FOOD FOR THOUGHT: THE EFFECTS OF FEEDING ON NEUROGENESIS IN THE
BALL PYTHON, PYTHON REGIUS

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ABSTRACT

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Hannah Fern Bow

Pythons are a well-studied model of postprandial physiological plasticity. Consuming a meal has been shown by past work to evoke a suite of physiological changes in pythons and elicit one of the largest documented increases in post-feeding metabolic rates relative to resting values. However, little is known about how this plasticity manifests in the brains of ball pythons, *Python regius*. Previous work using the cell-birth marker 5-bromo-12′-deoxyuridine (BrdU) has shown that cell proliferation in the python brain increases six days following meal consumption. This study aimed to confirm these findings and build on them in the long term by tracking the survival and maturation of these newly created cells across a two-month period. We investigated whether these cells differentiated into neurons using double-immunofluorescence for BrdU and a reptile-specific neuronal marker (Fox3). We did not find significantly greater rates of cell proliferation in snakes six days after feeding, but we did observe more newly created cells in neurogenic regions in fed snakes two months after the meal. Feeding did not influence neurogenesis, but feeding does appear to have a neuroprotective effect. More newly created cells survived in fed snakes two months later, particularly in the olfactory bulbs and lateral cortex. These findings shed light on the extent of postprandial plasticity and regional differences in the creation of new neural cells in the brains of ball pythons.
Keywords: postnatal neurogenesis, BrdU, postprandial plasticity, SDA, heat increment of feeding

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<tr>
<td>AC</td>
<td>Anterior Cortex</td>
</tr>
<tr>
<td>ACC</td>
<td>Nucleus Accumbens</td>
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<tr>
<td>AOB</td>
<td>Acessory Olfactory Bulb</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
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<td>DC</td>
<td>Dorsal Cortex</td>
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<tr>
<td>DVR</td>
<td>Dorsal Ventricular Ridge</td>
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<td>LC</td>
<td>Lateral Cortex</td>
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<td>MC</td>
<td>Medial Cortex</td>
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<td>NS</td>
<td>Nucleus Sphericus</td>
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<td>OB</td>
<td>Olfactory Bulb</td>
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<td>PC</td>
<td>Posterior Cortex</td>
</tr>
<tr>
<td>RMS</td>
<td>Rostral Migratory Stream</td>
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<td>SN</td>
<td>Septal Nucleus</td>
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Chapter 1

PYTHON REGIUS AS A MODEL OF POSTPRANDIAL NEUROGENESIS

1.1 Introduction

Ball pythons (Python regius) have long served the scientific community as important models of extreme physiological plasticity following the consumption of a meal. However, little attempt has been made to investigate the extent of the postprandial response in the brains of these animals. In this chapter I provide background information on the existing research on postnatal neurogenesis across taxa, on digestive functions in ball pythons specifically, and on the known connections between feeding behavior and neurogenesis. I also explain how my research helps to fill some of the gaps in our understanding of these topics.

1.2 History of Neurogenesis Research

Prior to the 1960s, the field of neuroscience was dominated by the belief that the creation of new neurons was limited to the embryonic stage. Joseph Altman’s pioneering studies documenting neurogenesis in rodents (Altman, 1962, 1963) were the first to contradict this ideology, but his findings constituted such a dramatic shift in the contemporary scientific understanding that they were met with deep skepticism. A few studies in the next two decades confirmed these results in rats (Kaplan & Hinds, 1977) and mice (Kaplan & Bell, 1984) and unsuccessfully tried to substantiate them in primates (Rakic, 1974, 1985), but Fernando Nottebohm’s definitive observations of adult neurogenesis in songbirds was one of the first publications to seriously challenge the prevailing dogma (Goldman & Nottebohm, 1983; Nottebohm, 1985). In the following decades, the development of techniques such as immunohistochemistry with 5-bromo-2’-deoxyuridine (BrdU) and confocal microscopy allowed for a sharp increase in the number
of studies documenting convincing evidence of adult neurogenesis across a wide range of taxa, including humans (Eriksson et al., 1998; Gross, 2000). By the year 2000, the accumulation of support for the existence of postnatal neurogenesis was so irrefutable that a paradigm shift took place in the scientific community’s understanding of neuroplasticity (Gross, 2000).

The majority of research on adult neurogenesis has been carried out in rodents due to their phylogenetic proximity to humans and public interest in novel treatments for neurodegenerative diseases and mental illness. However, most of these studies have found that postnatal neurogenesis is relatively limited in mammals (Figure 1; Paredes et al., 2016). Cell proliferation in the brains of adult mammals almost exclusively functions in the replacement of existing glial cells and, to a lesser extent, neurons, such that the net number of neurons in an animal’s brain remains stable throughout its lifetime (Kaslin et al., 2008). Additionally, cell proliferation in the telencephalon of adult mammalian brains has been found to be restricted to just two defined regions, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Fares et al., 2019; Kaslin et al., 2008). Some preliminary studies have found evidence of the potential for neurogenesis in a few other brain regions such as the neocortex (Dayer et al., 2005), hypothalamus (Kokoeva et al., 2005), amygdala (Bernier et al., 2002), midbrain (Zhao et al., 2003), dorsal vagal complex (Bauer et al., 2005), and spinal cord (Yamamoto et al., 2001), but more research is needed to substantiate these observations. By contrast, reptiles have been shown to undergo higher rates of postnatal neurogenesis in terms of both cell proliferation and cell survival.
Figure 1.1. Neurogenesis is more widespread throughout the brains of reptiles than in mammals. Left panel: Renderings of the dorsal view of the brains of representatives from several major taxa, scaled to the 1cm bar at bottom. Middle and right columns: Schematics of sagittal and coronal cross sections through each brain. Green shading indicates germinal centers, red dots indicate destinations of newly generated neurons. cb: cerebellum, cx: cortex, dvr: dorsal ventricular ridge, hc: hippocampus, lcx: lateral cortex, lv: lateral ventricle, mcx: medial cortex, mms: medial migratory stream, ob: olfactory bulb, rms: rostral migratory stream, tlv: temporal lobe of the lateral ventricle. (Image from Paredes et al., 2016).
1.3 Neurogenesis in Reptiles

1.3.1 Proliferation

In reptiles, cell proliferation occurs in the ependyma along the lateral ventricles, referred to here as the ventricular zone (Font et al., 2001). The ventricular zone is composed of a pseudostratified columnar epithelium of radial glial cells, so named due to their long processes that project radially away from the ventricle (Font et al., 2001). These radial glial cells serve as stem cells for both newly created glial cells and neurons. Mitotically active cells have been documented in all major regions of the reptilian telencephalon, including the olfactory bulbs, accessory olfactory bulbs, retrobulbar region, septum, dorsal ventricular ridge, striatum, nucleus sphericus, and all subdivisions of the cortex, but rates of cell proliferation vary between regions and across species (Font et al., 2001). In ball pythons, the highest rates of cell proliferation have been documented in the accessory olfactory bulbs and retrobulbar region (Bales, 2014; Habroun et al., 2018).

1.3.2 Migration

After cell division, newly created daughter cells may remain in the ventricular zone and become radial glial cells themselves. However, many will migrate along the long processes of the radial glial cells into the surrounding parenchyma. The proportion of cells that migrate away from the ventricles varies among taxa. In the medial cortex of the Iberian wall lizard *Podarcis hispanica*, 90% of newly generated cells migrate away from the ventricles, whereas only 30% of cells migrate away in the tropical lizard *Tropidurus hispidus* (López-García et al., 1990; Marchioro et al., 2005). In ball pythons, migration rates may be correlated with brain region, as migration away from the ventricles was found
to be higher in the accessory olfactory bulb, dorsal ventricular ridge, medial cortex, and nucleus sphericus (Bales, 2014).

In most cases, cells migrate short distances into the parenchyma directly adjacent to their birthplaces in the ventricular zone. However, there is evidence in lizards and ball pythons that cells may migrate longer distances to the olfactory bulbs and accessory olfactory bulbs from regions located more caudally in the telencephalon (Bales, 2014; Pérez-Cañellas et al., 1997; Pimentel et al., 2021). This migration pathway parallels the mammalian rostral migratory stream, the pattern by which newly created neurons migrate from the subventricular zone to the olfactory bulbs (Pencea et al., 2001; Sun et al., 2010). However, further study is required to determine the extent of the similarity across taxa (Font et al., 2001).

1.3.3 Maturation and Survival

In as little as a week after they are created, new cells incorporate into the parenchyma neighboring the ventricular zone (López-García et al., 1990). Postnatally generated neurons have been documented in every region of the reptilian telencephalon, including the olfactory bulbs, accessory olfactory bulbs, retrobulbar region, septum, dorsal ventricular ridge, striatum, nucleus sphericus, and all subdivisions of the cortex (Font et al., 2001). In ball pythons, survival rates have been found to be highest in the accessory olfactory bulbs, anterior lateral cortex, anterior medial cortex, and anterior nucleus sphericus (Bales, 2014). Newly created cells may differentiate into glial cells or neurons, and the proportion of each cell type varies across taxa. In lizards, the majority of newly created cells will differentiate into neurons, whereas in turtles, both neurons and glial cells are produced (López-García et al., 1988; Pérez-Cañellas et al., 1997). Prior to this study, the phenotype of postnatally
generated neural cells in snakes was unknown (Bales, 2014; Habroun et al., 2018; Maine et al., 2014).

Neurons created in adulthood survive and integrate into the existing neural network at much higher rates in reptiles than in mammals. In both mammals and birds, neuron numbers reach a plateau relatively early in life, but in lizards, neuron number increases indefinitely with age and size (Font et al., 2001; López-García et al., 1984). Few to no degenerating cells have been found in the brains of reptiles studied, suggesting that the cells produced are relatively long-lived. This contrasts with postnatally generated mammalian neural cells, the majority of which undergo apoptosis without ever integrating into the neural network (Font et al., 2001; López-García et al., 1990). It has been hypothesized that the structure of mammal brains may contribute to this difference in neurogenic capacity. The greater complexity of mammalian neural networks may more tightly restrict the integration of newly created neurons in comparison to reptiles, leading to mass death of cells that are unable to form life-sustaining connections with other neurons (Paredes et al., 2016).

1.3.4 Regenerative Capacity

Low levels of constitutive neurogenesis contribute to the constant lifetime growth of the reptilian brain, but there is evidence to suggest that acute neuronal damage can spark a high rate of neurogenic activity and regeneration. The neurotoxin 3-acetylpyridine (3AP) has been used in many studies to induce widespread apoptosis affecting up to 95% of neurons throughout the medial cortex and, to a lesser extent, other cortical areas (Font et al., 1991; López-García et al., 1992, 2002; Molowny et al., 1995). These massive cell death events are succeeded within a week by high rates of cell proliferation and migration of
replacement neurons, resulting in regeneration of damaged areas (Font et al., 1991; López-García et al., 1992, 2002; Molowny et al., 1995). Treated Podarcis hispanica were indistinguishable from controls in as little as four weeks after 3AP treatment. This remarkable capacity for postnatal regeneration in reptiles makes them important models for understanding the mechanisms that govern this process, in addition to the functional applications of postnatal neurogenesis. However, studies of the reptilian reactive neurogenesis response have mainly been confined to lizards, and very little is known about how other taxa, including snakes, respond to acute neurogenesis triggers.

1.4 Pythons as Models of Specific Dynamic Action

Pythons in particular have been extensively studied for another physiological capacity for which they are especially well-adapted. These snakes dramatically modulate their physiology in response to the ingestion of a meal, resulting in a widely documented surge in metabolic rate (Cox & Secor, 2008; Secor, 2008; Wang & Rindom, 2021). All vertebrates undergo a temporary increase in metabolism after feeding called specific dynamic action (SDA) or heat increment of feeding, but the shift is especially pronounced in pythons that engage in sit-and-wait predation and can go for weeks or months between meals (Figure 2; Secor, 2008). While they wait, pythons maintain extremely low metabolic rates by suppressing their digestive organs into a quiescent state. Once they do consume a meal, they must rapidly resurrect these systems to be able to digest and absorb the ingested energy. The contrast in the level of exertion between fasted and fed pythons is reflected in their metabolic rates, which can increase as much as 687% after consumption of a meal weighing 25% of the snake’s body mass. For comparison, the maximum postprandial
increase in metabolic rate that has been measured in fish is 136%, and only 25% in humans (Secor, 2009).

Many studies have attempted to define where exactly energy from specific dynamic action is allocated in postprandial pythons, and at the same time have elucidated the truly dynamic nature of the python’s physiology. The transformation begins nearly as soon as the snake’s multi-hinged jaw has successfully “walked” up the length of the prey and enclosed its meal. The volume of the meal inflicts significant pressure on the python’s blood vessels and internal organs, necessitating rapid action by the digestive system. Unlike animals that feed frequently, the stomach of a ball python does not constitutively produce gastric acid or digestive enzymes. The parietal cells along the inner epithelium of the stomach immediately begin releasing HCl, bringing the gastric pH from 7 down to 2 in just 24 hours, while chief cells pump out pepsinogen to be converted into the protease pepsin in the presence of low pH (Secor, 2003). The stomach maintains this acidic environment despite the large buffering capacity of the meal itself, while its muscular walls churn the previously intact prey animal into a liquid chyme that subsequently passes into the small intestine. This transition begins in the first 24 hours after ingestion and is nearly complete within a week (Secor, 2003).

By the time the meal enters the small intestine, the organ and its accessory structures are prepared. The pancreas releases sodium bicarbonate into the small intestine, neutralizing incoming acidic chyme to a pH of 6.5 within a few centimeters. The pancreas also begins accelerating production of trypsin and amylase, two digestive enzymes which peak in their activity on day four post-feeding (Cox & Secor, 2008). This upregulation in pancreatic function is reflected in the organ’s mass, which doubles over the course of
digestion (Secor & Diamond, 1995). The entry of chyme into the small intestine triggers
the release of cholecystokinin, glucose-dependent insulinotropic peptide, and neurotensin,
which have been attributed to the subsequent release of stored bile from the gallbladder
into the small intestine (Cox & Secor, 2008). Additionally, the enterocytes that make up
the intestinal mucosa grow up to 40% in size, leading to a quadrupling of the length of
microvilli on the apical surface 24 hours after ingestion, and a 70% increase in total
intestine volume over the course of digestion (Cox & Secor, 2008; Secor & Diamond,
1995). This increase in surface area expands the interface between chyme from the stomach
and the membrane-bound brushborder enzymes aminopeptidase-N and maltase, both of
which peak in capacity by the third day after the meal was consumed (Cox & Secor, 2008;
Secor, 2005). The longer microvilli also allow for more efficient absorption of nutrients
such as L-leucine, L-proline, and D-glucose, as reflected in a peak in their uptake rates by
the second day after a meal (Cox & Secor, 2008). Increases in the density of amino acid
and glucose transporters may also support higher rates of absorption (Ferraris et al., 1992).
Any unabsorbed material passes from the small intestine into the large intestine and is
eventually excreted through the cloaca.

This upregulation of digestive activity is supported by an accompanying increase
in the activity of the cardiovascular system. At its peak after a meal, breathing frequency
is five times greater than resting values, and tidal volume increases significantly to
facilitate more efficient gas exchange (Secor et al., 2000). Heart rate increases three to four
times resting values. The mass of the heart itself increases by 40% over the course of
digestion, yielding an increase in stroke volume of 50%. Combined, these changes allow
for a five-fold increase in cardiac output (Secor & White, 2010). In the small intestine and
liver, vasodilation facilitates greater volumes of blood flow, accelerating gas exchange as well as nutrient absorption, processing, and dispersal (Starck & Wimmer, 2005).

![Figure 1.2](image)

**Figure 1.2.** Pythons undergo dramatic physiological changes following ingestion of a meal. **A.** Comparison of SDA coefficients of several vertebrate species (Data from Secor, 2008). **B.** Python microvilli quadruple in length over the first 24 hours following feeding. Black bars represent 1μm. **C.** Python gastric pH drops from over 7 to 2 in the first 24 hours after meal consumption, then remains stable over the course of digestion in spite of the large buffering capacity of the meal. Error bars represent ±1 s.e.m. **D.** Wet mass of the heart, pancreas, liver, and kidneys increase by 40%, 94%, 106%, and 72%, respectively (Images B-D from Secor, 2008).

Just as quickly as these systems have peaked, they begin their decline back to the dormancy of fasting. Within six days after feeding, affected organs have all begun to return to their previous quiescent states (Cox & Secor, 2008). With most digestion completed, some newly absorbed energy may be allocated towards growth and maintenance of major organs,
including the brain. Cell proliferation in the ball python brain increased six days after feeding compared to fasted controls or two days after feeding (Habroun et al., 2018). However, it was unknown if newly created cells survived in the future and matured into functional neurons.

1.5 Food Consumption and Neurogenesis

Studies that aim to characterize the relationship between feeding and neurogenesis have yielded mixed results. Most of these studies focus on chronic effects of feeding strategies that are relevant to the health of human populations, rather than acute feeding events (Landry & Huang, 2021). For example, zebrafish subjected to overfeeding to induce obesity were found to have high rates of cerebral oxidative stress and neuroinflammation along with low levels of neurogenesis (Ghaddar et al., 2020).

Additionally, there is a significant body of research on the somewhat ambiguous effects of calorie restriction on neurogenesis and cognitive function. Studies in adult mice show that dietary restriction (feeding on alternate days) is associated with increased neurogenesis in the dentate gyrus, along with higher levels of the neurogenesis-supporting proteins brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Lee et al., 2000; Lee et al., 2002). Further, in a mouse model of traumatic brain injury, intermittent fasting increased the number of neural progenitor cells in the subgranular zone of the dentate gyrus and improved performance in the Morris water maze (Cao et al., 2022). However, another study found that calorie restriction had no effect on neuronal proliferation in adult mice, but did lead to an increase in the survival of glial cells in the hilus of the dentate gyrus (Bondolfi et al., 2004). Importantly, any potential benefits of calorie restriction are believed to be limited to older adult animals, as calorie restriction
was found to decrease new neuron survival in the dentate gyrus of young adult mice and young rats (Cardoso et al., 2016; Staples et al., 2017). Additionally, rats subjected to protein restriction during weaning and lactation maintained fewer progenitor cells in the neurogenic niches of the subgranular zone and the subventricular zone as adults (Godoy et al., 2013). In one of the few non-mammal studies, food restriction was also found to reduce rates of neurogenesis in young chickens (Robertson et al., 2017).

Other perspectives have investigated the role of postnatal neurogenesis, particularly in the hypothalamus, in the regulation of appetite. Inhibition of hypothalamic neurogenesis has been shown to induce reduced appetite and weight loss, while the same effects have been observed via the stimulation of hypothalamic neurogenesis in response to treatment with the neuropeptide ciliary neurotrophic factor (CNTF) (Kokoeva et al., 2005; Lee et al., 2012; Pierce & Xu, 2010). Many of the details of this complex relationship remain to be described.

Beyond the direct link between feeding and neurogenesis, numerous studies have aimed to identify key factors and hormones that may be involved in the regulation of both processes. For example, brain-derived neurotrophic factor (BDNF) is a well-studied neuropeptide that is essential for maintaining basal neurogenesis (Bartkowska et al., 2007; Jaewon Lee et al., 2002). However, it has also been implicated in the regulation of appetite. BDNF protein content was found to decrease in the dorsal vagal complex 48 hours after food deprivation and increase after refeeding, while infusion of BDNF into the dorsal vagal complex reduced food intake and induced weight loss (Bariohay et al., 2005). BDNF was also found to be a necessary facilitator of leptin signaling (Liao et al., 2012). Similarly, the peptide vascular endothelial growth factor (VEGF) has been shown to stimulate
hippocampal neurogenesis, especially in response to an enriched environment, as well as improve hippocampus-dependent learning (Cao et al., 2004; Licht et al., 2006). VEGF has also been shown to respond to metabolic input, as prolonged consumption of a high-fat diet has been shown to increase levels of VEGF in the brain (Jais et al., 2016). Further, glial cell-derived neurotrophic factor (GDNF) is a neuroprotective neuropeptide that promotes the survival of dopamine neurons and enhances locomotor ability in mouse models of Parkinson’s disease (Littrell et al., 2013). This factor also has a relationship to feeding in that high levels of glucose availability have been shown to induce increases in expression of GDNF (Aldhshan et al., 2022).

Well-studied regulators of appetite and metabolism have also been increasingly implicated in the control of postnatal neurogenesis. The orexigenic hormones ghrelin and neuropeptide Y have both been shown to stimulate cell proliferation and neuronal differentiation in the hippocampus of adult mice (Chung et al., 2013; Decressac et al., 2011). However, the anorexigenic hormones leptin and α-Melanocyte stimulating hormone (α-MSH) have also been associated with higher rates of hippocampal neurogenesis. Administration of leptin was shown to increase hippocampal neurogenesis in mice both in vivo and in vitro (Garza et al., 2008). A direct effect of α-MSH on neurogenesis has not yet been described, but the hormone was shown to have a neuroprotective effect in the hippocampus of mouse models of Alzheimer’s disease, facilitating the survival of new and existing cell populations (Giuliani et al., 2014). Additionally, the metabolic hormone insulin-like growth factor 1 (IGF-1) has been shown to increase proliferation in adult rat hippocampal cells (Åberg et al., 2003).
There is copious evidence for the interdependence of energy balance and postnatal neurogenesis, and yet much of the complexity of this relationship remains to be elucidated. Namely, the existing literature largely ignores non-mammal study species (Landry & Huang, 2021). This oversight fails to take advantage not only of the high neurogenic potential of reptile species, but also of their greater potential for fasting. In mammals, the window of opportunity for studying the relationship between feeding and neurogenesis is constrained, because their high metabolic demands prevent them from fasting for long periods of time without starving (Careaux et al., 2014). This complicates their use in feeding studies because the distinctions between fed, fasted, and starving animals are blurred. Reptiles generally have lower energetic demands, and ball pythons in particular employ an infrequent feeding strategy that is characterized by long periods of fasting punctuated by discrete feeding events (Secor & Diamond, 1995). Pythons undergo dramatic physiological and metabolic changes in the shift between a fasted and fed state, clearly demarcating an animal’s physiological state (Secor, 2008). The extreme contrast between fasted and fed ball pythons makes them well-suited candidates for understanding the physiological changes induced by feeding. And yet, few studies have applied this unique advantage to investigate the postprandial processes that take place in the brain.

The only paper to date to study the effects of feeding on the creation of new neural cells in pythons was published by Habroun et al. in 2018 (Figure 3). This study found significantly more newly created neurons in the telencephalon of juvenile ball pythons six days after feeding compared to two days after a meal or in fasted controls. These results provide encouraging insights into the python’s heightened capacity for neurogenesis following meal consumption, but they are limited in that they capture only the initial spike
in cell proliferation acutely after feeding. It is as yet unknown if these newly created cells survive in the longer term or if they differentiate into neurons. Previously, this latter question would have been virtually impossible to answer due to the lack of a commercially available antibody that effectively targets neural proteins in snakes. Fortunately, recent work has led to the development of an antibody against the product of the python Fox3 gene, a marker of neuronal nuclei (Kim et al., 2009; Strand et al., unpublished data). This antibody allows for the analysis of the fate of cells created in the snake brain, and enables me to investigate the question central to Chapter 2: does feeding induce neurogenesis in ball pythons?

Figure 1.3. Feeding induces acute increases in cell proliferation in the brains of ball pythons. A. Mean overall density of BrdU-labeled cells in the telencephalon of snakes that were fasted (C, n=7), sacrificed two days after feeding (D2, n=6), and sacrificed six days after feeding (D6, n=7). Snakes had significantly more BrdU-labeled cells six days after feeding than the two-day or fasted control groups. Error bars represent ±1 s.e.m. B. Mean density in five brain regions. Snakes in the D6 group had higher density of BrdU-labeled cells across all regions. These results are encouraging, but do not capture the eventual fate of these newly created cells. AOB/OB: accessory olfactory bulb/olfactory bulb, RB: retrobulbar region, ctx: cortex, DVR/ns: dorsal ventricular ridge/nucleus sphericus, SN: septal nuclei (Images A and B from Habroun et al., 2018).
Chapter 2

THE EFFECTS OF FEEDING ON NEUROGENESIS IN THE BALL PYTHON, *PYTHON REGIUS*

2.1 Introduction

Postnatal neurogenesis is a process that has well-documented implications for spatial learning and memory, neurodegenerative disorders including Alzheimer’s and Parkinson’s diseases, mental health conditions such as anxiety and depression, and the brain’s capacity for regeneration after traumatic injury (Berger et al., 2020; Gomes-Leal, 2021; Lieberwirth et al., 2016; Redell et al., 2020; Wakhloo et al., 2022). This phenomenon’s ramifications for cognitive function and brain health prompt the need for a thorough understanding of the factors that regulate it. Voluntary exercise and environmental enrichment are among the most well-documented stimuli of neurogenesis (Birch & Kelly, 2019; Grońska-Pęski et al., 2021; Okamoto et al., 2021; Trinchero et al., 2019), and many studies have shown that stress significantly decreases neurogenesis (Briones & Gould, 2019; Du Preez et al., 2021). However, the creation of new neurons requires a significant energetic investment (Beckervordersandforth, 2017; Heiden et al., 2009), so it stands to reason that metabolic state is another regulatory factor. The relationship between food intake and postnatal neurogenesis is thus the subject of a growing body of research (Landry & Huang, 2021).

The results of these studies are somewhat mixed. Caloric restriction has been found to increase rates of neurogenesis in some mouse models, including a mouse model of traumatic brain injury (Cao et al., 2022; Lee et al., 2000, 2002). However, other investigations have found that similar diet regimes have no effect on neurogenesis, or
even decrease new neuron survival, particularly in young animals (Bondolfi et al., 2004; Cardoso et al., 2016; Staples et al., 2017). These findings are additionally complicated by studies demonstrating the neurogenic roles played by many hormones classically associated with appetite regulation and metabolism, including ghrelin, neuropeptide Y, leptin, insulin, and IGF-1 (Chung et al., 2013; Decressac et al., 2011; Dicou et al., 2001; Nieto-Estévez et al., 2016; Tofighi Zavareh et al., 2022). These studies and more illustrate the complex interdependence of energy status and neurogenesis, and yet a detailed understanding of this relationship remains elusive. One of the largest limitations of this work so far is the fact that the vast majority of these studies are conducted in mammalian models, while non-mammals are often overlooked. Among the few exceptions, one study found that calorie restriction in chickens led to decreased rates of neurogenesis, while another used a zebrafish model of diet-induced hyperglycemia to demonstrate the antineurogenic effects of overfeeding (Ghaddar et al., 2020; Robertson et al., 2017). These studies are a start, but the particular value of non-mammals in studying this relationship is thus far largely underutilized. Endotherms have a limited capacity for fasting, and therefore the distinctions between fed, fasted, and starving animals are indiscrète. By contrast, many ectotherms can survive for weeks or months without food and undergo significant physiological changes between fasted and fed conditions, making them useful subjects to highlight the contrast between metabolic states. Additionally, many non-mammal species have demonstrated a great potential for neurogenesis and therefore serve as important examples for understanding the mechanisms behind this phenomenon (Paredes et al., 2016).
Specifically, there is a large body of evidence illustrating the impressive neurogenic capacity of reptiles. In adult mammals, there are only two neurogenic niches in the telencephalon, and the net number of neurons does not increase over the lifetime (Font et al., 2001; Kaslin et al., 2008; Paredes et al., 2016). By contrast, cell proliferation in reptiles has been documented in every major brain region and net number of neurons increases indefinitely (Font et al., 2001; Kaslin et al., 2008). Furthermore, reptiles have an impressive regenerative ability, as evidenced in studies in which the lizard *Podarcis hispanica* was subjected to a chemical lesion of up to 95% of cells throughout the medial cortex. These lizards are able to generate new neurons that return the damaged area to its previous state within just a few weeks (Font et al., 1991; López-García et al., 1992, 2002; Molowny et al., 1995). Postnatal neurogenesis studies in reptiles provide valuable insight into the functions of this process and the mechanisms driving it. And yet, they are not widely used in studies examining the relationship between feeding and neurogenesis.

Pythons in particular make an interesting subject for this endeavor because they are a well-established model of extreme postprandial plasticity. All animals undergo an increase in metabolic rate after feeding to meet the energetic costs of digestion and absorption, termed specific dynamic action (SDA) or heat increment of feeding. However, Burmese pythons have the largest SDA response documented in any vertebrate, with an increase of as much as 687% from basal metabolic rates after a meal weighing 25% of the animal’s bodyweight (Secor, 2009; Secor & Diamond, 1995). For comparison, the largest known increases in postprandial metabolic rates in fish and humans are 136% and 25%, respectively (Secor, 2009). The dramatic SDA response
exhibited by many python species is explained by the fact that these animals employ a strategy of infrequent feeding, allowing for weeks or months of fasting between each meal. During these extended periods without food, pythons maintain an extremely low metabolic rate and conserve energy by downregulating their digestive organs into a state of dormancy (Secor, 2008). Once a python consumes a meal, a massive energetic investment is needed to quickly rouse the digestive system to break down the food and absorb nutrients. This systemic upregulation of activity is reflected in the python’s immense SDA.

Determining the ways in which energy is allocated during the SDA response in pythons is itself an entire field of study. Virtually every major organ is affected by the rapid transformation from quiescence to activity. Gastric pH drops from 7 down to 2 within 24 hours, and digestive enzymes are pumped into the stomach and small intestine (Cox & Secor, 2008; Secor, 2003). The surface area available for absorption in the small intestine is dramatically augmented as the microvilli lining the luminal space quadruple in length, allowing for nutrient uptake rates to increase 3- to 10-fold (Cox & Secor, 2008; Secor, 2005; Secor & Diamond, 1995). Elsewhere, heightened activity in accessory organs is evidenced by mass increases of 94% in the pancreas, 106% in the liver, and 72% in the kidneys (Secor, 2008; Secor & Diamond, 1995). This upregulation of digestive activity is supported by an accompanying surge in the activity of the cardiovascular and respiratory systems. At its peak after a meal, breathing frequency is five times greater and heart rate three to four times greater than resting values (Cox & Secor, 2008; Secor et al., 2000). The mass of the heart increases by 40% over the course of digestion, yielding an increase in stroke volume of 50% (Cox &
Secor, 2008). Altogether, there is ample evidence illustrating systemic plasticity in pythons during the postprandial period. And yet, until recently, it was completely unknown whether this response extends to the brain.

At present, the only investigation into the effects of feeding in the brains of ball pythons is described by Habroun et al. (2018). This study documented higher rates of cell proliferation in ball pythons six days post-feeding compared to snakes two days post-feeding or fasted controls. These results provide encouraging insights into the python’s heightened capacity for neurogenesis following meal consumption, but they are limited in that they capture only the initial spike in cell proliferation acutely after feeding. It was the aim of the current study to determine if these newly created cells survive in the long-term and if they differentiate into neurons. We hypothesized that feeding increases neurogenesis not only in terms of the proliferation of new cells in the brain, but also their maturation into neurons and long-term survival. We tested this hypothesis using two different time points. First, we predicted that snakes would demonstrate higher rates of proliferation six days after feeding compared to fasted controls sacrificed at the same time. Second, we predicted that snakes would have higher numbers of newly created neurons two months after feeding compared to fasted controls at the same time.

2.2 Materials and Methods

2.2.1 Animal Care

We obtained 24 hatchling ball pythons (*Python regius*) from Strictly Reptiles, Inc. (Hollywood, Florida) April 23, 2021. Upon arrival, snakes were weighed and randomly assigned a numeric label. Each snake was housed on a 12:12 light:dark cycle in a clear 54-
quart plastic bin furnished with a sheet of newspaper and a hide made from an eight-inch segment of PVC pipe cut in half lengthwise. Newspaper was replaced as necessary and water was supplied in a glass dish *ad libitum* throughout the duration of the experiment. Heated wire was plugged into the wall via an electric timer and run along one side beneath each plastic bin to create a thermal gradient between the hours of 8:00AM and 7:00PM. Snakes were acclimated in the laboratory for a period of five months. One snake died in the first 48 hours after arrival in the laboratory. About once a week during the 5-month acclimation period, snakes were offered a rodent meal (either thawed frozen fuzzies or live fuzzies) weighing approximately 10% of their body mass. Snakes who still had not eaten two months after arrival in the vivarium were force-fed fuzzies thawed from frozen. Three snakes died during this acclimation period and seven still refused to voluntarily eat. To replace the snakes who had died and those who would not willingly eat, eleven additional ball pythons of approximately the same age/size were obtained from Strictly Reptiles, Inc. on July 21, 2021. These snakes were weighed, randomly assigned numeric labels, and maintained under the conditions previously described for an acclimation period of approximately one month. Five of these snakes refused a rodent meal in any form for at least two consecutive weeks during this time. On August 25, 2021, all snakes were fed a rodent meal weighing 10% of their body mass. Thirteen snakes had not willingly eaten 48 hours later and were force-fed. All snakes were subsequently fasted for a period of one month before the beginning of treatment. Four snakes (three of them from the new shipment) died during the fasting period, leaving a sample size of 25.
2.2.2 Experimental Design

Animals were assigned to four treatment groups (proliferation-fasted, n=5; proliferation-fed, n=5; survival-fasted, n=7; and survival-fed, n=8) in a stratified random manner. Mass did not differ between groups (p = 0.77). At the beginning of the experimental period, snakes in the fed treatment groups were fed live mice weighing 20% of snake body weight. Two snakes that had consistently refused live rodent meals were force-fed. Six days after feeding, snakes in all treatment groups received a subcutaneous injection of 100mg/kg of 5-bromo-2’-deoxyuridine (BrdU; Fisher Scientific, cat. no. BP25081) suspended in reptile Ringer’s solution at a concentration of 10mg/mL. Previous work has shown a significant increase in proliferating cells in the brains of ball pythons six days after feeding, so this time point was chosen to confirm this initial response (Habroun et al., 2018). Snakes in the proliferation-fed and proliferation-fasted groups were sacrificed 24 hours after BrdU injection. These groups established the difference in cell proliferation between fasted and fed groups during the initial response to feeding.

Snakes in the survival-fed and survival-fasted groups were maintained in lab conditions as previously described. All remaining snakes were fed a rodent meal weighing approximately 10% of their body mass one month after the initial feeding event to maintain homeostatic conditions. These snakes were sacrificed by the same method two months after BrdU injection. The survival-fed and survival-fasted groups allowed for the comparison of rates of cell survival and neuron maturation between fasted and fed snakes.

2.2.3 Tissue Preparation

24 hours after injection with BrdU, snakes in the proliferation-fed and proliferation-fasted groups were sacrificed. After injection with ketamine (25 mg/kg) suspended at a
concentration of 5mg/mL in 0.9% NaCl, snakes were anesthetized via isoflurane inhalation. Once each snake was unresponsive to a tail pinch, final mass and snout-vent length were measured, sex was recorded, and the animal was transcardially perfused with wash buffer (0.9% NaCl, 0.1% NaNO₂ in 0.1 M phosphate buffer) followed by 4% paraformaldehyde and 0.1% NaNO₂ in 0.1 mol/L phosphate buffer (PB). Brains were dissected then submerged in 4% paraformaldehyde for 24 hours, then transferred to 0.1 mol PB for the following 24 hours. Brains were next soaked in a 4% gelatin solution for several minutes before being embedded in 8% gelatin blocks. After the gelatin was set, blocks were submerged in 4% paraformaldehyde for 21 hours. The next day, gelatin blocks were transferred to a 10% sucrose solution for 24 hours, followed by a 30% sucrose solution until gelatin blocks sank to the bottom of their jars, about 48 hours. Gelatin blocks were then frozen in dry ice and maintained at -80°C until sectioning. Sectioning was performed on a cryostat (Bright OTF-5000) at -16°C. Brains were sliced into 8 series of 35μm-thick coronal sections. The first series was directly mounted onto microscope slides (Fisher Scientific, cat. no. 12-550-003) treated with Vectabond (Vector, cat. no. NC9280699) and hydrated with mounting solution to smooth sections onto the slide. Sections were left to dry at least 48 hours before staining with cresyl violet. The remaining series were stored at -20°C in cryoprotectant (20% glycerin in 0.1mol/L PB) until immunohistochemical staining.

2.2.4 Immunohistochemistry

The second series of each brain, beginning with the olfactory bulbs and ending at the caudal end of the telencephalon, was used for immunohistochemistry. Cryoprotectant was first washed off brain sections using phosphate-buffered saline (PBS), then sections
were washed in PBS three times for five minutes. Brain sections were then incubated in 2N HCl at 37°C for 30 minutes for antigen retrieval. Sections were washed in PBS for five minutes, 3.68% sodium borate (sodium tetraborate decahydrate NF in distilled water) for ten minutes, then PBS three times for five minutes each time. Next, sections were incubated in the primary antibodies: mouse anti-BrdU (DSHB cat. no. G3G4; concentration 1:1000) and rabbit anti-Fox3 (Cal Poly Bio Department; concentration 1:4000) diluted in Animal-Free Blocker (Vector, cat. no. SP-5030) for 22 hours on a shaker table. Commercially available antibodies against the neuronal marker NeuN, the product of the Fox3 gene (Kim et al., 2009), do not typically work well in snakes (C.R.S., personal observation). A polyclonal anti-NeuN antibody was successfully used in leopard geckos (McDonald & Vickaryous, 2018), but the rabbit anti-Fox3 antibody used in this study was designed with specificity for the product of the Fox3 gene in rattlesnakes. Sections were rinsed in PBS with 0.3% Triton x-100 (PBST) three times for five minutes each before incubating in secondary antibodies: horse anti-mouse Dylight488 (Vector, cat. no. DI-2488; concentration 1:400) and horse anti-rabbit Dylight594 (Vector, cat. no. DI-1094; concentration 1:200) diluted in PBST for 1 hour. Finally, sections were rinsed in PBS three times for five minutes each before mounting on microscope slides and coverslipped using Vectashield hardset mounting medium (Vector, cat. no. H-2000-10).

2.2.5 Data Collection

Study regions were identified in accordance with Holding et al. (2012), Smeets (1988), and Bales (2014). Brain sections were observed on a fluorescent microscope (Olympus BX53) and colocalization was confirmed using an epifluorescent microscope (Zeiss Axio Observer Z1 inverted epifluorescence microscope). BrdU-positive cells were
observed under a FITC filter and Fox3-positive cells were observed under a TXRED filter. Brain regions were determined by direct observation of Fox3-labeled cells under the TXRED filter. Cell counts were conducted in specific brain regions in one hemisphere from each section. The hemisphere counted was alternated throughout the brain except when one hemisphere was damaged.

New cells are created along the walls of the ventricles throughout the telencephalon in reptiles (Font et al., 2001). For snakes in the proliferation groups, BrdU-labeled cells were counted by a treatment-blind observer in the ventricular zone of each region of interest (Fig. 1) beginning in the olfactory bulbs and spanning through the telencephalon. Cells were considered to be within the ventricular zone if they were within 50μm of the ventricle. Few BrdU-labeled cells were observed outside the ventricular zone in snakes in the proliferation groups, as expected; cells that incorporated BrdU during proliferation would likely not have had time to migrate away from the ventricular zone in the 24 hours between BrdU injection and sacrifice. Those BrdU-positive cells that were observed in the parenchyma were most likely endothelial cells and were not included in the analysis. Cell totals per study region were determined for each brain.

Newly created cells may migrate away from the ventricles into the parenchyma of the telencephalon and olfactory bulbs (Font et al., 2001). Here, they may differentiate into mature neurons. For snakes in the survival groups, BrdU-labeled cells were counted by region in the ventricular zone from the olfactory bulbs through the telencephalon. BrdU-labeled cells were also counted throughout the parenchyma of each study region. BrdU-labeled cells that were identified in the parenchyma were examined for colocalization with Fox3. Cells that were BrdU- and Fox3-double labeled were determined to be newly created
neurons. Cell totals in the ventricular zone and in the parenchyma of each study region were calculated for all brains in the survival groups. As expected, no cells in the ventricular zone were colocalized with Fox3.

Images were taken of the sections with the TXRED filter (Fox3 positive cells). The length of the ventricular zone was measured from these images by study region on every brain section using the freehand line tool in ImageJ (NIH, version 1.3a). In the brains of the snakes in the survival treatment groups, the surface area occupied by the parenchyma of each study region was also measured on every section using the freehand selection tool in ImageJ. The lengths of the ventricular zone were totaled for every study region within a particular brain for all treatment groups. The surface area of the parenchyma was totaled for each study region within the brains of snakes in the survival groups. Cell density in either the ventricular zone or parenchyma of a given study region was calculated by dividing the cell total by the dimensions (length or surface area, respectively) for that region. Cell densities were calculated for the ventricular zones of all regions of interest in the proliferation groups, and for the ventricular zones and parenchyma of all regions of interest in the survival groups. Additionally, estimated cell totals were calculated as described in Maine et al., 2014. For each brain region within an individual snake, the number of observed BrdU-ir cells was divided by the total number of usable sections containing that region. This number was further multiplied by the average number of brain sections containing a given region across all animals. These estimated cell totals were used for proportion calculations.
2.2.6 Statistical Analysis

All data were analyzed using JMP Pro software (SAS Institute, Inc., version 16.0.0). Cell densities were log transformed to meet the normality assumption. Cell densities from the ventricular zone of the proliferation groups, the ventricular zone of the survival groups, and the parenchyma of the survival groups were analyzed separately. To assess differences between fasted and fed snakes across five main brain regions simultaneously, we performed a multivariate analysis of variance (MANOVA). When the MANOVA exhibited a significant difference between fasted and fed snakes, we performed separate univariate analyses of variance (ANOVA) for each brain region to determine which of the regions was individually affected by the treatment. A Bonferroni correction ($\alpha=0.01$) was applied to account for the increased risk of false positives after conducting multiple pairwise comparisons. We determined differences in cell population proportions between fasted and fed snakes using a chi-square analysis for each brain region.
Figure 2.1. A. Schematic depicting location of sections B-F throughout the telencephalon of the python brain. B-F. Cresyl violet stained coronal sections depicting the location of brain regions of interest. AC, anterior cortex; ACC, nucleus accumbens; AOB, accessory olfactory bulbs; DC, dorsal cortex; DVR, dorsal ventricular ridge; LC, lateral cortex; MC, medial cortex; NS, nucleus sphericus; OB, olfactory bulb; PC, posterior cortex; RB, retrobulbar region; SN, septal nuclei.
2.3 Results

2.3.1 Treatment Effects on Cell Proliferation

Six days after feeding, BrdU-labeled cells were observed in the ventricular zone throughout the telencephalon. As expected, these cells were not colocalized with the neuronal marker Fox3 (Fig. 2). Previously, Habroun et al. (2010) demonstrated increased rates of ependymal zone proliferation in ball pythons fed a meal six days prior compared to fasted controls. Although we did not find a statistically significant increase in cell proliferation six days after feeding, our data did follow a similar trend (p=0.136; Fig. 3).
Figure 2.2. Maximum intensity projection of Z-stack sections, obtained via wide-field microscopy, of BrdU labeling in the ventricular zone of the telencephalon of ball pythons magnified 630 times. BrdU labeling (green) was observed under the FITC filter and Fox3 (red) labeling was observed under the Cy3 filter. Cells were considered to be in the ventricular zone if they were within 50µm of the ventricle.
2.3.2 Treatment Effects on Survival of Ventricular Zone Cells

Two months after feeding, the majority of BrdU-labeled cells were maintained in the ventricular zone (Fig. 4). These BrdU-labeled cells were not colocalized with Fox3. At this point in time, fed snakes had significantly more BrdU-labeled cells in the ventricular zone than fasted snakes (p<0.0001), and there was a significant interaction between treatment and brain region (p<0.0007; Fig. 5). Individual ANOVA tests for each brain region revealed that the effect of treatment was not significant in the ob/aob (p=0.0159;
Fig. 5) but that fasted snakes had significantly more BrdU+/Fox3+ cells in the ventricular zone in the rb, sn/acc, dvr/ns, and cortex (p<0.0001 for all; Fig. 5).

**Figure 2.4.** Distribution of cell populations between the ventricular zone and parenchyma in each brain region of interest for fasted controls and snakes fed a meal two months prior to sacrifice. Estimated cell totals were calculated as described in Maine, et al., 2014. The proportion of cell types was calculated as the average number of a given cell type in the region of interest across all snakes in the treatment group divided by the average number of all cell types in the region (fasted snakes: n=7, fed snakes: n=8). Chi-square tests on each brain region revealed no significant differences between fasted and fed snakes (df = 2 for all; OB/AOB: p = 0.972; RB: p = 0.977; SN/ACC: p = 0.980; DVR/NS: p = 0.925; CTX: p = 0.967). OB/AOB, olfactory bulbs/accessory olfactory bulbs; RB, retrobulbar region; SN/ACC, septal nuclei/nucleus accumbens; DVR/NS, dorsal ventricular ridge/nucleus sphericus; CTX, cortex.
Mean (±s.e.m.) density of BrdU+/Fox3− cells in the ventricular zone of ball pythons two months after feeding for each brain region of interest. Snakes fed a meal two months prior to sacrifice (n=8) had significantly more BrdU-labeled cells in the ventricular zone than fasted controls (n=7; ***p<0.0001). Individual ANOVA tests for each brain region with Bonferroni correction (α=0.01) revealed no significant difference in the ob/aob (p=0.0159), but significantly greater cell densities in the rb, sn/acc, dvr/ns, and ctx (**p<0.0001 for all). OB/AOB, olfactory bulbs/accessory olfactory bulbs; RB, retrobulbar region; SN/ACC, septal nuclei/nucleus accumbens; DVR/NS, dorsal ventricular ridge/nucleus sphericus; CTX, cortex.

2.3.3 Treatment Effects on Neurogenesis

Two months after feeding, a minority of BrdU-labeled cells migrated away from the ventricles into the surrounding parenchyma (Fig. 4). Few of these cells were colocalized with the neuronal marker Fox3 and were thus identified as newly created neurons (Fig. 6). There was not a significant effect of feeding on the density of BrdU+/Fox3+ cells (new neurons) in any brain region (p=0.1601; Fig. 7). We also found no significant differences in the density of newly created neurons among brain regions (p = 0.3932). Combining average cell density for all snakes regardless of treatment revealed significant differences
among brain regions, and the lateral cortex had a significantly greater density of new neurons than several brain regions (p=0.0012; Fig. 8).

**Figure 2.6.** Maximum intensity projection of Z-stack sections, obtained by wide-field microscopy, of BrdU and Fox3 colocalization in parenchymal cells (arrows) of ball python brain, magnified 630 times. BrdU labeling was observed under the FITC filter and Fox3 labeling was observed under the Cy3 filter. These markers were used to identify newly created neurons.
Figure 2.7. Mean (±s.e.m.) density of BrdU+/Fox3+ cells in the parenchyma of ball python brains two months after feeding for each brain region of interest. There were no significant differences between fasted (n=7) or fed snakes (n=8) in any region (p=0.1601). OB/AOB, olfactory bulbs/accessory olfactory bulbs; RB, retrobulbar region; SN/ACC, septal nuclei/nucleus accumbens; DVR/NS, dorsal ventricular ridge/nucleus sphericus; CTX, cortex.
2.3.4 **Treatment Effects on Cell Survival**

The majority of BrdU-labeled cells that migrated into the parenchyma two months after feeding were not colocalized with Fox3 (Fig. 4). The identity of these cells is at present unknown (Fig. 9). Fed snakes had significantly more of these unidentified newly created cells than fasted controls (p=0.0001; Fig. 10). Individual ANOVA tests for each brain region revealed no significant effect of treatment in the rb (p=0.0122) and significant differences in all other regions (ob+aob: p=0.0040; sn+acc: p=0.0008; dvr+ns: p<0.0001; ctx: p=0.0036).
Figure 2.9. Maximum intensity projection of Z-stack sections, obtained by wide-field microscopy, of a BrdU-ir nucleus in the parenchyma of ball python brains that was not colocalized with Fox3, magnified six hundred thirty times. BrdU labeling was observed under the FITC filter and Fox3 labeling was observed under the Cy3 filter. The identity of this cell is unknown.
Figure 2.10. Mean (±s.e.m.) density of BrdU+/Fox3- cells in the parenchyma in ball pythons fed a meal two months prior to sacrifice (n=8) and fasted controls (n=7). Fed snakes had significantly more BrdU+/Fox3- parenchymal cells than fasted controls (**p=0.0001). Individual ANOVA tests for each brain region with Bonferroni correction (α=0.01) revealed no effect of treatment in the rb (p=0.0122) and significant differences in the ob/aoB (**p=0.0040), sn/acc (**p=0.0008), dvr/ns (**p<0.0001), and ctx (**p=0.0036). OB/AOB, olfactory bulbs/accessory olfactory bulbs; RB, retrobulbar region; SN/ACC, septal nuclei/nucleus accumbens; DVR/NS, dorsal ventricular ridge/nucleus sphericus; CTX, cortex.
Figure 2.11. Cell survival in ball pythons fed a meal two months prior (n=8) and fasted controls (n=7). Estimated cell numbers for each region were calculated as described in Maine, et al., 2014. Two month survival was calculated as the estimated number of BrdU-ir cells in the parenchyma of a given region two months after injection with BrdU divided by the average number of BrdU-ir cells in the ventricular zone of a given region one day after injection with BrdU. For the anterior cortex, which does not extend to the ventricular zone, the average number of cells in the ventricular zone of the adjacent retrobulbar region was used. OB, olfactory bulbs; AOB, accessory olfactory bulbs; RB, retrobulbar region; ACC, nucleus accumbens; SN, septal nuclei; DVR, dorsal ventricular ridge; NS, nucleus sphericus; AC, anterior cortex; MC, medial cortex; DC, dorsal cortex; LC, lateral cortex; PC, posterior cortex.

2.4 Discussion
2.4.1 Treatment Effects on Cell Proliferation

The day after BrdU injection, previous observations in ball pythons have noted BrdU-labeled cells localized to the ependyma lining the ventricles throughout the telencephalon (Bales, 2014; Habroun et al., 2018). Our findings followed a similar pattern. Virtually no cells in the parenchyma of the telencephalon were labeled with BrdU, and the few that were labeled were most likely endothelial cells. No BrdU-labeled cells were colocalized with Fox3 (Fig. 2).
Additionally, the pattern of cell proliferation across brain regions in our study was consistent with previous neurogenesis studies in ball pythons (Bales, 2014; Habroun et al., 2018). Virtually no BrdU labeling was observed in the diencephalon, and the retrobulbar region had the highest density of BrdU-labeled cells for both fasted and fed snakes. High levels of proliferation in the retrobulbar region have been noted across reptilian species, and it has been suggested that this region serves a homologous role to the mammalian rostral migratory stream, acting as the birthplace for cells that will ultimately migrate to the olfactory bulbs (Pérez-Cañellas et al., 1997).

Although the differences in cell proliferation between the fed and fasted snakes was not significant as in Habroun et al. (2018), there was a trend for feeding to increase neural cell proliferation (Fig. 3). It is possible that the slightly smaller sample size used for this study (n=10 total compared to n=14 for Habroun et al., 2018) may have contributed to the lack of a significant difference. Additionally, a major difference between our study and that of Habroun et al. (2018) is the size of the animals upon arrival in our facilities. Juvenile ball pythons were used for this study for the sake of consistency with previous work on ball python neurogenesis, but most reptile suppliers offer very little information about the exact age or developmental history of the animals being sold. At the start of this experiment, the animals used in this study had an average mass of 65.0±3.4g, whereas the animals used by Habroun et al. (2018) had an average mass of 286.3±16.8 g. Although we cannot know for sure, it stands to reason that the animals used in our study were younger than those used previously, and this may have impacted the magnitude of the response to feeding.
It is notable, however, that two months after feeding, BrdU labeling was significantly higher in the ventricular zone in fed snakes than in fasted controls (Fig. 5). This suggests that the cells initially labeled in the postprandial period continued to divide at a faster rate in fed snakes in the following two months. The delayed emergence of this difference supports the conclusions previously drawn by Habroun et al. (2018), that feeding induces an increase in cell proliferation in pythons after the completion of digestion. The ingestion of a meal rapidly induces a dramatic increase in a python’s metabolic rate, but this response usually peaks by the second day post-feeding and returns to baseline levels within a week, as the majority of digestion and absorption is completed by that time (McCue et al., 2015; Stephen M. Secor, 2009). Our findings suggest that some of the newly acquired energy from the recent meal may be allocated towards cell proliferation in the telencephalon in the weeks or months following the meal. The mechanisms responsible for this timeline remain to be elucidated, though there are several possibilities. For instance, pythons rapidly oxidize proteins from food, but the oxidation of lipids is drawn out over the weeks following feeding (McCue et al., 2015). It is possible that telencephalic cell proliferation is fueled by the oxidation of ingested lipids, and the number of BrdU-labeled cells increased as more energy became available to fed snakes. Additionally, the delay in this response suggests that the effect might be mediated by an indirect hormonal pathway. For example, the hormone leptin has been studied extensively for its role in satiety, but it also has a documented role in neurogenesis. Leptin administration was shown to increase cell proliferation in the dentate gyrus of adult mice, but this effect only emerged after 14 days of injection with the hormone (Garza et al., 2008). The anorexigenic effects of leptin
have been documented in reptiles, but the relationship between leptin and neurogenesis in reptiles requires further study (Niewiarowski et al., 2000).

2.4.2 Treatment Effects on Cell Migration

Ependymal cells lining the ventricles in the telencephalon are destined for one of two possible fates after undergoing division: some cells will remain along the ventricles to undergo further division, reinforcing the existing population of neural progenitor cells, while others will migrate into the adjacent parenchyma where they may incorporate into the neural network and mature into functional neurons (Font et al., 2001). In ball pythons, BrdU-labeled cells have been documented in the parenchyma as early as two weeks after their creation in the ventricular zone (Bales, 2014). Two months after BrdU injection, many cells in the ependyma lining the ventricle still maintain the BrdU label, but the majority of these cells have migrated away from the ventricle and into the parenchyma (Bales, 2014). A similar migration timeline has been observed in the lizards Podarcis hispanica and Tarentola mauritanica (Font et al., 2001; Pérez-Cañellas & García-Verdugo, 1996). Our findings deviate from this previous work in that a surprising number of BrdU-labeled cells were retained along the ventricles for both fasted and fed snakes (Fig. 4). Many cells did migrate into the parenchyma, but the majority of BrdU-labeled cells in all brain regions were still localized along the ventricles two months after BrdU injection for both fasted and fed groups. This pattern conflicts with previous work in ball pythons and instead more closely resembles the pattern of migration that has been documented in turtles, in which only a minority of cells migrate away from the ventricles and into the parenchyma as much as six months after their creation (Pérez-Cañellas et al., 1997). It is unclear exactly why this might have occurred, but the age of our animals may again be responsible for this
difference. Our animals were presumably younger than those used by Bales (2014) (65.0±3.4g at the start of our experiment compared to 121.4±5.9g in the previous study), and this may have had an impact on the rate of migration of cells away from the ventricle. In young animals, the ventricular zone itself may undergo a higher rate of growth to build up the proliferative capacity that will sustain neurogenesis throughout the animal’s lifetime. The lizard P. hispanica exhibits such a pattern, as perinatal animals retain a higher proportion of new cells in the ventricular zone after a four-week survival time compared to young animals nearing adulthood and fully mature animals (López-García et al., 1988). What remains unclear is whether the cells retained at the ventricular zone in the two-month survival group would eventually migrate into the parenchyma and become neurons themselves, or whether they would stay there for the duration of the animal’s life, augmenting the animal’s telencephalic proliferative capacity. Ultimately, more information is needed to refine our understanding of postnatal brain development in reptiles.

Cells that do not migrate radially away from the ventricles may follow another well-documented movement pattern. In many reptile species, the main and accessory olfactory bulbs exhibit high rates of neurogenesis despite low rates of cell proliferation (Font et al., 2001; Pimentel et al., 2021). By contrast, the adjacent retrobulbar region demonstrates especially high rates of proliferation while the survival of cells in this region is relatively low. These observations suggest that a high proportion of cells created in the retrobulbar region migrate into the main and accessory olfactory bulbs. This reptilian migration system has been posited as a possible homologue to the mammalian rostral migratory stream, which has been documented in rodents and primates (Pencea et al., 2001; Xie et al., 2018). The rostral migratory stream is hypothesized to be particularly important in animals that
rely on olfactory cues and pheromones for food-seeking behaviors, navigation, and mating (Curtis et al., 2009). The constitutive addition of new neurons to these brain regions is thought to facilitate the continuous integration of novel sensory stimuli over the course of an animal’s life. In this study, the olfactory bulbs of both fasted and fed snakes had the highest proportion of two-month-old cells relative to the number of cells in the ventricular zone six days after feeding (Fig. 11). Two-month-old BrdU-labeled parenchymal cells outnumbered cells that had previously been labeled in the ependyma by 620% for fed snakes and by 223% for fasted controls. At the same time, the adjacent retrobulbar regions exhibited two-month survival rates of only 18.6% for fed and 14.4% for fasted snakes. These data suggest that cells migrated into the olfactory bulbs from the ventricular zones of more rostral brain regions and indicate that this pattern is particularly dominant in snakes that have consumed a meal. Functionally, this influx of cells after feeding may facilitate an increase in the uptake of olfactory information. For the purpose of predation, this indicates a positive feedback loop by which receiving food may reinforce the associated olfactory stimuli. This pattern may also support pythons’ ability to detect the cues necessary for courtship and mating when energetic resources are abundant in the environment. However, the majority of the cells that made this migration did not express the markers of mature neurons, and so it is unclear exactly what role they serve to this end.

The lateral cortex is another region with a high rate of cell survival relative to proliferation, with 243% of cells that had previously been labeled in the ventricular zone occurring in the parenchyma of the lateral cortex two months later. Again, the high rate of survival suggests the possibility that cells migrated into the lateral cortex after undergoing proliferation in other regions, but such a migration pattern has not been documented in ball
pythons or other reptiles (Bales, 2014; Font et al., 2001). It remains a possibility that feeding induces such a pattern in parallel with the olfactory bulbs, which is supported by evidence that the lateral cortex functions in olfactory processing (Lanuza & Halpern, 1997). Ultimately, future studies that include additional time points between six days and two months after feeding will be necessary to conclude with certainty how feeding affects the migration of cells throughout the telencephalon.

The final region to exhibit a particularly high survival rate in fed snakes was the septal nuclei, in which BrdU-labeled cells in the parenchyma two months after feeding numbered 362% of the BrdU-labeled cells in the ventricular zone six days after feeding. Although there is no direct evidence of migration into the septum from other regions, the high number of surviving cells relative to proliferating cells in the region suggests that some surviving cells may have migrated from other regions. Previous studies have found low rates of constitutive cell migration to the septal nucleus across reptile taxa, including the lizard *Podarcis hispanica*, the turtle *Trachemys scripta*, and ball pythons themselves (Bales, 2014; Font et al., 1997; Pérez-Cañellas et al., 1997) However, there is evidence for a commissural migration route through the septum in the tropical lizard *Tropidurus hispidus* (Pimentel et al., 2021). These authors found tracts of DCX+ cells migrating through the septum along commissural fibers, but ultimately these cells were seemingly bound for a final destination in the nucleus sphericus rather than the septal nucleus itself.
2.4.3 Treatment Effects on Neurogenesis

Cells that migrate away from the ventricles into the surrounding parenchyma will either mature into functional neurons and incorporate into the existing neural circuitry, become glial cells, or undergo apoptosis. The least ambiguous way to determine the phenotype of newborn cells is to use immunohistochemistry targeting specific markers. In snakes, previous studies have been limited in their ability to characterize the phenotype of newly created cells in the brain due to a lack of commercially available antibodies that accurately label snake neurons. In this study, we used an antibody produced against rattlesnake Fox3 (Fig. 6).

We hypothesized that feeding would induce an increase in neurogenesis in terms of cell survival and maturation, but our data do not support this hypothesis (Fig. 7). This is not necessarily surprising, given the lack of a significant difference in proliferation between fasted and fed snakes six days after the meal. However, a difference in proliferative rates along the ventricles did emerge two months after feeding, which suggests the possibility that a difference in neurogenesis may follow at a time further removed from the initial feeding event than previously anticipated. Future studies would benefit from time points that allow for a longer term of investigation.

Nevertheless, our data suggest an interesting trend in neurogenesis that persists regardless of treatment. In both fasted and fed snakes, the survival rates of newly created neurons were highest in the lateral cortex (Fig. 8). As discussed previously, the lateral cortex is considered homologous to the mammalian olfactory cortex (Lanuza & Halpern, 1997). Squamate reptiles have highly developed chemosensory systems that integrate information from two separate sensory organs (Halpern, 1992; Schwenk, 1995). The first
is the olfactory epithelium, located inside the nasal cavities, which detects volatile odorants in the environment and transmits this information to the main olfactory bulbs (Halpern, 1992; Schwenk, 1995). The second is the vomeronasal organ, a specialized structure within the roof of the mouth that contains sensory epithelium sensitive to pheromones and prey chemoattractants (Halpern, 1992; Halpern & Frumin, 1979). Pythons engage in tongue-flicking to pull these molecules into the mouth and into contact with the vomeronasal organ, which transmits the information to the accessory olfactory bulb (Halpern, 1992). Both the main and accessory olfactory bulbs project to the lateral cortex, where the information is integrated and relayed to the hypothalamus, effecting behavioral change (Hoogland & Vermeulen-Vanderzee, 1995). The vomeronasal organ is especially well-developed in snakes and has been shown to be critical for predation, courtship, and mating (Halpern & Frumin, 1979; Kubie et al., 1978). A high rate of neurogenesis in the lateral cortex, further supported by a relatively high rate of neurogenesis in the accessory olfactory bulbs, is consistent with the critical functional role of this system.

2.4.4 Treatment Effects on Cell Survival

Two months after injection with BrdU, most BrdU-labeled cells present in the parenchyma of both fasted and fed snakes did not express the neuronal marker Fox3 (Fig. 9), suggesting that only a small minority of cells had matured into functional neurons (Fig. 4). This pattern contrasts that which has been documented in adult lizards, in which the majority of cells that are newly created in the ependyma mature into neurons (Font et al., 2001; López-García et al., 1988; Pérez-Cañellas & García-Verdugo, 1996). However, adult turtles undergo relatively low rates of neurogenesis in favor of higher rates of gliogenesis (Pérez-Cañellas et al., 1997). This study is the first to investigate the phenotype of
postnatally generated cells in the brains of a snake model. The low rates of neurogenesis documented here may suggest that a majority of newly created cells are instead maturing into glial cells, following a testudine development pattern. Antibodies specific to snake glial markers would be an invaluable development to answer this question.

Alternatively, many of the young cells might have been on their way to becoming neurons, but they were not yet mature enough to express detectable amounts of the Fox3 protein. Seven days is the minimum amount of time needed for cells to undergo division, migration, and neuronal maturation in the lizard *Podarcis hispanica*, and reports across many reptile species find that one month is adequate for the majority of newly created cells to follow suit (Austin et al., 2022; Font et al., 2001; López-García et al., 1990). But this timeline is not universal, as the lizard *Gallotia galloti* reportedly requires as many as three months to form mature neurons (Delgado-Gonzalez et al., 2011). Along a similar vein, it has been hypothesized that a substantial proportion (as many as 20%) of newly created cells in the brain may “lie in wait” in an undifferentiated state until they are recruited to fill a given functional role as needed (Czéh et al., 2002; Prickaerts et al., 2004). It is unclear why neurons in different species, and even certain neurons within a given animal, may differ in their rates of maturation. Nevertheless, it is possible that ball pythons undergo postnatal neurogenesis at a rate higher than that which we have documented in this study, but that more time is needed to fully capture the effect.

Although we did not find higher numbers of newly created neurons in fed snakes, feeding does appear to increase survival rates in neural cells. Significantly more BrdU+/Fox3- cells survived in fed snakes two months after feeding (Fig. 10). The mechanisms behind this response remain to be elucidated, but there is certainly evidence
for a number of possible mediators. For example, the hormone leptin, which has been well-
studied for its role in appetite suppression, also has a strong anti-apoptotic effect in the
brains of mice (Dicou et al., 2001; Zhang et al., 2007). Another anorexigenic hormone, a-
MSH, has been shown to prevent cell death in neuron populations (Giuliani et al., 2014).
Additionally, insulin and IGF-1 are not only important metabolic mediators after a meal,
but also promote survival of both neurons and glial cells. High levels of apoptosis in
cortical cells in mouse models of type 1 diabetes have been attributed to an inadequate
supply of insulin (Li et al., 2009; Tofighi Zavareh et al., 2022; Yu et al., 2008), and IGF-1
has anti-apoptotic effects in the brain (Nieto-Estévez, 2016). Both leptin and insulin
stimulate the translation of the neuropeptide BDNF in the hypothalamus (Liao et al., 2012).
BDNF is an anorexigenic signaling molecule itself, and it is also a critical promoter of cell
survival in the brain (Hofer & Barde, 1988; Kalcheim & Gendreau, 1988; Rui, 2013). High
levels of these or any number of other key hormones in fed snakes may have protected
newly created cells from degradation in the weeks following the meal.

2.4.5 Conclusions and Future Directions

The findings of this study do not support our initial hypothesis that feeding
increases neurogenesis in terms of both the creation and survival of new neurons. We did
ultimately document a higher rate of cell proliferation in the brains of fed snakes, although
the difference emerged at a later time point than previously documented. However, we did
not find that feeding produced greater numbers of newly created neurons two months after
the meal. Instead, feeding is here shown to have a positive effect on cell survival, but the
identities of these newly minted cells remains a mystery.
There are many possible avenues for future research to build on these findings and answer the numerous questions that still remain. For one, the long-term fate of newly created cells could be illuminated by an expansion of the timeline of the present study to include more datapoints after the second month post-feeding. Additionally, the identity of newly created cells could be identified through the use of immunohistochemistry targeting markers of immature neurons, such as DCX, or of glial cells, such as GFAP. Finally, further research on the mechanisms that connect changes in metabolic state to neurogenesis in reptiles would be a welcome addition to the scientific literature.
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