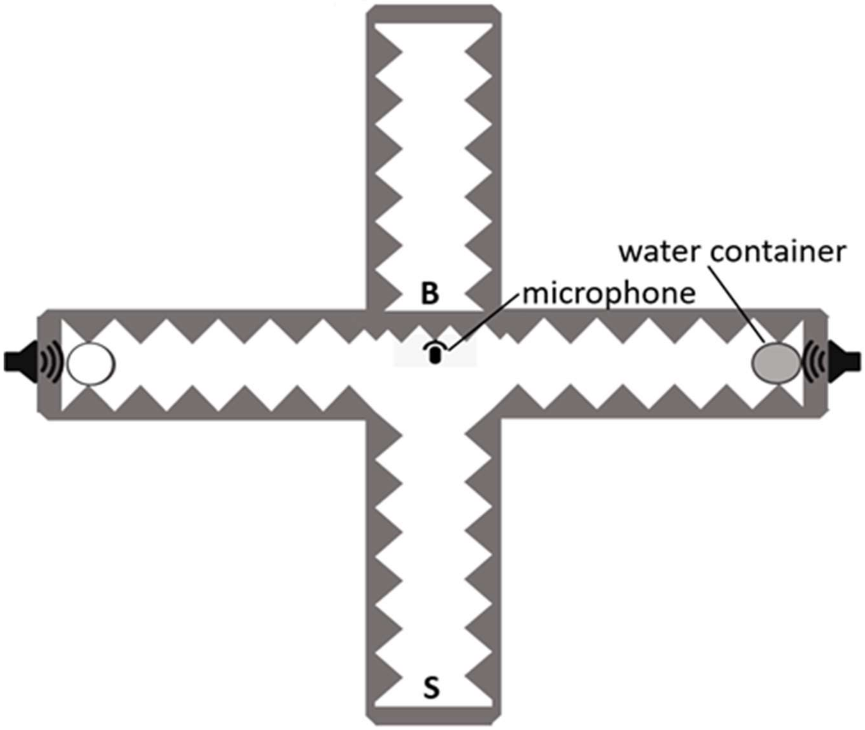

Figure 1. Plus-maze diagram. Walls are shaped with foam wedges, a microphone placed at the center area, and loudspeakers and water containers at the arms endings. The gray color container represents reward choice (accessible water). White container represents non-accessible water. Blocked arm (B) and starting area (S).

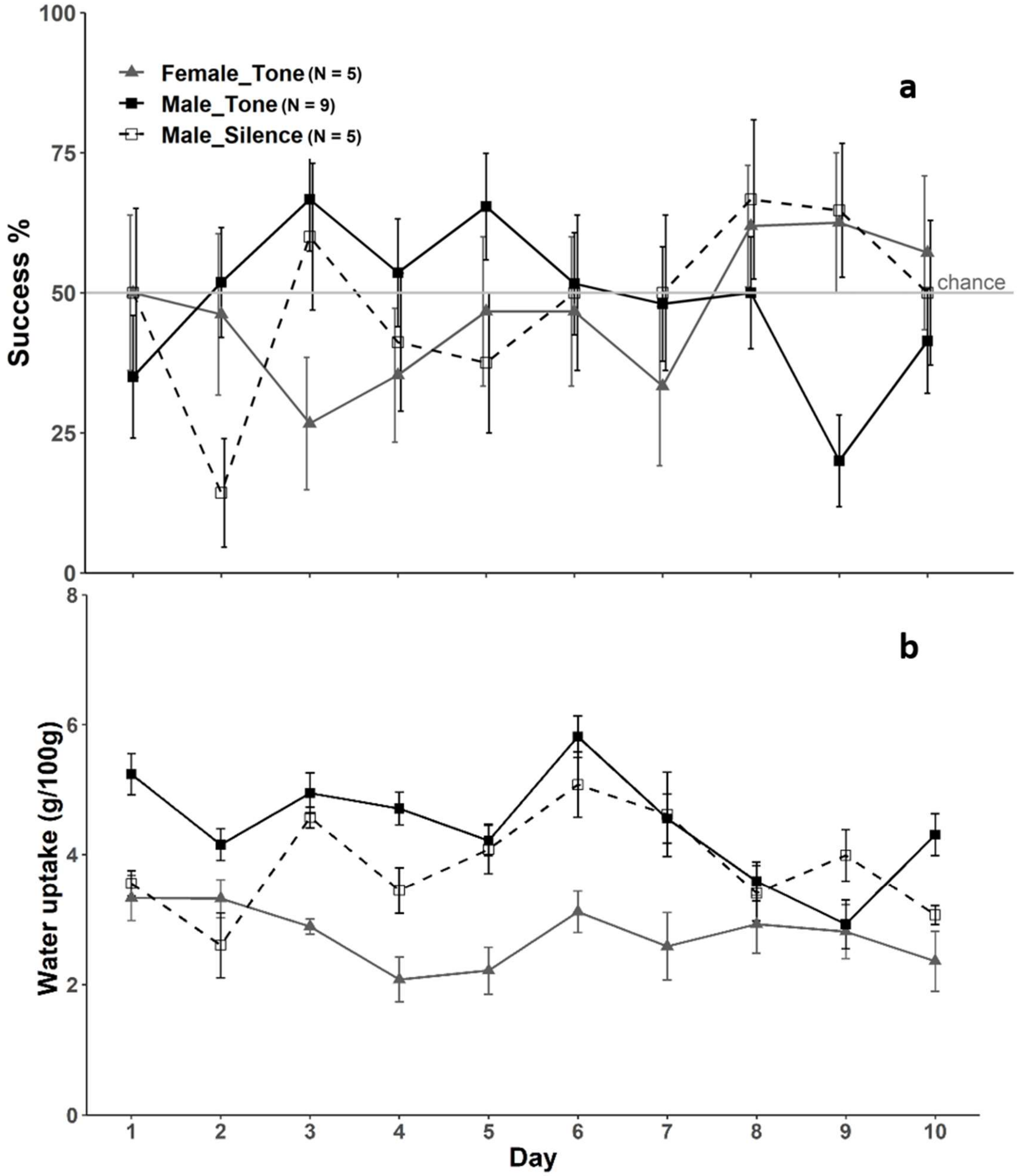

Figure 2. Toad group performance during the ten days of the experiment. Mean and standard error of (a) success rate, showing success rate varying at the chance level, and (b) the water uptake demonstrating the relative weight variation.

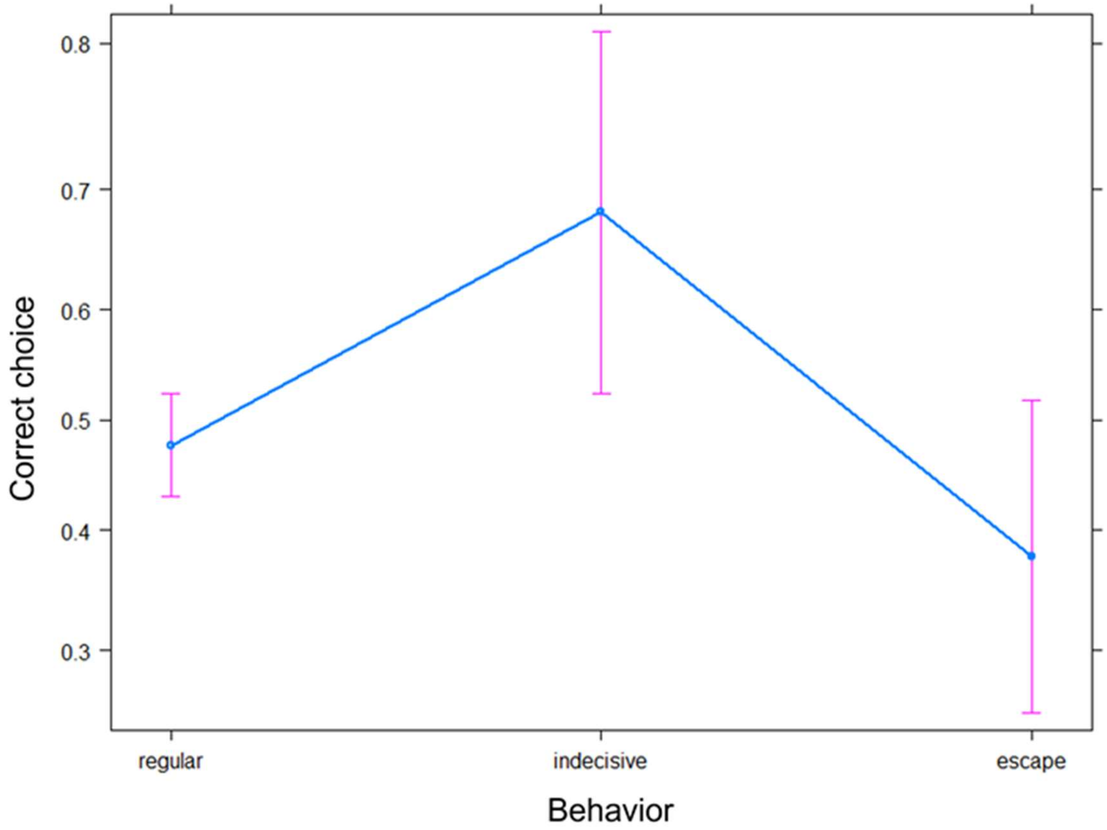

Figure 3. Effect of toad behavior during the maze task on the probability of making the correct choice in trials (N) in which toads behaved: regular (N=440), indecisive (N=43) and escape (N=50). Vertical line indicates 95% CI on GLMMs model.

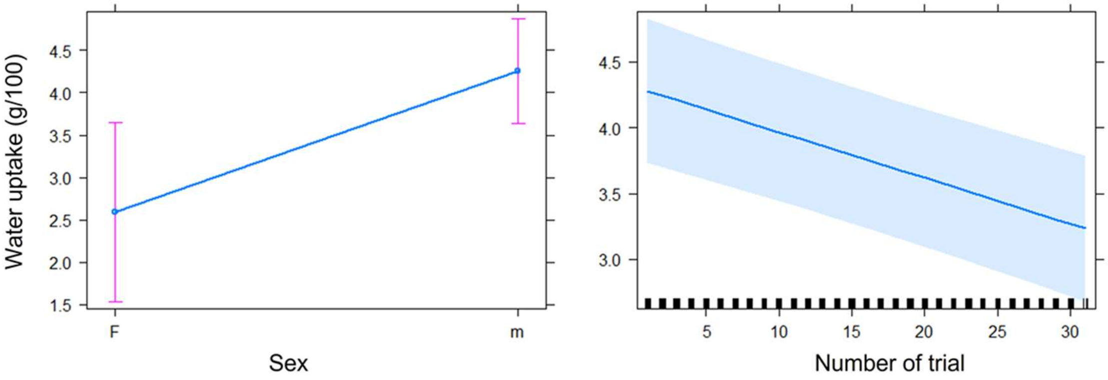

Figure 4. The effect (LMMs, 95% CI) of sex and number of trials on water uptake by toads during the experiment. Left. Sex[males] (χ2(1) =30.51, p < 0.0001). Right. The number of trails (LMMs: χ2(1) =5.62, p=0.01006).

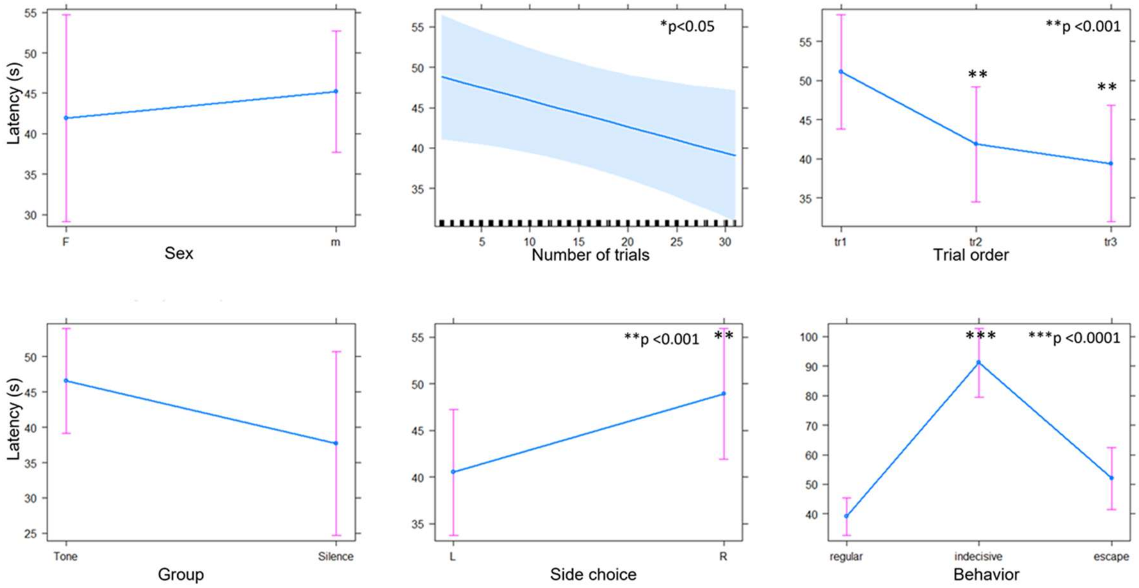

Figure 5. Regression (LMMs, (LMMs, 95% CI)) effects of experimental parameters (x-axis) on response time to the trial (Latency [y-axis]).

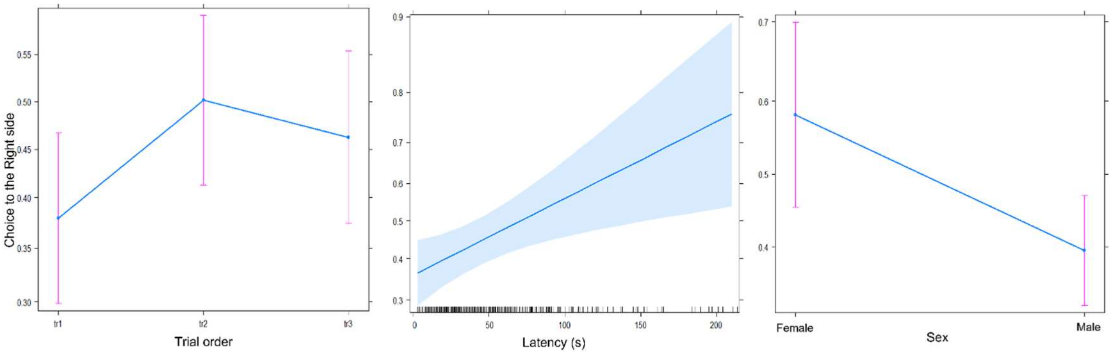

Figure 6. Predictors effects ((GLMMs, 95% CI) on proportion of toad response to the right side of the maze (y-axis). The Independent parameters are trial order (right), number of trials (middle) and sex (left).

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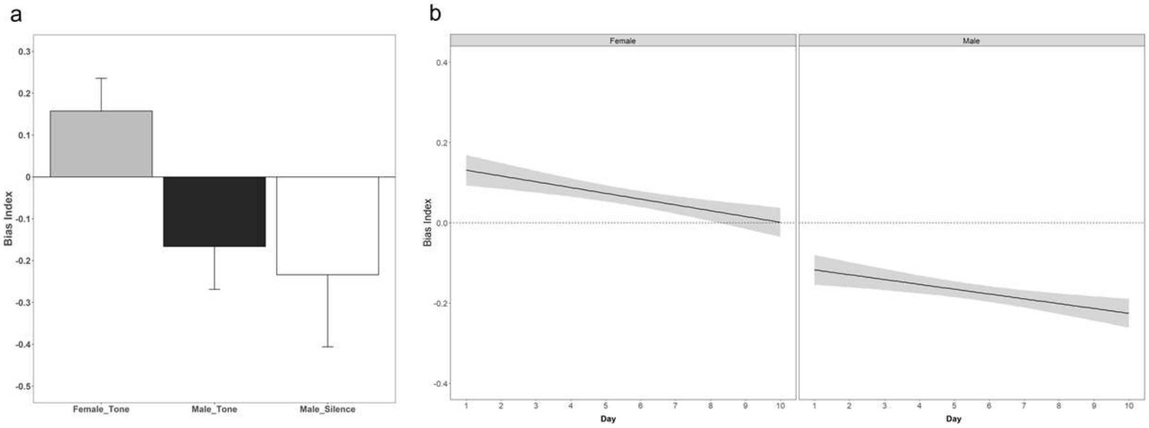

Figure 7. Bias Index average. a. Bias Index and SE of three groups of toads trained in the maze Positive value (y-axis) indicates the right side and negative values indicate the left side. Female toads (gray) and males (dark) trained to associate the tone and the males trained opposed to the tone (white). b. Bias Index and 95% CI of difference between female (left panel) and male (right panel).

4.6 References

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CHP 5 – CONCLUSION

In this dissertation, I reviewed the status of learning and cognition studies in understudied vertebrates and ran two experimental tests to further develop methods for sound learning in amphibians. Specifically, for the literature review I investigated the progress that the fields of comparative psychology and cognition have made to describe mental skills among the ectothermic vertebrates (i.e., fish, amphibians and reptiles) and evaluated the efforts to increase species diversification. My review found that among the three ectothermic vertebrates, amphibians were the most neglected in this regard, The paucity of such studies in anurans, such as frogs and toads, is explained to some extent by the rarity of methodological approaches designed specifically for them. To address this issue, I conducted one study focused on spatial learning in anurans to evaluate tasks associated with sound cues that are not representative of their natural vocalizations but using effective training protocols demonstrated in other frogs in studies using natural calls and access to water as a conditioning reward This study and another one using a food reward were unsuccessful in conditioning my subjects – American toads – to move toward the source of an acoustic stimulus despite the extensive use of acoustic signals to locate and reproduce with conspecific individuals during the breeding season. Nevertheless, the results from my investigations do provide interesting insights for designing future protocols for research in sound learning and cognition in anurans.

In Chapter two, I reviewed the animal cognition literature over the last six decades. This review was inspired by a growing tendency to shift attention from overstudied mammal and bird models to a wider variety of species (Beran et al. 2014; Blaser and Bellizzi 2014; Bräuer et al. 2020; Domjan and Krause 2002; Healy 2019; Krasheninnikova et al. 2020; Papini 2002; Shettleworth 2010 and 2009; Stevens 2017; Vonk 2016). By increasing the diversity of animals studied we will better be able to fill gaps of knowledge that are required if we are to take a true evolutionary approach in comparative psychology and cognition. One particular group of organisms that remain understudied and underrepresented are the ectothermic vertebrates (fish, reptiles, and amphibians), which ended up being the primary focus of my review. I found that the number and advances of cognitive topics on fish and reptiles has increased (Matsubara et al. 2017; Pouca and Brown 2017; Miller 2017; Salena et al. 2021; Szabo et al. 2021; Wilkinson and Huber 2012), but amphibians were still neglected. In my quantitative survey, I found the number of studies that assessed learning in these animals increased over the last 60 years, along with the efforts of some, mainly new and non-tradition journals to publish these studies. Specifically, studies on fish have been growing rapidly in the last 30 years, although this growth still lacks species diversification, with most studies focusing on zebrafish and goldfish (Salena et al. 2021). The goal of species diversity can be achieved with phylogenetic tactics that would expand studies to lesser-studied but closely related taxa to those already studied (Krasheninnikova et al. 2020). Nevertheless, model organisms, like zebrafish and goldfish, are important species for testing and promoting expansion of learning methods to be applied to other kinds of ectothermic vertebrates (Gerlai 2020; Luchiari et al. 2021; Sison and Gerlai 2010). The trend in the growth of investigations of reptiles and amphibians were much less than in fish, with the greatest deficiency in amphibians. Indeed, I could not find even one general review of cognitive studies devoted to amphibians. An increase in studies of learning and cognitive in amphibians will require collaborative efforts (e.g., zoological and conservation aspects (Shaw et al. 2021) and replication of successful approaches based on ecological relevance of the tasks to be learned and the nature of the conditioning stimuli (Bräuer et al. 2020; Krasheninnikova et al. 2020). It is also important to take into consideration the culture of science by discouraging the tendency to avoid publishing negative results and unsuccessful protocols. The dearth of research on the learning and cognition of amphibians and this “cultural” issue inspired my research for the next two chapters of my dissertation. My hope is that enough progress can be made in the near future to counter the apparent reluctance to fund studies of non-charismatic animals (Lambert et al. 2021, Many Primates et al. 2019; Rosenthal et al. 2017).

In chapter three, I evaluated the ability of a terrestrial anuran to move toward the source of a tone burst cue (non-natural signal) in a T-maze task. I used American toads (*Anaxyrus americanus*) and adapted previously successful methods that had been applied to test spatial learning in poison frogs (*Dendrobates auratus*), such as shelter and aversive maze conditions (Liu et al. 2016; 2020). Toads that were part of the treatment group that received a pre-training phase, three trials a day and 1500 Hz target-tone were expected to perform more accurately and faster than the treatment group that was trained only 2 trials/day, received no pre-training and a 1000 Hz target-tone. My results were inconclusive, as fewer toads from the pre-training-1500-Hz group passed the single-tone association training compared to the naïve-1000-Hz group. Surprisingly the pre-training-1500-Hz toads reached the criterion more quickly than the naïve-1000-Hz toads (within the first 9-10 trials), while the naïve-1000-Hz group required more trials (30-56) but with only a single toad failing the criterion (see methods of chapter 3 for considerations on the criterion). The interpretation of the criterion performance must be qualified because of the likelihood that some proportion of the toads reached the criterion by chance. Even though establishing arbitrary and flexible criteria of acquisition are appropriate ways to avoid overtraining on multiple experimental phases, such as reversal learning (Brucks and Bayern 2020; Fuss et al. 2019; Lucon-Xiccato and Bisazza 2014; Warren 1960), the performances I observed here are not sufficient to conclude that the toads were successfully conditioned to respond to the acoustic stimuli. Instead results from this study highlight the need for more exploratory studies and reinforce the challenges of implementing and creating successful experimental protocols and relevant parametric measures. For instance, latency to respond was the only significant experimental element that differed between the groups. Baseline data from the first 6 trials showed that pre-training-1500-Hz toads responded to the trial more rapidly showing familiarity with the task. Moreover, with regard of latency, my descriptive observation was that successful toads (reached the criterion) in both groups followed the speed-accuracy trade off pattern reported in other animal learning assays (Jones et al. 2020; Mazza et al. 2018; Reinagel and Clark 2012). This pattern of long trial and a higher probability of making the correct decision found here suggests a critical aspect of the protocol to be tested for future development. A similar consideration is concerning the discrimination training phase that showed the three pre-training-1500-Hz toads were more consistent in the number of errors in trials before reaching criterion through the training levels. The result of this phase is promising, but only a single individual (Hf12) advanced through all three levels of discrimination level training, repeating the performance regarding the proportion of errors and trials to criterion. More studies and large sample size are needed to confirm sound discrimination learning in this type of procedure. In addition, probe or reversal trials are needed to strengthen the association skills in order to test learning and psychophysical hypothesis.

In Chapter Four, I applied an established protocol based on partial-dehydration and a water reward that was replicated from Sotelo et al. (2019). The only difference was the species used and the sound stimuli. I tested this method with the same purpose as chapter 3: to train American toads to respond to tone burst cues in order to find the reward. Once again, the replication failed, and American toads were not successfully conditioned to the task. Here several reasons for why this might not have worked for American toads. First, American toads do not live in the desert conditions experienced by the Argentinian toads, which use natural vocalizations to locate breeding aggregations (Sanabria et al. 2007); thus, a water may not have been a relevant reward for them. Second, toads may lack spatial learning skills and are an unreliable model to test behavior flexibility in a training context. This would support the general assumption made by some authors that amphibians are stimulus-response organisms and therefore are hard to condition (Megela-Simmons and Moss 1995, 1985; Strother 1962). Third, it is possible the toads were unresponsive to any sounds – even natural advertisement calls – because hormonal mechanisms prime their auditory system for mating calls might be inactive during the non-breeding season when my study was done (Capranica and Moffat 1983; Gerhardt and Schwartz 2001; Simmons et al. 2007; Walkowiak et al. 1981; Wilczynski and Lynch 2011; Wilczynski and Burmeister 2016). Fourth, non-breeding sounds such as tone bursts may not be effective in any circumstances because natural selection is the mechanisms whereby they recognize and move to conspecific rather than heterospecific calls in natural breeding aggregations. Interestingly, there are studies showing responses to non-breeding sounds, which however occur in nature and lead to adaptive consequences (Grafe at al. 2002; Jaeger 1976). Understanding the mechanisms involved with the reasons above will require more studies even performed on model species and methods that have shown successful navigation on mazes as in the studies of Argentine toads and poison frogs (Liu et al. 2016, 2020; Sotelo et al. 2019). Finally, the fact that American toads failed in my study does not mean that they or some other anurans cannot be conditioned, but an extensive search for appropriate conditioning motivators, stimuli, and protocols will be necessary. In order to test American toads in future work, it is necessary to correct for side biases and sex differences in side preference.

In summary, it is fundamental to embrace the challenges and to develop and reassess the experimental designs that are used to investigate learning in species like American toads. The methods used to train toads to sound association, although not conclusive for learning determination, set the stage to underline methodological confounds and parameters involved in the process that can lead to the final metrics of conditioning. These aspects need to be in considered and applied in future studies if we are to establish a reliable procedure that will answer broad questions on anuran auditory learning and function beyond mating purposes.

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APPENDIX A

A1. List of articles compiled in animal learning

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APPENDIX B

SUMMARIES OF STATISTICS FOR WATER REWARD EXPERIMENT (CHP4)

Figure B1. The mean and standard error (SE) of body weight (SBW[g]) and body size (SVL [mm]) among groups of toads trained on maze task for tone association with water reward.

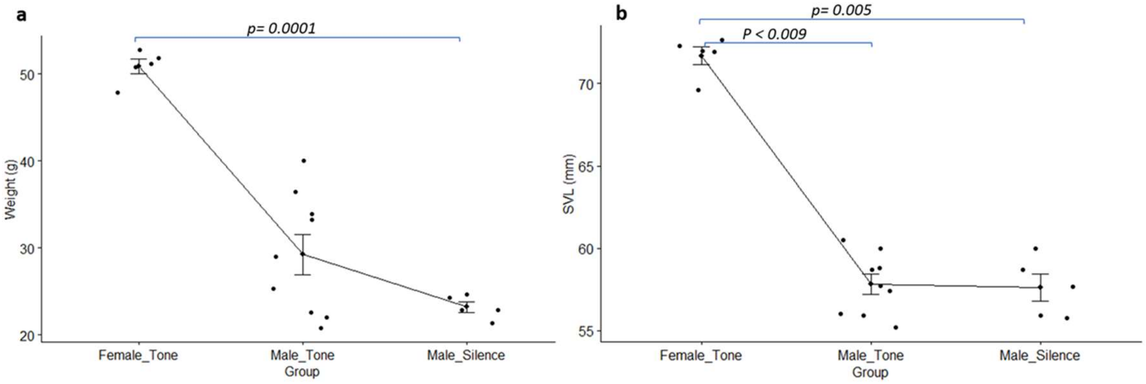

Table B2: Toads groups summary statistics. The number of toads (N), the (Mean ± sd) of the toad body length (SVL), body weight (SBW), and latency.

Group	N	SVL (mm)	SBW (g)	Latency (s)
Female-tone	5	71.68 ± 1.19	50.84 ± 1.82	44.3±36.3
Male-Tone	9	57.80 ± 1.86	29.23 ± 6.98	47.2±37.0
Male-silence	5	57.62 ± 1.81	29.23 ± 6.98	37.9±36.5

Table B3: GLMMs outcomes from 534 observations and number of toads (N).

Dependent variable	N	Fixed effects	Estimate	SE	Z	*p*
Correct choice	19	Intercept	-0.101	-0.101	-0.369	0.7122
		Sex (male)	0.084	0.212	0.396	0.6920
		Total trials	0.0058	0.010	0.556	0.5784
		Second trial (tr2)	-0.274	0.214	-1.281	0.2001
		Third trial (tr3)	-0.274	0.215	-1.271	0.2038
		Group (Silence)	-0.120	-0.120	-0.555	0.5786
		Side choice (right)	0.157	0.157	0.876	0.3810
		Behavior (Indecisive)	0.856	0.352	2.428	0.0152 *
		Behavior (Escaping)	-0.410	0.308	-1.328	0.1842
		Random effect		Variance	Std.Dev.	
		Toad (ID)	(Intercept)	0.047	0.216	
Side choice (Right)	19	Intercept	-0.095	0.353	-0.271	0.7862
		Sex (male)	-0.755	0.314	-2.406	0.0161 *
		Total trials	-0.007	0.010	-0.695	0.4873
		Second trial (tr2)	0.499	0.225	2.222	0.0263 *
		Third trial (tr3)	0.342	0.228	1.500	0.1335
		Group (Silence)	-0.072	0.321	-0.224	0.8223
		Latency (s)	0.008	0.002	2.795	0.0052 **
		Behavior (Indecisive)	-0.330	0.392	-0.842	0.3998
		Behavior (Escaping)	-0.603	0.327	-1.841	0.0656
		Random effect		Variance	Std.Dev.	
		Toad (ID)	(Intercept)	0.163	0.40	

Table B4. Estimates for predictors of latency in linear mixed model analysis (LMMs) from 534 observations. The p-value extracted through Likelihood Ratio Test. Significant parameters (*)

Dependent variable	N	Fixed effects	Estimate	SE	t value
Latency (s)	19	Intercept	46.8750	6.6358	7.064
		Sex (male)	3.2230	7.1073	0.453
		Trial number	-0.3256	0.1632	- 1.995*
		Second trial (tr2)	-9.2497	3.3126	-2.792**
		Third trial (tr3)	-11.7398	3.3422	-3.513**
		Group (Silence)	-8.8771	7.1414	-1.243
		Side choice (right)	8.4054	2.8507	2.949**
		Behavior (Indecisive)	52.3069	5.3494	9.778***
		Behavior (Escaping)	12.8594	4.7371	2.715
		Random effect		Variance	Std.Dev.
		Toad (ID)	(Intercept)	978.2	31.28

*p<0.05; **p <0.001; *** p < 0.0001

VITA

Deise Cruz Santos was born in 1983 in the city of Salvador, Bahia, Brazil. She grew up in an urban area where her mother raised her and her sister in a two-bedroom apartment, with her working-class father being the sole-provider for the family. As first-generation college student, Deise decided to major in biological science mainly in part to her high-school professor's teaching style which was inspiring and easy to understand. She got her both Bachelor's degree (2009) and master's degree (2011) at the Universidade Federal da Bahia, Brazil, where she researched natural history, taxonomy and the ecology of frogs from the Atlantic Forest in the laboratory of Dr. Marcelo Napoli. Deise, then, came to the USA to learn the language the new culture. She decided to volunteer in the herpetology laboratory at Cal Academy, San Francisco, supervised by Dr. David Blackburn, working with taxonomy of tadpole and adult species of frogs from Western Africa. She started graduate school at the University of Missouri where she majored in Biological Sciences with a focus on ecology, behavior and evolution. Under the mentorship of Dr. Carl Gerhardt, Deise worked with the auditory behavior, cognition, and perception of frogs. This work became the theme of her dissertation, presented here. One thing Deise learned at her time in Mizzou is her passion for education equality, which led her in 2020, during the pandemic, to create a network of volunteers that remotely tutored 200 students from public schools in Brazil. She is continuing to work with her organization, and now her goal is to have a future career that engages low-income communities with science as well as assure equal education opportunities worldwide.