Effects of Food Consumption on Cell Proliferation in the Brain of *Python regius*

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ABSTRACT

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Neurogenesis is an important and vastly under-explored area in reptiles. While the ability to generate new brain cells in the adult mammalian brain is limited, reptiles are able to regenerate large populations of neuronal cells. Pythons exhibit a characteristic specific dynamic action (SDA) response after food intake with an increase in metabolic rate that facilitates processing the meal. Associated with this change in SDA, pythons (*Python* spp.) also exhibit impressive plasticity in their digestive and cardiovascular physiology due to the sheer magnitude of the increase in organ growth that occurs after a meal to speed digestion, absorption, and assimilation of nutrients. While this systemic growth in response following food consumption is well documented, whether the python brain exhibits associated changes in cell proliferation following food consumption and digestion is currently unexplored. For this study, juvenile male ball pythons (*Python regius*) were used to test the hypothesis that postprandial neurogenesis is associated with food consumption. We used the thymidine analog 5-bromo-12'-deoxyuridine (BrdU) to quantify and compare cell proliferation in the brain of fasted snakes and at two time points: two days and six days after a meal, which span time periods of during and after SDA response, respectively. Quantification of BrdU-labeled cells in the ventricular regions relealed that – consistent with other reptile species – the retrobulbar and olfactory regions had the highest numbers of proliferating cells in the python brain, regardless of sampling time. Throughout the telencephalon, cell proliferation was significantly greater in the six-day post-feeding group, with no difference between the two-day post-feeding group and controls. Most other postprandial systemic plasticity occurs within a day or two after a meal and decreases thereafter; however, the brain displays a more delayed response, with a surge of cell proliferation after most of the digestion and absorption is complete. Our results support our hypothesis that food consumption does affect cell proliferation in the python brain, and indicates that the degree of increased proliferation is dependent on the time since feeding.

Keywords: Python, snake, BrdU, neurogenesis, specific dynamic action, proliferation, brain, feeding

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Chapter 1: Digestive and metabolic response and gastrointestinal performance in pythons after food consumption

1.1 Pythons as a Model for Extreme Digestive Physiology

One of the most basic necessities of life is the need to eat to provide energy for all life functions. The need for an efficient digestive system is necessary for all physiological processes, and the ability to capture prey, acquire food, and obtain energy affords the basis for necessary functions such as growth and movement (Wang 2001). Taxonomic variation in digestive efficiency and digestive physiology can therefore have consequences for an animal's performance, such as feeding, energy expenditure, and digestive efficiency (Karasov and Diamond 1988; Hume and Biebach 1996; Karasov and Hume 1997; Witmer 1998).

Snakes in the genus *Python* are model organisms for the study of extreme digestive and metabolic physiology due to their commercial availability, experimental manageability, and the exceptional magnitude of their digestive response (Secor et al. 1994; Secor and Diamond 1998; McCue et al., 2005). Pythons, who are infrequent feeders, have the capacity for extensive gastrointestinal and cardiovascular plasticity during periods of fasting and food consumption (Secor and Diamond, 2000; Ott and Secor, 2007). Food consumption in these organisms stimulates remarkable changes in form and function at the tissue and organ levels. These same responses are rapidly reversed at the completion of the digestion of a meal, lending to the impressive capacity for extreme gastrointesinal plasticity in these animals (Secor and

Diamond, 1998; Cox and Secor, 2008; Secor 2008). Pythons also have a particularly high postprandial metabolic response, which has been investigated in depth in many studies (Secor et al. 1994, 2000, 2002; Secor and Diamond 1995, 1997; Starck and Beese 2001; Overgaard and Wang 2002; Wang et al. 2002; Secor 2003; Starck et al. 2004).

This chapter will be a detailed review of the literature surrounding the digestive physiology and accompanying metabolic response of pythons. I will first address using the python as a model for digestive physiology and go through the specific foraging mode of these animals. Then I will then discuss the details of the digestive process and physiology, followed by a discussion of the metabolic responses to digestion. This will include a description of the specific dynamic action (SDA), which is the accumulated energy expended on all postprandial activities including ingestion, digestion, absorption, and assimilation of a meal and its determinants (Jobling 1994). I will end with a summary of what we know about the digestive physiology in pythons, as well as suggestions for future directions to consider for further research.

1.2 Python Foraging Mode: Sit and Wait Predatory Behavior

There are generally two different types of foraging behavior: sit-and-wait foraging and widely active foraging (Pianka 1966; Schoener 1971; Gerritsen and Strickler 1977). Unlike many snake species that forage for prey and feed quite often, pythons are sit-and-wait predators who can spend long periods of time

between meals (Greene 1997). As opportunistic and infrequent feeders and unlike frequent foragers, when pythons do capture prey they are capable of ingesting over half of their own body mass (Greene 1997; Secor 2008). These differences in foraging mode between species are due to ecological pressures leading to evolutionary changes in life history and physiology (Secor and Diamond 2000). Active foragers, for example, typically have greater distances of movement and daily higher rates of energy expenditure than sit-and-wait species (Greene 1997). The sit-and-wait foraging mode results in organisms going weeks or months between meals, and thus balancing lower foraging costs with modest energy consumption to allow this successful adaptation.

Secor and Diamond (2000) measured post feeding regulatory responses from eight different snake species: four frequent feeders with small meals and four infrequent feeders with large meals. During fasting, infrequent feeders had lower metabolic rates and organ masses compared to during digestion, when these animals displayed postprandial increases in energy expenditure, metabolic rate, and organ masses (Secor and Diamond, 2000). These characteristics remain after phylogeny is taken into account, where energy savings motivates the evolution of decreased form and function of organs during fasting and large postprandial regulatory responses in infrequent foragers. Notably, of the four infrequent feeders – Burmese python (*Python bivittatus*), boa constrictor (*Boa constrictor*), sidewinder rattlesnake (*Crotalus cerastes*), and rosy boa (*Lichanura trivirgata*) – pythons had a significantly higher SDA and increase in organ mass one day post feeding (DPF) than any of the other three (Secor and Diamond,

2000).

The up and downregulation of the python gastrointestinal (GI) tract allows the python to manage its infrequent foraging mode. This strategy reduces the maintenance costs of the gastrointestinal (GI) organs during fasting periods and to allow the quick upregulation of the digestive physiology immediately upon food consumption (Slip and Shine, 1988; Secor and Nagy, 1994; Greene 1977). The scale of these rapid, reversible morphological and physiological responses to feeding and fasting is what sets pythons apart from actively foraging snakes.

1.3 The Digestive Process in Pythons

1.3.1 Introduction

The reptilian gastrointestinal tract and accessory organs are composed of the esophagus, pancreas, stomach, gall bladder, appendix, small and large intestines, and cloaca. The general structure is similar in vertebrates, but the individual components of the gastrointestinal tract can vary considerably depending on factors such as diet (Hume 1989; Alexander 1991; Horn and Messner 1992; Karasov and Hume 2010; Karasov et al. 2011).

1.3.2 Prey Capture

When a python is in wait for its next meal, its metabolic rate is at basal levels and its GI tract and other associated organs are quiescent. The GI tract is inactive, including the microvilli and cells of the intestine, which are in an atrophied state. Nutrient transporter and enzyme levels are reduced, and other

organs such as the heart, pancreas, kidneys, and liver have depressed activity (Secor and Diamond, 1995; Secor et al., 2000b; Starck and Beese, 2001; Secor, 2003; Lignot et al., 2005; Cox and Secor, 2008). All of these organs are in dormant and reduced morphological and physiological states when not in active digestion.

Once the python has spotted its prey, it captures it with an open mouth, piercing with its teeth while coiling itself around it to kill the animal by constriction (Secor and Diamond 1988; Secor 2001). This constriction induces rapid prey death as a result of circulatory arrest (Bobak 2015). After the prey has been killed, the python initiates swallowing, often with the prey head-first, using its teeth to grasp the prey as it "walks" its head over the prey. Pythons have the capacity to consume prey items that may be many times larger than their own heads, with skulls that have an impressive amount of flexibility (Secor and Diamond, 1997; Secor and Diamond 1998). In this way, they use this mobility to move the mouth over the prey until the prey is swallowed (Secor and Diamond, 1997; Secor 2008).

1.3.3 Stomach

In reptiles, meal entry into the stomach initiates the gastric phase and activates the release of acid, lipases, and proteases (Johnson et al. 1991; Sanford 1992). During long periods of fasting, gastric acid secretion stops in pythons, but within 24 hours after a meal the contents of the gastric lumen decrease from pH 7 to pH 2 due to an increase in HCl production (Cox and

Secor, 2007; Secor, 2003; Secor and Diamond, 1998). Stomach pH can decline even further and is maintained in the range of pH 1.5 – 2 during digestion, despite the great buffering capacity of the meal (Bessler and Secor 2012). Pepsin is released in its inactive form, pepsinogen, from gastric zymogen granules (Secor 2003; Cox and Secor 2008). This proteolytic enzyme is activated in an acidic environment within the gastric lumen where the breakdown of connective tissue, skeleton, and muscle is begun by the combined effect of the low luminal pH environment and pepsin (Secor 2003; Cox and Secor 2008; Sanford 1992). This low pH also protects the GI tract from bacterial infections (Johnson et al. 1991; Sanford 1992; Cox and Secor 2008). Once the prey is inside the stomach, an immediate and regulated digestive response is necessary, as the dead prey is fully intact and will start to putrefy (Secor 2003). If digestion does not occur quickly, the python's body will swell as bacteria produce gas (Secor 2003; Secor 2008). The python's expanded stomach and esophagus can also put pressure on the lungs, making breathing difficult during digestion of a huge meal (Stark et al, 2004; Secor 2008).

1.3.4 Intestines, Liver, Gall Bladder, and Pancreatic Secretions

As the intestinal phase beings and the meal is digested, gastric chyme moves from the stomach to the small intestine through the pyloric sphincter. After the chyme has arrived at the small intestine, it is quickly neutralized by the bicarbonate to the point that its pH will increase from 2.5 to 6.5 (Secor et al., 2006), protecting the epithelium of the intestine (Secor et al., 2006; Cox and Secor 2008). In addition, bile produced in the liver is released from the

gallbladder to aid in the assimilation, breakdown, and absorption of lipids from the meal (Secor 1995; Secor et al. 2000b). This fluid allows for the emulsification of fat, as well as for the formation of micelles to increase the surface area for digestive action of lipases secreted from the pancreas (Secor 1995; Secor et al. 2000b).

The chemical breakdown of proteins, lipids, and carbohydrates is carried out by the pancreatic liquid, which contains a mixture of enzymes and bicarbonate (Johnson 1977; Sanford 1992). Lipase, amylase, trypsin, and enzyme proteases are released by the pancreas to aid in digestion (Secor 1995; Secor et al. 2000b). Cox and Secor (2008) found that pythons have a postprandial 6-fold increase in trypsin activity and a 20-fold increase in amylase activity within four days post feeding as the prey continues to the intestine. During fasting periods, enzymes are down-regulated to diminish energy expenditure between meals (Cox and Secor 2008). To do this, pythons must be able to restore digestive enzyme capacities after food consumption, which they do by increasing enzyme production and activity, as well as the surface area of the secretory organs (Cox and Secor 2008).

Within twenty-four hours after food consumption, the python's small intestine can increase in mass by 70% (Figure 1) (Secor and Diamond, 1995; Lignot et al., 2005). Enterocytes double their microvilli length and rate of amino acid uptake within 6h after a meal, and by 24 h they quadruple in length, contributing to a ten-fold increase in nutrient uptake (Secor and Diamond, 1995; Lignot et al., 2005; Cox and Secor, 2008). At this time, the small intestine has

greatly increased its synthesis of oleolethanolamide. Oleoylethanolamide has been shown to inhibit feeding in rodents, and is known to induce between-meal satiety (Astarita et al. 2006). Astarita et al. (2006) quantified the postprandial levels of oleoylethanolamide in the small intestine of Burmese pythons using isotope-dilution liquid chromatography/mass spectrometry (LC/MS). They observed a 300-fold increase in oleoylethanolamide levels in the small intestine of fed pythons compared with fasted animals (Secor and Diamond, 1995; Lignot et al., 2005; Astarita et al., 2006; Cox and Secor, 2008). Overall, these data demonstrate the matched and coordinated regulation and massive increases in GI form and function.

Through the last leg of the digestive process, unabsorbed material starts to enter the large intestine, which occurs around two days after meal consumption in juveniles (Lignot et al., 2005). As each day of digestion proceeds, the large intestine and cecum continue to be filled with additional unabsorbed material, much of which is hair, as it moves to the cloaca (Cundall et al., 1993). Urate deposits coming from the kidneys begins to accrue in the cloaca, and a combined bolus of urate and feces leave the cloaca at the conclusion of this process (Secor et al., 2006; Cox and Secor, 2008).

1.3.5 Conclusion of Digestion

When the last of the prey has gone through the stomach, usually six to seven DPF, the downregulation of gastrointestinal organ structure and function begins. The stomach rises above pH 6, enzyme activity in the pancreas

decreases by half, the mass of the kidney, liver, and intestines decrease, and the intestinal microvilli length and nutrient uptake rates decrease substantially, all back to levels similar to those prior to meal consumption (Figure 3) (Secor et al., 2006; Cox and Secor, 2008). With the final excretion of wastes from the meal, the pythons are able to swiftly downregulate gastrointestinal form and function at the organ and tissue levels.

1.4 Cardiovascular Response

There is a similarly large cardiovascular response that supports the increase in metabolism after food consumption in pythons. In response to digestion, cardiac mass (Figure 2), heart rate, and stroke volume all experience significant increases as systemic resistance decreases, all while blood pressure is maintained (Secor et al., 2000a,b; Wang et al. 2001b; Starck and Wimmer 2005; Skovgaard et el. 2009; Enok et al. 2012).

Pythons exhibit up to 11-fold increases in blood flow to the heart after a meal (Secor and White; 2010). This substantial increase in blood flow in these animals compared to other vertebrates is due to the much greater increase in oxygen consumption after a meal (Wang and Skovgaard 2008). In pythons, postprandial cardiac hyperemia mediates increased blood flow to the heart, which allows an accompanying increased blood flow to the intestines (Secor and White 2010). In some fishes and mammals exhibiting infrequent feeding, intestinal hyperemia also occurs after food consumption, but on a much smaller scale (Rees et al. 1982; Axelsson et al. 2000; Madsen et al. 2006; Secor and

White, 2007; Altimiras et al. 2008; Eliason et al. 2008; Seth et al. 2009). Intestinal hyperemia is driven by neurotensin, a regulatory peptide released during digestion that has been shown to decrease intestinal vascular resistance in pythons (Wang et al. 2000; Secor et al., 2001; Skovgaard et al., 2007). Plasma concentrations of neurotensin increase more than three-fold after food consumption, allowing the increase in intestinal blood flow during digestion in pythons (Hicks et al. 2000; Secor et al. 2000a). As metabolism increases after a meal, pythons also display up to five-fold increases in ventilation and cardiac output, which can exceed four-fold increases (Secor et al., 2000b; Secor and White, 2007). There is a substantial increase in heart rate, and stroke volume doubles, all while cardiac mass can increase by up to 40% (Secor and Diamond, 1995; Andersen et al., 2005).

Digestion in pythons and the secretion of gastric acid also causes increases in plasma bicarbonate ion concentrations (Secor and Diamond, 1997a; Overgaard et al., 1999; Overgaard and Wang, 2002). The postprandial increase in bicarbonate ions in blood effects an alkalosis, also referred to as an “alkaline tide.” The degree of this alkaline tide can be managed, in part, by an accompanying increase in the partial pressure of carbon dioxide (Overgaard et al., 1999; Overagaard and Wang, 2002). These together allow the arterial pH to be maintained, with no significant change after feeding (Overgaard et al., 1999; Overgaard and Wang, 2002). While ventilation increases, it is not greater than the increase in metabolic carbon dioxide also produced during digestion (Overgaard et al., 1999; Busk et al., 2000a, b; Arvedsen et al., 2005).

1.5 Mechanisms of Regulation and Integrated Efforts

Digestion in pythons requires both a substantial metabolic investment as well as the organized interactions of tissues to be able to quickly regulate gastrointestinal structure and function to process a meal. Pythons are able to do this by many different mechanisms. After food consumption, plasma concentrations of many gastrointestinal peptides increase significantly (Secor et al., 2001). Cholecystokinin (CCK), which is released from the small intestine in response to feeding, is one such peptide and is a hunger suppressant (Rindi et al., 2004; Murphy and Bloom 2006; Simpson et al; 2008). In pythons, CCK increases 25-fold after a meal, whereas in humans there is only a three to fourfold postprandial increase in this same peptide (Secor et al; 2001).

A large amount of the energy of digestion is used during the first or second day after food consumption, when the prey is still within the stomach of the python (Secor and Diamond 1995). Much of this energy is derived from the python's stored energy reserves, as opposed to only from the absorbed energy content of the prey (Secor and Diamond 1995). This is evidenced by significant increases in plasma triglycerides after feeding, especially within the first 24 h after food consumption (Secor and Diamond, 1995; Starck et al., 2004).

The small intestine, as mentioned before, increases greatly in size after food consumption. This large increase in mass is mainly due to the hypertrophy of epithelial cells in the small intestine with an accompanying hyperplasia (increase in cell proliferation) of the epithelium (Lignot et al., 2005). Enterocytes

can increase by 40% in width, with lengthening of the villi and thickening of the mucosa (Lignot et al., 2005). The absorption of lipids from the meal has been found to promote this increase in width (Starck and Beese, 2001; Lignot et al., 2005). Additionally, while python enterocytes continue to replicate during fasting periods, it is after a meal that replication rates increase significantly (Secor et al., 2000a; Starck and Beese, 2001; Lignot and Secor, 2003).

Cellular replication rates in the python gastrointestinal tract are modulated by the snake's digestive status, with significant increases immediately following food consumption followed by decline after digestion is complete (Helmstetter et al., 2009). Within hours after feeding, overall cell proliferation rates increase, but the exact timing is organ-specific (Helmstetter et al., 2009). Cell proliferation in the stomach begins to increase upon ingestion of the meal and peaks at three days post-feeding. Oxyntopeptic (acid-secreting) cells in the stomach take on a greater workload after food consumption when there is a need more for turnover of cells in the gastric glands, as do the pepsinogen-secreting cells described above (Helmstetter et al., 2009). There is also extensive cell turnover in the small intestine, which has the adaptive capacity to alter form and function to changes in digestive demand quickly and efficiently (Lignot et al., 2005). In the small intestines, Helmstetter et al. (2009) found that cell proliferation can quadruple within twelve hours after a meal and increase almost ten-fold at one day post-feeding, with peak rates at three days post-feeding (Helmstetter et al., 2009). These rates of cell proliferation are substantially greater than at fasted levels, and no significant change in rates were observed at six days after a meal

(Helmstetter et al., 2009). In the large intestine, feeding also initiates a large increase in cell proliferation, doubling at twelve hours post-feeding and increasing up to thirteen times more at one day post-feeding, where it reached a plateau (Helmstetter et al., 2009). New cell density in the epithelium remains at this plateau through the third day and only starts to decrease at six at one days post-feeding (Helmstetter et al., 2009).

1.6 Metabolic response

One of the most impressive features of the digestive response in pythons is the accompanying postprandial increase in metabolism, which can increase up to 44-fold during this time (Secor and Diamond, 1997b). This SDA response is defined as the accumulated energy expended on all postprandial activities – ingestion, digestion, absorption, and assimilation – relating to the digestion of a meal (Jobling, 1994). While the metabolic responses of both invertebrate and vertebrate species have been well studied for many years, the python stands out because of the sheer magnitude of its metabolic response (Secor et al., 1994; Secor and Diamond 1997; Cox and Secor 2007; Secor 2008). To put this into perspective, the average maximum increase in metabolic rate for humans during digestion is about 25% and in fishes is 136%, but for pythons consuming a meal 25% of its body mass, this number is an extraordinary 687% (Westerterp 2004; Secor 2009). This capacity is a necessary effect of consuming extremely large meals. For this reason, the python is an iconic physiological model for studying massive and rapid increases in postprandial metabolism (Secor and Diamond, 1997b.; Secor 2008).

1.7 Specific Dynamic Action (SDA)

The first documented SDA profile carried out in a reptile was done in 1910, examining the *Boa constrictor* (Buytendijk 1910). Later, Francis Gano Benedict was a pioneer in studies examining reptile metabolism and SDA in 1932, when he described the metabolic response after food consumption in three snakes species (Benedict 1932a,b). After his research, there was very little interest in the reptile SDA until the mid 1990's, when several studies reported large postprandial metabolic responses in snakes (Andrade et al. 1997; Secor et al. 1994, Secor and Diamond 1997). This led to renewed interest in research on the SDA in reptiles, with many studies carried out in snakes, specifically pythons (Secor 2009).

SDA is a critical part of an organism's energy budget and has remained a topic of interest for many years since the 1990's, having been studied in all major vertebrate groups (Andrade et al., 2005; McCue 2006; Secor 2009). The SDA response is characterized by a rapid postprandial increase in oxygen consumption. This response has typically been divided into three different phases: preabsorptive, absorptive, and postabsorptive (McCue 2006). The first phase, the preabsorptive phase, includes everything from prey capture to gut peristalsis, enzyme and acid secretion, and initial intestinal changes (McCue 2006). Nutrient transport and intestinal absorption distinguish the absorptive phase, and the postabsorptive phase is characterized by glycogen and urea production, protein synthesis, renal excretion, and subsequent growth (McCue 2006).

SDA is usually measured as the total amount of oxygen consumed and is expressed as an SDA coefficient, represented by the SDA percentage (%). This coefficient gives the cost of digestion as a percent of the energy ingested. This allows the comparison of differences in meal size and composition in relation to how much energy is expended (McCue 2006). Many factors affect the postprandial metabolic response, such as the size and type of meal, its composition, and the python's body temperature during digestion (Secor and Diamond 1997).

1.8 SDA Determinants in a Python

The main determinants of SDA are the meal size and composition and temperature (Overgaard et al., 1999), other factors can also influence SDA % values. For instance, increasing the size of the meal or the protein content of the meal each contribute to a greater SDA response (Secor and Diamond, 1997b; Wang et al., 2003; McCue et al., 2005).

1.8.1 Meal Size and Composition

Pythons have the capacity to consume very large meals, and after a python has fed it may experience difficulty moving about, increasing the possibility of predation. Thus, rapid and efficient digestion is desirable (Garland and Arnold 1983). Meal size is positively associated with the metabolic response after a meal (Andrade et al., 1997; Secor and Diamond 1997; Tsai et al., 2008; Bessler et al., 2010). In many species, such as fishes, the metabolic response to

increased meal size reaches a plateau as the maximum oxidative capacity of the gastrointestinal tissues approaches (Jobling and Davies 1980). Pythons, however, do not appear to show this plateau: oxygen uptake increases after a meal continuously, up to 100% of the animal's body weight (Andrade et al. 1997; Secor and Diamond 1997; McCue and Lillywhite 2002; Toledo et al. 2003).

The SDA response is also influenced by the meal composition. McCue et al. (2005) examining this response in Burmese pythons using different protein, carbohydrate, and fat-based meals. This study found that protein-based meals yielded a signficiantly higher SDA response than those with higher amounts of fats or carbohydrates (McCue et al., 2005). SDA response generally rises with increasing protein content in the meal and not with the ingestion of fat (Coulson and Hernandez 1979; McCue et al. 2005). Protein synthesis is a major contributor the SDA response in other reptiles, birds, amphibians, and fishes as well (Bonnet 1926; Karst et al. 1984; McCue et al; 2005; et al. 2006; Tandler and Beamish 1980; Weiss and Rapport 1924). Various specific proteins also elicit different responses during digestion, with more complex proteins causing a larger response than simpler ones (Secor et al., 2002). Small sugars are digested and cause similar metabolic responses, comparable to proteins (Secor et al., 2002; McCue et al., 2005). Other components like large carbohydrates, such as starch and cellulose, are not easily digested by pythons (McCue et al., 2005).

1.8.2 Temperature

Ectotherms depend on temperature for many physiological functions, and

ambient temperature has a strong influence on the postprandial SDA response. Snakes favor higher temperatures for digestion and may even regurgitate a meal if the temperature decreases too low (Wang et al., 2002; Tsai et al., 2008). Both the standard metabolic rate and maximum oxygen uptake increase with rising temperatures as the duration and time to reach that maximum uptake decrease (Wang et al., 2002; Toledo et al., 2003; Bessler et al., 2010). Wang et al. (2003) found that both the standard metabolic rate and the maximum oxygen uptake increase with temperature in pythons, similar to the factorial increase in metabolism after a meal (Wang et al. 2002).

1.9 Future Directions

Regulation of gastrointestinal morphology and function during the postprandial digestive response is not a novel process in pythons; it is documented in many different vertebrates (Hammond and Diamond, 1994; Secor and Diamond, 2000; McWilliams and Karasov, 2001; Kroghdahl and Bakke-Mckellep, 2005; Secor, 2005a). However, pythons, who are infrequent and opportunistic feeders, set themselves apart due to the scale of their regulatory response. The rapid and substantial increases in GI form and function and accompanying metabolic response after a meal, followed by a decline back to fasting levels after digestion has completed, is what makes this response in pythons especially unique (Secor, 2005b; Ott and Secor, 2007). Infrequent feeders such as pythons, who use the sit-and-wait foraging method, benefit from

having a reduced rate of basal energy expenditure. The GI tissues are very expensive to maintain in general, especially in a python consuming large meals between long periods of fasting (Reenstra and Forte, 1981; Cant et al., 1996; Nyachoti et al., 2000). Selection favoring the depressed and downregulated activity of the gastrointestinal tract during fasting periods can be seen in this way as an adaptive response to conserve energy during fasting periods (Secor and Diamond, 2000; Secor, 2005b).

Pythons have unique and remarkable digestive capabilities with impressive changes in gastrointestinal morphology and physiology after a meal. They rapidly upregulate form and function of gastrointestinal tissues and organs following food consumption, with massive increases in metabolic rate to account for the large energy costs that comes with the consumption of particularly large meals. While much has been discovered about digestive physiology in pythons, there is still much to learn about the responses of other organ systems to feeding and the mechanisms underlying digestive physiology and associated organ growth.

One area of physiology clearly lacking in reptiles, and specifically in pythons, is how food availability and energy balance may affect brain cell proliferation. This would be especially interesting to study in pythons considering that their digestive physiology is changing in a very uniform and matched manner after food consumption. Given the impressive digestive physiology of pythons, the influx of energy after food consumption is very likely important to supporting the growth and function of other critical tissues, such as the brain. However, the

brain remains one of the only unstudied organs in terms of post-prandial response. Cell proliferation rates in the python gastrointestinal tract increase after a meal to aid in cell renewal after digestion, as detailed above, but whether proliferation rates are also increasing at the same time in the brain has not yet been examined. This would be beneficial in order to further understand neuroplasticity in relation to different factors such as energy balance and metabolism in an evolutionary context.

Chapter 2: A BrdU Study of the effects of food consumption on brain cell proliferation in the ball python, *Python regius*

2.1 Introduction

The ability to generate new neurons throughout life has been documented in all vertebrate classes. Neurogenesis occurs beyond early development across a wide range of taxa, but at a much higher rate and broader distribution in several non-mammalian vertebrates as compared to mammals (Barker et al., 2011; Migaud et al., 2016). Adult neurogenesis in the mammalian brain is well studied but limited to only a few brain regions, while in reptiles it is widespread throughout the telencephalon, but has not been examined in comparable detail (Goffinet, 1983; Lopez-Garcia et al. 1988; Lopez- Garcia et al., 1990; Pérez-Cañellas and García-Verdugo, 1996; Bond et al., 2015). Since reptiles are exceptional neural regenerators with extensive neural plasticity, it is surprising that there is not more research investigating neurogenesis in this group of vertebrates (Font et al. 2001; González-Granero, Lezameta, and García-Verdugo 2011).

The production of new brain cells in reptiles occurs primarily at the ventricular zone (VZ) along the ventricle walls in the telencephalon, and the new cells migrate away from the VZ towards different regions of the telencephalon (Alvarez-Buylla et al., 1990; Lopez-Garcia et al., 1988; Lopez-Garcia et al., 1990). Many factors, such as season, temperature, and behavior, are known to affect neurogenesis, and these effects are not uniform throughout the brain. In Algerian psammodromus lizards (*Psammodromus algirus)*, cold temperatures

reduced cell proliferation and migration significantly throughout the telencephalon (Penafiel et al., 2001). Delgado-Gonzalez et al. (2008) found that in Tenerife lizards (*Gallotia galloti*), proliferation of new cells in the olfactory regions was higher during the spring and summer than winter and autumn. Other studies found the highest rates of proliferation in the medial cortex of the brain, which have been associated with memory and spatial navigation (Perez-Canellas and Garcia-Verdugo, 1996). In common garter snakes (*Thamnophis sirtalis parietalis*), Maine et al. (2014) found that the number of new cells in the VZ were higher in the fall than in the spring, demonstrating that neurogenesis may play a role in mediating seasonal rhythms in migratory or reproductive behaviors.

To date, the majority of research on neurogenesis in reptiles has been done in lizards, and only a few studies have examined neurogenesis in snakes (Holtzman and Halpern, 1990; Holding et al., 2012; Maine et al., 2014). More research on brain cell proliferation in snakes would be useful to establish a better comparative perspective between reptiles, as well as among vertebrates. Furthermore, in order to learn more about how different physiological factors, such as food availability and energy balance, affect brain cell proliferation, more research examining snakes would be especially valuable. Examining this in pythons may provide critical new insights into understanding the relationship between energy balance and availability in relation to the cell proliferation in the brain, contributing to our understanding of neurogenesis in vertebrates in a broader context.

What sets pythons apart from other snake species is their specific dynamic action (SDA) response after food intake, where metabolic rate increases to process a meal (Jobling, 1994). While most organisms have a postprandial increase in metabolic rate, it is the magnitude of the python response that makes them unique (Houlihan 1991; McCue 2003; Secor 2009). Pythons are able to go for long periods of time without eating, but then are capable of ingesting considerably large prey relative to their body size (Secor, 2008). When a python is waiting for its next meal, the GI tract is inactive, nutrient transporters and enzymes are reduced, and other organs such as the pancreas, kidneys, and liver have depressed activity (Secor and Diamond, 1995; Secor et al., 2000b; Starck and Beese, 2001; Secor, 2003; Lignot et al., 2005; Cox and Secor, 2008). All of these organs are in a significantly reduced in their morphological and physiological state when not in active digestion. Within twenty-four hours of ingesting prey, each of these organs is upregulated in form and function (Figure 3) with rapid increases cell proliferation as the snake undergoes large a rise in metabolic rate and gastrointestinal (GI) performance (Secor and Diamond, 1995; Lignot et al., 2005; Cox and Secor, 2008). The cardiovascular system is also upregulated at this time, with up to five-fold increases in ventilation, cardiac output, and heart rate within one day post feeding (Stark et al, 2004). Organ form and function are up-regulated to allow the python to carry out digestive process and contribute to the massive energy cost associated with the consumption of meals (Secor, 2008). This upregulation incurs a metabolic cost, causing the large SDA response. Despite the impressive magnitude of SDA and organ responses

to feeding, the one organ that has not been investigated in this light is the brain. Given that the brain is a fundamental central regulator of feeding activity and metabolism, and that cell proliferation in the brain in response to feeding is a likely response, it is surprising that this important organ has never been investigated before and after feeding in the python model.

Reptiles can regenerate large populations of neuronal cells through adulthood, although overall postnatal neurogenesis decreases with age, making the juvenile stage an important one to study (Capilla-Gonzalez et al., 2015; Dennis et al., 2016). Further, decreases in postnatal neurogenesis during the juvenile stages may have long-lasting effects on brain function, which makes this phase an important time frame to study (Lee et al., 2014; Nieto-Estévez et al., 2016). Also, changes in GI physiology with age are common and vary among organisms (Saffray 2013). Understanding other physiological processes that could be associated with this GI plasticity during the pre-adult phase is important to examine because this is a critical life stage during development (Saffrey 2013; Kotrschal 2014). The majority of studies in pythons examining digestive physiology have therefore used juveniles as study animals (Andrew et al., 2015; Cox and Secor, 2007; Helmstetter et al., 2009; Lignot et al., 2005; Secor and Diamond, 1997, 2000, Secor et al., 2000, 2012; Wang et al., 2002). Nutrition also plays a large role in growth and development in general, which points to its possible importance for neurogenesis related to brain growth in the juvenile and sub-adult stages (van der Meeren et al., 2009; Nieto-Estévez et al., 2016; Turkmen et al., 2017).

To understand more about postprandial brain plasticity in reptiles, we investigated the effects of food consumption on new brain cell proliferation in *Python regius*. We separated pythons into treatment groups based on time given to digest a meal to test the hypothesis that food consumption affects brain cell proliferation. If post-prandial neural cell proliferation follows similar patterns to systemic upregulation during digestion, we would expect to see an increase in dividing cells within two days after a meal. We look to examine if cell proliferation does occur in response to feeding in pythons, and if so, determine the post-prandial time course.

2.2 Materials and Methods

2.2.1 Animals and Experimental Design

Twenty-one juvenile male *P. regius* were purchased from Reptile City in Honey Grove, Texas. All procedures were approved by the California Polytechnic State University Institutional Animal Care and Use Committee (protocol #1625). Upon arrival (March 4, 2016) we measured body mass (mean: 286.3 +/- 16.8 g; range: 190.7-430.2 g) and snout-vent length (mean: 63.93 +/- 1.37 cm; range: 53.5-77.0 cm). Snakes were maintained individually in clear 54-quart containers with an 8-inch-long, lengthwise cut polyvinyl chloride piping (6” diameter) as a hide box in each enclosure, with newspaper lining the bottom. A heating cable was placed under one end of each enclosure to provide a thermal gradient within the enclosures. Lights were turned on at 7:00AM and turned off at 17:00PM,

however, there was also ambient light from a window, which provided natural light before and after the lights in the room were on (approximately 11.5-14h light: 12.5-10h dark throughout the experiment). Each python was given water *ad libitum* and fed every two weeks (live mice, 10% of body weight) during a month-long acclimation period before starting the experiment. After the acclimation period, all snakes were fasted for 30 days.

Pythons were haphazardly assigned to one of three groups (n=7 per group), and mass within each group was not significantly different (p=0.767, t-test). Following the 30-day fast, one group (Day 6, abbreviated D6) was fed a large meal (live mice, 20% of body weight) and allowed to digest for six days before sacrifice. The D6 group had completed digestion by 6DPF, as evidenced by defecation observations at this time point during the acclimation period. A second treatment group (D2, abbreviated D2) was fed four days later (live mice, 20% of body weight) and allowed to digest for two days before sacrifice. A control group (C, n=7) continued to be fasted throughout the experiment (Figure 4). We chose these two time points in order to examine cell proliferation during and after active digestion (D2 and D6, respectively). The day before sacrifice, all pythons were given an intraperitoneal injection of 100mg/kg of 5-bromo-2'-deoxyuridine (BrdU; Fisher BioReagents, Fair Lawn, New Jersey) diluted to a concentration of 20mg/ml in reptile Ringer's solution to label proliferating cells (Holding et al. 2012).

2.2.2 Sacrifice, Perfusion, & Embedding

Twenty-four hours after BrdU injection, all snakes were injected with ketamine (50mg/kg) then deeply anesthetized by isofluorane inhalation. After snakes were non-responsive to inversion or tail pinch, they were transcardially perfused with a wash solution (0.9% NaCl, 0.1% $NaNO_2$, 0.1M phosphate buffer (PB)) followed by 4% paraformaldehyde in 0.1M PB. After perfusion, brains were extracted and immersed in 4% paraformaldehyde for twenty-four hours, followed by another twenty-four hours in 0.1M PB solution. Each brain was then embedded in gelatin overnight to form blocks around the brain tissue, then placed again in 4% paraformaldehyde. Gelatin blocks were added to a 30% sucrose solution in 0.1M PB and kept at 4°C until each gelatin cube sank. These blocks were frozen in dry ice and stored at -80°C until sectioning.

2.2.3 Tissue Sectioning and BrdU Immunohistochemistry

Brains were cut on a cryostat (Bright OTF-5000) into eight series of 36μm thick coronal sections. The first series was directly mounted onto slides (Fisherbrand Double Frosted Microscope Slides treated with Vectabond, Vector Laboratories, Burlingame, CA, USA), hydrated with mounting solution to flatten the sections on the slides, and allowed to dry overnight. These sections were then stained with cresyl violet after drying. All other sections were stored in cryoprotectant (20% glycerin in 0.1M PB) at -20°C until immunohistochemical processing.

New cell proliferation was examined by using immunhistochemistry for BrdU as in Holding *et al.* (2012). Every eighth section through the olfactory bulbs and telencephalon (adjacent sections from the cresyl violet-stained sections) were used. Free-floating sections were washed with phosphate-buffered saline (PBS) three times for five minutes each, followed by an incubation in 4N HCl for 15 minutes to initiate DNA denaturation. Following this, all sections were rinsed once for 5 minutes in PBS before being added to a 3.8% sodium borate wash for ten minutes. Sections were rinsed in PBS three times for ten minutes each, then immediately placed in a blocking solution composed of 5% normal horse serum (Vector Laboratories) and 0.5% hydrogen peroxide in PBS with 0.3% Triton-X 100 (PBST) to reduce nonspecific binding for 60 minutes on a rotating shaker table. Immediately following blocking, sections were incubated in primary antibody (mouse anti-BrdU, Dako Laboratories, clone: Bu20a) at a dilution of 1:1000 in PBST for 24 hours on a rotating shaker table. After this period, sections were rinsed in PBST three times for five minutes each and immediately incubated in 1:100 secondary antibody (biotinylated horse anti-mouse antibody, Vector Laboratories, cat no: BA-2000) for 60 minutes on a rotating shaker table. Sections were rinsed in PBST three times for five minutes each before being incubated in avidin-biotin-peroxidase solution (Vector Laboratories, Elite ABC kit, cat no: PK6100) for one hour on a rotating shaker table. Sections were then washed in PBST three times for five minutes each, and then immersed in a chromagen and hydrogen peroxide solution in PBS (Vector Laboratories, cat no: SK-4700) for 4 minutes to visualize primary antibody binding. Following this

staining, all sections were washed in PBS two times for five minutes each to terminate the reaction. All sections were mounted onto slides and cover slipped after drying for 24 hours. Negative control tests were also carried out; these consisted of excluding the primary antibody and the antigen retrieval step and blocking staining with excess BrdU. All negative controls had no staining.

2.2.4 Measurement and Cell Density

Study regions in the brain were based on the work of Halpern (1980) and Smeets (1988) and determined by direct observation of cresyl violet stained sections under a microscope (Leica DM750). All cresyl violet stained sections were photographed with a dissecting microscope (Leica EZ4HD) at 16X magnification. These digital images were used to determine and delineate the study regions of the brain including the accessory olfactory and olfactory bulbs making up the olfactory region (AOB/OB), the retrobulbar regions (RB), cortex, dorsal ventricular ridge and nucleus sphericus (DVR/NS), and septal nucleus (SN) (Figure 5). The DVR and NS were combined and analyzed together due to our inability to distinguish these regions (Maine *et al.* 2014) and because we quantified cell proliferation only, not migration, so cells born along the ventral side of the ventricle could migrate into either the DVR or NS. The lateral, medial, and dorsal regions of the cortex were grouped and analyzed together (Maine *et al.* 2014).

All anti-BrdU stained sections were observed under a microscope, and the observer was blind to treatment groups. Cell counts were carried out in the pre-

determined regions listed above along the ventricle along the ependymal surface. A cell was considered ependymal if it was within 50μm of a ventricle (Almli and Wilczynski, 2007; Maine et al. 2014; Figure 6). Cells were counted on both the right and left hemispheres of each section per brain to obtain a total cell count per pre-determined region. Cell counts were performed once at 40X magnification and a second time at 100X magnification. A recount was performed at 100X magnification if the counts differed by 1-3 cells. As expected, labeled cells in the parenchyma were rare, because cell proliferation occurs along the ventricles (Lopez-Garcia et al., 1988; Lopez-Garcia et al., 1990). Total cell counts were calculated for each region and for each individual brain. All anti-BrdU stained sections were photographed using a dissecting microscope (Leica EZ4HD) at 16X magnification and saved as digital images. The length of the ventricle in mm in each region was measured using ImageJ (Image Processing and Analysis in Java, National Institutes of Health). To obtain cell densities (cells/mm), we counted the number of BrdU-positive cells found in each region along the length of the ventricle in that region. Overall cell density per brain was calculated with the same method using all brain regions.

2.2.5 Statistics

We used a one-way multivariate analysis of variance (MANOVA) using JMP Pro software (SAS Institute Inc.; Version 12.2.0) to determine if there were treatment effects on new cell densities in all brain regions simultaneously. We

also used profile analysis in MANOVA to test for parallelism or non-parallelism between treatment groups.

When MANOVA gave a significant main effect, we performed an analysis of variance (ANOVA) to compare new cell densities in each pre-determined study region by treatment, and each significant ANOVA was followed by a post-hoc Tukey-Kramer HSD test. A separate ANOVA was also performed to examine overall total new cell density by treatment group. Cell densities were log-transformed to meet the assumption of normality.

One python brain in group D2 showed inconclusive BrdU staining and was excluded from all analyses, leaving this group with a reduced sample size (n = 6). There was also one python from the control group that had a portion of the olfactory bulb damaged during sectioning, causing several tissue sections to be unusable for further staining. Therefore, we did not include the olfactory bulb cell density of that snake in our analysis, but did include data from all other brain regions of that individual.

2.3 Results

BrdU-ir cells were found along the VZ, as expected, in every brain region in the telencephalon. Treatment had a significant effect on total new cell density ($F_{2,17}$=37.06; p<0.0001; Figure 7), with total new cell density higher in group D6 than both groups C and D2 (p<0.0001), with no significant difference between groups C and D2 (p=0.9421).

A one-way MANOVA indicated that treatment had a significant main effect on cell proliferation ($F_{10,24}$=4.43; p=0.0013). We then used profile analysis to examine these effects further, where profiles are parallel if the differences in new cell density by region are the same across the treatment groups. Significant non-parallelism would indicate that the differences in new cell densities by region are not the same across the treatment groups. Profile analysis on our data demonstrated non-parallelism in the profiles across treatments, representing a significant differential regional effect ($F_{8,26}$ =4.43; p=0.0426, supplemental figure S.1.). Significant non-parallelism indicates differential treatment effects on brain region for new cell density (Figure 8). Within each brain region, treatment had a significant effect on the mean new cell density within each of the five brain regions (AOB/OB: $F_{2,16}$=15.38; p=0.0002; RB: $F_{2,17}$=11.09; p=0.0008; Cortex: $F_{2,17}$=15.95; p=0.0001; DVR/NS: $F_{2,17}$=24.28; p<0.0001; SN: $F_{2,17}$=17.90; p<0.0001). In every brain region measured, the D6 group had significantly higher new cell density than both the D2 and C groups, and there was no significant difference between the C and D2 groups (Table 1). Post-hoc tests to examine regional differences by treatment between brain regions failed to identify significant differences by region (p>0.05).

2.4 Discussion

We demonstrate that food consumption affects brain cell proliferation in juvenile pythons. Our results support the hypothesis that food consumption

affects new cell growth in the brain, with proliferation largely increased after most of the digestion and absorption is complete, compared to during active digestion or without food consumption.

Our study only examined cell proliferation, not migration or survival, in the brain and did not determine whether new cells were neurons or glial cells. Common neural markers, such as doublecortin (DCX) and NeuN, have not been successful in labeling cells in the brain tissue of snakes (Nomura et al., 2013; Bales 2014, unpublished results). Currently, there are no known antibodies that label mature neurons in snakes, which limits the ability to identify BrdU-labeled cells in reptile brains (Nomura et al., 2013).

2.4.1 Treatment Effects on New Cell Density

Treatment had a significant effect on overall cell proliferation, with the D6 group having a significantly higher amount of cell proliferation than the C or D2 groups. New cell density was not different between the D2 and control groups. These results may be understood more clearly by considering when energy expenditure would be most beneficial to the python after feeding in relation to proliferation of new brain cells compared to the rest of the body at any point after meal consumption.

Pythons exhibit postprandial systemic plasticity and metabolic upregulation immediately after ingesting a meal, with rapid and pronounced growth of organs contributing to digestive and cardiovascular function (Secor et al., 2000; Ott and Secor, 2007). We found that at this time (D2), python brains do

not yet experience an increase in cell proliferative activity. In contrast, after most of the digestion and absorption is complete (D6), digestive system physiology at the organ and cell level decreases after the SDA response (Secor 2008); this was when we saw the highest density of proliferating cells.

Our results may be explained by a physiological tradeoff between differences in energy balance and metabolic performance needs at certain postprandial time points. During a python's initial SDA response, when significant postprandial metabolic energy is necessary, brain cell proliferation is low. The python's physiological state and energy input here is focused toward the immediate need: digesting a large meal. The python must use the mobilized energy for its proximate needs after food consumption: the digestion, absorption, and assimilation of the meal (Cox and Secor, 2007; Helmstetter et al., 2009; Ott and Secor, 2007; Secor and Diamond, 1997; Secor et al., 1994). While there was some proliferative activity in the brain, it is possible that such proliferation is not extensive because it is more efficient to put energy into the digestive needs of the python at that point. But after this response has passed, so has the need for extensive investment into the gastrointestinal (Secor and Diamond, 1995; Starck and Beese, 2001; Secor, 2003; Lignot et al., 2005; Cox and Secor, 2008). The delay in brain cell proliferation that we saw in the D6 group may be an adaptive response where energy may be diverted to growth or maintenance of other systems, including the brain, after the immediate need of processing a meal has finished.

2.4.2 Total and Regional New Cell Density

The pattern from highest to lowest overall proliferation rate by region was identical across treatment groups: highest in the retrobulbar region and olfactory region, followed by the septal nucleus, dorsal ventricular ridge/nucleus sphericus, and lowest in the cortex region, and this order was seen consistently within each treatment group (Supplemental Figure S.2.).

New cell proliferation has been found in all major subdivisions of the reptile telencephalon, with a substantial amount of neurogenesis documented in the main and accessory olfactory bulbs, compared to the rest of the brain (Font et al., 2001). Our results corroborate this, with these two regions having the highest new cell density in each treatment group. Proliferative activity was increased in all brain regions examined in the D6 group compared to both the D2 and control groups.

We observed that the retrobulbar region of pythons showed significantly increased cell proliferation 6 days following food consumption. This region, which has also been called the rostral forebrain, or anterior olfactory nucleus in lizards, is directly linked to the olfactory bulb (Halpern, 1980). This area has been shown to have high rates of neurogenesis in lizards (Shao et al.,2012, Delgado-Gonzalez et al., 2008) and snakes (Bales 2014, unpublished results), and is one of the highest areas of neurogenesis in reptiles studied so far (Font et al., 2001). It is speculated that this region is where new cells proliferate to migrate toward the olfactory bulbs for future processing for incorporation into pathways associated with olfactory processing, a mechanism resembling the rostral

migratory stream (RMS) in mammals (Lois and Alvarez-Buylla, 1994; Pérez-Cañellas and Garcia-Verdugo, 1996; Font et al., 2001). However, while the RMS has been studied in mammals, it is poorly documented in reptiles, and the effects of food consumption and digestion on this region are unknown.

Comparing cell proliferation among reptiles, our data was similar to existing patterns seen across different taxa. The SN, Cortex, and DVR/NS displayed the lowest new cell densities of the five brain regions investigated. This is somewhat in contrast to previous studies in lizards that found more proliferative activity in these regions than we found in ours. Perez-Canellas and Garcia-Verdigo (1996) found that *T. mauritanica* exhibited highest cell proliferation in the medial cortex, while the NS had the highest proliferative activity in *P. hispanica* (Perez-Sanchez et al., 1989). However, the olfactory bulbs were also quantified in these studies and were the next highest region of cell proliferation. Maine et al. (2014) found that the SN had the highest proliferative activity in T*. sirtalis parietalis*, followed by the cortex, with the DVR and NS regions having the lowest proliferation; however, they did not quantify proliferation in the olfactory and retrobulbar regions.

The lack of a common pattern of cell proliferation rates across brain regions in different taxa among reptiles could be due to several factors. There is a possibility that these differences may be species-dependent or also reliant upon other factors such as sex or seasonal effects. Increased neurogenesis in specific regions may be related to different functions that are more important for those species. Furthermore, there is a need for future research to investigate a

wider range of factors that may affect neuroplasticity, some of which may be species-specific. Comparative studies that look at differences among reptiles may be important to understand more about proliferative activity differences and to give insight into the evolution of brain cell proliferation.

2.4.3 Implications for Further Research

While our research is an important starting point to investigate the effects of food consumption on neurogenesis, many other aspects of these data could be built upon to gain a greater understanding of this phenomenon. Future studies should examine different time points after food consumption and possibly in adults as well as in juveniles to gain a broader understanding of neurogenesis in pythons. While we examined juvenile pythons, we would expect to find the same results if this were carried out in adults, but the effects may not be as pronounced because neurogenesis tends to decrease with age (Capilla-Gonzalez et al., 2015; Dennis et al., 2016). In the adult mammalian brain, aging is considered an intrinsic factor that has been shown to affect the developmental potential of the ventricular zone where cell proliferation occurs (Capilla-Gonzalez et al., 2015). Considering that reptiles continue to add neurons at a high rate through adulthood, the decrease in neurogenesis with age exhibited by other vertebrates may not be as distinct in these animals (Font et al., 2001; Capilla-Gonzalez et al., 2015).

Neurogenesis in mammals has focused on developing new regenerative treatments for neurological and psychiatric diseases and is commonly studied in

mice (Apple et al., 2016; Lindsey and Tropepe 2006). Other research has examined neurogenesis in relation to obesity and metabolic disorders and has suggested that newly generated neurons may contribute to metabolism and energy balance associated with weight regulation (Migaud et al., 2016). There is also evidence that decreased neurogenesis may worsen cognitive deficits that accompany neurodegenerative and metabolic disease in humans (Apple et al., 2016; Migaud et al., 2016). The general trend is that in diseased conditions, there is less neurogenesis in the brain than in healthy conditions. While more research is needed to understand why this occurs, future research building upon our study may give more insight into the mechanisms underlying this phenomenon. There is a possibility that the D6 group may not have had such a dramatic increase in neurogenesis if the pythons were subjected to a more food-limited state, for example, if they had been fasted for longer than the 30-day period prior to feeding in our experiment. In this case, and in conditions such as disease, energy limitations may decrease the amount of energy available for neurogenesis. Understanding more about the mechanisms that stimulate proliferation may clarify how neurogenesis is regulated in different vertebrate species, and provide a better understanding of the functional significance of this trait. (Ruan et al., 2014; Lindsey and Tropepe 2006).

2.4.4 Conclusion

This is the first study to consider how feeding affects cell proliferation in the brain of a reptile. Our study demonstrated an increase in cell proliferation in the python brain six days following food consumption, a time period typically after

the SDA response to feeding. The python's increasing brain cell proliferation after food consumption is highest when peak GI plasticity ends, after digestion is complete. Given the digestive physiology of these animals after a meal, these results closely align with the likely physiological tradeoff between differences in energy balance and metabolic performance needs at certain postprandial time points.

Pythons in particular have several unique features such as an impressive digestive physiology and large, integrated metabolic responses. These characteristics may contribute to understanding more about the evolutionary physiology of vertebrates. This is especially true in pythons, whose sit-and-wait predatory behavior has allowed them to have a distinctive digestive physiology that is well-suited to it. Further research comparing the timing of brain cell proliferation after feeding across several taxa of snakes with different foraging tactics, and therefore divergent SDA responses, would be of great benefit to better understand neuroplasticity in an evolutionary context and how energy balance and metabolism may affect neurogenesis. The roles and possible benefits of neurogenesis may be better elucidated if there is greater understanding of the physiological processes that affect neural cell proliferation and survival across vertebrates.

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APPENDICES

Appendix A Tables

Table 1. Summary of univariate ANOVA test results from post-hoc Tukey HSD tests to compare new cell densities (cells/mm) across treatments for each of the five brain regions and on overall new cell density. Analyses were performed on log-transformed values.

Brain Region		Comparisons			p-value
AOB/OB Density		D6	/	D2	0.0002
$F_{2,16}$ =	p-value	D6	/	C	0.0036
15.38	0.0002	C	/	D2	0.3756
RB Density		D6	/	D2	0.0021
$F_{2,17}$ =	p-value	D6	/	C	0.0025
11.09	0.0008	C	/	D2	0.9711
Cortex Density		D6	/	C	0.0003
$F_{2,17}$ =	p-value	D6	/	D2	0.0007
15.95	0.0001	D2	/	C	0.9654
DVR/NS Density		D6	/	C	<.0001
$F_{2,17}$ =	p-value	D6	/	D2	0.0001
24.28	<.0001	D2	/	C	0.6786
SN Density		D6	/	C	<.0001
$F_{2,17}$ =	p-value	D6	/	D2	0.0015
17.90	<.0001	D2	/	C	0.4404
Overall Cell Density		D6	/	D2	<.0001
$F_{2,17}$ =	p-value	D6	/	C	<.0001
37.06	<.0001	C	/	D2	0.9421

Appendix B Figures

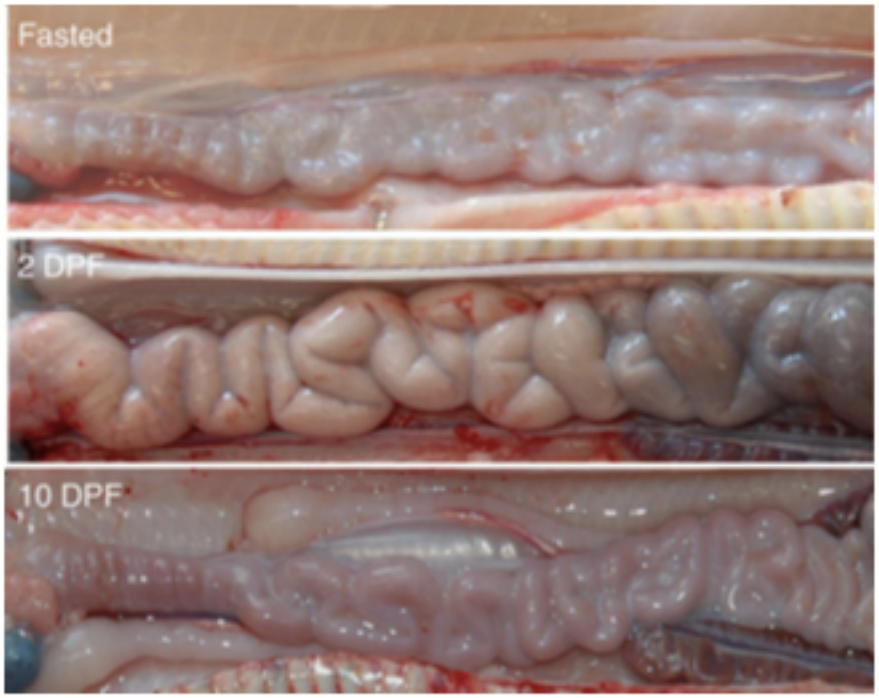


Fig.1. Images of the small intestine of similar-sized Burmese pythons fasted and at 2 and 10days postfeeding (DPF). By 2DPF, the intestine has increased in diameter due primarily to hypertrophy of the epithelial cells; a response that has reversed by 10 DPF.

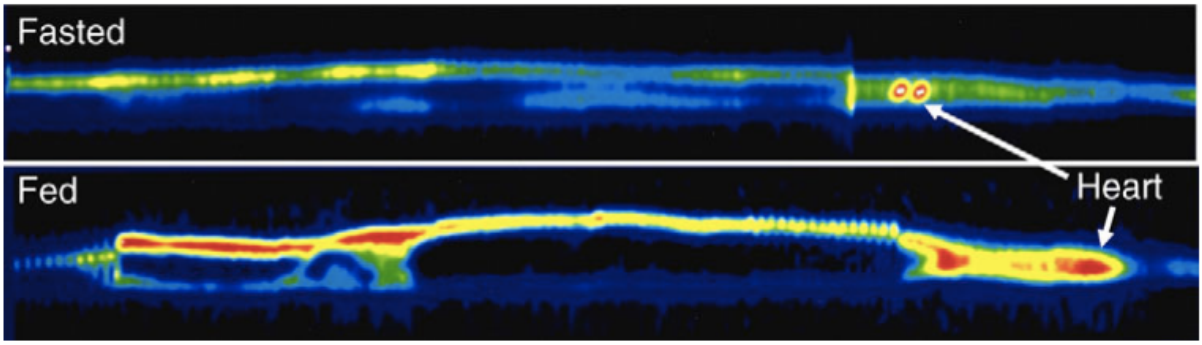


Fig.2. Positron emission tomography (PET) images of a fasted and fed (1 day postfeeding) Burmese python. Snakes were injected with 2-[18F]fluoro-2-deoxyglucose prior to scanning. Bright areas signify regions experiencing high rates of glucose metabolism. The difference between the two images is actually greater given that the intensity of the fasted image had to be increased 1000-fold in order to view the entire snake.

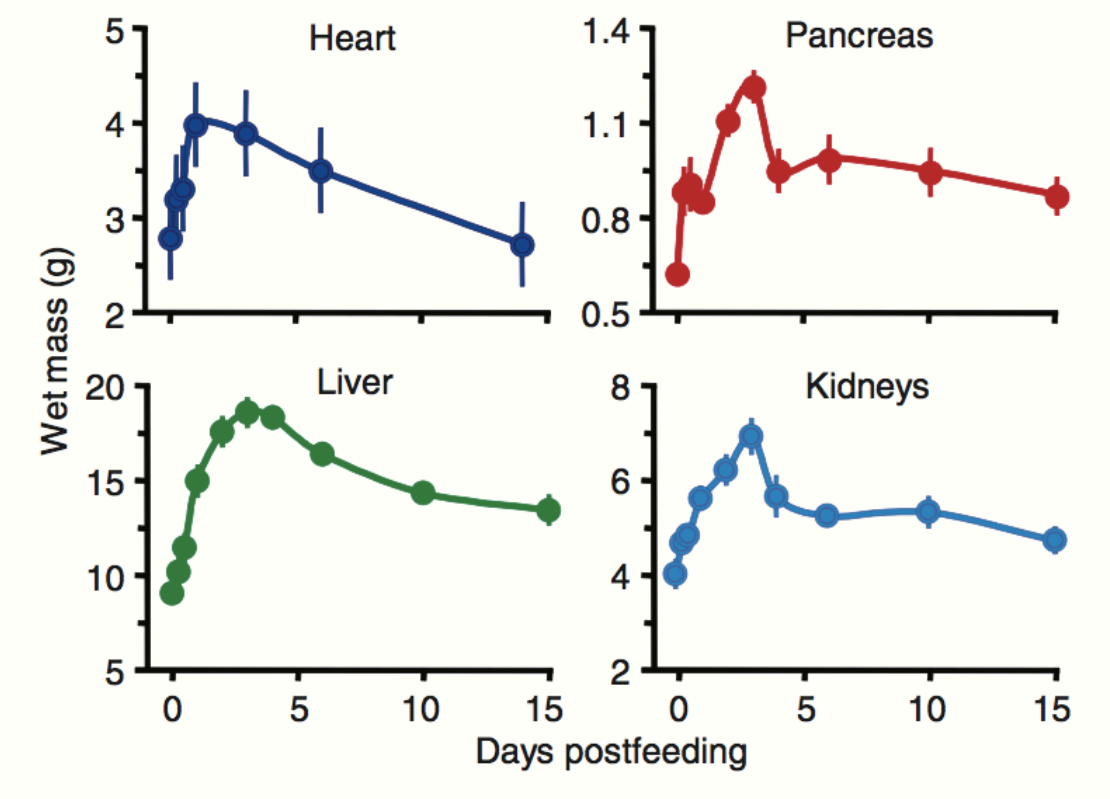


Fig.3. Wet mass of the heart, pancreas, liver and kidneys plotted against time postfeeding for Burmese pythons fasted (0) and following the consumption of rodent meals equal to 25% of the snake's body mass. Feeding generates respective increases in wet mass of 40%, 94%, 106% and 72% for the heart, pancreas, liver and kidneys.

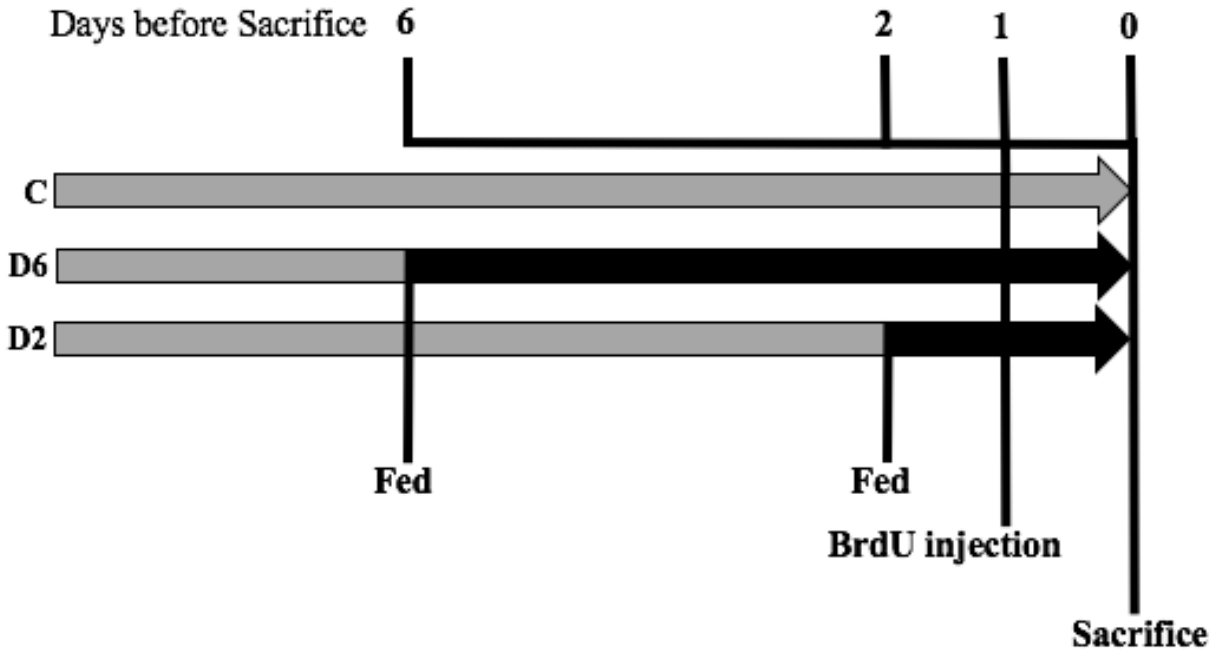


Fig.4. Timeline showing the fasting and feeding periods per treatment group in *Python regius* (C, D2, D6; n=7 per group) after a 30-day acclimation period. BrdU administration occurred in all snakes one day before sacrifice. Areas in grey are periods of fasting and areas in black are periods after feeding.

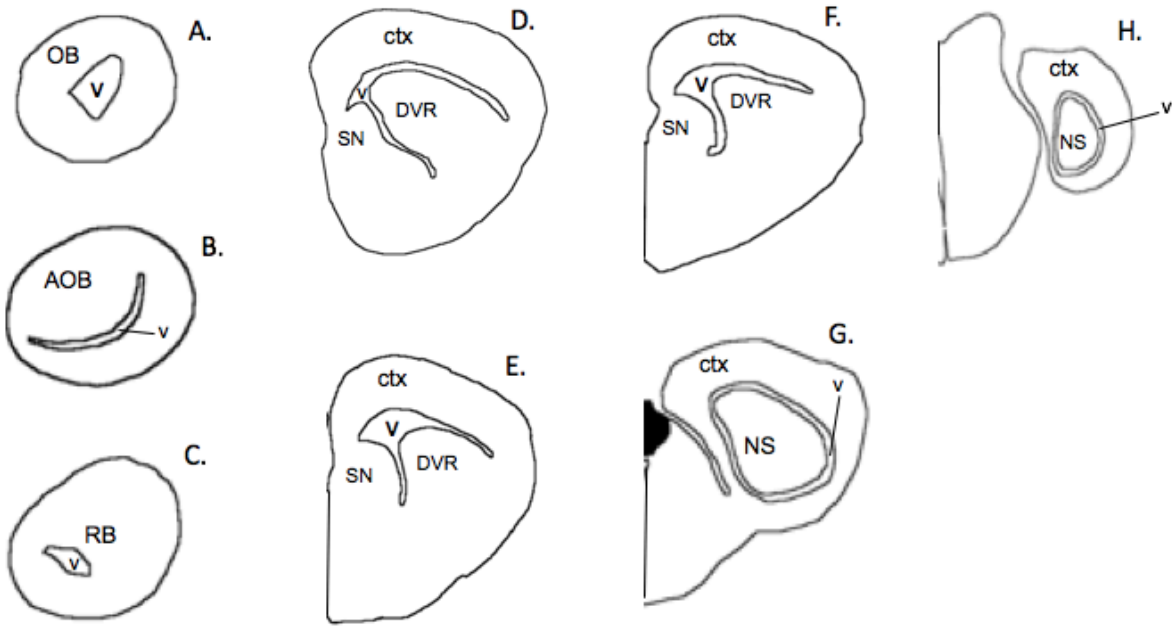


Fig.5. Schematic drawings showing coronal hemi sections of *Python regius* in order from the rostral portion of the brain through the telencephalon. Sections A-H depict the subdivisions used for cell counts, in order of location through the brain. BrdU-labeled nuclei were found and counted along the ventricles, labeled "v," within each section above. AOB: accessory olfactory bulb; ctx: cortex; DVR/NS: dorsal ventricular ridge/nucleus sphericus; OB: olfactory bulb; RB: retrobular; SB: septal nucleus. Sections A-H were outlined from stained images in GNU Image Manipulation Program (GIMP; free software; version 2.8.18).

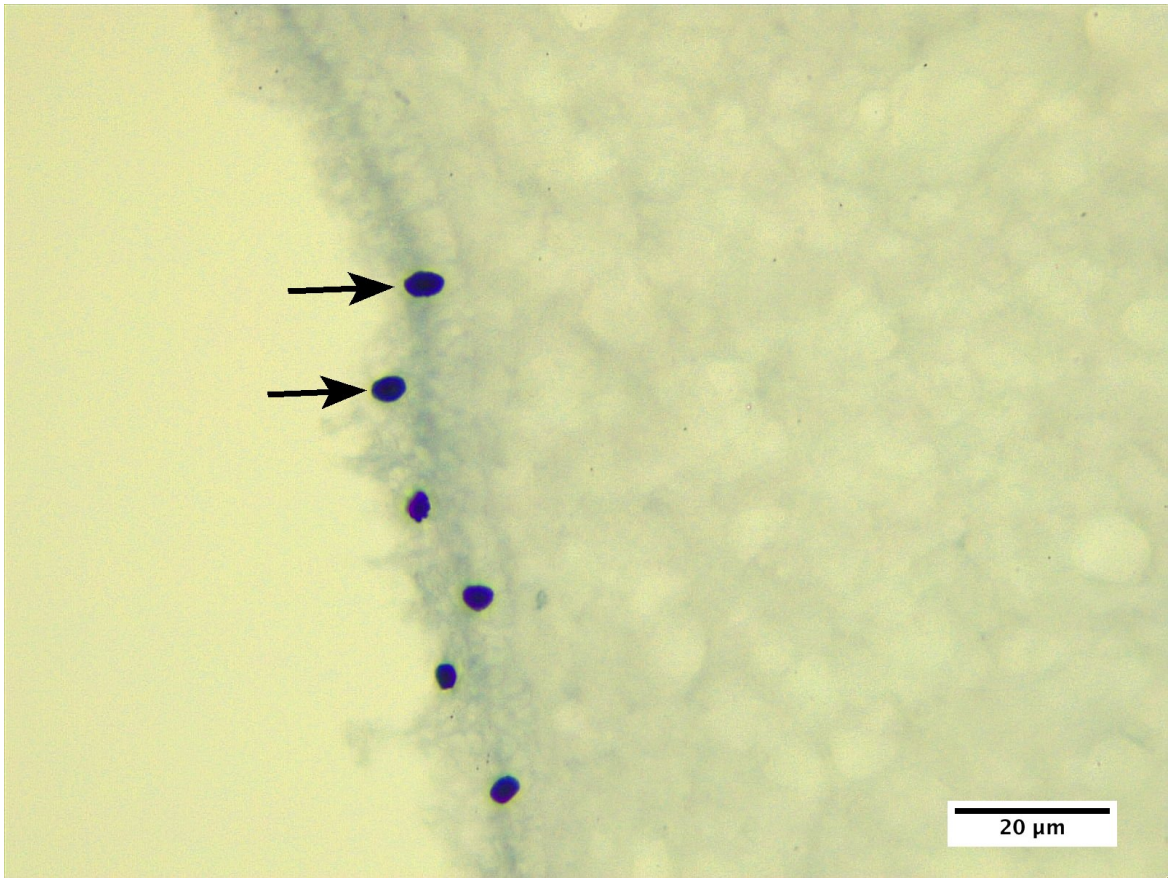


Fig.6. Image of BrdU-stained nuclei in a brain tissue section of *Python regius.* The arrows indicate the BrdU-stained nuclei along the ventricle; scale bar = 20µm. Cells were considered within the ventricular zone if they were within 50µm of the ventricle.

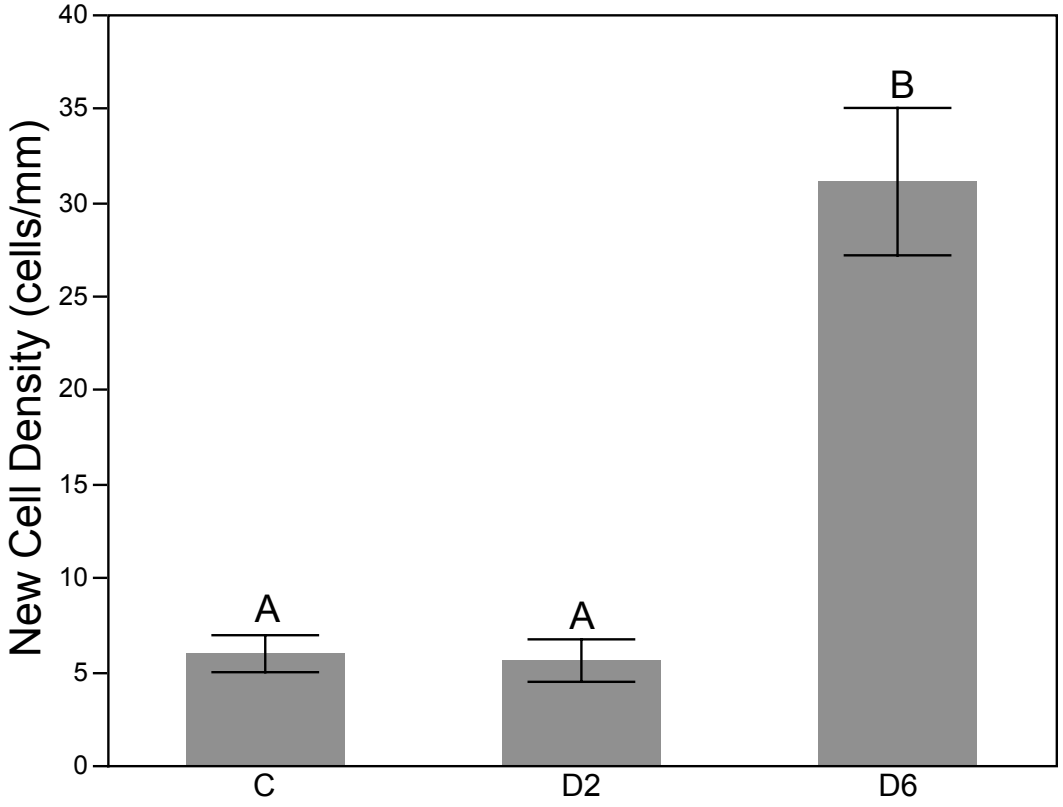


Fig.7. Mean +/- 1 SEM density of all BrdU-ir cells in the telencephalon for each treatment group. Bars that do not share a letter are significantly different from each other ($p<0.0001$).

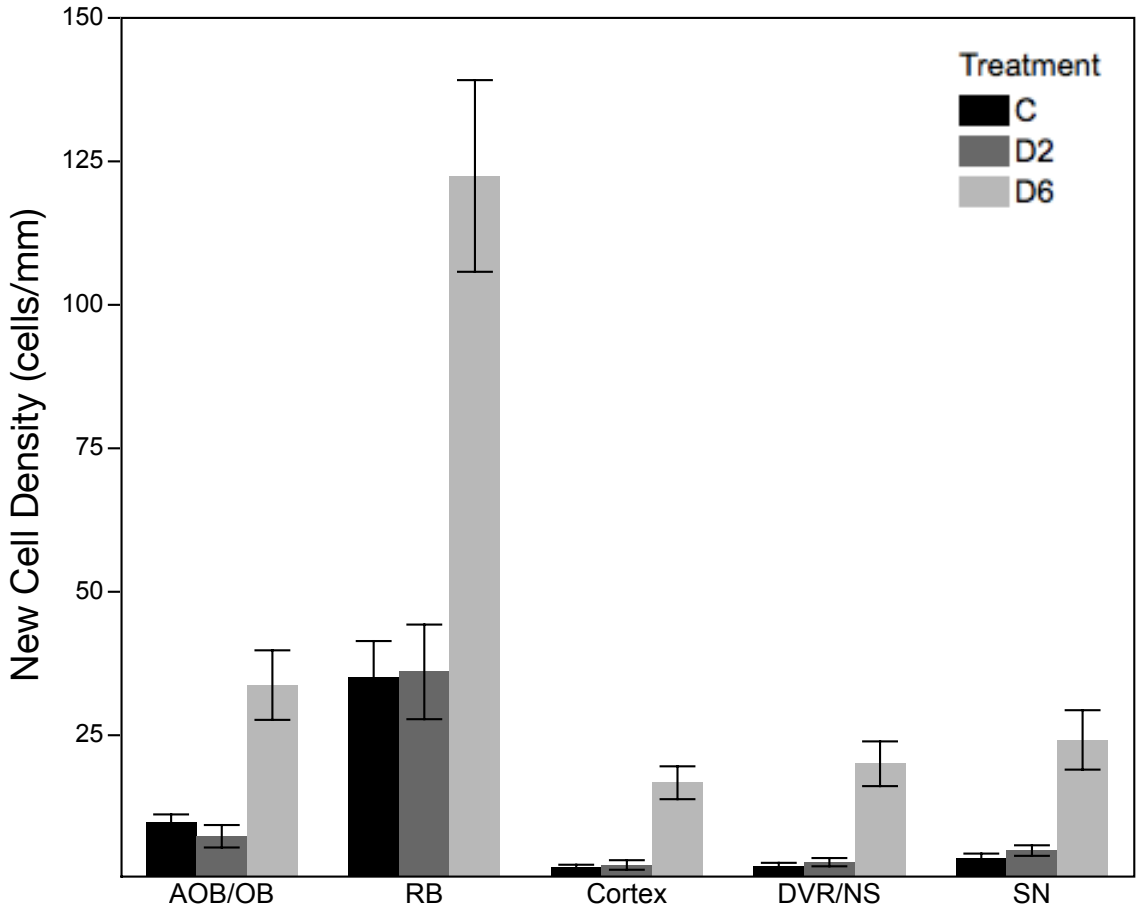
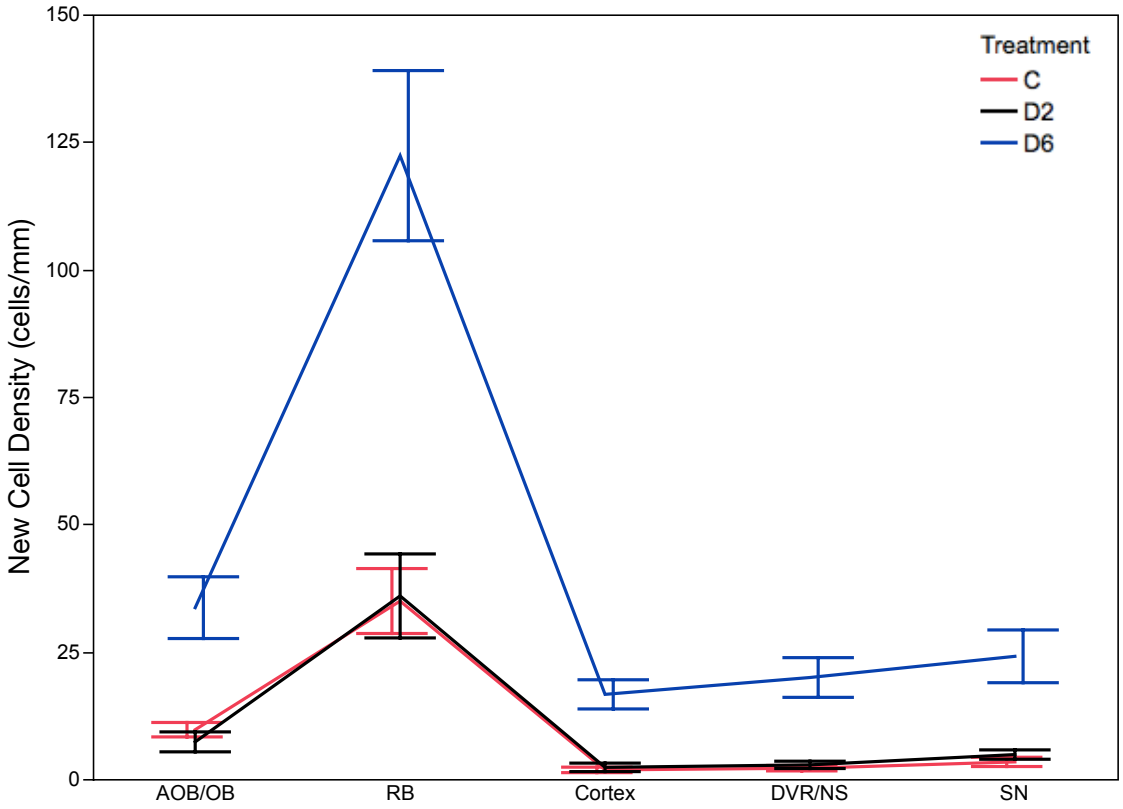
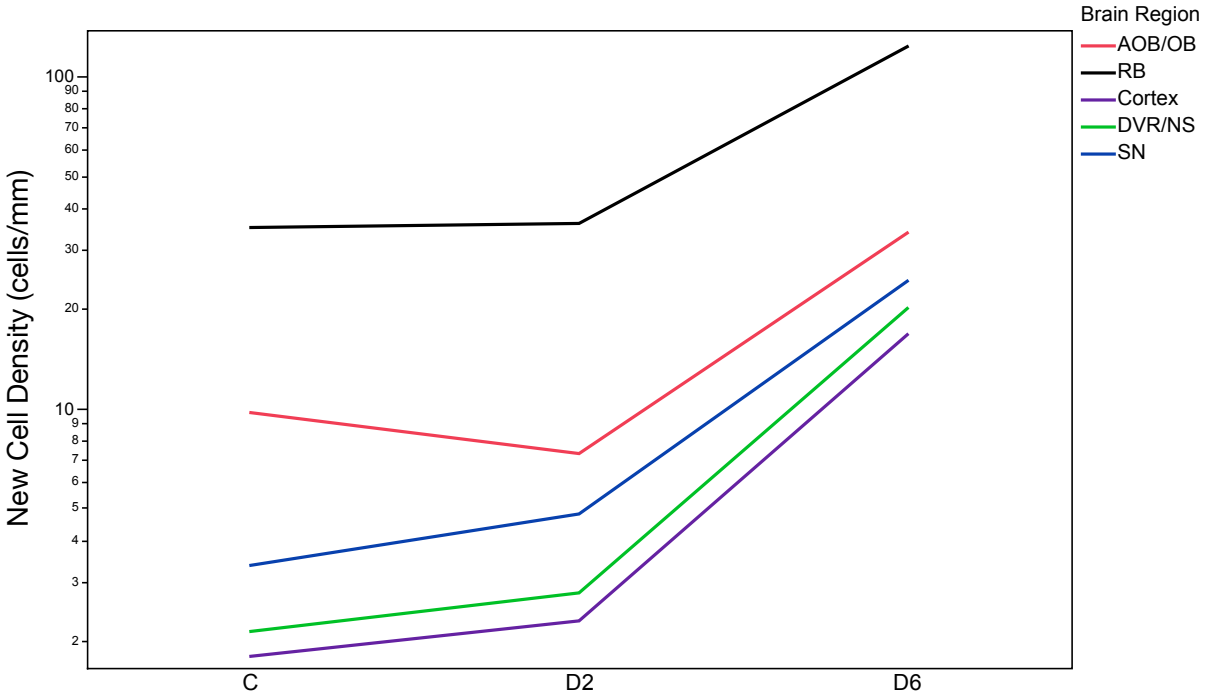


Fig.8. Treatment differences in mean density of BrdU-ir cells (cells/mm, +/- 1 SEM) across all five brain regions.

Appendix C Supplemental Figures



S.1. Mean density of BrdU-ir cells (cells/mm +/- SE) for each brain region (AOB/OB, RB, Cortex, DVR/NS, and SN). The D6 group had significantly more BrdU-ir cells than both the D2 and control groups (p=0.0013), and this effect was not parallel (results from a one-way MANOVA using profile analysis).



S.2. Trends in mean density of BrdU-ir cells (cells/mm) by all five brain regions across treatments. From highest to lowest proliferation by region, respectively: RB, AOB/OB, SN, DVR/NS, SN. This order was seen consistently within each treatment group. Data is on a log-transformed scale.