INTERACTIONS BETWEEN
NEUROANATOMY, COGNITION, AND
ECOLOGY IN *ANOLIS* LIZARDS

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by

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DEDICATION

This dissertation is dedicated to my family and friends, without whom I would not have grown into the person I am today.

To my parents, Jody and Mark Storks, who always supported my passion for science, no matter how odd.

To my grandmother, Shirley Phyllis Storks, who planted a seed of curiosity and introduced me to the term ‘zoologist.’

And to my husband, Kevin, who has been my lighthouse through it all.

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ABSTRACT

The interactions between an organism and its environment are mediated by cognition, the substrates of which are in the brain. Cognition is ubiquitous across vertebrates, yet we still know very little about the factors shaping its evolution, particularly outside of birds and mammals. In natural environments, cognition likely impacts organism fitness. Behavioral flexibility, which enables an animal to modulate its behavior to match its environment, may facilitate success in novel habitats, such as in dispersal to islands, biotic invasions, and urban adaptation. Cognitive specializations may also be associated with specific ecological traits, such as habitat complexity. Furthermore, our understanding of the neural substrates of cognition in the brain is primarily limited to studies of relative brain size. The first chapter provides a more in depth introduction to these topics.

In this dissertation, I explore the interactions between cognition, neuroanatomy, and ecology in *Anolis* lizards. *Anolis* lizards exhibit a diversity of habitat specializations, which is the result of adaptive radiation in the West Indies. As mentioned above, cognitive mechanisms in anoles may play a role in adjusting to novel environments and exploiting new niches. In the second chapter, I modified a detour task to evaluate whether wild, free-living *Anolis sagrei* can solve a novel detour problem under natural conditions. In the second chapter, I compare the neuron and nonneuron number and density of *Anolis*

cristatellus and *Anolis evermanni* to see whether differences in neuroanatomy reflect their differential performance on an extractive foraging task. I also explore how these data relate to published observations from other vertebrates. Finally, in the fourth chapter I expand upon two previous studies by evaluating whether neuron number follows the predictions of concerted or mosaic evolution in five species of Puerto Rican *Anolis* and whether habitat complexity explains differences in neuron density between species. I conclude in the final chapter by summarizing my results and outlining future directions.

Taken together, the results presented in this dissertation demonstrate the potential for studying cognition and neuroanatomy in an evolutionary context. The methods applied in my second chapter can be used in the future to explore the connection between cognition and fitness in lizards, which are a tractable model for such studies under natural conditions. My third and fourth chapters took a novel approach by studying neuroanatomical differences between species in neuron number and density, and generated novel insights into brain evolution in *Anolis*. By studying cognition and the brain in lizards and other ectotherms, we can begin to finally understand factors shaping the evolution of cognition and neuroanatomy across vertebrates.

CHAPTER 1

INTRODUCTION

Animals live in environments that are variable over space and time. To effectively utilize variable resources and maximize fitness, an animal must modulate its behavior in response to the unique conditions present in its environment. Cognition describes the mechanisms through which an animal senses, processes, and acts on information from its environment, involving perception, learning, memory, and decision-making (Shettleworth 2010). Cognitive mechanisms are ubiquitous across animals and involved in many different behaviors. The specific selective pressures faced by animals within their unique environment and ecological niche favor different cognitive specializations (Shettleworth 2010).

Cognition has fascinated biologists for centuries. Ideas surrounding the nature of cognition in Western history can be traced back to European philosophers such as Aristotle and Descartes (Thagard 2020). Formal study of cognition arose in the nineteenth century with the advent of experimental psychology (Miller 2003; Thagard 2020). In this period, Edward Thorndike conducted the first systematic, comparative studies of cognition in animals using puzzle boxes designed to test problem-solving in different species (Thorndike 1898; Galef Jr. 1998). After an initial dominance of Behaviorism, by the 1950s experimental psychology was experiencing a cognitive revolution (Miller 2003; Thagard 2020). Behaviorism focused strictly on observable behavior (Skinner 1974; Shettleworth 2010; Graham 2019). On the other hand, cognitive psychology focused on subjective processing of behavior, including mental representations and consciousness, primarily focusing on humans (Miller 2003;

Shettleworth 2010). Current research on cognition is shaped by both theoretical frameworks. Although cognitive science has permeated research on the behavior of nonhuman animals (Real 1993), cognitive research still primarily revolves around species with complex faculties similar to humans, such as birds and mammals (Bitterman 1965; Shettleworth 2010).

More recently, cognitive research has been integrated with evolutionary ecology into the field of comparative cognition (Real 1993; Shettleworth 2010). This field brings the theoretical frameworks of cognitive science into research on the ecology and evolution of animal behavior in the wild (Real 1993). For example, studies on chickadees across their range have found that populations from more northern latitudes, where birds rely more heavily on cached food, show better performance on a spatial learning task than populations from southern latitudes that rely less on cached food (Pravosudov and Clayton 2002). This research connects the species' habitat to potentially adaptive variation in cognition. Like other areas of animal behavior research, the sorts of questions addressed in comparative cognition research can be divided into Tinbergen's four questions (Shettleworth 2010; Nesse 2013). Cognitive mechanisms that acquire and process information from the environment are the proximate causes of different behavioral responses, and mechanisms of learning and memory affect the development of an individual's future behaviors (Shettleworth 2010). Behavioral outcomes modulated by cognitive mechanism have ultimate effects on an organism's fitness, and the evolution of these mechanisms can potentially be traced through evolutionary history (Shettleworth 2010).

One major area where cognitive mechanisms have been implicated is in an animal's response to novel environments (Sol 2009; Sutter and Kawecki 2009; Dukas 2013). Behavioral flexibility describes the propensity of an individual to modulate its behavior in response to changing conditions within its environment (Tebbich et al. 2010; Wright et al. 2010; Lea et al. 2020). This flexibility in behavior is based on an individual's experience and enables an individual to capitalize on opportunities or overcome challenges in its environment to potentially increase fitness (Lea et al. 2020). Behavioral flexibility has been implicated in biotic invasions, where the establishment and spread of an exotic species might depend on its ability to cope with the novel environment it is invading (Wright et al. 2010; Szabo et al. 2020). For example, bird species that show a greater propensity for innovation are more likely to become established invaders worldwide (Sol et al. 2005; see also Roudez et al. 2008; Damas-Moreira et al. 2019; Ortiz-Alvarado and Rivera-Marchand 2020). Similar mechanisms have also been connected to successful invasion of urban areas (Audet et al. 2015; Cook et al. 2017; Kozlovsky et al. 2017; Lee and Thornton 2021; Vrbanc et al. 2021). On a larger evolutionary timescale, behavioral flexibility has been suggested to facilitate adaptive radiation on islands by enabling species to persist in new niches that are eventually entrenched via selection (Tebbich et al. 2010). For example, avian clades with higher innovation rates show greater species diversity (Nicolakakis et al. 2003), and bird species living on islands have larger relative brain size, which is associated with behavioral flexibility, than their mainland sister species (Sayol et al. 2018).

Behavioral flexibility has been tested in two different ways. One approach is to quantify the number of published anecdotal reports on innovative behavior per species,

primarily in the context of foraging. This has been used only in avian and primate taxa (Lefebvre et al. 1997; Reader and Laland 2002). The second, and much more widespread, approach is to measure the performance of animals in problem-solving tasks (Griffin and Guez 2014; Lea et al. 2020). The types of tasks that have been applied to measure behavioral flexibility are diverse (Audet and Lefebvre 2017; Lea et al. 2020), and include extractive foraging tasks (i.e., Auersperg et al. 2011; Leal and Powell 2012), detour inhibition tasks (i.e., Boogert et al. 2011; MacLean et al. 2014), and reversal learning tasks (i.e., Boogert et al. 2010; Liu et al. 2016). The majority of experiments testing problem-solving performance occur in the laboratory, however an increasing number of studies are being conducted under natural conditions (Morand-Ferron et al. 2015; Pritchard et al. 2016; i.e., Benson-Amram and Holekamp 2012; Croston et al. 2017), conditions under which flexibility is likely to be ecologically relevant.

The substrates of cognition, like other aspects of behavior, are in the neural tissues of the brain and central nervous system. Most comparative work on the neural substrates of cognition focuses on relative brain size, a measure of the size of a brain or a brain region corrected for body size (Striedter 2005). Initial researchers recognized that the absolute size of the brain may not be an accurate measure of cognitive ability, as this would suggest species like elephants had greater cognitive ability than smaller-brained primates, including humans (von Bonin 1937; Lashley 1949; Jerison 1973; Dicke and Roth 2016). The preponderance of relative brain size can be traced back to Jerison (1973), who emphasized the importance of this measure, which he referred to as “encephalization,” in explaining variation in cognition across vertebrates. Species with greater relative brain size had larger brains than expected given their body size, resulting

in “excess” neural tissue beyond what was required for bodily function that could provide an increased information-processing capacity (Jerison 1973; Herculano-Houzel 2011; Dicke and Roth 2016). The popularity of this index of brain function can likely be attributed to the privileged place that it gave to humans, which are the most encephalized species among vertebrates despite not having the largest absolute brain size (Jerison 1973; Herculano-Houzel 2011; Dicke and Roth 2016). In the decades since, the relative size of the brain and specific brain regions has been widely used as a measure of neural function across vertebrate lineages and related to diverse aspects of vertebrate behavior, ecology, and evolution (Allman et al. 1993; Sol et al. 2005; Isler and van Schaik 2006; Reddon et al. 2016; De Meester et al. 2019; Macrì et al. 2019).

Although brain size has been studied extensively, a wider diversity of neurological mechanisms have been related to vertebrate cognition in recent years (Striedter 2005; Dicke and Roth 2016; Logan et al. 2018). The number of neurons and their connectivity in the brain has long been recognized as an important determinant of information processing capacity (Williams and Herrup 1988; Striedter 2005), however only recently has it been possible to estimate the number of neurons across a wide range of species (Herculano-Houzel 2011). This line of research has found that the number of neurons in major brain regions does not scale according to a shared rule across clades (Herculano-Houzel 2011; Herculano-Houzel et al. 2014; Olkowicz et al. 2016). For example, primate brains gain neurons at a faster rate with brain size than other mammalian clades, resulting in the highest neuron densities among mammals (Herculano-Houzel et al. 2014). The brains of specific lineages of birds have been shown to have much higher neuron densities than mammalian brains (Olkowicz et al. 2016).

This research has demonstrated that brains of diverse species do not share neuronal structure, as was assumed by early investigators (Jerison 1973) despite evidence to the contrary (Williams and Herrup 1988). The number of forebrain neurons shows a positive correlation with performance in a motor self-regulation task across multiple species of birds and mammals (Herculano-Houzel 2017). Similarly, larger numbers of hippocampal neurons are found in populations of chickadees from harsher climates that show greater performance on a spatial memory task (Pravosudov and Clayton 2002). However, no correlation was found between the number of neurons in different brain regions and performance on myriad tasks across individuals in mice (Neves et al. 2020).

Neuromolecular mechanisms of cognition have been explored in an increasingly large number of species. This is particularly apparent in the study of social behavior (O'Connell and Hofmann 2011), where highly conserved neural and genetic mechanisms are involved in sociality across disparate vertebrate clades (O'Connell and Hofmann 2012). Studies in poison frogs find increased hippocampal neuronal activation in frogs transporting tadpoles, which is cognitively demanding due to the navigation of complex environments to find suitable deposition sites (Fischer et al. 2019). Another study found upregulation of genes related to learning and memory, synaptic plasticity, and neurogenesis in the hippocampus of poison frogs, which may be related to their increased spatial cognitive abilities (Liu et al. 2020). In zebrafish, differences in fear learning between fish with different stress coping styles are associated with differences in the expression of the plasticity related gene *npas4a* in the basolateral amygdala, hippocampus, and bed nucleus of the stria terminalis (Baker and Wong 2020). These

studies demonstrate the detail with which we are beginning to understand the proximate neural mechanisms of cognition in a comparative context.

Cognitive research in animals has primarily focused on endothermic vertebrate lineages, which represent only a fraction of vertebrate diversity (Burghardt 2013; Roth II et al. 2019). This lack of taxonomically widespread data biases and constrains our general understanding of cognitive mechanisms in vertebrates and its evolutionary origins (Roth II et al. 2019). Research in reptiles, which are the focus of this dissertation, has found evidence of many cognitive mechanisms also found in birds and mammals (Roth II et al. 2019). Similarly, many brain regions found in reptiles are homologous with regions also found in the brains of birds and mammals, although the organization of reptile brains is simpler than that found in those lineages (Striedter 2005; Kabelik and Hofmann 2018). Because of this conserved cognition and neuroanatomy, combined with their simpler brain structure, reptiles provide a powerful and tractable model for uncovering fundamental mechanisms of cognition in vertebrates. Furthermore, data from across the vertebrate phylogeny is necessary before we can begin to understand the evolution of cognition across vertebrates.

Anolis lizards provide an ideal context for the study of cognitive mechanisms, their neural substrates, and their role in coping with novel environments. On the islands of the West Indies, anoles have repeatedly diverged and specialized on multiple arboreal habitat niches (Williams 1972; Losos 2009). Selection to exploit these unique habitat niches has shaped many aspects of anole biology, including morphology, communication, social behavior, and thermal physiology (i.e., Williams 1972; Leal and Fleishman 2004; Johnson and Wade 2010; Gunderson et al. 2018). The diversity of habitats occupied by

different species of anoles exposes them to environments which potentially differ in their cognitive demands, such as the degree to which movement is constrained by habitat complexity (Powell and Leal 2014). Furthermore, behavioral flexibility may play a role in the radiation of anoles into diverse niches in the West Indies (Leal and Powell 2012) and in their success as biotic invaders worldwide (Losos 2009; Winchell et al. 2020). Combined with a long history of study and extensive knowledge of their natural history (Losos 2009), *Anolis* provide an ideal context for exploring how cognitive mechanisms affect ecological and evolutionary patterns in nature.

In this dissertation, I explored the interactions between cognition, neuroanatomy, and ecology in *Anolis* lizards at multiple levels of biological organization. In Chapter 2, I established a novel paradigm to test behavioral flexibility in wild, free-living *Anolis sagrei* using a modified detour task. This study established a feasible paradigm for measuring behavioral flexibility through motor self-regulation in wild lizards, which can be used in future research to connect ecologically relevant variation in flexibility to fitness. Additionally, this study demonstrated that *A. sagrei* can modify its highly stereotyped foraging behavior, which was unexpected given its sit-and-wait foraging mode (Shettleworth 2010). In Chapter 3, I established the isotropic fractionator technique for use in *Anolis* and presented the first data on neuron number and density collected using the isotropic fractionator from a squamate brain. I found that the data from the *Anolis* brain followed similar patterns to those observed in other vertebrate brains. I then compared the neuroanatomy of *Anolis cristatellus* and *Anolis evermanni* in relation to their differential performance on an extractive foraging task. I found that *A. evermanni*, which showed better performance on the task, had a greater number of nonneurons in the

hind- and midbrain compared to *A. cristatellus*. Nonneurons consist primarily of glia, which have been shown to play a role in cognition (Roth et al. 2013; Fields et al. 2014). In Chapter 4, I compared the number and density of neurons in major brain regions of five species of Puerto Rican *Anolis* from divergent habitat niches. I found that neuron number varied across species following the predictions of concerted brain evolution, with some evidence of mosaic evolution in the telencephalon, hindbrain, and midbrain. I found that species differed in neuron density across the brain, and that these differences were associated with habitat complexity in the telencephalon, hindbrain, and midbrain. This suggested that habitat specialization in *Anolis* was reflected in the neural substrates of the brain at the level of neuron density, although such a difference was not apparent in previous studies at the level of brain volume (Powell and Leal 2012, 2014), highlighting the value of evaluating neuroanatomy at multiple levels. In Chapter 5, I conclude by summarizing the previous chapters.

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CHAPTER 2

THINKING OUTSIDE THE BOX: PROBLEM-SOLVING IN FREE-LIVING LIZARDS

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Abstract

Despite evidence that organisms are more likely to exhibit their full range of cognitive abilities under conditions found in nature, studies evaluating cognition under such conditions remain rare, particularly in vertebrate species. Here, we conducted an experiment to evaluate problem-solving and motor self-regulation in free-living arboreal lizards, *Anolis sagrei*, under natural conditions. We presented lizards with a novel detour problem which challenged individuals to circumvent a transparent barrier in order to obtain a food reward. Individuals varied in their ability to solve the detour problem. Furthermore, those that solved the problem were able to improve their performance across trials by modifying the natural response of attempting to strike the reward through the transparent barrier, providing evidence of motor self-regulation. Solving the problem required individuals to modify their typical foraging behavior, as approaching the prey in a single burst of movement that culminated with an attack was an unsuccessful strategy. Contrary to expectations, our findings provide evidence of motor self-regulation in a visually oriented, sit- and-wait predator under natural conditions, suggesting motor self-regulation is not limited by foraging strategy. Our results also underscore the need to evaluate the cognitive abilities of free-living organisms in the wild, particularly for taxa that perform poorly under laboratory conditions.

Introduction

Research evaluating the cognitive abilities of animals has flourished over the last decade, providing new insights into the potential mechanisms and evolutionary processes mediating inter- and intra-specific variation in cognition (Shettleworth 2010). A recurrent prediction of these studies is that the demands of a species' ecology have shaped the evolution of cognitive traits, including behavioral flexibility and problem-solving (Sol et al. 2005; Shettleworth 2010; Auersperg et al. 2011; Benson-Amram et al. 2016).

Observation of the full range of cognitive abilities for a species requires studies under conditions where subjects have access to the full repertoire of stimuli available in nature (Morand-Ferron et al. 2015; Pritchard et al. 2016). Thus, in order to observe a species' full range of cognitive abilities, researchers have called for more studies under conditions where individuals have access to the full repertoire of stimuli available in nature. Two approaches have been taken to achieve this goal. First, and more commonly, experiments are conducted under semi-natural conditions with captive individuals, providing an opportunity for the individuals to potentially experience a larger range of ecologically relevant conditions than under laboratory conditions (e.g., Healy and Hurly 2003; Noble et al. 2012; Schwarz et al. 2017). Second, and relatively rarer, are studies conducted with free-living organisms under natural conditions (e.g., Benson-Amram and Holekamp 2012; Morand-Ferron et al. 2015; Cauchoux et al. 2017; Croston et al. 2017). The latter approach provides an opportunity to measure cognition under the conditions in which selection has shaped the evolution of these traits and under which the ability to solve

novel tasks is likely to be a better predictor of behavioral flexibility and innovation (Shettleworth 2010; Morand-Ferron et al. 2015).

Detour tasks, using transparent barriers, have been commonly used to test the ability of animals to access a reward that can be seen but is not directly accessible. Because transparent surfaces are extremely rare in nature, incorporating these surfaces into the paradigm reduces the possibility that previous experience with those surfaces directly or indirectly impacts the ability of organisms to solve the task (Epstein et al. 1984; von Bayern et al. 2009; Griffin and Guez 2014; Logan 2016). This presents a novel problem that can provide insight into an organism's cognitive abilities (e.g., Boogert et al. 2011; MacLean et al. 2014; Lucon-Xiccato and Bisazza 2017; Szabo et al. 2019). In order to solve the task, the subject must inhibit the impulsive behavior of trying to reach the reward through the transparent surface and instead detour around the barrier to access the reward, what is known as motor self-regulation (Kabadayi et al. 2018). Motor self-regulation underpins self-control and problem-solving abilities (reviewed in Kabadayi et al. 2018). For example, corvids' performance in a detour-reaching task is associated with absolute and relative brain size (Kabadayi et al. 2016), which are positively correlated with general cognition (Sol et al. 2005). Detour tasks have been mostly used with birds and mammals (reviewed in Kabadayi et al. 2018; but see Lucon-Xiccato and Bisazza 2017; Szabo et al. 2019) and implemented under laboratory conditions. Furthermore, most of the species tested consume a significant portion of nonmoving prey items in their diet, because the general expectation is that motor self-regulation should be selected against in species that eat moving prey (Shettleworth 2010).

Detour tasks have been conducted using a variety of methods (reviewed in Kabadayi et al. 2018). A current trend for detour experiments employs the cylinder task, stemming from Boogert et al. (2011), in which subjects are first trained to retrieve a reward from an opaque cylinder and then tested on their ability to retrieve a reward from a transparent cylinder. Trials are only successful in the cylinder task if the subject does not touch the surface of the cylinder during both the training and testing phases (Boogert et al. 2011). Multiple recent studies have employed this procedure (MacLean et al. 2014; Kabadayi et al. 2016; Vernouillet et al. 2016; Szabo et al. 2019; Johnson-Ulrich and Holekamp 2020). However, this procedure has only become popular within the last decade and is far from the only published procedure to test detour performance. Many published detour tasks, especially those evaluating learning, have not employed an opaque training phase (Schiller 1949a; Spigel 1964; Scholes 1965; Scholes and Wheaton 1966; Santos et al. 1999; Wynne and Leguet 2004; Vlamings et al. 2010; Wilkinson et al. 2010), which is included to shape the response of subjects to the transparent cylinder. The requirement of subjects to make no contact with the apparatus during successful trials was also not present in previous paradigms of the detour-reaching task (Diamond 1990; Santos et al. 1999; Amici et al. 2008; Vlamings et al. 2010).

In our experiment, we present free-living naïve lizards with a transparent half-cylinder containing a reward, similar to the apparatus utilized in the studies described above. However, we do not use a training phase and score any trials in which the reward is accessed as successful. This is due to the natural history of *Anolis sagrei*, a visually oriented, sit-and-wait forager that would be unmotivated to interact with an opaque cylinder blocking the view of a reward. Because of this, we expected incremental

improvement as lizards learned to detour, and considered lizards to have successfully completed the task when reaching a pre-defined criterion, as employed in previous detour studies (Schiller 1949b; Spigel 1964; Scholes 1965; Scholes and Wheaton 1966; Boogert et al. 2011).

Compared with endothermic species, the cognitive abilities of reptiles have largely been overlooked (Burghardt 2013). This is surprising when considering their evolutionary history, species richness, and diverse natural history, which is likely to provide valuable insights into the evolution of heavily studied avian and mammalian cognition (Shettleworth 2010). In fact, recent studies have demonstrated that cognitive abilities of reptiles are more complex than recognized historically (Wilkinson et al. 2010; Davis and Burghardt 2011; Leal and Powell 2012; Burghardt 2013; Noble et al. 2014; Szabo et al. 2019), raising questions about previous hypotheses addressing the evolution of cognitive traits developed from studies on endothermic animals. For example, it has been demonstrated that having a complex social structure or exploiting a diversity of food resources, two of the main predictors of behavioral flexibility in endothermic species are not predictors of behavioral flexibility in the arboreal lizard *Anolis evermanni* (Leal and Powell 2012). In a similar vein, the ability to learn by observing the behavior of conspecifics has been shown in the skink *Eulamprus quoyii*, a mostly solitary lizard, suggesting that the potential for social learning is not limited to highly social species (see also Wilkinson et al. 2010; Davis and Burghardt 2011; Noble et al. 2014). Nevertheless, our ability to study reptile cognition has been hampered by their relatively low metabolic rate, which can limit the number of trials in training and testing phases when food is used as reward and the difficulty of creating laboratory conditions under which reptiles might

be expected to behave naturally (Burghardt 2013; Steinberg and Leal 2018). Thus, our current understanding is based primarily on a relatively small sample of the vast diversity of species of reptiles.

In this study, we evaluate if free-ranging individuals of *Anolis sagrei* can solve a novel detour problem under natural conditions. To do so, we administered a modified cylinder task, in which naïve individuals were presented with a transparent tube containing a reward. The task challenged individuals to circumvent a transparent barrier in order to obtain a food reward, without prior training, providing an opportunity to evaluate problem-solving and motor self-regulation (Kabadayi et al. 2018). Furthermore, by working with free-ranging individuals, we avoid the possibility of previous experience with transparent surfaces, thus presenting a novel problem to the individuals. As a novel problem, the expectation is that motor self-regulation will be exhibited incrementally, as individuals gain experience by interacting with the transparent surface (Vernouillet et al. 2016). Therefore, we predicted that individuals of *A. sagrei* would solve the detour problem and in doing so would decrease the number of attempts to secure the reward through the transparent surface as they gained experience with the novel problem.

Materials and Methods

Study system

We carried out this study from June 19 through July 21, 2016 in Marsh Harbour on the island of Great Abaco, Bahamas. *Anolis sagrei* ($n_{\text{male}} = 10$, $n_{\text{female}} = 13$) were sampled in a forest fragment of approximately 7500 m² located within the premises of The Abaco Beach Marina, which holds a high density of lizards.

Anolis sagrei is a small-to-medium-sized, sexually dimorphic (females to 39.5 mm; males to 51.9 mm), arboreal, territorial lizard that inhabits open and semi-shaded forest (Losos 2009). Individuals are most commonly observed within 1.5 m of the ground on the trunks of trees. From these perches, males and females signal to conspecifics in the surrounding habitat and forage for potential prey items, primarily insects (Schoener and Schoener 1982). *Anolis sagrei* is a sit-and-wait forager, and while foraging, individuals typically assume a survey posture—head-down position with the front limbs extended and head elevated—while scanning the ground for the movement of potential prey (Stamps 1977; Schoener and Schoener 1982). If a prey item is detected, individuals will sprint to the ground and capture the prey at the end of the run, which commonly occurs as one sequence of events (Moermond 1981).

Lizards were located by walking slowly through the forest. Once a lizard was found, it was captured using a noose and its snout–vent length (± 0.1 mm) and weight (± 0.1 g) were measured and recorded (Table A2.1). It was then marked by attaching two temporary queen bee tags (Bee Works, Ontario, Canada) with a small amount of cyanoacrylate adhesive to each shoulder, providing each individual with a unique color and number combination. Lizards were released at their site of capture, which was marked with flagging tape. We conducted experiments from 0800 to 1800 h, excluding periods of rain. The first trial for each lizard occurred at least 24 h after it was marked.

This research adhered to the guidelines of the Institutional Animal Care and Use Committee at University of Missouri, Columbia protocol (#8244). We followed the Recommendations for the Care of Amphibians and Reptiles (Pough 1991) in the treatment of all animals used in this study. This is a field study, and as part of this study,

animals were only individually marked and immediately released. The Bahamas Ministry of Agriculture and the Bahamas Environment, Science, and Technology Commission of the Ministry of the Environment provided permission to conduct this research.

Testing apparatus

A photo of the testing apparatus is presented in Fig. 2.1. The apparatus consisted of an acrylic half-cylinder (11.5 L $\times$ 8 W $\times$ 4 H cm) divided into three segments: a removable transparent center (5 cm) and two equally sized terminal segments (3 cm) that were attached to a green painted wood platform (11.5 L $\times$ 10.5 W $\times$ 2 H cm). Both ends of the half-cylinder were open and provided access to a live fly maggot used as a reward. The maggot was restrained at the center of the half-cylinder by tethering it to a small piece of dental floss (ca. 1.5 cm) with cyanoacrylate adhesive and then securing the other end between two strong magnets at the center of the platform. The movements of the maggot are very salient to anoles (Fleishman and Pallus 2010), usually eliciting a foraging response. Lizards were able to easily detach the maggot from the dental floss.

The terminal pieces of the half-cylinder were covered by one of two black-white patterns: vertical lines or checkered (Fig. 2.1). Those patterns provided cues for the lizards to associate a given pattern with access to the reward. However, it should be noted that the development of an association was not a requirement to solve the detour task. Nevertheless, providing this additional cue allows for the evaluation of this possibility, which has been shown before in other species of lizards including anoles (Leal and Powell 2012).

Testing

Lizards were located by walking slowly through the forest. Once a marked lizard was found, we walked slowly towards the lizard's perch and placed the apparatus perpendicular to the lizard's line of sight within a meter of the base of its perch. The orientation of the patterns was randomly determined for each trial relative to the lizard's line of sight; thus, from the lizard's perspective, a given pattern and the spatial position of an entrance were not correlated. Under field conditions, it was not possible to place the apparatus in the exact same position for every trial. Instead, the apparatus was placed in proximity to the focal individual, as described above, which likely corresponded to the territory of that lizard. Each individual completed 0–10 trials per day, and lizards were tested until they were no longer found, or they stopped approaching the apparatus.

Prior to presenting the lizards with the complete half-cylinder, lizards were habituated to the painted wood platform and the pattern sections of the half-cylinder by presenting the apparatus with the reward, but without the central section (Fig. 2.1a). This allowed lizards to access the reward unimpeded and controlled for the possibility that lack of participation in the experiment was due to neophobia. A total of 23 lizards completed this stage of the experiment after grabbing the reward in seven consecutive trials, exhibiting behavior typical of anoles while foraging. Individuals directly approached the reward, usually as part of a sprint that concluded with the lizard striking the reward, all happening as a single event.

After the lizards completed habituation, the central section of the half-cylinder was replaced (Fig. 2.1b), presenting the lizards with the novel detour problem of accessing the reward behind a transparent barrier. We defined a trial as a success

whenever the lizard accessed the reward. We predicted that the number of touches to the cylinder would decrease as lizards experienced the obstacle presented by the barrier and reached our learning criterion. Thus, our experiment is designed to measure problem-solving by trial-and-error learning with the expectation that motor self-regulation will be exhibited incrementally (Shettleworth 2010). The criterion to solve the detour problem was to reach the reward in 7 of their previous 8 trials, similar to criteria commonly used in learning experiments (e.g., Schiller 1949b; Spigel 1964; Scholes 1965; Scholes and Wheaton 1966; Anderson et al. 2017; Szabo et al. 2019).

A trial lasted a maximum of 15 min, beginning when the apparatus was placed in front of a lizard, and ending when the lizard took the reward or the 15-min period elapsed. Using a stopwatch and a notebook, we recorded all behaviors exhibited by the lizard, including the side by which the lizard entered the apparatus and the number of touches (i.e., the number of times a lizard placed its snout against the outside of the transparent barrier) made during each trial. To reach the reward, lizards had to place most of their body inside the apparatus. Trials in which individuals did not approach the apparatus within one body length are not included in the analysis. We evaluated the strategies employed by individuals in solving the detour problem by determining which cues were correlated with an individual's access to the reward. Individuals demonstrated preference for access using spatial (i.e., side) cues, pattern cues, or neither. We evaluated this by calculating a bias index as performed in Szabo et al. (2019). The magnitude of the bias index indicates the strength of the bias towards one cue, either spatial (left or right) or pattern (checked or striped). A preference was indicated when the bias index for one cue was greater than the other, and no preference was indicated when indices for each cue

were equal. Because we were working with free-living lizards and individuals can move outside the forest fragment while the study was going on, we only counted lizards as having participated in the experiment if they attempted at least seven detour trials. It was not possible to record data blind because we conducted focal observations of animals in the field.

Statistics

All statistical tests are two-tailed and were performed using R statistical software (R Development Core Team 2008) with the package nlme (Pinheiro et al. 2019). A linear mixed effects model was used to test for a decrease in number of touches as individuals proceeded through the detour task. In particular, the model evaluated if the number of touches decreased as a function of trial number, including only individuals that reached criterion in the detour task. Because each individual completed a different number of trials to reach criterion, we scaled trial number for each individual between 0 and 1 by dividing each trial number by the number of the last trial for each individual. Only successful trials in which individuals accessed the reward were included in this analysis. We used a linear mixed effects model with the number of touches as the response variable and scaled trial number as the predictor with individual as a random effect. Our data conformed to all assumptions of this model. The graph was generated using the package ggplot2 (Wickham 2009). The datasets generated during and analyzed during the current study are available in the Dryad repository,

<https://doi.org/10.5061/dryad.g79cnp5mq>.

Results

Twenty-three lizards completed habituation (Table 2.1). Of those, 17 lizards participated in the detour problem, and 9 of those lizards solved the problem. Sex, body size, and mass did not differ between the lizards that solved the detour problem and those that failed (two-sided Fisher's exact test, $n=17$, $P=0.15$; two-sided Wilcoxon signed rank sum test, $W=28.5$, $n=17$, $r=0.178$, $P=0.49$ and $W=27.5$, $n=17$, $r=0.199$, $P=0.44$ respectively). Lizards used three strategies to solve the detour problem. Five lizards solved the problem by entering more often through a given side (i.e., left or right entrance with respect to their line of sight). One lizard entered the half-cylinder more often under a given pattern (i.e., striped or checkered) irrespective of its orientation with respect to the lizard's perch site. Three lizards showed neither preference (Table A2.2).

Lizards that ultimately solved the detour problem improved their performance by reducing the number of touches committed during their approach to the reward, rather than through greater persistence (Fig. 2.2). The latter would have resulted in an increase in touches rather than a decrease. A significant negative fixed effect of scaled trial number on the number of touches was detected with individual as a random effect (Fig. 2.2, $n=74$, estimate $\pm$ standard error = -5.98 ± 2.91 , $df=64$, $P=0.04$), indicating that individuals decreased the number of touches made during trials as they progressed through the detour problem and reached criterion.

Discussion

Our findings demonstrate the feasibility of experimentally evaluating the cognitive abilities of free-ranging lizards in nature and of the ability of *Anolis sagrei* to exhibit

motor self-regulation in response to experience with a transparent surface, resulting in a decrease in the number of touches made as they progressed through the detour problem. The ability of *A. sagrei* to solve the detour problem provides further evidence that the cognitive abilities of lizards, and more specifically *Anolis*, can provide significant insights into potential factors shaping the evolution of behavioral flexibility and motor self-regulation (Leal and Powell 2012; Burghardt 2013). The solution to the problem required major changes to the highly stereotyped foraging behavior of *A. sagrei* (Losos 2009), which consists of scanning the habitat for moving prey and approaching them directly in a single burst of movement before striking. This was a behavioral strategy that was ineffective in our experiment because the transparent section of the half-cylinder served as a physical barrier between the reward and the lizard. Instead, lizards moved either towards an entrance before turning to enter the half-cylinder, that is, approached the apparatus at an angle away from the prey, or moved toward the prey and detoured away from it after reaching the apparatus in order to reach an open end. Therefore, regardless of the approach taken by the lizards, the prey was temporarily not visible (i.e., out of the line of sight), potentially resulting in a temporal mismatch between reaching the reward and decision-making.

Behavioral flexibility has received significant attention as a trait that contributes to species' invasive abilities, particularly in birds (Lefebvre et al. 1997; Sol et al. 2002, 2013), although there are conflicting ideas regarding the degree of flexibility needed for successful invasion (reviewed in Wright et al. 2010). *Anolis sagrei* is the most invasive anole species (Kolbe et al. 2014), and our findings open the possibility for further work to evaluate the potential contribution of behavioral flexibility to its invasive ability. Future

work, comparing the cognitive abilities of multiple species of anoles, including those with different degrees of invasiveness, should shed light on this hypothesis. Alternatively, a study evaluating problem-solving abilities between native and invasive populations of the same species can also shed light on the potential contributions of cognition to exploit novel habitats (Wright et al. 2010). Also, both *A. sagrei* and *A. evermanni* are members of the Caribbean anole radiation which is characterized by the independent evolution of ecological forms and convergence in morphological and behavioral traits across those forms (Losos 2009). Behavioral flexibility has been proposed as a contributor to the radiation of clades by facilitating the exploration of novel environments (Sol and Price 2008; Tebbich et al. 2010; Leal and Powell 2012).

Lizards used three strategies to access the reward. The most common strategy was for a lizard to preferentially enter the apparatus through the same side (i.e., right or left with respect to the individual's line of sight), regardless of the pattern of the entrance. The apparatus was not placed in the same spot for every trial, and thus we are unable to disentangle the attendance to allocentric vs. egocentric cues in our experiment. A less common strategy was a preference for entering the half-cylinder through an entrance covered by a given pattern (i.e., vertical lines or checkered) regardless of the side, suggesting the development of an association between access to the reward and one of the distinct patterns at each entrance. Both strategies might be expected of a territorial species, such as *A. sagrei*, where the ability to associate particular cues or landmarks with ecologically important information can contribute to an individual's fitness (Ladage et al. 2009; Leal and Powell 2012).

On average, the number of touches to the cylinder made by individuals of *A. sagrei* that solved the detour problem decreased significantly across trials (Fig. 2.2). This finding indicates that individuals are not only learning how to reach the reward by achieving our learning criterion but also to avoid using a natural behavior (i.e., striking through the transparent barrier) that is ineffective at securing the reward. Furthermore, as has been shown in corvid species (Kabadayi et al. 2016), although individuals of *A. sagrei* had no previous experience with transparent surfaces, relatively few presentations were needed to reduce their motor response and improve their ability to access a reward behind a transparent surface (Table 2.1). This reduction occurred even in the absence of a training phase with the opaque surface, which can shape the behavior of individuals as they interact with a transparent surface and increase the likelihood of accessing the reward without touching the cylinder (Santos et al. 1999; Vlamings et al. 2010; Isaksson et al. 2018). It should be noted that a reduction in the number of touches (i.e., motor self-regulation) is even more surprising when considering that individuals performed the tasks while facing all the potential distractions of a natural environment, including competition for food. The latter should select for quick, impulsive behavior, which is commonly exhibited when multiple individuals are chasing the same moving prey. However, individuals were able to modify the required behavior to solve the task. Nonetheless, individuals varied in their ability to inhibit striking at the reward through the transparent barrier (i.e., individual differences in the magnitude of the decrease in touches), which demonstrates that the paradigm used in this study can also be used to evaluate individual variation in cognitive performance under natural conditions.

The ability of individuals to inhibit the natural response of attempting to access a food reward through a transparent barrier has been measured across a diversity of endothermic species, and the findings suggest that in primates, dietary breadth predicts cognitive performance (MacLean et al. 2014; Kabadayi et al. 2016). However, compared with primates, the dietary breadth of *A. sagrei* is relatively narrow, suggesting that other aspects of species' ecology are also likely to contribute to the ability of species to inhibit natural responses. Furthermore, our findings also challenge the prediction that species like *A. sagrei*, for which success of prey capture is determined by their ability to surprise moving prey, are unlikely to exhibit motor self-regulation abilities (Shettleworth 2010). This finding provides further evidence of the potential insights that can be gained by studying lizard cognition.

Two factors that are commonly suggested to contribute to individual differences in performance on cognitive tasks are body size and neophobia (Shettleworth 2010; Wright et al. 2010). In lizards, including *A. sagrei*, body size can be correlated with age, thus potentially influencing previous experience (Noble et al. 2014). Age has been shown to affect behavioral diversity and neophobia in spotted hyenas, both of which are associated with increased problem-solving success (Benson-Amram and Holekamp 2012). The body size of *A. sagrei* was not significantly different between those individuals that solved the detour problem and those that failed. Furthermore, all the individuals that participated in the detour problem had completed the habituation period by grabbing the maggot from the same apparatus in seven consecutive trials. Therefore, it seems unlikely that differences in body size or neophobia account for our results.

Elucidating the shared ecological demands that have favored the evolution of cognitive traits across species is a long-standing goal of cognitive ecology. Our findings that *A. sagrei* exhibits motor self-regulation provide further evidence that the cognitive abilities of *Anolis* lizards, and more generally reptiles, have been underappreciated, as this ability is commonly suggested to underpin higher cognitive processes such as decision making and problem-solving (Kabadayi et al. 2016; Vernouillet et al. 2016). Furthermore, as discussed above, the dietary breadth, foraging behavior, and social structure of *A. sagrei* are unlike those previously associated with the evolution of motor self-regulation, suggesting that other aspects of species' ecology might also contribute to this behavior. One possibility is that motor self-regulation is necessary for the evolution of other cognitive traits, such as behavioral flexibility, including reversal learning, which requires the use of inhibitory responses and has been shown in multiple species of lizard (e.g., Day et al. 1999; Leal and Powell 2012; Szabo et al. 2019). Under this scenario, selection favoring the ability to modify pre-existing behaviors would also favor the evolution of motor self-regulation. More studies across a diversity of taxa, particularly with free-living organisms, are needed in order to develop a cohesive framework in which to evaluate the interactions between species ecological demands and the evolution of cognitive traits.

Our findings underscore the potential advantages of conducting cognitive experiments under natural conditions. Captivity causes subjects potential stress and presents conditions that greatly differ from those found in nature, both of which can affect behavioral responses in an unpredictable fashion (Steinberg and Leal 2018). In the case of *A. sagrei*, this approach also provides an opportunity in which unique factors of

the species' ecology, such as territoriality and spatial awareness, are more likely to be exhibited and to contribute to problem-solving ability.

More generally, the most commonly used version of the cylinder task includes a training phase with an opaque cylinder (see intro for references). This training step likely reduces the number of species that can be tested using the method, for example, sit-and-wait foragers or those that rely on visual motion cues for the detection of prey are unlikely to be able to perform the task due to lack of adequate stimulation. Our modification—elimination of the opaque cylinder phase— allowed us to use the cylinder task with a visually oriented, sit-and-wait forager, *A. sagrei*. Our adaptation of the paradigm illustrates the utility of using experimental paradigms that are rooted in an understanding of the natural history of the species of interest (i.e., their “umwelt”), which is a critical component for conducting experiments with free-living individuals. Furthermore, it provides a methodology to evaluate problem-solving through motor self-regulation by measuring the reduction in the number of touches made to the cylinder as individuals gain experience by interacting with the transparent surface (Vernouillet et al. 2016). The apparatus used in this study can also be used to evaluate associative and reversal learning under natural conditions in free-living animals by limiting the access to the reward to a given pattern and/or changing the reward contingency, providing an opportunity to evaluate performance of the same individuals across multiple cognitive tasks.

In summary, by implementing a modified version of the cylinder task, we demonstrate the feasibility of experimentally evaluating the cognitive abilities of free-living lizards, opening a new avenue for the study reptile cognition. Lizards have been

mostly studied under laboratory settings (Burghardt 2013), potentially limiting our ability to sample the diversity of taxa within this clade and consequently hampering our understanding of the cognitive abilities of a diverse group, which is characterized by using distinct niches and showing a wide range of life-history traits. Therefore, understanding variation in lizard cognition is likely to shed light on current efforts to elucidate shared ecological demands contributing to the evolution of cognitive traits across vertebrates. Furthermore, our results demonstrate that a sit-and-wait forager can modify its foraging strategy relatively quickly in the face of a novel problem, providing further evidence of behavioral flexibility in anoles (Leal and Powell 2012) and of motor self-regulation. Additionally, *A. sagrei* does not possess the traits previously associated with the evolution of motor self-regulation (MacLean et al. 2014; Kabadayi et al. 2016), suggesting that other aspects of species' ecology can also contribute to this ability.

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Tables and Figures

Fig. 2.1: Photos of the detour problem used in this study. a) Apparatus without the central section of the half-cylinder. b) Apparatus with the central transparent section of the half cylinder.

Table 2.1: Number of individuals of *Anolis sagrei* that participated in each of the stages and the mean number of trials required to complete each stage. Individuals that failed attempted at least seven trials but did not reach criterion.

Stage	Failed	Solved	Mean trials to solve	Range trials to solve
Habituation	-	23	7	7
Detour	8	9	11	7-19

Fig. 2.2: The relationship between scaled trial number and the number of touches made by individuals of *Anolis sagrei* that solved the detour problem. Trial number was scaled for each individual between 0 and 1 by dividing each trial number by the number of the last trial for each individual. Values are the number of touches made by one individual during one trial in solving the detour problem.

Appendix

Table A2.1: Summary of *Anolis sagrei* tested in this experiment. For each individual, the table describes the stages completed, sex, weight (g), and snout-vent-length (cm). For individuals that completed the detour problem, the number of trials to reach criterion in the detour problem is indicated. An N/A indicates an individual that did not complete at least seven trials in the detour problem.

ID#:	Habituation	Detour problem	Sex:	Weight (g):	SVL (cm):	Number of trials to reach criterion in detour problem
2	Pass	N/A	Male	3.5	5.2	-
10	Pass	Fail	Female	2.1	3.9	-
24	Pass	N/A	Female	1.7	3.8	-
30	Pass	Pass	Male	2.2	4.6	13
65	Pass	Pass	Male	3.5	4.8	7
67	Pass	Fail	Female	1.9	4.1	-
68	Pass	Pass	Female	1.4	3.9	7
73	Pass	Pass	Female	1.7	4.0	8
74	Pass	Fail	Male	3.5	4.9	-
79	Pass	N/A	Female	1.7	4.0	-
86	Pass	Pass	Female	1.5	4.0	13
87	Pass	Pass	Male	2.7	4.8	19
90	Pass	N/A	Female	1.4	3.8	-
92	Pass	Fail	Female	1.4	4.0	-
93	Pass	Pass	Male	3.1	4.8	16
97	Pass	Pass	Male	3.2	4.8	8
99	Pass	Fail	Male	3.2	4.9	-
102	Pass	N/A	Female	1.5	4.0	-
103	Pass	N/A	Male	3.3	5.3	-
106	Pass	Pass	Male	2.4	4.5	8
109	Pass	Fail	Female	1.5	4.0	-
110	Pass	Fail	Female	1.4	3.7	-
114	Pass	Fail	Female	1.8	4.2	-

Table A2.2: Summary of bias index analysis for each individual. The magnitude of the bias index for side (i.e., left or right entrance with respect to individual's line of sight) and pattern (i.e., checked or striped entrance) are presented. A bias is indicated as positive (+) when one index is of greater magnitude than the other, and no bias is indicated (-) when these indices are equivalent.

ID#:	Side Bias Index :	Pattern Bias Index :	Side bias:	Pattern Bias:
30	0.750	0	+	-
65	0.429	0.143	+	-
68	0.143	0.143	-	-
73	0.143	0.143	-	-
86	0.500	0	+	-
87	0.714	0	+	-
93	1.000	0.111	+	-
97	0.143	0.714	-	+
106	0.429	0.429	-	-

Videos of the detour task are available online as supplementary material at <https://doi-org/10.1007/s00265-020-02852-x>.

CHAPTER 3

PEEKING INSIDE THE LIZARD BRAIN: NEURON NUMBERS IN *ANOLIS* AND ITS IMPLICATIONS FOR COGNITIVE PERFORMANCE AND VERTEBRATE BRAIN EVOLUTION

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Abstract

Studies of vertebrate brain evolution have mainly focused on measures of brain size, particularly relative mass and its allometric scaling across lineages, commonly with the goal of identifying the substrates that underly differences in cognition. However, recent studies on birds and mammals have demonstrated that brain size is an imperfect proxy for neuronal parameters that underly function, such as the number of neurons that make up a given brain region. Here we present estimates of neuron number and density in two species of lizard, *Anolis cristatellus* and *A. evermanni*, representing the first such data from squamate species, and explore its implications for differences in cognitive performance and vertebrate brain evolution. The isotropic fractionator protocol outlined in this article is optimized for the unique challenges that arise when using this technique with lineages having nucleated erythrocytes and relatively small brains. The number and density of neurons and other cells we find in *Anolis* for the telencephalon, cerebellum, and the rest of the brain (ROB) follow similar patterns as published data from other vertebrate species. *Anolis cristatellus* and *A. evermanni* exhibited differences in their performance in a motor task

frequently used to evaluate behavioral flexibility, which was not mirrored by differences in the number, density, or proportion of neurons in either the cerebellum, telencephalon, or ROB. However, the brain of *A. evermanni* had a significantly higher number of nonneurons across the whole brain, which could contribute to the observed differences in problem solving between *A. cristatellus* and *A. evermanni*. Although limited to two species, our findings suggest that neuron number and density in lizard brains scale similarly to endothermic vertebrates in contrast to the differences observed in brain to body mass relationships. Data from a wider range of species are necessary before we can fully understand vertebrate brain evolution at the neuronal level.

Introduction

Vertebrate brains are neuroanatomically diverse. Although all vertebrate brains are composed of the same, conserved major regions, the structure and both relative and absolute size of different brain regions are highly variable (Striedter 2005). A major goal of comparative neuroanatomy is elucidating principles that describe the evolution of such diversity across vertebrates and determining how such variation is related to differences in behavior. In particular, there has been a growing interest in understanding how variation in neuroanatomy underpins the ability of animals to modify behavior (e.g., behavioral flexibility; Weir et al. 2002; Sol et al. 2005; Shettleworth 2010; Kabadayi et al. 2016; Olkowicz et al. 2016; Fristoe et al. 2017; Gutiérrez-Ibáñez et al. 2018; Ducatez et al. 2020; Sayol et al. 2020).

Historically, research evaluating general patterns of brain evolution have

focused on the relative or absolute size of the whole brain or its major regions. For example, early investigators proposed that vertebrates with larger than predicted brains for their body size were more “encephalized,” which they suggested supported a higher information processing capacity (Jerison 1973; Dicke and Roth 2016). This research emphasized that non-avian reptiles, amphibians, and fish were characterized by a relationship between brain and body mass that had a distinctly lower intercept than that of endothermic vertebrates, in other words, smaller brains for their body size than that would be expected for endothermic vertebrates (Jerison 1973; Striedter 2005; Northcutt 2013; Tsuboi et al. 2018). Research on the allometry of brain and body mass has led to an abundance of studies investigating the relationship between relative brain size and aspects of physiology, ecology, or behavior across vertebrate species (Allman et al. 1993; Sol et al. 2005; Isler and van Schaik 2006; Reddon et al. 2016; De Meester et al. 2019; Macrì et al. 2019). For example, larger-brained mammalian carnivore species have been found to be better at solving a novel problem (Benson-Amram et al. 2016). In a similar vein, bird species with relatively larger brain mass are more likely to adapt to novel conditions resulting from anthropogenic climate change (Fristoe et al. 2017), be more urban tolerant (Sayol et al. 2020), or modify their feeding behavior (Sol et al. 2005). Although brain size has received the most attention, other neural traits have also been shown to correlate with cognitive differences between species or populations (Chittka and Niven 2009; Shettleworth 2010; Healy and Rowe 2013; Dicke and Roth 2016).

Studies evaluating patterns in the evolution of vertebrate brain size assume that the volume or mass of the brain is a reliable proxy for neuronal parameters, such

as the number and size of neurons, that underly the function of the nervous system (von Bonin 1937; Lashley 1949; Jerison 1973; Williams and Herrup 1988; Dicke and Roth 2016). Recent studies, however, challenge this assumption, finding that the size of a brain or brain region is not consistently related to its number of neurons across species and clades (Herculano-Houzel 2011a; Herculano-Houzel et al. 2014; Nemec and Osten 2020). For example, in mammals, the number of neurons that compose the cerebral cortex follows a shared scaling rule in five major clades but diverges in primates, in which the cerebral cortex gains more neurons as it scales larger (Herculano-Houzel et al. 2014). This means that primate brains have higher densities of neurons in the cerebral cortex than typical mammal brains of similar size (Herculano-Houzel et al. 2014). The scaling relationships between brain size and number of neurons are less distinct or masked altogether in the scaling of brain mass with body size (Herculano-Houzel et al. 2014). These findings highlight the unique insights that can be gained by understanding the number of neurons in different regions of the brain (Roth et al. 2010; Dicke and Roth 2016; Herculano-Houzel 2017; Nemec and Osten 2020). For example, stereological studies have found that chickadees from harsh climates with better performance on a spatial memory task had more hippocampal neurons in addition to increased hippocampal volume (Pravosudov and Clayton 2002). In another stereological example, a greater number of newly generated neurons were observed in the medial cortex of territorial male side blotched lizards with increased spatial demands (LaDage et al. 2013). Nevertheless, studies evaluating neuronal traits in a comparative context are relatively rare.

Comparative studies evaluating brain evolution commonly face the difficulties

of untangling the effect of evolutionary history and species ecology on neuroanatomy. This has led to mixed results in studies that evaluate associations between neuroanatomy and species ecology or life history (Wasserman and Zentall 2006; Healy and Rowe 2007; Logan et al. 2018). In some instances, a strong signal of species ecology or life history is observed in neuroanatomy, whereas in other instances no signal is observed. For example, across squamates differences in cerebellar shape are associated with locomotor specialization (Macrì et al. 2019). However, a study comparing the neuroanatomy and habitat complexity of six closely-related species of *Anolis* lizards found that the complexity of the habitat most commonly used by each species did not have an effect on the volume of different brain structures (Powell and Leal 2014). It has been suggested that, due to their direct link to brain function, neuron numbers are a robust comparative metric across species (Williams and Herrup 1988; Roth et al. 2010; Dicke and Roth 2016; Herculano-Houzel 2017), which can provide an opportunity to untangle the intertwined effects of species ecology and evolutionary history on neuroanatomy.

Squamata is one of the most speciose vertebrate clades, exhibiting a diversity of ecological, physiological, and behavioral traits and providing a rich context for comparative studies of brain evolution (Northcutt 1978; Nomura et al. 2013; Hoops 2018). Although compared to endothermic vertebrates, studies of non-avian reptilian brain evolution are relatively rare, their findings suggest that reptile cognition is underpinned by much of the same neural patterns as mammalian cognition (Roth II et al. 2019). Homologs to cortical areas observed in other vertebrates have been identified in non-avian reptiles, and the reptilian cortex exhibits a similar laminar

structure to mammalian cortex, although with fewer layers (Northcutt 2013; Naumann et al. 2015). Neurogenesis—which is often implicated in cognition—is observed more widely in the brains of reptiles than in mammals (Font et al. 2001; Kaslin et al. 2008). An increasing number of studies evaluating the cognitive abilities of non-avian reptiles have found evidence of many of the cognitive processes also observed in birds and mammals (reviewed in Burghardt 2013; Northcutt 2013; Roth II et al. 2019). However, unlike findings in mammals, solitary squamate species have relatively larger brains than social species (De Meester et al. 2019). Squamates diverged recently from birds, which have exceptionally high neuron densities compared to other vertebrates studied (Olkowicz et al. 2016) and cognitive capacities comparable to many mammalian species (Kabadayi et al. 2016; Rössler et al. 2020). As such, the brains of squamates, with their relatively simpler structure and homology with avian and mammalian lineages, can provide unique insights into the evolution of neuronal structure in vertebrate brains (Striedter 2005; Naumann et al. 2015).

West Indian *Anolis* lizards provide an ideal group for studies of comparative neuroanatomy and cognition. Within each island of the West Indies, *Anolis* species have evolved into several ecomorph types, each specialized for a specific structural habitat (Williams 1972). Selection to effectively exploit distinct habitat types has driven many aspects of anole anatomy and ecology, including morphology, communication, and territoriality (e.g., Williams 1972, 1983; Losos 1990a, 1990b; Losos et al. 1994; Leal and Fleishman 2002, 2004; Johnson and Wade 2010; Gunderson et al. 2018). While species of *Anolis* primarily occupy different structural habitat types, they all exhibit the same general body form, foraging behavior, and

sensory abilities (Losos 2009). A recent study across 171 species of squamates found that anoles have some of the highest values for relative brain mass, which seems to be partially driven by their arboreal ecology (De Meester et al. 2019). In a series of papers, Powell and Leal (2012, 2014) took advantage of the correlated evolution between morphology, ecology, and behavior in anoles to evaluate if ecomorphology had an effect on neuroanatomy. These studies found that neither habitat complexity nor ecomorph had an effect on the volume of different brain structures across six species of *Anolis* lizards. Two potential explanations for these unexpected findings are that neuroanatomy is less evolutionary labile than morphological and physiological traits in anoles, or that measurements conducted at the gross level (i.e., volume of brain structures) are too coarse for evaluating potential differences in neuroanatomy due to habitat specialization in anoles.

Here, we extend previous neuroanatomical and cognitive work in anoles by documenting the number of neurons in the brain of two species of lizards, *Anolis cristatellus* and *A. evermanni*, representing the first published data of this type from squamate species. To measure cognitive performance, we use a behavioral paradigm that evaluates the ability of lizards to modify the motor component of prey-capture foraging behavior to access a food reward (Leal and Powell 2012). This paradigm provides an opportunity to evaluate problem solving and behavioral flexibility in an ecologically relevant context (Leal and Powell 2012) and has become a popular paradigm in studies evaluating lizard cognition (e.g., Clark et al. 2014; Noble et al. 2014; Szabo et al. 2019) and cognition more generally (Griffin and Guez 2014). We use these data to evaluate whether differences in cognitive performance or

ecomorphological variation in *A. cristatellus* and *A. evermanni* mirrors variation in the number of neurons or nonneurons in key regions of the brain. More generally, we discuss the number of neurons in the brain of these two species in the context of vertebrate brain evolution, with particular focus on common patterns we observe between our findings and published data from other vertebrate clades. In addition, we outline in our methods an isotropic fractionator protocol that has been optimized to deal with the unique challenges presented by tissues from small, ectothermic vertebrates that have nucleated erythrocytes.

Materials and Methods

Study species

We collected five adult male *A. evermanni* in the forest surrounding El Verde Field Station located in the Luquillo Experimental Forest in Río Grande, Puerto Rico and five adult male *A. cristatellus* near the Mata de Plátano Field Station in Arecibo, Puerto Rico in July 2019. *Anolis evermanni* is a medium sized trunk-crown anole that occupies large-diameter trunks and branches in the forest canopy (Rand 1964), whereas *A. cristatellus* is a medium sized trunk-ground anole that typically perches on moderate- to large-diameter tree trunks within 2 m of the ground (Rand 1964). All lizards were then transported to the University of Missouri where they were housed until euthanasia (August 2019). The research reported here was performed following the guidelines established by Duke University and University of Missouri Animal Care and Use Committee.

Neuronal analyses with the isotropic fractionator

Tissue preparation

We euthanized lizards with an overdose of isoflurane prior to intracardial perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Following perfusion, we postfixed whole heads in 4% paraformaldehyde for 24 h after which we dissected the brains and postfixed in 4% paraformaldehyde for another 48 h. To ensure consistency of dissection, we bisected the brainstem from the spinal cord at the foramen magnum. We then transferred whole brains to 30% sucrose until equilibrated and then to antifreeze solution (30% ethylene glycol 30% glycerol) until equilibrated. Tissues were kept at 4°C during fixation steps and for short term storage and transferred to 20°C for long term storage. This methodology is adapted from Herculano-Houzel and Lent (2005), Powell and Leal (2012), and Hoops (2015), but optimized for the tissues used in this study.

Prior to analysis, we dissected brains into three regions: telencephalon, cerebellum, and the rest of the brain (ROB; representing the diencephalon, mesencephalon, pons, and myelencephalon) (Northcutt 1978, 2013; Greenberg 1982). A dissecting scope and fine surgical tools were used for dissection of these regions. We dissected the telencephalon by separating the telencephalon from the diencephalon and then bisecting anterior to the optic chiasm (Northcutt 2013). The olfactory bulbs were not included as these were too fragile to dissect from the anole skull. We dissected the cerebellum by pulling the cerebellum away from the midbrain and then bisecting it at its base where the cerebellar peduncles meet the pons and medulla (Northcutt 2013). We measured the mass of each brain region to the nearest

± 0.0001 g. Prior to measuring mass, each region was manipulated gently over a Kimwipe to remove excess fixative. We combined the mass of each region to calculate total brain mass.

Tissue dissociation

We analyzed each region (i.e., cerebellum, telencephalon, ROB) separately following the isotropic fractionator protocol (Herculano-Houzel and Lent 2005; Ngwenya et al. 2016). The following protocol has been optimized for use with small, nonmammalian tissues, particularly for groups having nucleated erythrocytes. We used a 1 mL Tenbroeck tissue grinder (DWK Life Sciences, Wheaton) to dissociate each brain region. We ground tissue with dissociation solution (40 mM sodium citrate 1% Triton-X) until no visible tissue remained in the grinder. After dissociation, we collected the homogenate in a 15 mL centrifuge tube and then centrifuged at 3000 RPM for 10 min to collect nuclei in a pellet. The size of these graduated tubes was appropriate for our samples, but tissues any smaller may require a different approach, such as that described in Marhounova et al. (2019). We carefully removed excess dissociation solution with a transfer pipette, added DAPI at 4 mg/L dilution, and adjusted the volume with PBS. The total volume of the suspension for each brain region was adjusted with PBS to yield at least 50 nuclei in the central fields of the hemocytometer when counting (cerebellum, 0.5 mL; ROB, 3 mL; telencephalon, 2 mL), following previous methodology. This value was then recorded.

Counting cells

We estimated the total number of cells in the telencephalon, cerebellum, and ROB using a hemocytometer under a Leica 5500B microscope. Prior to loading each well of the hemocytometer, we inverted the suspension to ensure homogeneity before removing four 10 uL samples and placing them into a hemocytometer. We counted the number of nuclei in the 25 central fields of the hemocytometer (0.0001 mL). Nuclei are recognizable by their defined contours and heavy labeling with DAPI. Erythrocyte nuclei were recognizable from their small, regular oblong shape and autofluorescent and intact cell membrane (Fig. 3.1), which allowed us to exclude them from counts. We counted samples of each homogenate until the coefficient of variation across four samples was less than 0.15 (Herculano-Houzel and Lent 2005). We then calculated the average number of nuclei per milliliter across the four samples and multiplied by the total volume in each tube to estimate the total number of cells in each brain region.

Immunocytochemistry

After estimating the number of cells in the telencephalon, cerebellum, and ROB, we performed an immunoreaction to label neuronal nuclei. We removed a 1 mL sample of homogenate for the NeuN immunoreaction. For small tissues, this may require the total homogenate. The sample was spun down in a microcentrifuge for 15 min at 5000 RPM. This longer centrifugation, at lower rotations per minute (RPM), improves yield when centrifuging a smaller amount of tissue, as with the tissues analyzed in this study. Following centrifugation, we removed the supernatant, and

then rinsed the nuclei with PBS by resuspending the pellet in 500 uL of PBS, centrifuging again, and removing the supernatant. We only rinsed once at each wash step to minimize loss of nuclei. We then resuspended the pellet in 1 mL of 0.2 M boric acid and incubated for 45 min at 70°C for heat-induced epitope retrieval. This step is necessary when tissue is overfixed, and it greatly improved binding of the antibody. Overfixation may be more common in smaller tissues. Nuclei were then spun down again and rinsed once with PBS. The pellet was then resuspended in a 1:100 dilution of NeuN (ABN78C3, Millipore Sigma) and incubated under agitation overnight in the dark at 4°C. We then centrifuged the nuclei, rinsed with PBS, and resuspended in 200–400 uL for counting.

Counting neurons

We estimated the number of neuronal nuclei in the telencephalon, cerebellum, and ROB. To do so, we loaded the hemocytometer with 10 uL of the immunoreaction for viewing under the microscope as described above. We counted the number of NeuN positive nuclei out of at least 500 total DAPI-positive nuclei. We then estimated the total number of neurons by multiplying this proportion by the total number of cells estimated in that brain region. The total number of nonneurons was estimated by subtracting the number of neurons from the total number of cells in that brain region. Neuron and nonneuron density were calculated by dividing the number of neurons or nonneurons by the mass of each brain region in milligrams. To calculate values for the whole brain, the number of neurons for each region was added together as was the number of nonneurons for each region. Each value was then divided by total brain

mass in milligrams to calculate whole brain neuron and nonneuron density. The total number of nonneurons was divided by the total number of neurons to calculate whole brain nonneuron to neuron ratios.

Comparisons with other vertebrates

In order to compare our data to other vertebrates, we reviewed the literature for studies that used the isotropic fractionator protocol to estimate the number and density of neurons and nonneurons in the brains of other taxa. We present these data along with our findings in Figs. 3.2 and 3.3. Due to methodological differences in dissection between studies, our findings from *Anolis* cannot be directly compared to published data, as described below. Furthermore, we make inferences cautiously taking into account the minimal representation of squamates in the dataset. Therefore, these figures represent our initial step in evaluating potential patterns of neuronal scaling across vertebrates. We compiled data for the number and density of neurons and nonneurons for mammals from estimates presented in Herculano-Houzel et al. (2015). The mammalian cortex does not contain the subpallial regions included in the avian, fish, and reptile telencephalons presented here. Data for birds were gathered from Olkowicz et al. (2016). The avian and mammalian ROB contain the subpallium, which is included in the avian, fish, and reptile telencephalon. Data for the Nile crocodile were originally reported in Ngwenya et al. (2016), and individual level data were provided by the authors. Since these data were taken across an array of subadult individuals, the value presented in Figs. 3.2 and 3.3 is taken from only the largest individual in the dataset. For the Nile Crocodile, we calculated the value of the ROB

by combining values of the tectum, diencephalon, medulla, and pons. Data for the guppy were presented in Marhounova et al. (2019). In this case, we calculated a value for the ROB from the reported data by combining values of the tectum, diencephalon, and brainstem.

Cognitive tests

Subjects and housing

Males of *A. evermanni* (n=6) and *A. cristatellus* (n=7) were collected in the general vicinity of El Verde Field Station located in the Luquillo Experimental Forest in Río Grande, Puerto Rico in August 2013 and transported to our laboratory at Duke University. Anoles were housed individually in cages (29 L, 21 W, and 21 H cm) located within an environmental chamber which was kept under 12 h:12 h photoperiod cycle at 28°C and 60% relative humidity. Lizards were watered twice daily and fed crickets on a regular schedule throughout the experimental period. All individuals were experimentally naïve adult males.

Behavioral tasks

Behavioral experiments followed the procedure outlined in Leal and Powell (2012) and were performed from September to December 2013. Behavioral experiments were conducted in the cage where the lizards were housed. The behavioral testing apparatus was placed inside the cage, and lizards had 15 min to perform the task. The apparatus was an opaque gray platform (12 L, 5.5 W cm) containing two wells, each 1.6 cm in diameter and 1 cm deep. A reward (intact dead larva of *Hermetia illucens*)

was placed inside a well and the lizard had to remove an opaque disc covering the well to reach the larva. Individuals were habituated (i.e., habituation phase) to the testing apparatus before conducting the experiments. This phase was performed in four sequential steps: (1) the apparatus was placed inside the cage with a live larva of *H. illucens* inside one well as a reward (2) the apparatus was placed inside the cage with a larva of *H. illucens*, freshly killed by freezing, inside one well as a reward; (3) individuals were randomly assigned a uniform colored blue or yellow disc, which was positioned next to the well containing the reward; (4) the disc was positioned covering half of the well containing the reward. The color of the opaque disc was irrelevant for the performance of the motor task. Also, during this phase, individuals did not need to manipulate the disc to access the worm. Individuals advanced stages or completed an experiment when they correctly performed the task six consecutive times.

After completing the habituation phase, the disc was placed over one of the wells and the lizards were presented with the novel problem of dislodging the disc to access the larva (i.e., motor task). The disc was placed over the well completely blocking line of sight into the well. Only one disc was used, and its position was randomly determined before each trial. Individuals conducted one trial per day. Anoles show no response to larva odor (Leal and Powell 2012).

Statistical analyses

All measures of SVL, body mass, brain mass, number of neurons and nonneurons, density of neurons and nonneurons, and nonneuron to neuron ratio were log-10

transformed prior to analysis. Differences between brain regions were evaluated via two-way ANOVA followed by Tukey–Kramer post hoc comparisons and differences between species were evaluated via Student’s t-test. When our data did not conform to the assumptions of normality, homoskedasticity, or no outliers for ANOVA or Student’s t-test, we performed either a nonparametric Kruskal–Wallis rank sum test followed by Dunn’s Kruskal–Wallis test for post hoc comparisons; a Welch’s ANOVA followed by a Game-Howell test for post hoc comparisons; or a Welch’s t-test. We calculated all statistics in R statistical software (R Development Core Team 2008) and generated all graphs using the package ggplot2 (Wickham 2009). The individual level data underlying the findings in this article are available as Online Supplementary Information and in Table A3.1.

Results

Number of neurons

The mean body size, brain size, number and density of neurons and nonneurons, and nonneuron to neuron ratio for each species are presented in Table 3.1. No significant differences in SVL (Student’s t-test, $t(8)=0.034$, $n=10$, $P=0.97$) or weight (Student’s t-test, $t(8)=1.044$, $n=10$, $P=0.33$) were detected in our sample between *A. cristatellus* and *A. evermanni*. Neuron density was significantly different between brain regions across species (Kruskal–Wallis, $X^2(2, n=30)=21.835$, $P<0.0001$). Post hoc tests revealed that the cerebellum ($n=10$, mean= $403,000 \pm 41,400$ cells/mg) had a higher neuron density than both the ROB ($n=10$, mean= $90,700 \pm 8180$ cells/mg; Dunn’s test,

$P < 0.0001$) and the telencephalon ($n=10$, mean= $116,000 \pm 6710$ cells/mg; Dunn's test, $P=0.005$). The neuron density of the telencephalon and the ROB overlapped (Dunn's test, $P=0.12$). Nonneuron density was significantly different between brain regions across species (Kruskal–Wallis, $X^2(2, n=30)=13.107$, $P=0.001$). Post hoc tests revealed that nonneuron density for the ROB ($n=10$, mean= $50,400 \pm 2880$ cells/mg) was significantly higher than that of the cerebellum ($n=10$, mean= $29,200 \pm 4660$ cells/mg; Dunn's test, $P=0.003$) and telencephalon ($n=10$, mean= $31,100 \pm 2290$ cells/mg; Dunn's test, $P=0.006$), while the cerebellum and telencephalon did not significantly differ in nonneuron density (Dunn's test, $P=0.76$). Nonneuron to neuron ratio was significantly different between brain regions (ANOVA, $F_{2,27}=90.218$, $n=30$, $P < 0.0001$). Post hoc tests revealed that the cerebellum ($n=10$, mean= 0.075 ± 0.011) had a lower ratio than both the telencephalon ($n=10$, mean= 0.27 ± 0.025 ; Tukey–Kramer contrast, $P < 0.0001$) and the ROB ($n=10$, mean= 0.61 ± 0.074 ; Tukey–Kramer contrast, $P < 0.0001$) while the telencephalon had a lower ratio than the ROB (Tukey–Kramer contrast, $P < 0.0001$).

We found no differences between *A. cristatellus* and *A. evermanni* in the mass of the whole brain (Student's t-test, $t(8)=0.267$, $n=10$, $P=0.80$), the number of whole brain neurons (Kruskal–Wallis, $X^2(1, n=10)=1.32$, $P=0.25$), the whole brain neuron density (Student's t-test, $t(8)=0.532$, $n=10$, $P=0.61$), the whole brain nonneuron density (Student's t-test, $t(8)=1.505$, $n=10$, $P=0.17$), or the whole brain nonneuron to neuron ratio (Kruskal–Wallis, $X^2(1, n=10)=2.4545$, $P=0.12$). However, the number of nonneurons across the whole brain was significantly higher in *A. evermanni* than *A. cristatellus* (Student's t-test, $t(8)=2.433$, $n=10$, $P=0.04$).

There were no significant differences between the mass of the telencephalon (Student's t-test, $t(8)=0.685$, $n=10$, $P=0.51$), cerebellum (Kruskal–Wallis, $X^2(1, n=10)=2.2329$, $P=0.14$), or the ROB (Student's t-test, $t(8)=0.078$, $n=10$, $P=0.94$) between *A. cristatellus* and *A. evermanni*. Similarly, the number of neurons did not significantly differ between *A. cristatellus* and *A. evermanni* for the telencephalon (Student's t-test, $t(8)=0.497$, $n=10$, $P=0.63$), cerebellum (Student's t-test, $t(8)=0.235$, $n=10$, $P=0.82$), or the ROB (Kruskal–Wallis, $X^2(1, n=10)=0.098182$, $P=0.75$). The number of nonneurons for the telencephalon (Student's t- test, $t(8)=0.69$, $n=10$, $P=0.51$) and cerebellum (Student's t-test, $t(8)=0.783$, $n=10$, $P=0.46$) were not significantly different between *A. cristatellus* and *A. evermanni*. The number of nonneurons was marginally significantly higher in the ROB of *A. evermanni* than *A. cristatellus* (Student's t-test, $t(8)=2.275$, $n=10$, $P=0.052$). The density of neurons did not significantly differ between *A. cristatellus* and *A. evermanni* for the telencephalon (Student's t-test, $t(8)=0.095$, $n=10$, $P=0.93$), cerebellum (Student's t-test, $t(8)=0.354$, $n=10$, $P=0.73$), or the ROB (Kruskal–Wallis, $X^2(1, n=10)=0.98182$, $P=0.75$). The density of nonneurons did not differ significantly between *A. cristatellus* and *A. evermanni* for the telencephalon (Student's t- test, $t(8)=0.854$, $n=10$, $P=0.42$), the cerebellum (Student's t-test, $t(8)=1.085$, $n=10$, $P=0.31$), or the ROB (Student's t-test, $t(8)=1.658$, $n=10$, $P=0.14$). The nonneuron to neuron ratio did not differ significantly between *A. cristatellus* and *A. evermanni* for the telencephalon (Kruskal–Wallis, $X^2(1, n=10)=2.4545$, $P=0.12$), cerebellum (Student's t-test, $t(8)=0.897$, $n=10$, $P=0.40$), or the ROB (Student's t-test, $t(8)=0.8030$, $n=10$, $P=0.98$).

Cognitive tests

A summary of the number of trials required for each individual to complete the motor task is presented in Table 3.2. Of the six individuals of *A. evermanni* that were tested, one individual failed to complete the habituation steps. Of the seven individuals of *A. cristatellus* that were tested, only three completed the habituation steps. Of those lizards that progressed to the motor task, it was completed by 100% (five of five) of *A. evermanni* and 33% (one of three) of *A. cristatellus*.

Discussion

Elucidating the processes underlying the morphological diversity of the brain observed in vertebrates and its potential correlates with cognition have been a central goal of comparative neuroanatomy and a major theme of comparative cognition (see papers in this volume). Here, we present estimates of the number of neurons in the brains of two species of *Anolis* lizards, representing the first data of this type from squamate species. Above we have outlined a protocol for the isotropic fractionator optimized for use with vertebrates possessing small brains and nucleated erythrocytes, like those analyzed in this article. Below we explore whether these data help to explain differences between *A. cristatellus* and *A. evermanni* in their ability to solve a motor task which has been previously used to evaluate behavioral flexibility in a number of species, including anoles (Boogert et al. 2011; Leal and Powell 2012; Noble et al. 2014). Finally, we discuss our findings on the neuronal parameters in the *Anolis* brain in the context of current hypotheses on vertebrate brain evolution.

In general, our findings indicate that the *Anolis* brain follows similar patterns

in neuronal and nonneuronal density to those exhibited by the brains of other vertebrates. Neuron density was highest in the cerebellum, lower in the telencephalon, and lowest in the ROB (Table 3.1). This pattern is congruent with findings in other species of vertebrates (Herculano-Houzel et al. 2014; Ngwenya et al. 2016; Olkowicz et al. 2016; Marhounova et al. 2019) suggesting that shared mechanisms shape brain evolution across vertebrates. General patterns of brain evolution, particularly with regard to allometric scaling of brain size as a function of body size and development, are well known across vertebrates (see Finlay and Darlington 1995; Striedter 1997; Font et al. 2019; and references therein), supporting the possibility of similar evolutionary patterns in neuronal parameters.

The cerebellum is extremely small in *Anolis*, accounting for only 3–5% of total brain mass compared to 6–16% across birds (Olkowicz et al. 2016), 8–25% across mammals (Herculano-Houzel et al. 2015), 8% in the Nile crocodile (Ngwenya et al. 2016), and 10% in the guppy (Marhounova et al. 2019). Because of the high cerebellar cell density across vertebrates, it still accounts for 7–24% of total brain neurons in the anole species sampled in this study, which is smaller than 20–64% across birds (Olkowicz et al. 2016), 53–98% across mammals (Herculano-Houzel et al. 2015), 55% in the Nile crocodile (Ngwenya et al. 2016), and 40% in the guppy (Marhounova et al. 2019). The cerebellum shows extreme morphological diversity across vertebrates (Voogd and Glickstein 1998; Macrì et al. 2019), which is reflected in the variation observed in the numbers of neurons in the vertebrate groups described above. Within squamates, locomotor specialization plays a major role in cerebellar morphology at multiple levels (Macrì et al. 2019), and previous work in *Anolis*,

including *A. cristatellus* and *A. evermanni*, suggests that the cerebellum is more variable in terms of its proportion of brain volume than other brain regions (Powell and Leal 2012).

We found that the brains of *A. cristatellus* and *A. evermanni* had the highest nonneuronal density in the ROB with similar, lower nonneuronal density in the telencephalon and cerebellum (Table 3.1). This pattern of nonneuronal density differs from both birds (Olkowicz et al. 2016), in which the telencephalon has a lower density of nonneurons than the cerebellum and ROB, and mammals (Herculano-Houzel et al. 2014), in which nonneuronal densities are consistent across the brain. The nonneuronal density pattern observed in anoles also differs from both the Nile crocodile (Ngwenya et al. 2016) and guppy (Marhounova et al. 2019). Both these species exhibited the highest nonneuronal densities in the cerebellum with lower densities in the telencephalon and ROB.

In every region of the *Anolis* brain, the ratio between nonneurons and neurons does not exceed 1 (Table 3.1), indicating that in no brain region does the number of nonneurons outnumber neurons. Nonneuron to neuron ratios of less than 1 are observed in the cerebellum and telencephalon of most bird species (Olkowicz et al. 2016) and the cerebellum of most mammal species (Herculano-Houzel et al. 2014). Ratios less than 1 are observed in the cerebellum of many individual Nile crocodiles (Ngwenya et al. 2016). However, a nonneuron to neuron ratio of less than 1 across all brain regions, as we observed in *Anolis*, is observed only in a few species of birds (Olkowicz et al. 2016) and the guppy (Marhounova et al. 2019). Both *Anolis* and other vertebrates (Herculano-Houzel et al. 2014; Olkowicz et al. 2016; Marhounova

et al. 2019) follow the same trend in which the lowest nonneuron to neuron ratios are observed in the cerebellum, followed by the telencephalon, and then highest in the ROB. The population of nonneuronal cells in the brain includes glial, endothelial, and ependymal cells, however, it is hypothesized that the majority of these cells are glial cells (Herculano-Houzel 2011b; Herculano-Houzel and Dos Santos 2018).

Historically, the glia to neuron ratio was hypothesized to increase as a function of brain size, as larger neurons in larger brains require more glia for metabolic support (Hawkins and Olszewski 1957). However, more recent research has revealed that the density of glia is relatively constant across brain structures and across vertebrate species (Herculano-Houzel et al. 2014; Olkowitz et al. 2016) and that the glia to neuron ratio thus increases as a function of neuron size as a result of developmental constraints in neural tissue (Herculano-Houzel and Dos Santos 2018). Thus, the small nonneuron to neuron ratios found in *Anolis* and the guppy result from the interaction between the relatively constant density of nonneuronal cells across species and the high neuron densities found in the brains of smaller vertebrates due to their small, tightly packed neurons (Niven and Farris 2012; Herculano-Houzel and Dos Santos 2018). This relatively constrained glial cell density, and therefore size, suggests that glial cells play a fundamental role in neural tissue, although, due to the variable functions of glia, the nature of this constraint is still unclear (Herculano-Houzel and Dos Santos 2018). Despite similarities in size, there are still apparent differences between *Anolis* and guppy brains, which likely reflect differences in species ecology (Striedter 1997).

As can be observed in Figs. 3.2 and 3.3, the smallest mammalian brain for

which neuron number and density using the isotropic fractionator protocol has been documented is four times larger than the *Anolis* brains presented here with six times the number of neurons, and the smallest avian brain is eight times larger with 30 times the number of neurons. The scarcity of data on the neuronal parameters of the smallest vertebrate brains prevents a robust evaluation of evolutionary patterns across vertebrates at this time. Further studies, particularly in small size vertebrates, are needed to elucidate whether the smallest vertebrate brains show unique or shared neuroanatomical patterns compared to larger brains.

At least one individual of *A. cristatellus* and *A. evermanni* completed the cognitive tests presented to the lizards (Table 3.2). This strongly suggests that interspecific differences in performance were not the result of physical constraints or a problem that was beyond the limits of the cognitive abilities of the species tested (Shettleworth 2010; Auersperg et al. 2011). Instead, differences are likely to result from interspecific differences in the ability to modify existing behavior (Leal and Powell 2012), particularly prey-striking behavior. In *Anolis*, this behavior is characterized by a downward movement of the neck and head, followed by a sudden strike of the prey (Losos 2009), which was ineffective at solving the motor task. In order to solve the task, individuals either used a sideways motion of the head to bite the disc to remove it from the well or used their snout as a lever to dislodge the disc and reach the reward. We found that all the individuals of *A. evermanni* presented with the motor task were able to solve it, but only 33% of the individuals of *A. cristatellus* were able to do so. Individuals of *A. cristatellus* that failed the task commonly struck the top of the disc. This strategy was ineffective at dislodging the

disc because it was wider than their gape, yet this behavior mirrors natural prey-striking behavior. It was common to observe individuals strike the disc multiple times and then stand next to the apparatus looking attentively toward the disc. In the case of *A. evermanni*, it was common to observe individuals changing their natural striking behavior (i.e., strike the disc by the top) as the experiment progressed. It should be noted that both species have the same general diet and mostly catch their prey using a sit-and-wait foraging strategy (Losos 2009). Therefore, it is unlikely that differences in diet or foraging behavior contributed to the observed differences in the performance of the motor task.

We found no differences in the number of neurons or neuronal density between the brains of *A. cristatellus* and *A. evermanni*. However, *A. evermanni* had a significantly higher number of nonneurons across the whole brain than *A. cristatellus*. Although this difference in nonneurons was apparent across the whole brain, the brain region primarily driving this difference was the ROB, which was the only region to show a marginally significant higher number of nonneurons in *A. evermanni* compared to *A. cristatellus*. The greater mean number of nonneurons in the telencephalon and cerebellum of *A. evermanni* compared to *A. cristatellus* may also contribute to the effect across the whole brain, although this difference was not significant. Glial cells have been increasingly implicated in learning and memory (Suzuki et al. 2011; Fields et al. 2014). Chickadees from more harsh climates, that showed more accurate performance in a spatial memory task, had more hippocampal glial cells than birds from mild climates (Pravosudov and Clayton 2002; Roth et al. 2013). Neurons that are larger in size have also been associated with larger glia to

neuron ratios or larger numbers of glia cells (Hawkins and Olszewski 1957; Herculano-Houzel 2014; Dicke and Roth 2016; Herculano-Houzel and Dos Santos 2018). Although we did not detect evidence of larger neurons in *A. evermanni* compared to *A. cristatellus* in the form of significant differences in neuron density, larger neurons have been associated with more advanced cognition. Chickadees from harsher climates had larger hippocampal neurons than those from mild climates (Freas et al. 2013). Because the exact identity of nonneuronal cells is mixed, it is unclear exactly how these differences may contribute to the observed difference in problem solving ability between *A. cristatellus* and *A. evermanni*. The brain areas included in the ROB are not traditionally implicated in behavioral flexibility and are generally involved in processing sensory input and motor output. Our working hypothesis is that both species are able to associate the disc with a food reward, but individuals of *A. evermanni* exhibit a higher rate of plasticity in the motor component of prey striking behavior, which may be related to the interspecific difference observed in the ROB. In one study, New Caledonian crows, a species known for its exceptional tool use, were found to have a relatively larger tegmentum and septum compared to other corvids and passerines (Mehlhorn et al. 2010), both regions encompassed in the ROB. A more detailed study of the *Anolis* ROB is needed to localize which part of the brain is primarily responsible for the increased number of nonneurons in the ROB. It should be noted that after correcting for body size, the relative size of the brain and of the major areas of the brain of *A. cristatellus* and *A. evermanni* are not significantly different (Powell and Leal 2012).

The differences in performance on the motor task can also be indicative of

species differences in dexterity, which can be influenced by interspecific differences in ecology, including the complexity of the habitat preferred by the species (Shettleworth 2010). *Anolis evermanni* occupies a habitat with a higher level of structural complexity (i.e., tree trunk and canopy) than does *A. cristatellus*, which is commonly found using tree-trunks or the ground (Powell and Leal 2014). One possibility is that the need to navigate a more complex habitat favors dexterity of motor movements, which might explain the higher number of individuals of *A. evermanni* solving the motor task. Different ecomorphs of *Ctenophorus* dragon lizards show mosaic evolution in the tectum and rhombencephalon (Hoops et al. 2017), both regions of the ROB where we observed differences above.

We present our findings alongside published data to explore how the *Anolis* brain scales in relation to other vertebrate species. In Fig. 3.2, we see that the number of neurons and nonneurons in each region of the *Anolis* brain fall along a comparable slope as the data from other vertebrate species. This similarity suggests that, at the cellular level, *Anolis* brains are not fundamentally different than the brains of birds and mammals. A similar pattern has been suggested in the allometry of brain and body mass if the analysis considers morphological adaptations exhibited by birds, such as those that reduced body mass to facilitate flight (Font et al. 2019). This is contrary to what has been suggested in previous studies of brain size that highlighted differences between so-called “lower” and “higher” vertebrates (Jerison 1973). Data from a more diverse array of squamate species will be necessary before precise scaling rules can be established and compared to the published scaling rules from other vertebrates.

The densities of neurons and nonneurons in *Anolis* fall below that of birds but generally along the same slope as that of mammals (Fig. 3.3). Given their more recent evolutionary divergence from birds, it is unexpected that the neuron and nonneuron densities observed in *Anolis* are closer to those observed in mammals than they are in birds, and this may suggest that the exceptionally high neuron densities observed in birds (Olkowicz et al. 2016) are a derived characteristic of avian brains. Interestingly, despite similarities in size, the number and density of neurons and nonneurons in the brain of the guppy (Marhounova et al. 2019) are higher than that observed in *Anolis*, more closely following the trends observed in birds than that of mammals. Future studies on the neuronal parameters of non-avian reptile brains can provide insight into the evolution of this unique feature of avian brains, particularly in a phylogenetic context.

As the substrate of behavior, the brain and its components have a long tradition in comparative cognition. As part of this study, we demonstrated the feasibility of performing comparative neuroanatomy at the level of neurons and nonneurons in *Anolis* lizards. Furthermore, we uncovered a difference between species in the total number of nonneurons that may contribute to interspecific differences in the ability to solve a novel motor task. Such a finding highlights the potential insights that can be gained by integrating neuroanatomy at the level of neuronal traits and experimental cognition. It also shows that studies at this level can uncover differences that are not found when neuroanatomy is studied at the level of total brain size or volume (Powell and Leal 2012). In the case of anoles, more generally, our findings suggest that neuroanatomy at the level of neuronal parameters

can be as labile as other traits that are known to diverge between species adapted to distinct habitats. Finally, although limited to two species, our findings suggest that neuron number and density in lizard brains scale similarly to endothermic vertebrates (Herculano-Houzel et al. 2014; Olkowitz et al. 2016) in contrast to the differences observed in brain to body mass relationships (Jerison 1973; Striedter 2005; Northcutt 2013; Tsuboi et al. 2018; Font et al. 2019). However, it is still unclear as to how widespread our findings are across squamates. As is the case for many non-model species, our results provide further evidence for the need to increase species diversity in comparative neuroanatomy and neurobiology (Striedter 2019; Striedter and Preuss 2019) in order to gain a complete understanding of brain evolution, including neuronal parameters across vertebrates.

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Tables and Figures

Fig. 3.1: *Anolis* nucleated erythrocyte under fluorescence. Nucleated erythrocytes constitute a variable fraction of nuclei in non-mammalian brains and should be excluded from nonneuronal cell counts. Morphologically, they are recognized by their regular, oblong nuclei, and “phantom” cell membrane which remains intact through dissociation. Under the microscope erythrocytes are also autofluorescent, as is apparent in the figure by the clearly visible cell membrane in both the DAPI and Cy3 filters. The nuclei to the right are both nonneuronal nuclei, as evidenced by their labeling with DAPI but not NeuN. Scale bar is 25 μm .

Fig. 3.2: Scaling of neurons and nonneurons by brain region in *Anolis* (this study) and other vertebrates (Herculano-Houzel et al. 2015; Ngwenya et al. 2016; Olkiewicz et al. 2016; Marhounova et al. 2019). (A) The scaling of neurons with brain mass while (B) shows the scaling of nonneurons with brain mass. Each point represents the average for a species, with shape indicating brain region (CER, cerebellum; CXT, cerebral cortex; ROB, rest of brain; TEL, telencephalon) and color indicating the class to which that species is classified. Please note that some methodological differences mean that the data from *Anolis* cannot be directly compared to published data. The mammalian cortex does not contain the subpallial regions included in the avian, fish, and reptile telencephalon presented here. The avian and mammalian ROB contains the subpallium which is included in the avian, fish, and reptile telencephalon.

Fig. 3.3: Relationship between density and brain mass by region in *Anolis* (this study) and other vertebrates (Herculano-Houzel et al. 2015; Ngwenya et al. 2016; Olkiewicz et al. 2016; Marhounova et al. 2019). (A) The relationship between neuron density and brain mass while (B) shows the relationship between nonneuron density and brain mass. Each point represents the average for a species, with shape indicating brain region (CER, cerebellum; CXT, cerebral cortex; ROB, rest of brain; TEL, telencephalon) and color indicating the class to which that species is classified. Please note that some methodological differences mean that the data from *Anolis* cannot be directly compared to published data. The mammalian cortex does not contain the subpallial regions included in the avian, fish, and reptile telencephalon presented here. The avian and mammalian ROB contains the subpallium which is included in the avian, fish, and reptile telencephalon.

Table 3.1: Neuronal parameters by brain region in *Anolis*. Region indicates the region of the brain to which data in that row belong (BR = Whole brain, TEL = Telencephalon, CER = Cerebellum, ROB = Rest of Brain). Estimates of the mean and standard error across individuals are presented in the table. N indicates the number of neurons in each brain region. O indicates the number of nonneurons in that region. DN and DO are the densities of neurons and nonneurons respectively. O/N is the ratio between the number of nonneurons and neurons in each brain region.

Species:	Mean SVL (cm):	Mean Body Mass (g):	Region:	Mean Region Mass (mg):	Mean N x10 ⁶ :	Mean O x10 ⁶ :	Mean DN (cells/mg) x10 ⁶ :	Mean DO (cells/mg) x10 ⁶ :	Mean O/N:
<i>A. cristatellus</i> (n=7)	5.0 ± 0.51	5.6 ± 0.068	BR	44.6 ± 2.36	4.79 ± 0.564	1.74 ± 0.0759	0.108 ± 0.0136	0.0395 ± 0.00298	0.39 ± 0.065
			TEL	16.4 ± 1.14	1.88 ± 0.159	0.469 ± 0.0485	0.116 ± 0.0126	0.0295 ± 0.00420	0.26 ± 0.052
			CER	1.5 ± 0.037	0.623 ± 0.0734	0.0526 ± 0.0122	0.410 ± 0.0477	0.0344 ± 0.00794	0.085 ± 0.017
			ROB	26.6 ± 1.35	2.29 ± 0.374	1.22 ± 0.0835	0.0858 ± 0.0141	0.0461 ± 0.00367	0.63 ± 0.15
<i>A. evermanni</i> (n=5)	4.3 ± 0.37	5.7 ± 0.20	BR	43.7 ± 2.47	4.92 ± 0.0737	1.97 ± 0.0525	0.114 ± 0.00785	0.0457 ± 0.00272	0.40 ± 0.015
			TEL	15.4 ± 0.757	1.77 ± 0.0623	0.500 ± 0.0275	0.116 ± 0.00660	0.0328 ± 0.00212	0.28 ± 0.012
			CER	1.8 ± 0.17	0.666 ± 0.0903	0.0395 ± 0.00508	0.395 ± 0.0736	0.0241 ± 0.00464	0.066 ± 0.015
			ROB	26.5 ± 1.65	2.48 ± 0.126	1.43 ± 0.0409	0.0957 ± 0.00950	0.0548 ± 0.00380	0.58 ± 0.039

Table 3.2: Number of trials performed by individuals of *A. cristatellus* and *A. evermanni* to complete the habituation and motor task. A “--” indicates individual failed to complete the stage.

Individual:	Species:	Habituation 1	Habituation 2	Habituation 3	Habituation 4	Motor Task
E04	<i>A. evermanni</i>	6	6	6	6	7
E03	<i>A. evermanni</i>	6	6	36	6	19
E10	<i>A. evermanni</i>	6	18	6		--
E06	<i>A. evermanni</i>	6	6	6	6	6
E08	<i>A. evermanni</i>	6	6	6	6	33
E09	<i>A. evermanni</i>	6	7	11	10	17
C07	<i>A. cristatellus</i>	--				
C03	<i>A. cristatellus</i>	24	6	--		
C01	<i>A. cristatellus</i>	7	15	6	6	7
C11	<i>A. cristatellus</i>	30	13	--		
C02	<i>A. cristatellus</i>	22	17	28	--	--
C09	<i>A. cristatellus</i>	--				
C10	<i>A. cristatellus</i>	6	6	6	6	--

Appendix

Table A3.1: Individual level data used in this publication. Also available for download at <https://doi-org.prox/10.1093/icb/icaa129>.

Ind.ID	Ecomorph	Species	Sex	SVL,cm	Wt,g	Reg	RMass,g	Cells	N	O	DN_N,mg	DO_O,mg	P_Mass	P_N	O_N
MAC19006	TrunkGround	cristatellus	M	5.5	3.4	BR	0.0413	4303750	2607935	1695815	63146.13	41060.9	1.00	1.00	0.65
MAC19006	TrunkGround	cristatellus	M	5.5	3.4	CER	0.0016	476250	420052.5	56197.5	262532.8	35123.44	0.04	0.16	0.13
MAC19006	TrunkGround	cristatellus	M	5.5	3.4	ROB	0.0246	1837500	828712.5	1008788	33687.5	41007.62	0.60	0.32	1.22
MAC19006	TrunkGround	cristatellus	M	5.5	3.4	TEL	0.0151	1990000	1359170	630830	90011.26	41776.82	0.37	0.52	0.46
MAC19013	TrunkGround	cristatellus	M	5.5	4.8	BR	0.0373	7151250	5353928	1793733	143536.9	48185.59	1.00	1.00	0.34
MAC19013	TrunkGround	cristatellus	M	5.5	4.8	CER	0.0016	798750	741240	57510	463275	35943.75	0.04	0.14	0.08
MAC19013	TrunkGround	cristatellus	M	5.5	4.8	ROB	0.023	3847500	2558588	1288913	111242.9	56039.67	0.62	0.48	0.50
MAC19013	TrunkGround	cristatellus	M	5.5	4.8	TEL	0.0127	2505000	2054100	450900	161740.2	35503.94	0.34	0.38	0.22
MAC19009	TrunkGround	cristatellus	M	5.6	6.2	BR	0.0455	7701250	5822546	1878704	127968	41290.19	1.00	1.00	0.32
MAC19009	TrunkGround	cristatellus	M	5.6	6.2	CER	0.0015	911250	817391.3	93858.75	544927.5	62572.5	0.03	0.14	0.11
MAC19009	TrunkGround	cristatellus	M	5.6	6.2	ROB	0.0262	4125000	2854500	1270500	108950.4	48492.37	0.58	0.49	0.45
MAC19009	TrunkGround	cristatellus	M	5.6	6.2	TEL	0.0178	2665000	2150655	514345	120823.3	28895.79	0.39	0.37	0.24
MAC19010	TrunkGround	cristatellus	M	5.8	6	BR	0.05	7065000	5208743	1856258	104174.9	37125.15	1.00	1.00	0.36
MAC19010	TrunkGround	cristatellus	M	5.8	6	CER	0.0015	662500	633350	29150	422233.3	19433.33	0.03	0.12	0.05
MAC19010	TrunkGround	cristatellus	M	5.8	6	ROB	0.0293	3862500	2398613	1463888	81863.91	49962.03	0.59	0.46	0.61
MAC19010	TrunkGround	cristatellus	M	5.8	6	TEL	0.0192	2540000	2176780	363220	113374	18917.71	0.38	0.42	0.17
MAC19012	TrunkGround	cristatellus	M	5.8	4.6	BR	0.0488	6433750	4971548	1462203	101876	29963.17	1.00	1.00	0.29
MAC19012	TrunkGround	cristatellus	M	5.8	4.6	CER	0.0014	528750	502312.5	26437.5	358794.6	18883.93	0.03	0.10	0.05
MAC19012	TrunkGround	cristatellus	M	5.8	4.6	ROB	0.0301	3855000	2802585	1052415	93109.14	34963.95	0.62	0.56	0.38
MAC19012	TrunkGround	cristatellus	M	5.8	4.6	TEL	0.0173	2050000	1666650	383350	96338.15	22158.96	0.35	0.34	0.23
MAE19004	TrunkCrown	evermanni	M	5	3	BR	0.0368	7102500	5139345	1963155	139656.1	53346.6	1.00	1.00	0.38
MAE19004	TrunkCrown	evermanni	M	5	3	CER	0.0014	937500	883125	54375	630803.6	38839.29	0.04	0.17	0.06
MAE19004	TrunkCrown	evermanni	M	5	3	ROB	0.0217	4155000	2692440	1462560	124075.6	67399.08	0.59	0.52	0.54
MAE19004	TrunkCrown	evermanni	M	5	3	TEL	0.0137	2010000	1563780	1837260	121986.5	44378.26	1.00	1.00	0.36
MAE19003	TrunkCrown	evermanni	M	5.5	4	BR	0.0414	6887500	5050240	1837260	121986.5	44378.26	1.00	1.00	0.36
MAE19003	TrunkCrown	evermanni	M	5.5	4	CER	0.0017	377500	336730	40770	198076.5	23982.35	0.04	0.07	0.12
MAE19003	TrunkCrown	evermanni	M	5.5	4	ROB	0.0255	4140000	2848320	1291680	111698.8	50654.12	0.62	0.56	0.45
MAE19003	TrunkCrown	evermanni	M	5.5	4	TEL	0.0142	2370000	1865190	504810	131351.4	35550	0.34	0.37	0.27
MAE19005	TrunkCrown	evermanni	M	5.8	4.6	BR	0.0443	6783750	4820940	1962810	108824.8	44307.22	1.00	1.00	0.41
MAE19005	TrunkCrown	evermanni	M	5.8	4.6	CER	0.0017	791250	748522.5	42727.5	440307.4	25133.82	0.04	0.16	0.06
MAE19005	TrunkCrown	evermanni	M	5.8	4.6	ROB	0.0278	3562500	2162438	1400063	77785.52	50361.96	0.63	0.45	0.65
MAE19005	TrunkCrown	evermanni	M	5.8	4.6	TEL	0.0148	2430000	1909980	520020	129052.7	35136.49	0.33	0.40	0.27
MAE19006	TrunkCrown	evermanni	M	5.8	4.6	BR	0.0439	6925000	4765345	2159655	108550	49194.87	1.00	1.00	0.45
MAE19006	TrunkCrown	evermanni	M	5.8	4.6	CER	0.0016	705000	668340	36660	417712.5	22912.5	0.04	0.14	0.05
MAE19006	TrunkCrown	evermanni	M	5.8	4.6	ROB	0.0259	3825000	2291175	1533825	88462.36	59221.04	0.59	0.48	0.67
MAE19006	TrunkCrown	evermanni	M	5.8	4.6	TEL	0.0164	2395000	1805830	589170	110111.6	35925	0.37	0.38	0.33
MAE19001	TrunkCrown	evermanni	M	6.2	5.2	BR	0.052	6751250	4820355	1930895	92699.13	37132.6	1.00	1.00	0.40
MAE19001	TrunkCrown	evermanni	M	6.2	5.2	CER	0.0024	716250	693330	22920	288887.5	9550	0.05	0.14	0.03
MAE19001	TrunkCrown	evermanni	M	6.2	5.2	ROB	0.0318	3900000	2429700	1470300	76405.66	46235.85	0.61	0.50	0.61
MAE19001	TrunkCrown	evermanni	M	6.2	5.2	TEL	0.0178	2135000	1697325	437675	95355.34	24588.48	0.34	0.35	0.26

CHAPTER 4

NEURONAL EVOLUTION AND HABITAT COMPLEXITY ACROSS THE PUERTO RICAN ANOLE RADIATION

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Abstract

Understanding the evolutionary selective pressures shaping the diversity of neuroanatomy across vertebrates is a major goal of comparative neuroscience. One factor that has been associated with differences in neuroanatomy is habitat complexity. Habitats of increasing complexity are suggested to place increased demands on cognition. In this study, we measure neuron number and density in the telencephalon, cerebellum, and other brain regions (ROB) to evaluate the predictions of concerted and mosaic evolution in five species of Puerto Rican *Anolis* lizards. We then evaluate whether differences in neuron density between species are explained by differences in habitat complexity. We find that the brains of these five species evolve in primarily a concerted manner, with some evidence of mosaic evolution in the telencephalon and ROB. Furthermore, we find that differences in neuron density between species are explained by differences in habitat complexity in the telencephalon and ROB, but not in the cerebellum. This study demonstrates the unique insights that can be gained into the evolution of vertebrate brains by focusing on the number and density of neurons in major brain regions.

Introduction

A major goal of comparative neuroscience is deciphering the evolutionary selective pressures that shaped the diversity of neuroanatomy throughout vertebrate phylogeny. All

vertebrate brains are composed of the same major brain regions; however, the size and structure of those brain regions varies across species and clades (Striedter 2005). Two interacting frameworks have been proposed to describe evolutionary changes in neuroanatomy. Concerted evolution describes coordinated changes in neuroanatomy across all brain regions, which is suggested to be a result of conserved processes governing brain development (Striedter 2005; Shettleworth 2010). For example, in 14 species of *Ctenophorus* dragon lizards, most of the variation in the volume of six different brain regions was predicted by changes in body size, suggesting that the neuroanatomy of species in this clade follows a shared scaling relationship (Hoops et al. 2017). On the other hand, mosaic evolution describes changes in neuroanatomy in a single brain region independent of the rest of the brain (Striedter 2005; Shettleworth 2010). For example, in Chondrichthyan, the size of the medulla varies inversely with the size of the telencephalon, such that the size of these structures varies independently of the size of other brain structures (Mull et al. 2020). Both processes interact to shape the diversity of vertebrate brains observed today, although it is suggested that concerted evolution plays a larger role (Striedter 2005).

It is difficult to untangle the effects of species ecology and evolutionary history on neuroanatomy. A common approach is to compare neuroanatomy between closely related species that differ in a particular ecological trait of interest (Striedter 2005; Shettleworth 2010). Ideally, this approach minimizes the influence of confounding differences between species and maximizes the likelihood that differences observed are related to the ecological trait that varies across species. For example, within two families of birds, Corvidae and Paridae, species that exhibit specialized food-storing behavior

have larger relative hippocampi (Krebs et al. 1989; Sherry et al. 1989; Lucas et al. 2004). Because species within these families have similar ecology, the difference in hippocampus size has been suggested to facilitate memory for stored food. Many studies have taken a similar approach in evaluating the potential effects of ecological traits on neuroanatomy (i.e., Pravosudov and Clayton 2002; Gonzalez-Voyer et al. 2009; Ladage et al. 2009; Kotrschal et al. 2015; De Meester et al. 2019; Mull et al. 2020).

Despite a long history of study of the vertebrate brain, our understanding of the factors affecting the evolution of vertebrate brains is still developing (Striedter 2005). One factor that has been shown to contribute to differences in neuroanatomy is habitat complexity (Godfrey-Smith 2002; Shettleworth 2010; Mettke-Hofmann 2014). Different animals occupy habitats that vary widely in their degree of heterogeneity, placing differential demands on cognition (Godfrey-Smith 2002). An animal's habitat is intrinsically linked to its fitness because it is the context in which an organism performs the behaviors required to survive and reproduce. Species of chipmunks that are found in habitats with denser vegetation cover have larger relative brain size than those in more open habitats (Budeau and Verts 1986). Similarly, the relative size of the cerebellum, telencephalon, medulla, and olfactory bulb in Ectodini cichlids of Lake Tanganyika varies with the rugosity, or "roughness," of lake substrates (Pollen et al. 2007). Multiple studies have explored the connection, or lack thereof, between habitat complexity and neuroanatomy in a range of taxa (Bauchot et al. 1977; Abbott et al. 1999; Safi and Dechmann 2005; Powell and Leal 2014; Sobrero et al. 2016; Ahmed et al. 2017).

As with many studies of brain evolution, habitat complexity is typically compared with measures of the relative size of the brain or its regions. Measures of relative brain

size are intended to evaluate whether the volume or mass of a brain or brain region is larger than expected given body or brain size (Jerison 1973; Striedter 2005; Healy and Rowe 2007; Logan et al. 2018). Recent studies have demonstrated that the size of the brain is not a reliable proxy for underlying neuronal parameters, such as the number and size of neurons that make up a brain or brain region (Herculano-Houzel 2011; Herculano-Houzel, Manger, et al. 2014; Olkowitz et al. 2016). For example, in the mammalian telencephalon, the number of neurons follows a shared scaling rule in a number of clades but diverges in primates, which maintain higher densities of neurons at larger brain sizes than other clades (Herculano-Houzel, Manger, et al. 2014). Due to their direct role in nervous function, neuron number may be a more robust comparative metric across species (Herculano-Houzel 2017). However, few studies have explored the relationship between neuron number and ecological variables like habitat complexity (but see Kverkova et al. 2018).

Squamates provide an ideal context for studying adaptive specialization in the brain. Squamata is one of the most speciose vertebrate clades and exhibits diverse ecology, physiology, and behavior. Previous studies have shown that variation in cerebellar structure is related to locomotor strategy in squamates (Macrì et al. 2019). Unlike findings in other clades, solitary squamate species have larger relative brain size than social species (De Meester et al. 2019). Particularly important is that the brains of squamates share homologous features with the brains of heavily studied birds and mammals, including a laminar forebrain organization (Striedter 2005; Northcutt 2013; Naumann et al. 2015; Kabelik and Hofmann 2018; Roth II et al. 2019). However, due to the simpler organization of their brain, squamates, and other reptiles, provide a tractable

model to understand fundamental mechanisms of cognition and brain evolution that can be applied to other vertebrates (Striedter 2005; Kabelik and Hofmann 2018; Roth II et al. 2019).

In this chapter, we explore the influence of habitat specialization on the brains of *Anolis* lizards. Anoles have a long history of study in the West Indies, primarily because of their repeated adaptive radiation across the islands of the Greater Antilles that has resulted in the evolution of distinct ecomorphs (Losos 2009). Species of the same ecomorph are defined by their structural habitat niche which has resulted in convergent evolution in morphology, ecology, behavior, and physiology across species within the same ecomorph (Williams 1972, 1983; Losos 2009; Johnson et al. 2010; Gunderson et al. 2018). The divergent habitat specializations of different *Anolis* are likely to place differential demands on cognition, as habitats differ considerably in their structure (Godfrey-Smith 2002; Powell and Leal 2014). However, a previous study found that the relative size of six brain regions in six species of Puerto Rican *Anolis* does not vary in relation to habitat complexity (Powell and Leal 2014), and that these brain regions vary concertedly as a function of brain size with no divergence between species (Powell and Leal 2012). This suggests that neuroanatomy is not as evolutionary labile as other traits diverging among *Anolis* ecomorphs, or that relevant variation in neuroanatomy is not captured at the level of brain size.

In this study, we evaluate brain evolution at the level of neuron number and density in five species of Puerto Rican *Anolis* and compare neuronal variation to habitat complexity. This research expands on previous studies, mentioned above, of the Puerto Rican anole radiation (Powell and Leal 2012, 2014). As such, we predict that neuron

number varies concertedly across species as a function of total brain neurons, with all species following a shared scaling relationship. Also following the previous study, we predict that neuroanatomy at the level of neuron density will not reflect differences in habitat complexity.

Materials and Methods

Study Species

We collected adult males of five species: *Anolis cristatellus* (trunk-ground ecomorph; n=12), *A. evermanni* (trunk-crown ecomorph; n=10), *A. gundlachi* (trunk-ground ecomorph; n=8), *A. krugi* (grass-bush ecomorph; n=10), and *A. stratulus* (trunk-crown ecomorph, n=9). We collected *A. evermanni* and *A. gundlachi* in the vicinity of El Verde Field Station in Rio Grande, Puerto Rico. We collected *A. cristatellus* and *A. krugi* in the vicinity of Mata de Platano Field Station in Arecibo, Puerto Rico. *Anolis stratulus* were collected from both El Verde Field Station, Mata de Platano Field Station, and Guayanbo, Puerto Rico. These represent prime locales for each species with minimally disturbed habitat. Individuals were sexed upon capture via visual identification. Individuals were captured and transported to the University of Missouri where they were housed briefly prior to euthanasia (August 2019). This research followed all guidelines of the University of Missouri Animal Care and Use Committee.

Neuronal analysis

We followed the methods outlined in Storks et al. (2020; Chapter 3). Immediately prior to euthanasia, the snout-vent-length (SVL; ± 0.1 cm) in centimeters and weight in grams ($\pm$

0.1 g) of each lizard was measured. Briefly, lizards were perfused via intracardial perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Whole heads and then dissected brains were postfixed in PFA. When necessary, sex was confirmed during dissection based on presence or absence of testis. Brains were then cryoprotected and stored at -20°C until analysis.

Prior to analysis, the brain was dissected into the telencephalon (TEL), cerebellum (CER), and rest of the brain (ROB; representing the diencephalon, mesencephalon, pons, and myelencephalon). The mass of each brain region was measured prior to analysis. Each brain region was analyzed separately using the isotropic fractionator technique (Herculano-Houzel and Lent 2005). Each brain region was homogenized in dissociation solution (40 mM sodium citrate in 1% Triton-X) using a 1 mL Tenbroeck tissue grinder (DWK Life Sciences, Wheaton) and collected in a 15 mL centrifuge tube. Tubes were centrifuged to collect nuclei in a pellet, after which we adjusted the volume of the suspension with PBS and added DAPI at a 4 mg/L dilution.

Following dissociation, the concentration of cell nuclei in the suspension was counted. Nuclei were resuspended and agitated to make an isotropic suspension. Samples of this suspension were collected and the number of nuclei in multiple samples was counted using a hemocytometer under a fluorescence microscope (Leica 5500B). Erythrocyte nuclei were excluded from cell counts. The number of nuclei was counted until the coefficient of variation across four samples was <0.15 . The average number of nuclei per milliliter was calculated for these four samples and multiplied by the volume of the suspension to estimate the total number of cells in each brain region.

The number of neurons was estimated through immunohistochemistry. A 1 mL sample of the suspension was collected, underwent heat-induced epitope retrieval, and immunoreacted under agitation overnight at 4°C for NeuN (ABN78C3, Millipore Sigma) at a dilution of 1:100. This antibody labels neuronal nuclei (Mullen et al. 1992) and has been used in diverse vertebrate clades. Following the immunoreaction, nuclei were resuspending in PBS for counting. The proportion of NeuN-positive nuclei in the sample was calculated by counting the number of NeuN-positive nuclei out of ~500 DAPI-positive nuclei under a fluorescence microscope. We then multiplied this proportion by the total number of cells to estimate the number of neurons in each brain region. This number was subtracted from the total number of cells to calculate the number of nonneurons in each brain region. Neuron and nonneuron density were calculated by dividing the total number of neurons or nonneurons by the mass of that brain region in milligrams. Nonneuron to neuron ratio (O/N) was calculated by dividing the number of nonneurons by the number of neurons in each brain region. Estimates for each brain region differ in sample size due to some tissues lost during processing.

Habitat Complexity

Measures of habitat complexity were extracted for each species from Powell and Leal (2014). More negative values of relative habitat complexity (RHC) indicate more complex structural habitats.

Statistical Analyses

All statistical analyses were performed in R Statistical Software (R Development Core Team 2008). Data for SVL, weight, brain mass, neuron number, and neuron density were log-10 transformed prior to analysis. Differences in brain mass between species were evaluated via one-way ANOVA with type III sums of squares ($\text{MASS}_{\text{BRAIN}} \sim \text{Species}$). This model conformed to all expectations for linear regression.

To evaluate concerted and mosaic evolution among the Puerto Rican anole radiation, we modeled the relationship between the number of neurons in each brain region as a function of the total number of neurons in the whole brain and tested for differences between each species. Neuron estimates from each individual were used in the models. First, we evaluated the correlation between number of neurons in each brain region and total brain neurons across individuals using Pearson's product-moment correlation. A significant correlation between variables suggests that a standard major axis regression is appropriate. Following Powell and Leal (2012), we extracted the absolute value of the residuals for each individual estimate from a standard major axis regression of the number of neurons on the total number of brain neurons ($N_{\text{REGION}} \sim N_{\text{BRAIN}}$) for each brain region separately using the R package *smatr* (Warton et al. 2012). We used a one-way ANOVA with type III sums of squares to compare the value of the residuals across species ($N_{\text{RESID}} \sim \text{Species}$). The residuals extracted did not conform to a normal distribution. Although ANOVA is robust to violations of this assumption, we also found similar results in all cases using a nonparametric Kruskal-Wallis test. Significant differences between species would indicate a difference in intercept in the relationship between neurons and brain neurons, which would be evidence of mosaic evolution.

Because the test just described can produce more conservative results than other models (Maxwell et al. 1985), we also performed ANCOVA with number of neurons as the response variable and number of brain neurons and species as predictor variables ($N_{\text{REGION}} \sim N_{\text{BRAIN}} * \text{Species}$) for each brain region. ANCOVA models used met all assumptions for linear regression and we used type III sums of squares. A significant interaction in this model would indicate a difference in slope between neurons and brain neurons, and a significant main effect of species would indicate a difference in intercept between neurons and brain neurons, both of which would be evidence of mosaic evolution. We note that these methods do not account for phylogeny.

We evaluated differences between species using neuron density in the three major brain regions. We used density as a measure that is corrected for the allometric scaling of neuron number with brain mass. To test for differences in neuron density between species, we used a one-way ANOVA with type III sums of squares that predicted neuron density from species ($DN \sim \text{Species}$). As these data did not conform to the assumption of equal variance for linear regression, we used generalized least squares from the R package nlme (Pinheiro et al. 2019) that enabled the explicit estimation of variance structure between groups in the model. We used Tukey-Kramer post hoc tests to evaluate differences between species through the R package emmeans (Lenth 2021).

Next, we tested whether habitat complexity, species, or body size explained the differences in neuron density that we observed above. To do this, we used model comparison to test which of the following seven models best explained variation in neuron density (DN) in each brain region. These combinations of variables were chosen based on a priori hypotheses and to avoid autocorrelation between predictors.

- 1) $DN \sim \text{Species}$
- 2) $DN \sim \text{RHC}$
- 3) $DN \sim \text{SVL} + \text{Species}$
- 4) $DN \sim \text{SVL} + \text{RHC}$
- 5) $DN \sim \text{SVL} * \text{RHC}$

Due to unequal variance, we again modeled these relationships using generalized least squares from the R package nlme (Pinheiro et al. 2019) with the variance structure estimated using the grouping variable in each model. These models were fit via maximum likelihood so they could be used in model comparison procedures. All other assumptions of linear regression were met. We compared these models for each brain region using Akaike Information Criterion corrected for small sample sizes (AICc). This model selection approach ranks and weights the models according to support via AICc.

Results

Tables 4.1-4.5 present the sample size, relative habitat complexity (RHC) from Powell and Leal (2014), and mean SVL, weight, region mass, number of neurons, density of neurons, number of nonneurons, density of nonneurons, and nonneuron to neuron ratio within each brain region for each species. Species significantly differ in mean brain mass (ANOVA, $n=44$, $F_{4,39}=24.726$, $p<0.001$). Post hoc tests revealed that *A. stratulus* had the smallest brain mass compared to the other species (Tukey-Kramer contrasts, $p<0.001$), although the difference in brain mass between *A. stratulus* and *A. krugi* was marginal (Tukey-Kramer contrast, $p=0.051$). Post hoc tests also revealed that *A. krugi* had the next smallest brain mass compared to the other species (Tukey-Kramer contrasts, $p<0.01$).

Anolis cristatellus, *A. evermanni*, and *A. gundlachi* did not differ significantly in brain mass in post hoc tests (Tukey-Kramer contrasts, $p > 0.05$).

Concerted and mosaic evolution of neuron numbers

All brain regions showed a significant positive correlation across species between the number of neurons within that region and the number of whole brain neurons

($r(42)_{\text{TEL}}=0.84$, $p < 0.001$; $r(42)_{\text{CER}}=0.60$, $p < 0.001$; $r(42)_{\text{ROB}}=0.76$, $p < 0.001$), indicating that standard major axis regression was appropriate. No differences between species were detected among the residuals of the standard major axis regression via ANOVA in the telencephalon ($n=44$, $F_{4,39}=1.541$, $p=0.21$), cerebellum ($n=44$, $F_{4,39}=0.654$, $p=0.63$), or the ROB ($n=44$, $F_{4,39}=0.178$, $p=0.95$). The interaction between species and total brain neurons in the ANCOVA was not significant in any brain region, indicating a shared slope across species. Total brain neurons were a significant, positive predictor in the ANCOVA model of telencephalon neurons ($n=44$, $F_{1,38}=86.204$, $p < 0.001$), cerebellum neurons ($n=44$, $F_{1,38}=11.246$, $p=0.002$), and ROB neurons ($n=44$, $F_{1,38}=34.181$, $p < 0.001$). Species was a significant predictor in the ANCOVA model of telencephalon neurons ($n=44$, $F_{4,38}=2.715$, $p=0.04$), a marginally significant predictor of ROB neurons ($n=44$, $F_{4,38}=2.526$, $p=0.06$), and a nonsignificant predictor of cerebellum neurons ($n=44$, $F_{4,38}=1.061$, $p=0.39$). Post hoc tests revealed in the telencephalon a significantly higher intercept in *A. gundlachi* compared to *A. evermanni* (Tukey-Kramer contrast, $p=0.04$) and marginally significant higher intercept in *A. gundlachi* compared to *A. stratulus* (Tukey-Kramer contrast, $p=0.07$). Post hoc tests revealed in the ROB marginally significant differences with *A. evermanni* having a higher intercept than *A. cristatellus* (Tukey-

Kramer contrast, $p=0.09$) and *A. gundlachi* (Tukey-Kramer contrast, $p=0.08$). Taken together, these results partially support our hypothesis of concerted evolution through a shared scaling relationship in all brain regions and shared intercept in the cerebellum. Conversely, these results partially reject our hypothesis with evidence of mosaic evolution in the telencephalon and ROB through differences in intercepts among certain species. These results are summarized in Fig. 4.1.

Species differences in neuron density

The ANOVA model showed significant differences between species in neuron density in the telencephalon ($n=49$, $F_{4,44}=5.631$, $p<0.001$), cerebellum ($n=46$, $F_{4,41}=8.639$, $p<0.001$), and ROB ($n=47$, $F_{4,42}=19.20$, $p<0.001$). These results are shown in Fig. 4.2. Post hoc tests revealed in the telencephalon that *A. stratulus* had significantly higher neuron density than both *A. cristatellus* (Tukey-Kramer contrast, $p=0.01$), *A. evermanni* (Tukey-Kramer contrast, $p=0.03$), and *A. krugi* (Tukey-Kramer contrast, $p=0.03$) and a marginally significant higher neuron density than *A. gundlachi* (Tukey-Kramer contrast, $p=0.07$). Post hoc tests revealed in the cerebellum that *A. stratulus* had significantly higher neuron density than both *A. gundlachi* (Tukey-Kramer contrast, $p=0.003$) and *A. krugi* (Tukey-Kramer contrast, $p=0.03$) and marginally significant higher neuron density than *A. evermanni* (Tukey-Kramer contrast, $p=0.08$). Post hoc tests also revealed in the cerebellum that *A. cristatellus* had significantly higher neuron density than *A. gundlachi* (Tukey-Kramer contrast, $p=0.001$). Post hoc tests revealed in the ROB that *A. stratulus* has a significantly higher neuron density than *A. cristatellus* (Tukey-Kramer contrast, $p=0.002$), *A. gundlachi* (Tukey-Kramer contrast, $p=0.001$), and *A. krugi* (Tukey-Kramer

contrast, $p < 0.001$). Post hoc tests also revealed in the ROB that *A. gundlachi* had a significantly lower neuron density than *A. cristatellus* (Tukey-Kramer contrast, $p = 0.04$), *A. evermanni* (Tukey-Kramer contrast, $p < 0.001$), and *A. krugi* (Tukey-Kramer contrast, $p = 0.02$). Taken together, these results reject our hypothesis by showing differences between species in neuron density across all brain regions. We observed that *A. stratulus* consistently had the highest neuron densities and *A. gundlachi* and *A. krugi* consistently had the lowest neuron densities among the species examined.

Neuron density and habitat complexity

Each brain region had two to three models that received most of the AIC weight. The top models for each brain region are presented in Table 4.6, and results of the full model selection are reported in the appendix to this chapter in Tables A4.1-A4.3. In the telencephalon, the models containing the predictors RHC, SVL, and species were best supported. In the cerebellum, the models containing the predictors species and SVL were best supported. In the ROB, the models containing the predictors RHC, SVL, and the interaction between RHC and SVL were best supported. Lower values of RHC indicate more complex habitats, and as such RHC is negatively correlated with neuron density, such that species found in more complex habitats have higher neuron densities. SVL is negatively correlated with neuron density, such that species with greater SVL have lower neuron density. Taken together, these results reject our hypothesis by suggesting an association between habitat complexity and neuron density in the telencephalon and ROB. However, our hypothesis was supported in the cerebellum, where we did not find a strong association with habitat complexity. These results are summarized in Fig. 4.2.

Discussion

In this chapter, we present estimates of the number and density of neurons and nonneurons in the brains of five species from the Puerto Rican anole radiation. We found that the relative number of neurons in the telencephalon, cerebellum, and ROB primarily follow a shared relationship across species, providing further evidence for concerted brain evolution in Puerto Rican *Anolis*. We found that species differed in neuron density in all brain regions, with *A. stratulus* having the highest neuron densities and *A. gundlachi* and *A. krugi* having the lowest neuron densities. We found that RHC, SVL and species were the best predictors for neuron density across species in our sample. Together, these results provide new insights into brain evolution across the Puerto Rican anole radiation that were not apparent at the level of brain volume, suggesting a link between habitat complexity and neuroanatomy in these lizards.

Our results support our hypothesis by providing evidence for concerted evolution in the telencephalon, cerebellum, and ROB of Puerto Rican *Anolis*. The number of neurons varied as a shared function of total brain neurons across species in each brain region, suggesting a common scaling relationship across species. This aligns with the results of a previous study on brain evolution in Puerto Rican *Anolis*, which found that the volume of six major regions of the anole brain varied as a shared function of brain size (Powell and Leal 2012). The slope of the relationship between the number of neurons and total brain neurons in each brain region was qualitatively similar to those found in Powell and Leal (2012). Concerted evolution is thought to be the primary factor shaping brain evolution across vertebrates (Striedter 2005), and evidence of concerted

evolution has been found in many vertebrate clades (Herculano-Houzel, Manger, et al. 2014), including lizards (Hoops et al. 2017). The common scaling of the brain and its regions is thought to be the result of conserved processes governing development of the brain (Striedter 2005).

Although all species followed a similar scaling relationship, we found evidence of mosaic evolution in the telencephalon and ROB, indicated by some species having different intercepts. Interestingly, in the telencephalon, *A. gundlachi* shows a significantly higher intercept, indicating that *A. gundlachi* had a larger proportion of its total brain neurons in the telencephalon than *A. evermanni* and *A. stratulus*. However, the opposite trend is observed in neuron density, where *A. gundlachi* had a lower neuron density than *A. evermanni* and *A. stratulus*. This suggests that *A. gundlachi* has a greater quantity of neurons that are larger or more distributed compared to the other species examined here. This may be expected, given that the telencephalon in vertebrates typically increases disproportionately in size as brains get larger compared to other brain regions (Striedter 2005). It is possible that the large proportion of neurons we observed in the telencephalon is the result of the interaction between telencephalon allometry and body size in *A. gundlachi*. However, the telencephalon of *A. gundlachi* is similar in size to the telencephalon of *A. evermanni*, despite a difference in intercept observed between these species. Similarly, this does not explain the differences observed in the ROB between *A. evermanni*, *A. cristatellus*, and *A. gundlachi*, as these species all share similar brain and body size. Further evidence of neuronal scaling in *Anolis* is necessary before we can conclude that mosaic evolution is acting in the telencephalon and ROB. Future research

examining additional *Anolis* species on multiple island radiations may provide unique insights into mosaic and concerted evolution in the brains of these species.

We found differences in neuron density among the five species we examined. In general, *A. stratulus* consistently exhibited the highest neuron density in all brain regions and *A. gundlachi* and *A. krugi* consistently showed the lowest neuron density. These differences can be partially explained by a grade shift along the scaling relationship of neuron number and brain mass. Due to the negative allometry of neuron number with brain mass, larger brains tend to have lower densities of neurons than smaller brains (Changizi 2001; Herculano-Houzel, Manger, et al. 2014). In our dataset, *A. gundlachi* has the largest brain while *A. stratulus* has the smallest brain. However, this does not explain the differences between *A. krugi* and *A. stratulus*, as these species have similar brain mass and body size.

The differences we observed in neuron density could impact neural function. It has been suggested that higher neuron packing density reduces interneuronal distance, which may enable signals to transmit faster and more efficiently in the brain (Changizi 2001; Chittka and Niven 2009; Dicke and Roth 2016). It is well-established that primate brains (Collins et al. 2010; Herculano-Houzel, Manger, et al. 2014) as well as some bird brains (Olkowicz et al. 2016) have exceptionally high neuron densities, which may contribute to their advanced cognitive abilities. Neuron density may also be a strong correlate of connectivity, at least in primates (Beul and Hilgetag 2019). However, very few empirical studies have explored the implications of neuronal density on neural function. Mice with artificially increased densities of cortical neurons were found to be better at visual discrimination than wild type conspecifics (Fang and Yuste 2017).

Diurnal primates, which rely heavily on vision, have high neuron densities in visual areas of the cerebral cortex (Collins et al. 2010). Male zebra finches show dramatic increases in neuron density with age in their high vocal center, which is related to song learning (Walton et al. 2012). Although these studies provide some evidence that neuron density is related to differences in function, it is still largely unknown what functional implications increased neuron density has on behavior.

We used measures of relative habitat complexity from a previous study of brain evolution in Puerto Rican *Anolis* (Powell and Leal 2014). This variable can be described as the degree to which the habitat constrains movement in the microhabitats utilized by each species. In general, species living higher in the canopy experience the most complex habitats while species living in the understory, such as on trunks, grass, and shrubs, experience the least complex habitats. The cognitive demands experienced by a lizard are likely higher in more complex habitats, as the animal must coordinate its movement along a larger number of potential paths to accomplish foraging, territory defense, mating, etc. Other studies have linked variation in habitat complexity to neuroanatomy in diverse clades (Bauchot et al. 1977; Budeau and Verts 1986; Abbott et al. 1999; Safi and Dechmann 2005; Shumway 2010; Sobrero et al. 2016).

Our results reject our hypothesis that species differences in habitat complexity would not be reflected in neuroanatomy. We found that habitat complexity was a well-supported predictor of neuron density across species in the telencephalon and ROB. The telencephalon is responsible for the integration of sensory information from the environment and associated with diverse cognitive function. The increased neuron density in the telencephalon may provide increased sensory resolution that facilitates the

navigation of complex habitats in arboreal species, similar to how increased neuron density in visual areas of the cerebral cortex may support high acuity vision (Collins et al. 2010).

Habitat complexity was also a well-supported predictor of neuron density across species in the ROB. The brain regions contained in the ROB are generally involved in sensory input and motor output, and these regions are not typically implicated in cognition. It is possible that the neuronal changes in the ROB facilitate motor plasticity, which could be important in navigating complex habitats. New Caledonian crows, which are known for their exceptional tool use and ability to manipulate objects, have a larger tegmentum and septum compared to other species (Mehlhorn et al. 2010), both of which are regions encompassed in the ROB. A previous study found a larger number of glia in the ROB of *A. evermanni* compared to *A. cristatellus*, which correlated with better performance on an extractive foraging task (Storks et al. 2020). However, the ROB represents multiple different brain regions, making it difficult to conclude how it may relate to habitat complexity. Future research should further evaluate neuronal density of the separate brain regions included in the ROB in *Anolis* to begin to understand how these hind- and midbrain regions vary across species.

Cerebellum neuron density is best predicted by SVL and species, suggesting that habitat complexity does not explain variation in cerebellum neuron density as it does in the telencephalon and ROB. This is unexpected, as the cerebellum is responsible for coordinating motor activity (Powell and Leal 2012), which would presumably be important in coordinating motor movements in navigating complex habitats. It is possible that we were unable to detect such a trend in our dataset, as the cerebellum is extremely

small in *Anolis*, which makes it more difficult to weigh precisely resulting in greater variation in neuron density. Alternatively, neuron density in the cerebellum may be linked to body size or another factor that we did not consider in this study.

In every brain region, SVL was a well-supported predictor of neuron density. This is expected, as it is well known that body size is tightly linked with brain size (Striedter 2005). In the case of neuron density, increasing body and brain size is associated with decreased neuronal densities due to the negative allometry of neuron number and brain size (Changizi 2001; Herculano-Houzel, Manger, et al. 2014). The inclusion of SVL in the best supported models across brain regions suggests that SVL explains additional variation that is not explained by habitat complexity.

A previous study found that differences between species in habitat complexity were not reflected in the volumes of six brain regions (Powell and Leal 2014). Our results demonstrate the potential of evaluating neuroanatomy at finer scales in studying brain evolution, as has been emphasized by recent research exploring neuron numbers in various vertebrate clades (Herculano-Houzel 2011; Herculano-Houzel, Avelino-de-Souza, et al. 2014; Herculano-Houzel, Manger, et al. 2014; Ngwenya et al. 2016; Olkowitz et al. 2016). On the other hand, our data confirm the results of a previous study in the same species (Powell and Leal 2014) by finding evidence of primarily concerted evolution of neuron number. The study by Powell and Leal (2014) found a trend suggesting interspecific differences in the relative size of the cerebellum. In our study, the cerebellum is the only region showing no evidence of interspecific variation, although this could be due to the lower correlation of cerebellum neuron number with total brain neurons across species.

One potential implication of our findings is that there may be a tradeoff in brain evolution between gaining more neurons through increasing brain size and packing neurons at higher density through minimizing brain size. If increased neuronal density facilitates signal transmission between neurons in the brain (Chittka and Niven 2009; Collins et al. 2010; Dicke and Roth 2016), increases in brain size may have diminishing returns due to not only the increasing costs of neural tissue but the decreasing efficiency of signaling between neurons due to decreasing neuronal densities. Such a tradeoff may affect brain evolution within the allometric constraints of neuronal scaling, and potentially select for changes in neural development that deviate from allometric constraints. Further research is required to understand the implications of neuron density for neural function and brain evolution across species.

In conclusion, we find evidence for concerted evolution of neuron number in the telencephalon, cerebellum, and ROB across the Puerto Rican anole radiation. Furthermore, we find evidence that species differ in neuronal density in all brain regions examined and that these differences are partially explained by habitat complexity in the telencephalon and ROB. Together, our results reveal the unique insights that can be gained into brain evolution by evaluating neuron numbers (Nemec and Osten 2020). In the future, comparing neuron numbers across diverse species has the potential to expand our understanding of brain evolution and adaptive specialization in vertebrates.

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Tables and Figures

Table 4.1: Mean SVL, weight, and relative habitat complexity for each species. Means are presented with standard error. RHC is from Powell and Leal (2014).

Species	<i>n</i>	SVL (cm)	Weight (g)	RHC
<i>Anolis cristatellus</i>	12	5.6 ± 0.084	4.6 ± 0.25	2.6196
<i>Anolis evermanni</i>	10	5.6 ± 0.20	4.4 ± 0.41	-5.1598
<i>Anolis gundlachi</i>	8	6.0 ± 0.11	4.9 ± 0.31	8.1306
<i>Anolis krugi</i>	10	4.8 ± 0.097	2.5 ± 0.19	3.2436
<i>Anolis stratulus</i>	9	4.3 ± 0.13	1.6 ± 0.12	-8.3974

Table 4.2: Mean mass, number and density of neurons and nonneurons, and nonneuron to neuron ratio for the whole brain of each species. Means are presented with standard error.

Species	<i>n</i>	Mass _{BBR} (mg)	<i>N</i> × 10 ⁶	<i>O</i> × 10 ⁶	DN × 10 ⁶ (N/mg)	DO × 10 ⁶ (O/mg)	<i>O</i> · <i>N</i>
<i>A. cristatellus</i>	10	48.0 ± 1.77	5.13 ± 0.114	1.79 ± 0.126	0.108 ± 0.00526	0.0374 ± 0.00222	0.35 ± 0.021
<i>A. evermanni</i>	8	45.3 ± 3.20	4.86 ± 0.229	1.82 ± 0.128	0.109 ± 0.00546	0.0407 ± 0.00314	0.37 ± 0.021
<i>A. gundlachi</i>	8	50.5 ± 2.02	4.16 ± 0.270	1.64 ± 0.160	0.0836 ± 0.00684	0.0334 ± 0.00390	0.40 ± 0.040
<i>A. krugi</i>	9	36.0 ± 1.28	3.85 ± 0.222	1.16 ± 0.0760	0.108 ± 0.00839	0.0326 ± 0.00233	0.31 ± 0.018
<i>A. stratulus</i>	9	30.2 ± 0.875	4.60 ± 0.173	1.41 ± 0.0595	0.153 ± 0.00569	0.0466 ± 0.00171	0.31 ± 0.015

Table 4.3: Mean mass, number and density of neurons and nonneurons, and nonneuron to neuron ratio for the telencephalon of each species. Means are presented with standard error.

Species	n	Mass _{TEL} (mg)	N x 10 ⁶	O x 10 ⁶	DN x 10 ⁶ (N/mg)	DO x 10 ⁶ (O/mg)	O.N	Prop Mass	Prop N
<i>A. cristatellus</i>	12	17.8 ± 0.765	1.97 ± 0.0713	0.369 ± 0.0292	0.112 ± 0.00562	0.0210 ± 0.00186	0.19 ± 0.012	0.37 ± 0.0054	0.39 ± 0.011
<i>A. evermanni</i>	10	15.6 ± 1.03	1.73 ± 0.0800	0.441 ± 0.0305	0.113 ± 0.00650	0.0289 ± 0.00215	0.26 ± 0.013	0.36 ± 0.0043	0.35 ± 0.012
<i>A. gundlachi</i>	8	17.8 ± 1.10	1.70 ± 0.166	0.371 ± 0.0311	0.0993 ± 0.0128	0.0219 ± 0.00279	0.23 ± 0.023	0.35 ± 0.0098	0.40 ± 0.017
<i>A. krugi</i>	10	13.3 ± 0.603	1.37 ± 0.101	0.315 ± 0.0259	0.105 ± 0.00895	0.0243 ± 0.00235	0.23 ± 0.015	0.37 ± 0.0050	0.36 ± 0.019
<i>A. stratulus</i>	9	11.1 ± 0.276	1.64 ± 0.117	0.343 ± 0.0323	0.147 ± 0.00814	0.0312 ± 0.00324	0.22 ± 0.024	0.37 ± 0.0059	0.35 ± 0.017

Table 4.4: Mean mass, number and density of neurons and nonneurons, and nonneuron to neuron ratio for the cerebellum of each species. Means are presented with standard error.

Species	n	Mass _{CER} (mg)	N x 10 ⁶	O x 10 ⁶	DN x 10 ⁶ (N/mg)	DO x 10 ⁶ (O/mg)	O.N	Prop Mass	Prop N
<i>A. cristatellus</i>	11	1.8 ± 0.10	0.851 ± 0.0569	0.104 ± 0.0315	0.468 ± 0.0259	0.0526 ± 0.0133	0.11 ± 0.029	0.038 ± 0.0018	0.17 ± 0.014
<i>A. evermanni</i>	8	1.7 ± 0.12	0.619 ± 0.0622	0.0445 ± 0.00756	0.369 ± 0.0464	0.0270 ± 0.00463	0.076 ± 0.013	0.038 ± 0.0017	0.13 ± 0.012
<i>A. gundlachi</i>	8	2.4 ± 0.10	0.572 ± 0.0621	0.0453 ± 0.00963	0.249 ± 0.0333	0.0201 ± 0.00488	0.074 ± 0.011	0.047 ± 0.0012	0.14 ± 0.013
<i>A. krugi</i>	10	1.5 ± 0.075	0.532 ± 0.0787	0.0292 ± 0.00490	0.357 ± 0.0448	0.0195 ± 0.00308	0.056 ± 0.0083	0.041 ± 0.0019	0.14 ± 0.021
<i>A. stratulus</i>	9	1.3 ± 0.050	0.728 ± 0.0593	0.0621 ± 0.00886	0.544 ± 0.0425	0.0455 ± 0.00577	0.087 ± 0.012	0.045 ± 0.0021	0.16 ± 0.011

Table 4.5: Mean mass, number and density of neurons and nonneurons, and nonneuron to neuron ratio for the ROB of each species. Means are presented with standard error.

Species	n	Mass _{ROB} (mg)	N x 10 ⁶	O x 10 ⁶	DN x 10 ⁶ (N/mg)	DO x 10 ⁶ (O/mg)	O.N	Prop Mass	Prop N
<i>A. cristatellus</i>	11	28.0 ± 0.863	2.29 ± 0.109	1.29 ± 0.0697	0.0830 ± 0.00539	0.0460 ± 0.00198	0.58 ± 0.051	0.59 ± 0.0056	0.45 ± 0.020
<i>A. evermanni</i>	10	26.4 ± 1.68	2.62 ± 0.128	1.32 ± 0.0781	0.102 ± 0.00683	0.0511 ± 0.00364	0.51 ± 0.037	0.60 ± 0.0042	0.52 ± 0.016
<i>A. gundlachi</i>	8	30.3 ± 0.978	1.88 ± 0.0844	1.23 ± 0.137	0.0625 ± 0.00313	0.0414 ± 0.00336	0.65 ± 0.074	0.60 ± 0.0096	0.46 ± 0.016
<i>A. krugi</i>	9	21.0 ± 0.664	1.90 ± 0.132	0.814 ± 0.0675	0.0920 ± 0.00854	0.0388 ± 0.00306	0.44 ± 0.044	0.58 ± 0.0050	0.49 ± 0.022
<i>A. stratulus</i>	9	17.8 ± 0.666	2.23 ± 0.105	1.00 ± 0.0694	0.127 ± 0.00801	0.0562 ± 0.00296	0.46 ± 0.043	0.59 ± 0.0065	0.49 ± 0.019

Table 4.6: Top ranked models for each brain region. Models are included only if AICc weight is greater than 0.10.

<i>Rank</i>	<i>Model</i>	<i>df</i>	<i>AICc</i>	$\Delta AICc$	<i>Weight</i>	<i>logLik</i>
Telencephalon						
1	DN ~ RHC	7	-75.59	0.00	0.45	46.16
2	DN ~ SVL + RHC	8	-74.89	0.70	0.32	47.25
3	DN ~ Species	10	-73.09	2.51	0.13	49.44
Cerebellum						
1	DN ~ Species	10	-39.31	0.00	0.83	32.80
2	DN ~ SVL + Species	11	-35.97	3.34	0.16	32.87
ROB						
1	DN ~ SVL + RHC	8	-89.04	0.00	0.54	54.41
2	DN ~ SVL*RHC	9	-88.47	0.57	0.41	55.67

Fig. 4.1: Linear relationship between number of neurons per region and the number of neurons in the whole brain. All species follow a shared regression slope in each region. Significant differences in intercept between species are only observed in the telencephalon and cerebellum.

Fig. 4.2: Linear relationship of neuron density (N/mg) and relative habitat complexity (RHC). More negative RHC indicate more complex habitat. Means are colored by species and error bars represent standard error on the mean.

Appendix

Table A4.1: Results of model selection for the telencephalon. AICc was used due to small sample size. All models were generated using the gls function in the R package nlme (Pinheiro, 2019) and estimated using maximum likelihood prior to model selection. Output is generated from the aictab function in the R package AICcmodavg (Mazerolle, 2020).

<i>Rank</i>	<i>Model</i>	<i>df</i>	<i>AICc</i>	<i>ΔAICc</i>	<i>Weight</i>	<i>logLik</i>
1	DN ~ RHC	7	-75.59	0.00	0.45	46.16
2	DN ~ SVL + RHC	8	-74.89	0.70	0.32	47.25
3	DN ~ Species	10	-73.09	2.51	0.13	49.44
4	DN ~ SVL*RHC	9	-71.95	3.64	0.07	47.28
5	DN ~ SVL + Species	11	-69.80	5.79	0.02	49.47

Table A4.2: Results of model selection for the cerebellum. AICc was used due to small sample size. All models were generated using the gls function in the R package nlme (Pinheiro, 2019) and estimated using maximum likelihood prior to model selection. Output is generated from the aictab function in the R package AICcmodavg (Mazerolle, 2020).

<i>Rank</i>	<i>Model</i>	<i>df</i>	<i>AICc</i>	<i>ΔAICc</i>	<i>Weight</i>	<i>logLik</i>
1	DN ~ Species	10	-39.31	0.00	0.83	32.80
2	DN ~ SVL + Species	11	-35.97	3.34	0.16	32.87
3	DN ~ RHC	7	-29.40	9.91	0.01	23.17
4	DN ~ SVL + RHC	8	-27.00	12.31	0.00	23.45
5	DN ~ SVL*RHC	9	-23.98	15.33	0.00	23.49

Table A4.3: Results of model selection for the ROB. AICc was used due to small sample size. All models were generated using the gls function in the R package nlme (Pinheiro, 2019) and estimated using maximum likelihood prior to model selection. Output is generated from the aictab function in the R package AICcmodavg (Mazerolle, 2020).

<i>Rank</i>	<i>Model</i>	<i>df</i>	<i>AICc</i>	<i>ΔAICc</i>	<i>Weight</i>	<i>logLik</i>
1	DN ~ SVL + RHC	8	-89.04	0.00	0.54	54.41
2	DN ~ SVL*RHC	9	-88.47	0.57	0.41	55.67
3	DN ~ SVL + Species	11	-84.02	5.02	0.04	56.78
4	DN ~ RHC	7	-77.17	11.87	0.00	47.02
5	DN ~ Species	10	-72.55	16.48	0.00	49.33

CHAPTER 5

CONCLUSION

Throughout this dissertation, I have investigated the complex ways that cognition, the brain, and ecology interact in *Anolis* lizards. This body of work exemplifies my interest in connecting multiple levels of biological organization in an evolutionary context, including neuroanatomy, behavior, and habitat. I found that wild, free-living lizards could solve a novel detour task under natural conditions, exhibiting an unexpected degree of motor self-regulation. I found neuroanatomical differences in the brains of different species of *Anolis* associated with differences in cognition and habitat complexity. I developed and implemented novel techniques to evaluate behavioral flexibility under natural conditions and to evaluate the number and density of neurons and nonneurons in major regions of the *Anolis* brain. Although this research has presented many challenges, this work has generated novel insights into cognition and the brain of *Anolis* lizards and establishes new directions for future investigation.

Summary of Research Results

CHAPTER 2

THINKING OUTSIDE THE BOX: PROBLEM-SOLVING IN FREE-LIVING LIZARDS

- Wild, free-living *Anolis sagrei* were able to solve the novel detour problem under natural conditions. Individuals varied in their performance.
- Individuals that solved the detour problem improved their performance across trials. This was unexpected given the sit-and-wait foraging behavior of *Anolis*.

- This study provides evidence of motor self-regulation in a sit-and-wait predator and underscores the need to evaluate cognition under natural conditions, particularly in species with poor performance in the laboratory.

CHAPTER 3

PEEKING INSIDE THE LIZARD BRAIN: NEURON NUMBERS IN *ANOLIS* AND ITS IMPLICATIONS FOR COGNITIVE PERFORMANCE AND VERTEBRATE BRAIN EVOLUTION

- This study presents estimates of neuron and nonneuron number and density of the telencephalon, cerebellum, and ROB in *Anolis cristatellus* and *Anolis evermanni*, representing the first such data published from squamate reptiles.
- We present an isotropic fractionator protocol optimized for the unique challenges that arise in species with nucleated erythrocytes and relatively small brains.
- The density of neurons in the telencephalon, cerebellum, and ROB followed similar patterns as that observed in other vertebrates.
- A higher proportion of *Anolis evermanni* individuals solved the extractive foraging task compared to *Anolis cristatellus*.
- These differences between species were not mirrored in the number or density of neurons in any brain region. However, the brain of *Anolis evermanni* had a greater number of nonneurons than *Anolis cristatellus*, which was driven primarily by a greater number of nonneurons in the ROB.
- Nonneurons are primarily glia, which could contribute to differences in performance observed in the extractive foraging task.

- These results underscore the potential of comparing neuroanatomy at the level of neuron number and density. Data from more species is necessary before we can fully understand the evolution of neuron number across vertebrates.

CHAPTER 4:

NEURONAL EVOLUTION AND HABITAT COMPLEXITY ACROSS THE PUERTO RICAN ANOLE RADIATION

- The brains of five species of Puerto Rican *Anolis* primarily follow the predictions of concerted evolution in their neuronal scaling relationships.
- Some evidence of mosaic evolution is found in the telencephalon and ROB with some species showing differing intercepts.
- These five species differ in neuron density in the telencephalon, cerebellum, and ROB. Across brain regions, *Anolis stratulus* had consistently higher neuron density than *Anolis gundlachi* and *Anolis krugi*.
- These differences are explained by habitat complexity in the telencephalon and ROB, but not the cerebellum. Both SVL and species were also significant predictors.
- This study builds on previous investigation of brain evolution in the Puerto Rican anoles that focused on brain volume. Our results demonstrate the unique insights that can be gained from evaluating neuron numbers in the context of brain evolution.

Future Directions

Taken together, the results presented in this dissertation demonstrate the potential for studying cognition and neuroanatomy in an evolutionary context. The methods applied in my second chapter can be used in the future to explore the connection between cognition and fitness in lizards. Although cognition has been suggested to contribute to fitness in many different ways, studies connecting cognition and fitness are rare. Due to their small size and relatively short lifespan, lizards are a tractable model for studying cognition and fitness under natural conditions. My third and fourth chapters took a novel approach by studying neuroanatomical differences between species in neuron number and density, and generated novel insights into brain evolution in *Anolis*. Neuron numbers are potentially a more informative comparative metric for studying species differences in the brain, as these parameters are more closely tied to neural function. Future work on more species of *Anolis* from additional island radiations could expand upon the data presented here and provide insight into how ecomorphological differences in habitat specialization affect brain evolution in *Anolis* and vertebrates in general. Further study of cognition and the brain in lizards and other ectotherms will expand our understanding of the evolutionary selective pressures shaping cognition and neuroanatomy across vertebrates.

VITA

Levi Storks was raised in Clinton Township, Michigan. As a child, he loved to watch animals, and this eventually led to him amassing a large collection of reptiles and amphibians. He still has one of his first pet turtles over 20 years later. After attending Chippewa Valley Schools and graduating at the top of his class, Levi attended Michigan State University where he pursued a degree in zoology. Although at first he intended to pursue veterinary school, his plans quickly changed when he found research. During his undergraduate career Levi worked in the lab of Dr. Juli Wade, where he assisted with projects exploring the neuroscience of sex differences in zebra finches and anole lizards. Later in his undergraduate career he found an interest in animal behavior, which led to him working as a research assistant at Kellogg Biological Station. Here he completed a project on calling behavior in gray treefrogs and was introduced to field work. At the same time, Levi worked as an undergraduate learning assistant in a chemistry course. This journey fostered a love of both research and teaching and led to him pursuing graduate school with Dr. Manuel Leal at the University of Missouri. As a graduate student, Levi has further developed his skills in research, teaching, and outreach, all of which now contribute to his identity as a scientist. Levi is the first person in his family to complete a PhD. Next, Levi will begin a postdoctoral position with Dr. Ryan Wong at the University of Nebraska Omaha, where he will further pursue his passions of research, teaching, and outreach.