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Replicative senescence and apoptosis are two cellular processes that have been linked repeatedly to life expectancy in many organisms. However, aging research at the cellular level in the novel model *Apis mellifera* (honey bees) has been limited. This study tests the hypothesis that cellular proliferation will be higher and apoptosis will be lower in bees with high natural life expectancy (queens, reproductive workers and workers in the winter) than in bees with low life expectancy (drones, normal summer workers). The DeadEnd Colorimetric TUNEL assay was used to investigate apoptosis, but I observed no quantifiable results. A 5-bromo-2'-deoxyuridine (BrdU) incorporation assay was used to examine cellular proliferation in relation to age, caste, season, and reproductive status. I focused on the midgut because it was the only tissue that showed consistent cellular proliferation and is crucial for organismal survival. Cellular proliferation decreased significantly with age in summer workers and queens but it was highest in drones at an intermediate age. In winter workers, cellular proliferation was most similar to that of middle-aged summer workers, which is also true for their behavior and physiology. No direct link was found between reproduction and cellular proliferation in workers. These results suggest that there is no direct link between the amount of cellular proliferation in the midgut and honey bee longevity. Instead, the observed patterns in proliferation may reflect the variation in intestinal activity. I propose the new digestive demand hypothesis. However, the results do not exclude replicative senescence of the intestinal stem cells over time as an important determinant of honey bee life expectancy.

A STUDY OF CELLULAR PROLIFERATION AND APOPTOSIS IN SHORT- AND LONG-LIVED HONEY BEES, *APIS MELLIFERA.*

by

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TABLE OF CONTENTS

Page

LIST OF TABLES iv

LIST OF FIGURES v

CHAPTER

I. INTRODUCTION 1

Aging 1
Honey Bee Biology 7
Honey Bee Aging Studies 10

II. METHODS AND MATERIALS 14

Cellular Proliferation Assay 14
Apoptosis Assay 17
Experiment 1: Evaluating the Tissues of Interest 20
Experiment 2: Caste Differences 21
Experiment 3: Seasonal Differences 22
Experiment 4: Reproductive Development 22
Analyses 24

III. RESULTS 25

Experiment 2: Caste Differences 25
Experiment 3: Seasonal Differences 27
Experiment 4: Reproductive Development 27
Apoptosis 28

IV. DISCUSSION 30

V. CONCLUSION 40

REFERENCES 42

APPENDIX. TABLES AND FIGURES 49

LIST OF TABLES

Page

Table 1. Kolmogorov-Smirnov test results for normality of all experimental groups for cellular proliferation....50

Table 2. Mean and standard deviation of number of proliferating cells in the midgut for evaluating caste differences (experiment 2)....51

Table 3. Mean and standard deviation of number of proliferating cells in the midgut for evaluating seasonal differences (experiment 3)....52

LIST OF FIGURES

Page

Figure 1. Cross section of a representative 11-day-old honey bee worker midgut with immuno-labeled cells that have incorporated 5-bromo-2'-deoxyuridine, indicating DNA synthesis and thus cellular proliferation....53

Figure 2. Five point scale of worker ovarian development....54

Figure 3. Average cellular proliferation in the midgut for all three castes at the three investigated ages....55

Figure 4. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the three experimental age groups of drones....56

Figure 5. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the three experimental age groups of workers....57

Figure 6. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the three experimental age groups of queens....58

Figure 7. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the young age group for all three castes....59

Figure 8. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the middle-age group for all three castes....60

Figure 9. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the old age group for all three castes....61

Figure 10. Comparison of the amount of cellular proliferation between winter workers and the three age groups of summer workers (mean and standard deviation are given)....62

Figure 11. No relation between ovarian development and the amount of cellular proliferation in the midgut in workers under queen-less conditions....63

Figure 12. Cumulative survival curve of workers in queen-less hive and workers in queen-right hive....64

Figure 13. Pictures of apoptosis assays in honey bee midgut....65

CHAPTER I

INTRODUCTION

Aging

Aging is a widespread process of biological systems that leads to senescence (Arking 1998). Senescence can be defined in two ways: first, the functional decline of an organism with age and second, when the organism's mortality risk approaches 100% (Arking 1998). Because aging is such a heterogeneous and complex process, there are many, non-mutually exclusive theories of aging. These theories fall into two categories, evolutionary (ultimate) and mechanistic (proximate) (Amdam and Rueppell 2006).

There are three main evolutionary theories of aging: disposable soma, mutation accumulation, and antagonistic pleiotropy (Finch 1990, Rose 1991). The first theory, the disposable soma theory, proposes that natural selection favors individuals that devote more energy to their germ line than to maintaining their somatic tissue indefinitely (Kirkwood 1977). Aging therefore occurs through damage to the organism's somatic cells (Rando 2006). The next two theories are based on the assumption that the force of natural selection declines in aging populations because older individuals contribute less to reproduction in most populations than young ones (Finch 1990, Rose 1991). The mutation accumulation theory states that alleles with detrimental effects may not be selected against if the allele's detrimental effects are not expressed until later in life, after the organism's reproductive peak (Medawar 1952). The antagonistic pleiotropy theory

postulates that some alleles that have detrimental effects later in life may actually be selected for and accumulate in the population if the allele's expression early in life was beneficial (Williams 1957). In both the mutation accumulation and antagonistic pleiotropy theories, it is the late onset of negative genetic effects that cause aging in the old organism. These evolutionary theories do not address the proximate mechanisms that lead to aging. There are numerous mechanistic theories of aging (Arking 1998) and many follow the idea that damage accumulates with age at the molecular level due to environmental insults and/or metabolic by-products. This accumulation of damage results from the organism's inability to repair posttranslational protein modification or a buildup of DNA mutations (Arking 1998, Finch 1990, Harman 1956, Hughes and Reynolds 2005). This damage accumulation can be exacerbated by hyperglycemia: when glucose levels are high, glucotoxicity can occur through multiple pathways (Mooradian 2006). These pathways have a similar result, the creation of free radicals through mitochondrial metabolism and subsequent molecular damage to the cells (Brownlee 2001).

One way to decrease this buildup of damage is through caloric restriction (CR). CR is the only environmental intervention that has been shown experimentally to increase consistently the life expectancy of many organisms (Masoro 2005). Some research suggests that CR decreases the amount of free radicals created during metabolism and therefore decreases the amount of oxidative damage experienced by the organism and thus increases longevity (Sohal and Weindruch 1996). CR animals exhibit less DNA damage accumulation (Raffoul *et al*. 1999, Sohal and Weindruch 1996). On the other

hand, CR may simply cause a shift in the allocation of energy from maintenance of the germ line to the soma (Raffoul *et al.* 1999). Expression of genes such as SIRT1 (Cohen *et al.* 2004), TOR and Sch9 (Kaberlein *et al.* 2005), and IGF-1 (Gredilla and Barja 2005) may regulate the increased lifespan seen in CR animals.

When molecular damage accumulates to a certain degree and cannot be repaired, cellular aging occurs. Cellular aging makes two cellular processes, cellular proliferation and programmed cellular death, crucial for the maintenance of functional tissues and thus organismal longevity. In tissues where the two cellular processes are out of balance, tumorigenesis or aging occurs (Campisi 2003). The accumulation of molecular damage (e.g. through oxidative radicals), affects these two cellular maintenance processes directly and generally causes a decrease in cellular proliferation and an increase in cell death with age (Campisi 2003, Zhang and Herman 2002).

A decrease in cellular proliferation (replicative senescence) results in a cessation of tissue renewal that may contribute to age-related dysfunction and organismal senescence (Campisi 1996, Finch 1990). Cell culture studies have suggested that cellular aging can be caused by the shortening of telomeres during DNA replication (Hezel *et al.* 2005; Von Zglinicki 2003). This causes the inactivation of genes at the ends of chromosomes or genomic instability and leads to replicative senescence (Hezel *et al.* 2005; Von Zglinicki 2003). Replicative senescence leads to a limited number of functional cell replications, now known as the "Hayflick limit" (reviewed in Hayflick 2000). The idea that replicative senescence is related to organismal aging is derived from two central lines of evidence. First, aging individuals in general have less replication

occurring in their tissues than young individuals (Campisi 1996, Finch 1990, Kirkwood 1984). Second, cells derived from organisms with short life spans will become senescent after fewer replications than cells from organisms with long life spans (Campisi 1996, Finch 1990, Kirkwood 1984). Supporting data come from studies of human cells *in vitro* and *in vivo* (Effros *et al.* 2005, Kirkwood 1984), as well as from birds and mammals (reviewed in Campisi 1996, Finch 1990, Haussman *et al.* 2003).

Replicative senescence is best known from normal somatic cells in culture (Hayflick 2003), but may also affect stem cells (Warner 2007). Stem cells are important in organismal aging because they can renew populations of aging cells (Whirledge *et al.* 2006). Adult tissues such as nervous tissue, kidney, and heart often have very low cell turnover and therefore aging of stem cells probably plays a smaller role in their aging phenotype compared to tissues such as epidermis, gut epithelium, and blood cells that are frequently replaced and whose stems cells have high proliferative ability (Rando 2006). Adult stem cells are found in most mammalian tissues (Rando 2006) and were thought to be mainly absent from somatic insect tissues (Finch 1990). Recent studies have shown that *Drosophila melanogaster* has intestinal stem cells in the adult midgut (Micchelli and Perrimon 2006, Ohlstein and Spradling 2006). Regenerative cells are also present in the midgut of larvae of the stingless bees *Melipona quadrifasciata* (Martins *et al.* 2006). Earlier, Snodgrass (1956) had reported that cellular proliferation occurs in the crypts of the midgut of adult honey bees. He presents a basic histological characterization of these cells and based on the cells' location, shape, and function this which suggests that they represent stem cells homologous to those in *Drosophila melanogaster*.

The buildup of molecular damage may cause an age-related increase in cell death that leads to tissue decay (Campisi 2003, Kujoth *et al.* 2005; Zheng *et al.* 2005). Cell death includes necrosis and programmed cell death. Necrosis results when a cell encounters severe changes that may disrupt the production of ATP, such as changes in osmolarity, pH, or the presence of oxidants or toxins. Cells that receive pathological insult that results in lysis of the plasma membrane will also undergo necrosis. In all cases, an inflammatory response occurs (Zakeri and Lockshin 2004). Programmed cell death, on the other hand, is regulated by the cell and works by very different pathways (Zakeri and Lockshin 2004).

Apoptosis is a highly conserved and regulated form of programmed cell death (Kerr *et al.* 1972, Zakeri and Lockshin 2004). Most forms of apoptosis involve distinct stages which are characterized by the following events (Kerr *et al.* 1972): Apoptosis may be initiated by an extracellular signal such as tumor necrosis factor or by altered mitochondrial function and the release of cytochrome c. Both events cause the activation and recruitment of several caspases, enzymes that cleave cellular proteins and lead to condensation of the cytoplasm and chromatin (Lockshin and Zakeri 2004). The DNA is then degraded by endonucleases into distinct fragments (Kerr *et al.* 1972, Potten and Wilson 2004). During this process of DNA fragmentation, the cell begins to split into smaller pieces called apoptotic bodies (Potten and Wilson 2004). These cellular remains are then removed by macrophages through the process of phagocytosis (Kerr *et al.* 1972). Apoptosis ensures that these apoptotic bodies are encapsulated and then removed. Therefore, the cells do not release degradative enzymes and cause tissue destruction and

inflammation (Potten and Wilson 2004). Multiple mechanisms for measuring apoptosis exist which can detect DNA fragmentation, the level of caspase activity, and morphological changes of the cell (Watanabe *et al.* 2002).

Apoptosis plays an important role in an organism's development, maintenance, and senescence (Potten and Wilson 2004). In aged tissue with a higher accumulation of damage in cells, apoptosis is responsible for the removal of these cells (Kirkwood 2005). In mice, an increase in apoptotic markers such as levels of cleaved caspase-3 and nuclear fragmentation was found in response to experimentally-induced aging (Kujoth *et al.* 2005). Satellite cells, which are the descendents of stem cells in skeletal muscle, have increased amounts of activated caspases and fragmented DNA in old rats than satellite cells in their younger counterparts (Jejurikar *et al.* 2006). In *Drosophila melanogaster*, aging is coupled with a gradual increase in apoptosis in the muscles and activation of apoptosis in fat cells (Zheng *et al.* 2005). In humans, increased apoptosis has been found in enterocytes of elderly subjects (Ciccocioppo *et al.* 2002). Thus, there is ample evidence that apoptosis is associated with organismal aging in several different organisms, but it is unknown whether it can account for naturally occurring differences in within species aging rates, such as those observed in honey bees.

In sum, cellular proliferation decreases and apoptosis increases in many organisms with increasing chronological age, resulting in organismal senescence (Campisi 2003). Because aging is a heterogeneous process that is experienced by all organisms, albeit at very different rates, it is essential that many different aging models be examined (Finch 1990). Therefore, this study examined the amounts of cellular

proliferation and attempted to examine the amounts of apoptosis in honey bees, which exhibit striking, natural differences in aging, as discussed in the following section.

Honey Bee Biology

Advanced sociality is characterized by the evolution of reproductively distinct castes, cooperative brood care and overlapping generations (Hölldobler and Wilson 1990). In honey bees, queens have evolved to be reproductively specialized while workers do not reproduce under normal circumstances. The honey bee colony represents a functional unit comprised of sterile workers that engage in kin-selected helping (the somatic tissue of the colony "superorganism") and the queen (or germ line) (Wilson 1971). In good conditions, a honey bee colony can consist of 10,000 to 30,000 workers, a reproductive queen, and a few hundred to a few thousand males (Winston 1987).

Drones (male bees) live on average 25 to 40 days (Page and Peng 2001). They develop from haploid eggs and are only present in the reproductive season (spring and summer) (Winston 1987). They do not contribute to any hive tasks and will stay in the hive until they are sexually mature at approximately 10 days of age (Rueppell ***et al.*** 2005). At this point, the drones leave the hive periodically to attempt to mate with a virgin queen in flight and ultimately die after mating (Winston 1987). Most drones, however, are not successful in mating with a queen and either die during mating flights from predation or exhaustion, or they are expelled from the colony at the end of the summer by workers (Page and Peng 2001). Thus, drones have a generally low life expectancy, even though they represent the "germ line" of the colony (Wilson 1971).

Queens are the reproductive entity of the colony and are responsible for laying eggs that will develop into workers, new queens, and drones (Winston 1987). Queens have an average lifespan of one to three years, which is much longer than that of workers. Yet, they develop from fertilized eggs that are identical to eggs that develop into workers. The alternative queen and worker phenotypes arise through the quantity and quality of food provided to the female larvae by the nurse bees (Page and Peng 2001). The same genotype can lead to a short-lived worker or a long-lived queen phenotype. There may be an underlying cellular mechanism that allows queens to live much longer than workers. The queen-worker comparison is especially interesting because queens live longer than workers despite their high fecundity, while fecundity and longevity are negatively correlated in most other species (Arking 1998, Finch 1990). Although queens must face this reproductive cost, they do not have to leave the hive except for mating flights and are taken care of for the rest of their lives by other workers in the colony, which reduces many environmental insults that may contribute to aging (Rueppell *et al.* 2004).

Worker bees are functionally sterile females that perform all of the maintenance tasks of the colony. Workers divide these tasks according to age: they exhibit age polyethism (Winston 1987). Newly emerged adult bees begin their lives cleaning cells, usually from the area that they recently emerged (Winston 1987). Young workers have a soft exoskeleton, are unable to fly, and rarely sting. As workers age, their hive duties shift from cleaning cells to feeding larvae, nest cleaning, comb building, ventilation of the hive, feeding the queen, drones, and other workers, and guarding the hive entrance

(Gary 1992). Workers that perform in-hive tasks are called nurse bees (Winston 1987). After two to three weeks of life, workers will shift from nursing to foraging (Page and Peng 2001). Foragers leave the hive to collect nectar, pollen, propolis (tree sap), and water (Winston 1987). This is the last task that worker bees will perform before they die (Page and Peng 2001). These three age classes of workers (young hive bee, mature hive bee, and forager) are distinguished not only by differences in behavior but also in physiology (Robinson 1992, Snodgrass 1956).

In a hive with an egg-laying queen, the reproduction of workers is suppressed to the point of sterility by pheromones from the queen and the brood she produces (Harris and Woodring 1995). However, workers in a queen-less colony may activate their ovaries and begin laying unfertilized eggs which will develop into haploid drones, who do not differ from drones produced by a queen (Hoover *et al.* 2005). This usually occurs in about 23 to 30 days after queen loss (Winston 1987). Not all workers will begin laying eggs because workers that begin to lay eggs will suppress ovary development in other workers similar to that of a true queen (Harris and Woodring 1995). The laying workers also resemble the queen in behavior and physiology (Winston 1987), but the consequences for lifespan are unknown.

In general, worker lifespan is very dependent on the season. Summer bees live on average 15 to 38 days, whereas winter bees can survive for 140 days or longer (Omholt and Amdam 2004, Page and Peng 2001). In the winter months, brood production, foraging, and many other in-hive tasks cease. As the temperature decreases, the bees will cluster together in the hive. The bees consume honey to fuel the thermoregulation of this

cluster until spring arrives when foraging and brood production will resume (Gary 1992). Workers exhibiting winter physiology can be obtained in the summer if brood (offspring) is removed and no foraging is allowed (Haydak 1963, Omholt and Amdam 2004), suggesting that it is not just a change in temperature that increases the worker lifespan. Workers bees in the winter are thought to undergo no physiological or internal aging and also face lower external mortality risks (Omholt and Amdam 2004). Therefore, it may be informative to compare possible internal causes of aging between long-lived winter workers and short-lived summer workers.

Honey Bee Aging Studies

Social evolution has strong effects on organismal lifespan (Carey 2001). Because the honey bee colony can be regarded as a superorganism, with workers acting as somatic tissue and the queen the germ line (Wilson 1971), the colony will devote less resources to maintaining the workers and more to the queen (Amdam and Page 2005). Therefore, queens are predicted to have evolved an increase in lifespan compared to workers (Carey 2001, Finch 1990, Keller and Genoud 1997).

Within the worker population, age polyethism strongly affects lifespan. As a worker honey bee grows older, her mortality risk does not change significantly until she begins to forage (Sakagami and Fukuda 1966, Tofilski 2002). However, the proximate causes for the increased worker mortality after the initiation of foraging are unresolved (Rueppell *et al.* 2004). Foragers experience an increased risk of environmental insults (Page and Peng 2001) but other, regulatory mechanisms have also been proposed. For

example, the amount of time a worker spends foraging and thus her lifespan may be pre-determined by the glycogen stores in the flight muscles. Glycogen levels and the worker's ability to restore them decrease with the onset of foraging, limiting flight performance and forager lifespan (Neukirch 1982). Honey bees have 38 antioxidant genes in their genome (Corona and Robinson 2006) which suggests that, as in the case with other animals, honey bee aging may also be influenced by free radicals generated during metabolism. Honey bees also have telomeres similar to that of humans (The Honeybee Genome Sequencing Consortium 2006) and therefore telomere loss and replicative senescence may play a role in honey bee aging as seen in other organisms (Hezel *et al.* 2005, Von Zglinicki 2003)

Furthermore, protein metabolism plays a significant role in honey bee longevity. In particular, the lipoprotein vitellogenin (together with juvenile hormone) is known to affect honey bee behavioral plasticity and is associated with the worker transition from hive bee to forager (Amdam and Omholt 2002, Huang *et al.* 1991), as well as from summer bee to winter bee (Amdam *et al.* 2005, Huang and Robinson 1995). Vitellogenin and juvenile hormone levels have an inverse relationship – vitellogenin being high in hive bees and winter bees and juvenile hormone being high in foragers (Omholt and Amdam 2004). A larger accumulation of vitellogenin (and therefore lower titers of juvenile hormone) promotes longer lifespan in honey bees (Amdam *et al.* 2004, Omholt and Amdam 2004) and prevents oxidative stress (Seehus *et al.* 2006).

Most of the research done on honey bee aging has been done at the colony level as well as the molecular level. In contrast, cellular mechanisms that result in or reflect

aging have not been adequately addressed. In almost all adult insects, the tissues are believed to be post-mitotic (Finch 1990) which has lead to the neglect of cellular aspects of aging in this important in-vertebrate group. However, there are important exceptions to the post-mitotic nature of adult insects (Malaterre *et al.* 2003, Micchelli and Perrimon 2006, Ohlstein and Spradling 2006), including honey bees (Finch 1990, Silva de Moraes and Bowen 2000, Snodgrass 1956). Specifically, an increase in juvenile hormone, as seen in foragers, causes immunosenescence in honey bees through apoptosis of hemocytes (Amdam *et al.* 2005), which suggests that apoptosis may be associated with aging in adult honey bees. However, more general studies at the cellular level are needed to understand the different processes that contribute to aging in the honey bee, and perhaps to the evolution of differential aging in general.

The purpose of this study was to investigate the relationship between honey bee lifespan and the amounts of cellular proliferation and cell death. Because it is impractical to quantify all forms of cell death simultaneously, the study focused on apoptosis, an important and well characterized form of programmed cell death that has been repeatedly implicated in aging (Amdam *et al.* 2005, Ganeshina *et al.* 2000, Gregorc and Bowen 1997, Silva de Moraes and Bowen 2000). This project was designed around the central idea that the amounts of cellular proliferation and apoptosis affect honey bee lifespan (aging rates). Therefore, I hypothesized that honey bees with long lifespans (queens, winter workers, and reproductive workers) will have more cellular proliferation and/or less apoptosis than honey bees at comparable ages with short lifespans (summer workers

and drones). Secondly, I hypothesized that cellular proliferation decreases and/or apoptosis increases with chronological age in honey bees.

CHAPTER II

METHODS AND MATERIALS

To examine how cellular regeneration and cell death relate to life expectancy in the honey bee, I studied the amounts of cellular proliferation and apoptosis in honey bees with different rates of aging. After identifying the midgut as the focus of my studies (experiment 1), I compared the two cellular processes between:

- three age groups (1-3 day old, 10-15 day old, 41-51 day old) of queens, workers, and drones (caste and age differences: experiment 2)
- summer and winter workers (seasonal differences: experiment 3)
- reproductive workers with different degrees of ovarian development (reproductive differences: experiment 4).

I used the following two assays to quantify cellular proliferation and apoptosis in all experiments.

Cellular Proliferation Assay

To examine the amounts of cellular proliferation occurring in the tissues of my experimental groups, I used 5-bromo-2'-deoxyuridine, BrdU. BrdU is a synthetic thymidine analog that is incorporated into the DNA of replicating cells and thus labels cells that have undergone DNA synthesis and therefore implies cellular proliferation (Gratzner 1982). It has been used successfully in insects (Fahrbach *et al.* 1995,

Lomassese 2000, Martins *et al*. 2006) to measure cellular proliferation. Honey bees of the three age groups and castes were collected and the bees were harnessed in plastic straws so that they could not escape and their proboscises (feeding apparatus) were accessible. All bees were starved for 30 minutes to one hour to ensure that they would consume all of the BrdU mixture and absorb it quickly (e.g. so the workers would not store food in their honey stomach). The bees were then fed 5µL of a 1:1 mixture of 10X BrdU solution (10mg/mL BrdU in S2 cell medium, both from Sigma) in 50% sucrose. Bees that did not consume the entire 5 µL were discarded. The bees were then placed in a 34°C incubator overnight for a total of 16 to 20 hours to allow BrdU to incorporate into replicating cells.

After the BrdU treatment was administered, bees of the three age groups and three castes were dissected in bee saline (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7) and all of their accessible tissues were removed. The tissues removed included glandular tissue, reproductive organs, nervous tissue, the heart, fat body, intestinal and excretory tissues, and muscle. The tissues were then fixed in Carnoy's fixative (60 mL 100% ethanol, 30 mL chloroform, 10 mL acetic acid) for one hour (Fahrbach, personal comm.). Next, the samples were dehydrated in 100% ethanol three times, placed once in a 1:1 mixture of xylene and ethanol, once into 100% xylene twice, once into a 1:1 mixture of xylene and paraplast wax (Fisher), once into 100% paraplast, each step for fifteen minutes. Finally the tissues were placed in fresh paraplast to harden overnight for 16 to 20 hours. The paraplast was remelted at 60°C and then the tissues were embedded in paraffin and

sectioned at a thickness of 10 μm. The paraffin was subsequently removed from the tissue sections with three washes of xylene and then rehydrated with a series of ethanol in distilled water mixtures (100%, 100%, 95%, 70% ethanol) each step for five minutes.

After the rehydration, the sections were rinsed briefly in distilled water to remove any excess ethanol and then permeabilized with 1X PBS (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) with 0.05% Triton X-100 (PBS-T) twice for five minutes. The DNA was denatured in 1X PBS with 0.05% Triton X-100 and 2 N HCl (PBS-H) for one hour and washed twice in PBS-T for five minutes each. Tissues were blocked for non-specific binding with PBS-T with 10% normal goat serum (Biomeda) and 20 mg of non-fat dried bovine milk (Sigma) in 8 mL of blocking solution for one hour. Anti-BrdU mouse antibody (Phoenix Flow Systems) diluted 1:500 in PBS-T were added to the slides and they were left to incubate horizontally overnight for 16 to 20 hours at 4°C. The samples were then washed three times in PBS-T for ten minutes each time before adding the goat anti-mouse peroxidase conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:40 in PBS-T and incubated horizontally for two hours. They were rinsed three times with PBS-T and twice with 1X PBS each for ten minutes. Tissues were incubated in diaminobenzidine (Sigma) for five to ten minutes until slides were visibly stained. Following the diaminobenzidine incubation, the samples were rinsed three times in distilled water for one minute each and then dehydrated using a series of ethanols (50%, 70%, 95%, 100%, 100%) for five minutes each. Citrus clearing solvent (VWR) was used three times for five minutes each to prepare the tissues for coverslipping. Coverslips (Fisher #12-545-88) were mounted

with Permount (Fisher) and the slides were allowed to dry horizontally for one week. Tissues were viewed under a light microscope with a 40X objective. Negative controls included the omission of HCl to maintain DNA structure and therefore inhibit antibody access to incorporated BrdU and omission of the anti-BrdU antibody.

For each bee, one section was randomly selected from a 28 mm by 25 mm grid (four 7 mm rows and five 5 mm columns) that represented the center of the slide. The section was picked from the middle of the slide to ensure full coverage of all solutions throughout the assay. Only slides that contained tissues from the center of the midgut were used to ensure homogeneity. The number of labeled nuclei was counted from that section and the quantification was done blindly. The nuclei were darkly stained and usually clustered together in crypts. Between group comparisons can be made because I saw no visible difference in morphology of the midgut or staining of the nuclei between the different experimental groups.

Apoptosis Assay

The DeadEnd Colorimetric TUNEL System (Promega catalog # G7131) was used to detect apoptosis in honey bee tissues. The terminal dUTP nick end-labeling (TUNEL) method uses labeled nucleotides that bind to the 3' hydroxyl ends of DNA fragments formed during apoptosis (Wantanbe *et al.* 2002). Tissues were dissected in bee saline and then fixed in 4% paraformaldehyde in 1X PBS at pH 7.50 for 16 to 20 hours overnight. They were then embedded in paraffin and sectioned as previously described in the BrdU protocol. The paraffin was removed from the tissues sections in two five

minute washes of xylene and rehydrated in a series of ethanols (100%, 100%, 95%, 85%, 70%, 50%) each for five minutes. The samples were then rinsed briefly in distilled water to remove any excess ethanol, washed in 0.85% NaCl and 1X PBS each for five minutes, and fixed in 4% paraformaldehyde in 1X PBS at pH 7.50 for fifteen minutes. Afterwards, the samples were permeabilized horizontally with 400 µL to 800 µL of a 20 µg/mL Proteinase K solution (10 mg/mL Proteinase K diluted 1:500 in 1X PBS) and covered with plastic coverslips (both provided by Promega) for 30 minutes at room temperature. The tissues were washed in 1X PBS for five minutes and then the fixation was repeated in 4% paraformaldehyde in 1X PBS at pH 7.50 for five minutes. They were then washed twice in 1X PBS for five minutes each. The samples were equilibrated horizontally in 200 µL to 400 µL of equilibration buffer (provided by Promega) and covered with plastic coverslips for five to ten minutes. They were then labeled with 100 µL of recombinant terminal deoxynucleotidyl transferase (rTdT) reaction mix (98 µL of equilibration buffer, 1 µL of biotinylated nucleotide mix, 1 µl rTdT enzyme, all provided by Promega) by incubating slides horizontally. Slides were covered with plastic coverslips and incubated for one hour at 37°C and 70% relative humidity. The rTdT reaction was then stopped by removing the plastic coverslips and immersing slides into 2X SSC solution (20X SSC solution provided by Promega) for 15 minutes. After three washes in 1X PBS for five minutes each, the tissues were blocked for endogenous peroxidases in 0.3% hydrogen peroxide for five minutes. They were washed three more times in 1X PBS for five minutes each and then incubated horizontally in 200 µL to 400 µL of horseradish peroxidase-labeled streptavidin (provided by Promega) diluted 1:500

in 1X PBS and covered with plastic coverslips for 30 minutes at room temperature. They were then washed in 1X PBS three times for five minutes each and then stained horizontally with 100 µL to 200 µL of diaminobenzidine mixture (50 µL DAB substrate 20X buffer, 950 µL deionized water, 50 µL DAB 20X chromogen, 50 µL 20X hydrogen peroxide, all provided by Promega) and covered with plastic coverslips for 10 minutes until tissues were stained. The slides were washed three times in deionized water for one minute each and then coverslips were mounted onto the slides with Permount (Fisher) and allowed to dry for one week horizontally. Slides were viewed with a light microscope with a 40X objective. Negative controls omitting the rTdT enzyme from the rTdT reaction mix were performed. Positive controls were created by treating tissues with 400 µL of DNase I buffer (Promega) and then covering them with plastic coverslips and incubating them horizontally for five minutes. Then 400 µL of DNase I buffer containing 10 unit/mL of DNase I (Promega) was added and the sections and they were incubated horizontally covered with plastic coverslips for ten minutes. The sections were washed three to four times with distilled water, and then washed for five minutes in PBS to induce DNA fragmentation. Positive biological controls included the midguts of *Drosophila melanogaster* larvae with known apoptosis (Mills ***et al.*** 2006). Quantification of labeled apoptotic nuclei was carried out as previously described in the cellular proliferation assay.

Experiment 1: Evaluating the Tissues of Interest

In my first experiment, I examined the following bee tissues for their patterns of cellular proliferation and apoptosis to determine which tissues show cellular turn-over that may be related to life expectancy: hypopharyngeal glands, salivary glands, brain and ganglia, the heart and pericardial cells, fat body, trachea, flight muscles, reproductive organs, Malpighian tubules (excretory system) and the entire digestive system (crop, midgut, rectum). These tissues would be the subject of the subsequent experimental comparisons between long- and short-lived honey bees.

To obtain honey bee worker and drone brood (offspring), empty combs were placed into a hive to induce egg-laying (Laidlaw and Page 1997). The brood was removed from the hive upon emergence and emerging bees were marked with paint (Textor™) on their thoraces to identify their age and then re-introduced into their colony. Queens were obtained by grafting first and second instar larvae into artificial queen cells (Graham 1992). The queens were removed from the colony one to five days prior to their emergence and placed in a 34°C incubator until they emerged. Queens were also marked with paint and then each was placed into a queen-less colony.

Workers of three different ages were collected (1 to 3 days old, 10 to 15 days old, and 41 to 51 days old). These ages correspond to the three main stages of worker life history: young hive bees, mature hive bees, and foragers (Winston 1987). Queens and drones of similar ages were used for comparison. After collection, a sample of bees was immediately dissected and multiple tissues (listed above) were removed. All tissues were processed as described above to investigate cellular proliferation and apoptosis. The only

tissue used in subsequent experiments was the midgut of the honey bee because it was the only tissue that showed clear, consistent cellular proliferation (Figure 1) and evidence of apoptosis. Thereafter, only the midgut was dissected from all bees in the following experiments and then processed for cellular proliferation and apoptosis.

Experiment 2: Caste Differences

To compare amounts of apoptosis and cellular proliferation between workers, drones, and queens under summer conditions, I generated worker and drone cohorts and grafted queens as previously described between May and August 2006. I marked emerging workers and drones and re-introduced them into full hives with a queen (queen-right). Grafted queens were individually placed into small, queen-less nucs. Nucs are small hives that consist of a few frames and a much smaller number of worker bees and less food than a regular colony (Ambrose 1992). For each caste, I collected the three age groups (1-3 days, 10-15 days and 41-51 days old) after the appropriate amount of time had passed. When sufficient queens that could not be successfully grafted and raised to collection age, we obtained additional queens from Wilbanks Apiaries in Claxton, Georgia and Miksa Honeybee Farms in Groveland, Florida at appropriate ages. For workers and drones, twenty individuals from each age group for each assay were collected randomly from their hives. The midgut of each bee was dissected, embedded, sectioned, examined, and number of labeled proliferating and apoptotic nuclei were quantified using the cellular proliferation and apoptosis assays described above.

Experiment 3: Seasonal Differences

To compare the amounts of apoptosis and cellular proliferation of long-lived winter bees and short-lived summer bees, I used winter bees shipped overnight from Greg Hunt at Purdue University in Indiana in February 2006. These workers were not rearing brood and were clustering, and therefore were exhibiting winter physiology (Omholt and Amdam 2004). Twenty winter workers for each assay were collected immediately after arrival and processed for cellular proliferation and apoptosis as described above. The three age groups of summer workers collected in experiment two were used for comparison.

Experiment 4: Reproductive Development

To compare the amounts of apoptosis and cellular proliferation of workers with varying levels of ovarian activation, I collected, marked with individual, colored number tags, and introduced 1000 newly emerged bees (as described above) into an observation hive in which the queen was removed in April. After the first week, I observed egg-laying behavior by these workers daily. After 25 days to allow ovarian activation (Winston 1987), I collected workers that I recorded exhibiting egg-laying behavior as well as random workers for a total of twenty workers for each assay. I collected workers at age 25 and 31 days. While quantifying cellular proliferation and apoptosis in the midgut, I assessed the reproductive status of these individuals by ovarian dissection using a 5-point modified Velthuis (1970) scale developed by Pernal and Currie (2000). Ovaries were classified with a score of 0 if undeveloped (small ovarioles), 1 if oogenesis started

(swelling at the top of the ovariole), 2 if slightly developed (egg volume similar to that of nutritive follicle), 3 if moderate development (egg volume larger than nutritive follicle), and 4 for highly developed (fully elongated eggs) (Figure 2).

In addition, two observation hives were populated with approximately 500 randomly aged bees and 1000 individually tagged newly emerged workers. One hive had a functional queen and the other did not. These two hives were used to compare the life expectancy of workers in the queen-right and queen-less observation hives. Each hive was maintained throughout the study with similar amounts of workers, brood, pollen, and honey. To control the amount of brood in the queen-right colony, the queen was caged until the workers in the reproductive colony began to lay eggs. I observed the control and experimental hive three times weekly at night with the help of Preston Gardner (an undergraduate research assistant). During each census, all tagged bees that were alive were recorded by noting their individual tag color and number until all the bees were dead. Concomitantly, foraging data was obtained three days weekly from 2 to 4pm to determine which workers were foraging and if this affected longevity of the hive. Age of first foraging was recorded as the date the bee was first seen leaving the hive to forage. Hivespan was determined for each tagged bee as period of time between emergence and first recorded flight. Flightspan was recorded for foragers as the time period between first recorded flight to day last seen in the hive.

Analyses

In each experiment, I quantified the BrdU and DeadEnd Colorimetric TUNEL labeling by counting all labeled nuclei in one randomly selected tissue section per bee (Figure 1). For all experiments, I tested the data for normality using a one-sample Kolmogorov-Smirnov test. When all experimental groups were tested together, the data significantly deviated from normality ($z=1.524$, $N=209$, $p=0.019$). When tested group-wise, only the data for middle-aged drones deviated marginally from normality ($z=1.377$, $N=16$, $p=0.045$) (Table 1). For consistency among and within experimental analyses, I therefore used parametric statistical tests. However, the results were compared to nonparametric tests where possible, and any discrepancies between parametric and nonparametric methods are indicated and discussed.

I evaluated caste and age differences (experiment two) with a 2-factorial ANOVA with appropriate post-hoc tests and Kruskall Wallis rank tests. For experiment three (seasonal differences), I evaluated the data with student's t-tests and Mann-Whitney U tests. In experiment four (reproductive development), I used a Pearson's (parametric) and Spearman's rank correlation (nonparametric) to assess the relationship between reproductive status and cellular proliferation and I used Mantel-Cox log rank tests (Kaplan-Meier survival analysis) to test differences in life expectancy, age of first foraging, flightspan, and lifespan of non-foragers between workers in the queen-less and queen-right colony.

CHAPTER III

RESULTS

Experiment 2: Caste Differences

The influence of caste and age on cellular proliferation in the midgut was complex (Table 2 and Figure 3). The two-factorial ANOVA revealed a significant effect of age ($F=11.371$, $df=2$, $p<0.001$) and caste ($F=11.732$, $df=2$, $p<0.001$). Dunnett's T3 post hoc tests were performed because equal variances could not be assumed according to Levene's test of equal error variances ($F_{(8,153)}=2.921$, $p=0.005$). These post hoc tests revealed that drones had significantly less cellular proliferation than workers when averaged over the three age groups and both young and middle-aged bees had significantly more cellular proliferation than old bees. However, the two factors also interacted significantly ($F=11.371$, $df=4$, $p<0.001$). Therefore, the effect of caste was analyzed separately for each age group, and the effect of age was analyzed separately for each caste. Within drones, age was found to have a significant effect on the amount of cellular proliferation ($F_{(2,53)}=4.486$, $p=0.016$). Middle-aged drones had more cellular proliferation than young drones, which had in turn more cellular proliferation than old drones (Figure 4). However, Scheffé's post hoc test (since equal variances could be assumed: Levene's $F_{(2,53)}=1.845$, $p=0.168$) indicated that only the difference between middle-aged and old drones was significant. For workers, age was also found to have a significant effect on cellular proliferation ($F_{(2,49)}=49.610$, $p<0.001$) with young workers

having the most cellular proliferation, then middle-aged, then old (Figure 5). Scheffé's test (Levene's indicated equal variances: $F_{(2,49)}=2.527$, $p=0.090$) indicated that all age groups were significantly different from each other. Age had also a significant effect in queens ($F_{(2,51)}=28.489$, $p<0.001$) similar to that in workers (Figure 6). Using Dunnett's T3 test (Levene's $F_{(2,51)}=5.012$, $p=0.010$), both young and middle-aged queens were found to exhibit significantly more cellular proliferation than old queens.

Possible caste effects were also analyzed for the separate ages. Within young bees, there was a significant effect of caste ($F_{(2,48)}=18.184$, $p<0.001$). Workers showed most cellular proliferation, then queens, and then drones (Figure 7). Using Scheffé's test (Levene's $F_{(2,48)}=2.793$, $p=0.071$), young drones had significantly less cellular proliferation than both young queens and young workers. Within middle-aged bees, there was no significant effect of caste ($F_{(2,49)}=1.094$, $p=0.343$; Figure 8). Caste had a significant effect in old bees ($F_{(2,56)}=4.477$, $p=0.016$). Old drones had more cellular proliferation then workers and then queens (Figure 9) but Scheffé's test (Levene's $F_{(2,56)}=1.539$, $p=0.224$) indicated that only the difference between old males and old queens was significant.

In contrast to these results from parametric analyses, the Kruskall Wallis rank test of caste effect across all ages did not indicate a significant effect ($\chi^2=4.965$, $df=2$, $p=0.084$). Furthermore, for the middle-aged bees, the Kruskall Wallis test showed that there is a significant difference in cellular proliferation between the three castes ($\chi^2=8.796$, $df=2$, $p=0.012$), unlike the ANOVA result.

Experiment 3: Seasonal Differences

Winter worker data was collected in experiment three and compared to data for summer workers in experiment two. Winter workers showed significantly less cellular proliferation than young summer workers (student's t-test: $t=5.727$, $df=30$, $p<0.001$) and significantly more than old summer workers ($t=-4.234$, $df=38$, $p<0.001$). Winter workers and middle-aged summer workers were most similar in amount of cellular proliferation. The parametric test indicated no significant difference ($t=1.785$, $df=38$, $p=0.082$) and the non-parametric Mann-Whitney U test indicated only a marginally significance ($Z=-2.016$, $p=0.044$) with winter workers slightly below middle-aged summer workers (Table 3 and Figure 10).

Experiment 4: Reproductive Development

The amount of cellular proliferation in the midguts of workers in a queen-less hive was not significantly correlated to the degree of ovarian activation using Pearson's correlation ($r=0.215$, $N=27$, $p=0.140$, one-tailed test) or Spearman's test ($r=0.176$, $N=27$, $p=0.189$) (Figure 11). Using a Kaplan Meier survival analysis and a Mantel-Cox log rank test, workers in a queen-less hive were found to be significantly longer-lived than workers in a queen-right hive by approximately 4 days ($\chi^2=24.631$, $df=1$, $p<0.001$) (Figure 12). Queen-less workers had a median life expectancy of 30 days ($N=501$, 28 to 32 days 95% confidence interval) and queen-right workers had a median life expectancy of 26 days ($N=500$, 24 to 28 days 95% confidence interval) (Figure 12). Foraging data for both colonies were also analyzed. For the age of first foraging, no difference was

found between workers in queen-less and queen-right hives (χ^2=1.078, df=1, p=0.299). Workers in the queen-less hive started foraging at a median of 20 days (N=159, 18 to 22 days 95% CI) and the same was true for workers in a queen-right hive (N=149, 17 to 23 days 95% CI). There was also no difference in the flightspan (lifespan as a forager) between the two colonies (χ^2=0.097, df=1, p=0.755). Workers in the queen-less colony had a median flightspan of 8 days (N=159, 6 to 10 days 95% CI) as did workers in the queen-right hive (N=149, 6 to 10 days 95% CI). However, the lifespan of non-foragers (hivespan) was significantly different between the two colonies (χ^2=22.193, df=1, p<0.001). Queen-less hive bees had a higher median lifespan of 28 days (N=342, 26 to 30 days 95% CI) than queen-right hive bees with a median lifespan of 24 days (N=351, 22 to 26 days 95% CI).

Apoptosis

No clear, quantifiable DNA fragmentation was found in the midgut in any of the experimental groups using the DeadEnd Colorimetric TUNEL assay (Promega catalog # G7131) (Figure 13). Some possible light staining was seen in random sections in old queens, middle-aged workers, and winter workers. This staining was unlike staining seen in the positive controls treated with DNase (Figure 13) and no clear cell body was located around the possible nuclear labeling. Because DNA fragmentation occurs early in the apoptotic pathway, the cellular membrane should still be visible. No positive staining was found in positive biological *Drosophila melanogaster* controls either. Therefore, the

amount of apoptosis was not quantified in any of the experimental groups and a comparison between long-lived and short-lived phenotypes was not possible.

CHAPTER IV

DISCUSSION

Overall, the results did not support my main prediction that longer-lived honey bee phenotypes exhibit more cellular proliferation than short-lived phenotypes. Furthermore, the predicted decline of cellular proliferation with age was found in queens and workers, but not in drones. For apoptosis, the experiments did not yield any data, and therefore I can neither refute nor support the hypothesis that the amount of apoptosis is higher in short-lived honey bee phenotypes. Nevertheless, some interesting patterns of cellular proliferation in the honey bee midgut emerged.

The most basic, expected pattern occurred in summer workers: Cellular proliferation in the midgut significantly declined with age from young to middle-age to old. This decrease in the amount of cellular proliferation with age mirrors results found in other organisms (Campisi 1996, Finch 1990, Kirkwood 1984) and agrees with results from cells in culture (Hayflick 1977). It is consistent with our prediction that as the worker bee ages, cellular proliferation in the intestine would decrease because workers are the somatic tissue of the "superorganism" and are easily replaced (Wilson 1971). They are therefore, according to the disposable soma theory, disposable and less energy should be used to maintain them at old ages (Kirkwood 1977). Also, workers have increased external mortality pressures at older ages because they frequently leave the

hive. Therefore there less investment into old bees since they have an high external probability of dying (Page and Peng 2001).

The results from the comparison between winter and summer workers also fit the hypothesis that longer-lived bees will have more cellular proliferation than short-lived bees. Indeed, winter workers, although over two months old, had amounts of midgut cellular proliferation similar to that of 10 to 15 day old summer workers. Taken together, these results could be interpreted as evidence that in honey bee workers cellular proliferation is linked to longevity.

The remainder of the results, however, did not fit the prediction that longer-lived bees have more cellular proliferation than short-lived bees. Cellular proliferation declined with age in queens, similar to workers. At all three ages sampled, queens showed even less (although not significantly) cellular proliferation than workers. Following studies from other organisms (Campisi 1996, Finch 1990, Kirkwood 1984), I expected that queens would have had significantly more cellular proliferation than age-matched workers. Therefore, the hypothesis that longer-lived bees have more cellular proliferation is not supported by the comparison between the two female castes.

It is a possibility that queens have less cellular proliferation as they age because they have fewer dying cells in need to be replaced. Unfortunately, this could not be determined, as I was not able to compare the amount of apoptosis between workers and queens. If cellular death is higher in workers than in queens, then workers should display more cellular proliferation because of an increased need for cellular replacement. Cell death and thus the need for cellular replacement may be directly related to intestinal

activity. Increased food consumption requires increased activity for digestion from secretory and absorptive cells in the honey bee midgut. The higher activity of these cells will cause them to senesce quicker and consequently they have to be replaced more frequently to maintain a functional tissue (Snodgrass 1956). This scenario would explain a positive relationship between a bee's nutritional intake and the amount of midgut cellular proliferation.

This "digestive demand" hypothesis could explain why queens, although much longer-lived, have slightly less cellular proliferation than workers. Queens and workers have very different diets. Queens are constantly fed by other workers a high quality diet of royal jelly. Royal jelly is pre-processed food, created in the hypopharyngeal glands of workers, and is composed of 67.1% water, 11.9% protein, and 4.3% lipids (Herbert 1992). Workers, in contrast, eat nectar for their carbohydrates and pollen for their proteins and lipids. Unlike queens, they process this food completely on their own (Herbert 1992). Pollen walls are extremely resistant to digestion (Roulston and Cane 2000) and this demanding digestion process may cause the destruction of intestinal cells that must be replaced (Snodgrass 1956). This damage to the intestinal cells is generated by the increased secretory and digestive activity in the gut, and not from actual abrasion to the intestinal walls because food in the honey bee midgut is enclosed in the peritrophic membrane (McFarlane 1985). Therefore, even though queens may ingest quantitatively more food than workers (Winston 1987), the qualitative difference in diet may explain why workers have slightly more cellular proliferation than queens: to replace dying cells due to increased digestive activity. The digestive demand hypothesis also predicts the

observed age-related decline in cellular proliferation in workers, because young workers ingest more pollen and nectar for their maturation and brood food production than older workers (Winston 1987). The digestive activity of queens could follow a similar trend, but little is known about the age-related changes in queen digestive activity. However, if this new hypothesis is correct, queens should maintain cellular proliferation in their midguts throughout their lives. To verify this prediction, older queens should be examined for their intestinal cellular proliferation in the future.

The relatively high level of cellular proliferation in winter workers is also compatible with the hypothesis that increased intestinal activity demands more cellular replacement. Winter workers do not lie dormant in the winter. Instead, they cluster together to maintain a core temperature of around 20°C and so must consume large amounts of honey to thermoregulate throughout the winter (Gary 1992, Winston 1987). Although winter bees are not consuming as much pollen as middle-aged summer bees, they do consume large quantities of honey and their intestinal activity is probably elevated due to increased food intake related to an increased energy demand. In general, winter bees are most similar to middle-aged summer workers not only in cellular proliferation, but also in behavior and physiology: While winter bees and middle-aged summer bees may perform different tasks based on the season, they are both mature hive bees and have low levels of juvenile hormone and high levels of vitellogenin (Amdam *et al.* 2004, Omholt and Amdam 2004). They also have similar glycogen stores (Panzeböck and Crailsheim 1997). Middle-aged summer workers are indeed similar enough to winter

workers that in the absence of brood, these summer workers can maintain a winter-like lifespan of over 130 days (Haydak 1963).

The continuous age-decline of cellular proliferation that was observed in queens and workers was not observed in drones. Young drones have significantly less cellular proliferation than both young queens and workers, which may be due to their lower demand for food in combination with the fact that they are fed by workers (Winston 1987). Middle-aged drones may have more intestinal cellular proliferation than young and old drones because around 7 to 10 days of age, drones begin to become sexually mature and go on mating flights (Rueppell *et al.* 2005). Because mating flights are energetically costly, drones must consume a large amount of food. Furthermore, drones feed themselves at this age and consume up to 14 mg of sugar per hour (reviewed in Winston 1987). In addition, they increase their protein content within 9 to 12 days after emergence (reviewed in Hrassnigg and Crailsheim 2005). Therefore, middle-aged drones may have more cellular proliferation than young drones and old drones because at middles age they have the highest nutritional demands, are not fed by workers anymore, and therefore experience the most intestinal activity and a high demand for cellular replacement.

Old drones have slightly more cellular proliferation in their midguts than old workers and queens. Old drones may have more cellular proliferation at old ages than workers because drones are part of the "germ line" and workers represent the somatic tissue (Wilson 1971). According to the disposable soma hypothesis, more resources should be devoted to maintenance of the germ line than the soma (Kirkwood 1984,

Rando 2006). Also, old workers (foragers) eat less than other workers, related to their onset of foraging (Amdam and Omholt 2003). Old drones may have more cellular proliferation compared to old queens as well, as queens are fed pre-processed royal jelly and old drones are not (Winston 1987). Therefore, because drones have more intestinal activity than both old workers, who consume little, and old queens, who are fed pre-processed food, they have a greater need for cellular replacement.

Because reproductive workers are similar to queens in behavior and physiology (Winston 1987), I hypothesized that they would also be similar in lifespan. Based on my original hypothesis and preliminary data that suggested queens had more cellular proliferation than workers, I furthermore predicted that this increased lifespan in reproductive workers could be associated with an increased amount of cellular proliferation. Therefore, I predicted positive correlations between worker life expectancy, amount of intestinal cellular proliferation, and ovarian activation. Reproductive activity was indeed associated with an increase in lifespan (see below). However, the degree of ovary activation was not significantly correlated with the amount of cellular proliferation in the midgut of these queen-less workers. The results from this experiment suggest that there is no direct relationship between intestinal cellular proliferation and a key marker of reproduction in honey bees. Because there was no significant difference in intestinal cellular proliferation between workers and queens who have the greatest reproductive differences, it is understandable that there would be no difference in cellular proliferation between reproductive and non-reproductive workers.

Also, although I am not aware of any research examining reproductive worker feeding behavior, reproductive and non-reproductive workers probably experience similar digestive demands. Therefore, their intestinal activity and cellular replacement would also be similar based on the digestive demand hypothesis. Interestingly, the average amount of cellular proliferation for queen-less workers, averaged over all ovarian activation classes, falls in between the average for middle-aged summer workers and old summer workers. Because these queen-less workers were collected after 25 days to allow for ovarian activation, the amount of cellular proliferation had already begun to decline to levels that were intermediate between the middle-aged and the old age summer workers. These results fit the age-related decline seen in summer workers, even though reproductive workers show a different behavioral profile (Hoover *et al*. 2005).

Although there was no evidence to support a correlation between reproduction and cellular proliferation, the workers in the queen-less hive were significantly longer-lived than workers in the queen-right control treatment, as predicted. While the observed four-day difference is not a significant amount of time when examining the queen-worker longevity differential, it is a significant difference considering that workers on average in the summer live only 15 to 38 days (Page and Peng 2001). The age-specific survival remains similar in both reproductive and non-reproductive workers until around 25 days. Afterwards, the queen-less workers have a higher overall survival rate. This age of 25 days corresponds well with the age when reproduction begins in queen-less workers (Winston 1987) as well as the beginning of foraging (Page and Peng 2001). Several possible explanations have to be considered to explain the increase in life expectancy

under queen-less conditions. First, if the absence of the queen delays foraging, workers could live longer because they stay in the protected hive environment longer (Page and Peng 2001, Winston 1987). This was not the case because workers in both the queen-right and queen-less hive initiated foraging at the same age and under both experimental conditions similar numbers of workers foraged. Second, workers in the queen-less hive may be longer-lived because they foraged less intensely than workers in the queen-right hive and total foraging effort is directly related to lifespan (Neukirch 1982). If workers in the queen-less hive foraged less intensely, they would be able to forage longer (Neukirch 1982). However, this was also not the case because foragers in both hives foraged for approximately the same number of days. The analyses show that queen-less workers live longer than queen-right workers because of differences within the hive. The development of worker ovaries in the absence of a queen is presumably accompanied by an increase of their stores of the yolk protein vitellogenin. Vitellogenin is known to promote longer lifespan in honey bees (Amdam *et al.* 2004, Omholt and Amdam 2004) and protect them from oxidative stress (Seehus *et al.* 2006) and thus may be a plausible explanation for the increase of worker life expectancy in a queen-less hive.

To measure apoptosis, the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) method was used in attempt to detect DNA fragmentation. This assay had been used successfully in previous studies examining honey bee larval fat body (Gregorc *et al.* 2004) and ovaries (Capella and Hartfelder 1998), pupal brains (Ganeshina *et al.* 2000), as well as adult hemocytes (Amdam *et al.* 2005). When using the assay during my initial examination of the honey bee tissues,

there may have been some positive staining in the midgut and certainly there was positive staining in the positive controls treated with DNase. Because our positive controls using DNase to induce DNA fragmentation worked and positive results had been achieved in other honey bee tissues in other studies, we decided to use the TUNEL assay for this study as well. However, after all the experimental groups were processed, it was apparent that the staining seen was not nuclear and thus not quantifiable. Therefore, it is not clear whether apoptosis through DNA fragmentation is occurring in any of our experimental groups. Also, because the integrity of the tissue sections was poor, I was unable to determine if the nuclei were pycnotic which would also imply apoptosis was occurring (Kerr *et al.* 1972). It is possible that cells are sloughing off at the apex of the villi in the midgut and undergo other forms of cell death that do not involve DNA fragmentation (Potten and Wilson 2004) which could be detected by other assays (Watanbe 2002).

In general, parametric statistical tests were used because most data did not deviate significantly from the statistical assumptions. For consistency, I also employed parametric tests in the few cases where the statistical assumptions were violated. However, all results were verified with nonparametric analyses and in general the two analyses agreed. The three exceptions were between the ANOVA and Kruskall Wallis rank test for the overall effect of caste on cellular proliferation and the effect of caste on middle-aged bees' cellular proliferation, as well as between the student's t-test and a Mann-Whitney U test comparing cellular proliferation in middle-aged summer workers and winter workers. None of these differences in the results crucially affect the

conclusions that can be drawn from this study, and significant values did not differ by a wide margin. A larger sample size should be examined in the future to resolve these statistical discrepancies.

CHAPTER V

CONCLUSION

Overall, the amount of cellular proliferation in honey bees is not associated with life expectancy. Queens live one to three years, whereas workers live 15 to 38 days (Page and Peng 2001), and reproductive workers were found to live 4 days longer than non-reproductive workers, and both differences are not accompanied by any significant differences in the amount of cellular proliferation in their only actively dividing, somatic tissue, the midgut. Instead, I propose the new hypothesis that the observed differences in cellular proliferation reflect the need for cellular replacement to maintain a functional intestine, driven by digestive demands. This hypothesis explains correctly the patterns of cellular proliferation in all three experimental comparisons. However, I could only speculate on the demand for cellular replacement because apoptosis could not be quantified in this study.

If a functional digestive system is crucial for longevity (Drozdowski and Thomson 2006), aging may be determined not by the amount of cellular proliferation occurring but instead how long cellular proliferation can match the demand for cellular replacement. The proliferating cells may be homologous to the intestinal stem cells recently characterized in *Drosophila melanogaster* (Micchelli and Perrimon 2006, Ohlstein and Spradling 2006). Nevertheless, these intestinal stem cells could have a different replicative capacity in the different castes or sexes, giving rise to different life

expectancy. Aging may occur when cellular proliferation can not match the demand for cellular replacement and nutrients cannot be properly absorbed anymore. The individual would thus starve without an apparent shortage of food (Neukirch 1982). Therefore, the replicative capacity of these stem cells may play a role in aging (Patil *et al.* 2005, Sethe *et al.* 2006, Van Zant and Liang 2003, Warner 2007) whereas the amount of cellular proliferation does not. Controlled cell culture studies of the replicative capacity of worker and queen derived intestinal stem cells should be performed to test whether replicative senescence relates to the natural differences in organismal life expectancy (Hayflick 1977, Hayflick 2000) of honey bees. If these experiments indicate a role for replicative capacity of the intestinal stem cells, future research should focus on manipulations of their activity and capacity, using the natural differences between honey bee castes.

Although the current results do not indicate an involvement of cellular replication in honey bee aging, complimentary evidence that nutritional status of the individual is important comes from other studies: The major hemolymph protein vitellogenin has been found to promote increased longevity in honey bees (Amdam *et al.* 2004, Omholt and Amdam 2004, Seehus *et al.* 2006) and glycogen may is known to be a limiting factor in the flightspan of foragers (Neukirch 1982). If intestinal cellular replacement plays a role in honey bee aging, these two nutrients may be diminished in bees with limited intestinal functionality. Further studies are needed to decipher the regulation of the unparalleled aging plasticity in honey bees.

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APPENDIX

TABLES AND FIGURES

Table 1. Kolmogorov-Smirnov test results for normality of all experimental groups for cellular proliferation.

Group	N (number of bees)	K-S z	p
All Bees	209	1.524	0.019*
Young Drones	20	0.752	0.624
Middle Drones	16	1.377	0.045*
Old Drones	20	0.566	0.906
Young Workers	12	0.469	0.980
Middle Workers	20	0.727	0.666
Old Workers	20	0.84	0.480
Young Queens	19	0.83	0.496
Middle Queens	16	0.966	0.308
Old Queens	19	0.717	0.684
Winter Workers	20	0.815	0.520
Reproductive Workers	27	0.831	0.495

* significant at the 0.05 level

Table 2. Mean and standard deviation of number of proliferating cells in the midgut for evaluating caste differences (experiment 2).

Group	N (number of bees)	Mean Number of Proliferating Cells	δ
Young Workers	12	303.6	88.6
Middle Workers	20	181.1	66.4
Old Workers	20	59.7	54.1
Young Queens	19	249.4	124.1
Middle Queens	16	172.5	71.4
Old Queens	19	43.3	28.1
Young Drones	20	115.9	50.3
Middle Drones	16	143.7	94.7
Old Drones	20	81.9	33.6

Table 3. Mean and standard deviation of number of proliferating cells in the midgut for evaluating seasonal differences (experiment 3).

Group	N (number of bees)	Mean Number of Proliferating Cells	δ
Young Summer Workers	12	303.6	88.6
Middle Summer Workers	20	181.1	66.4
Old Summer Workers	20	59.7	54.1
Winter Workers	20	142.9	69.2

Figure 1. Cross section of a representative 11-day-old honey bee worker midgut with immuno-labeled cells that have incorporated 5-bromo-2'-deoxyuridine, indicating DNA synthesis and thus cellular proliferation.

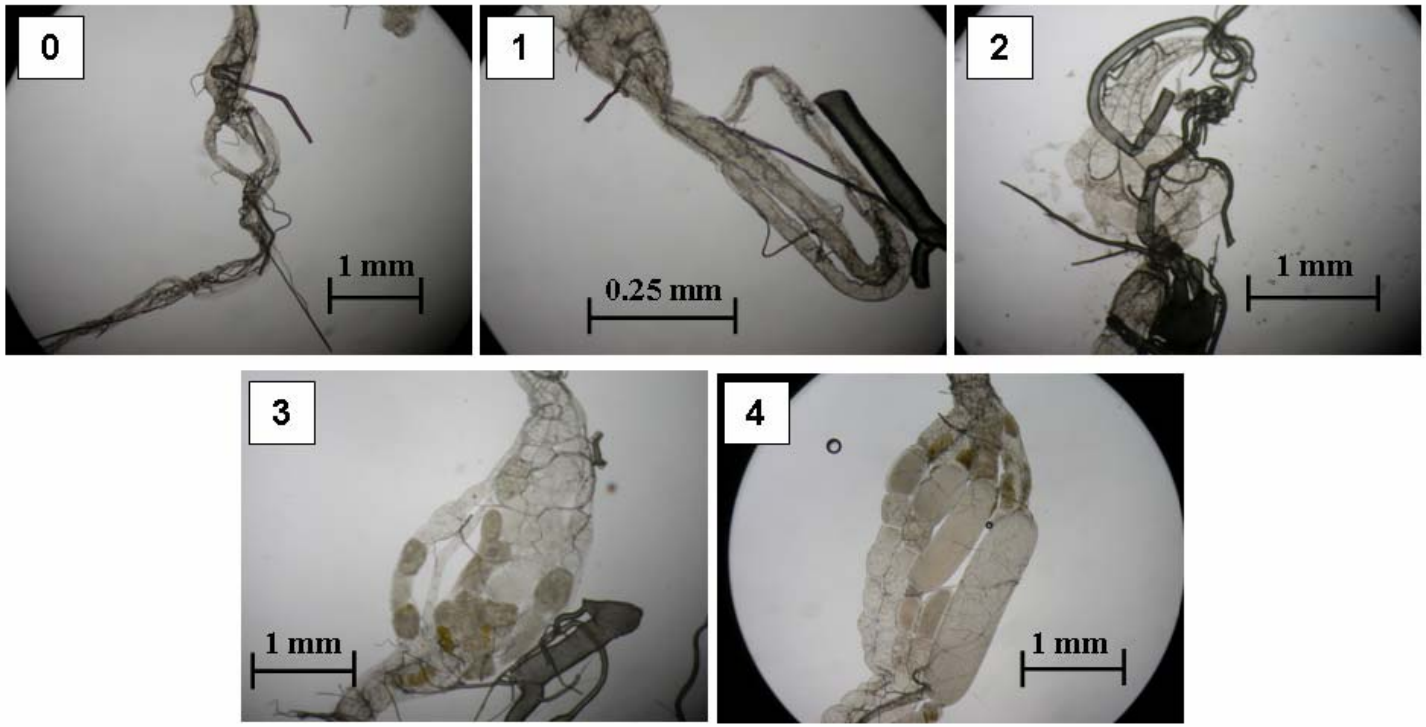

Figure 2. Five point scale of worker ovarian development. Used in experiment 4 to assess reproductive activity (following Pernal and Currie 2000). This scale takes the ovariole swelling and size of developing eggs into account.

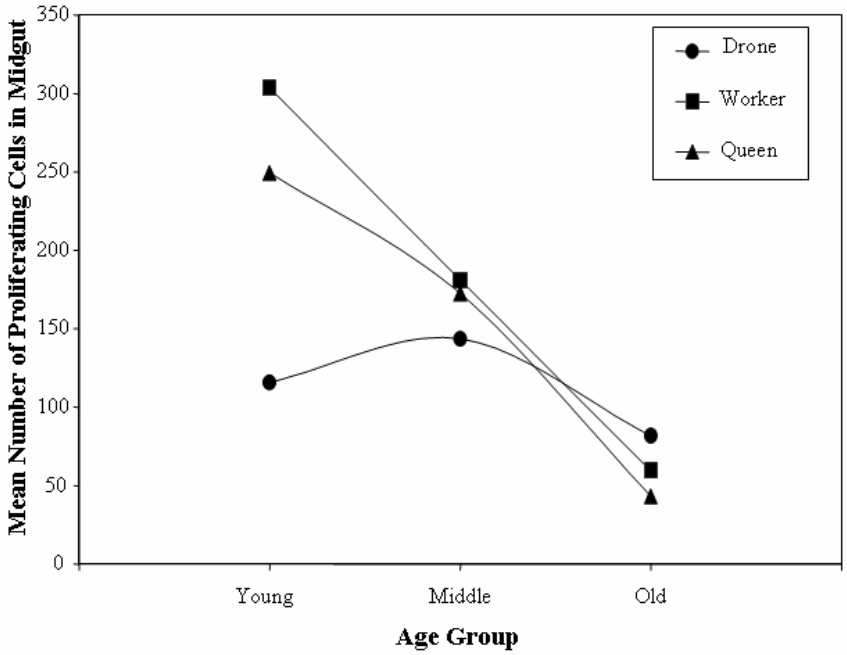

Figure 3. Average cellular proliferation in the midgut for all three castes at the three investigated ages.

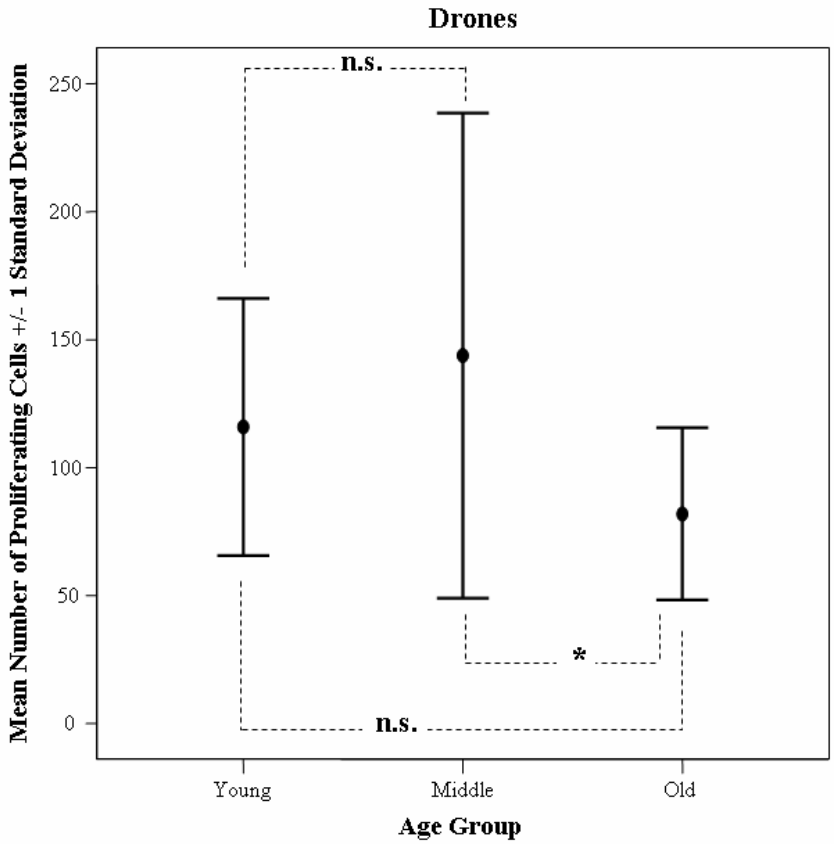

Figure 4. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the three experimental age groups of drones. Statistical differences are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

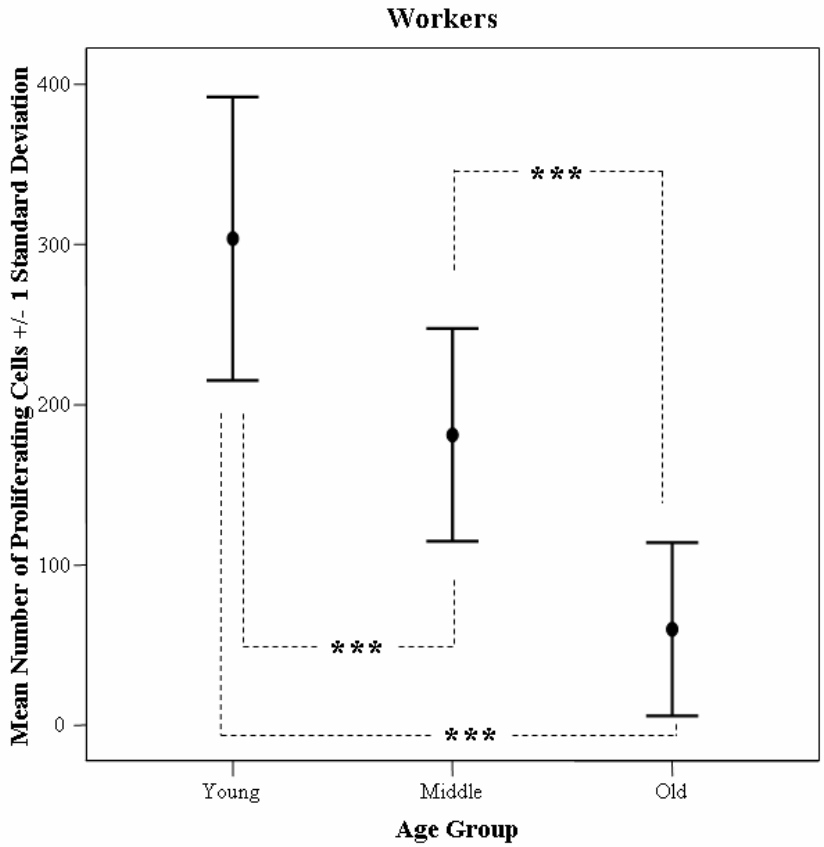

Figure 5. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the three experimental age groups of workers. Statistical differences are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

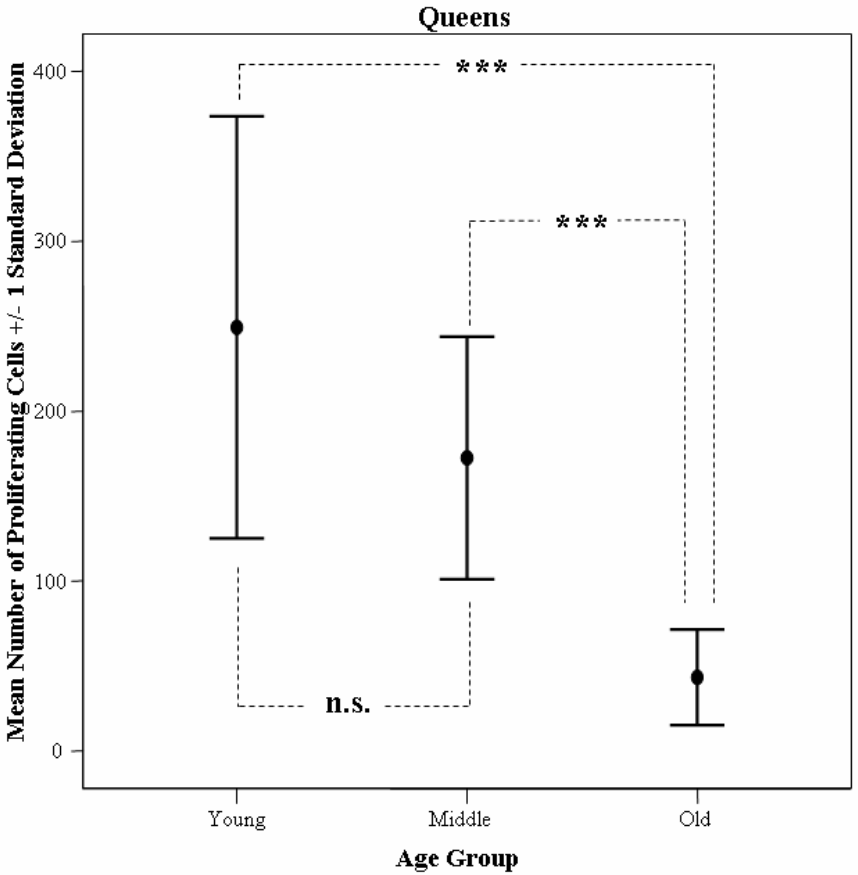

Figure 6. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the three experimental age groups of queens. Statistical differences are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

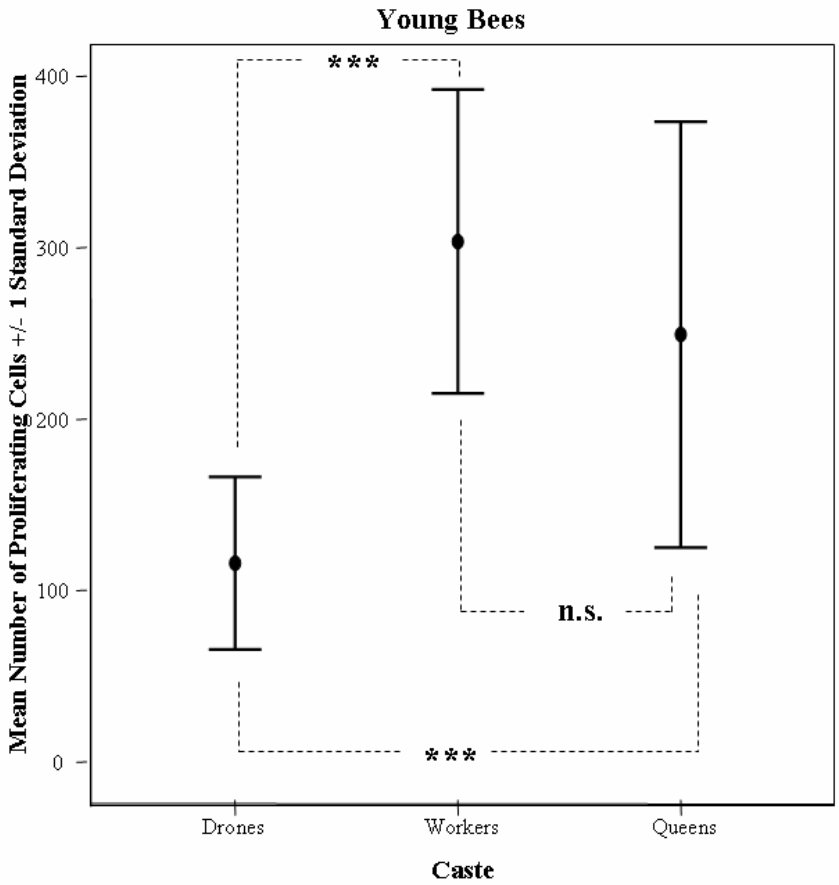

Figure 7. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the young age group for all three castes. Statistical differences are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

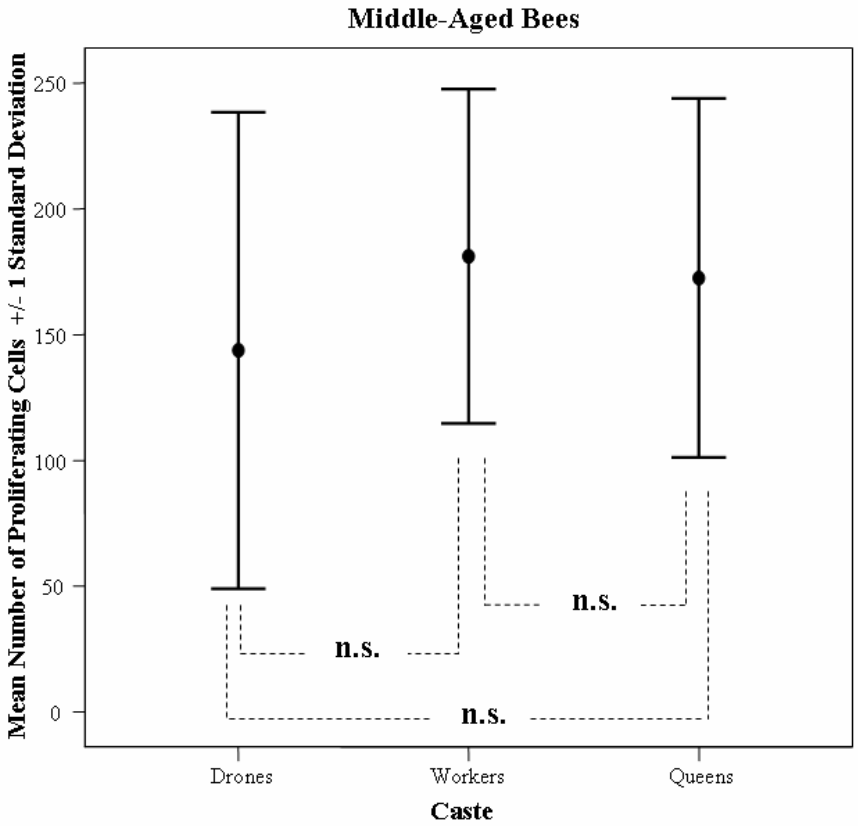

Figure 8. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the middle-age group for all three castes. Statistical differences are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

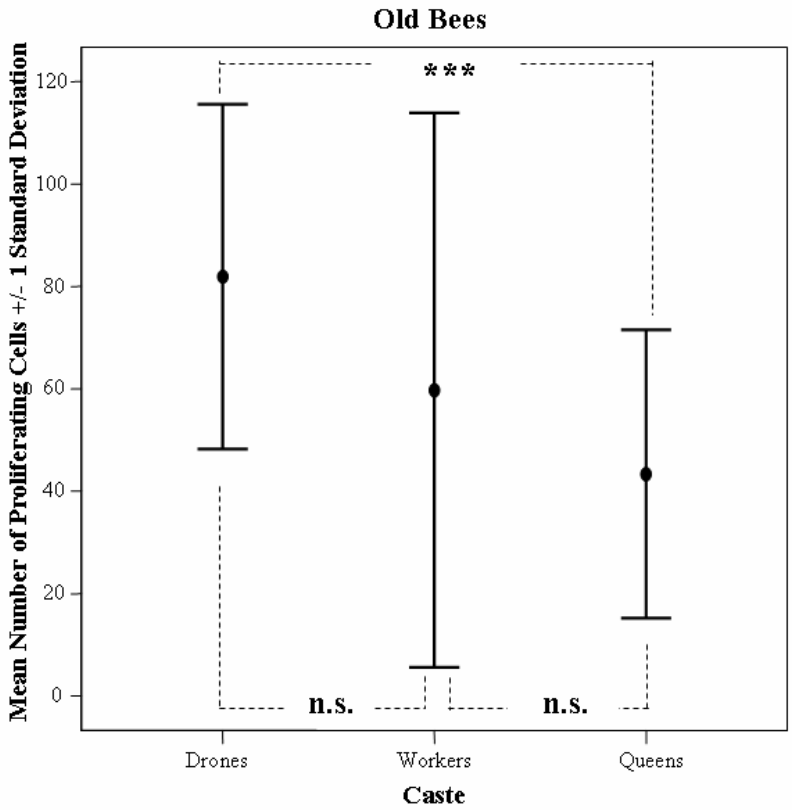

Figure 9. Comparison of the amount of cellular proliferation (mean and standard deviation are given) for the old age group for all three castes. Statistical differences are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

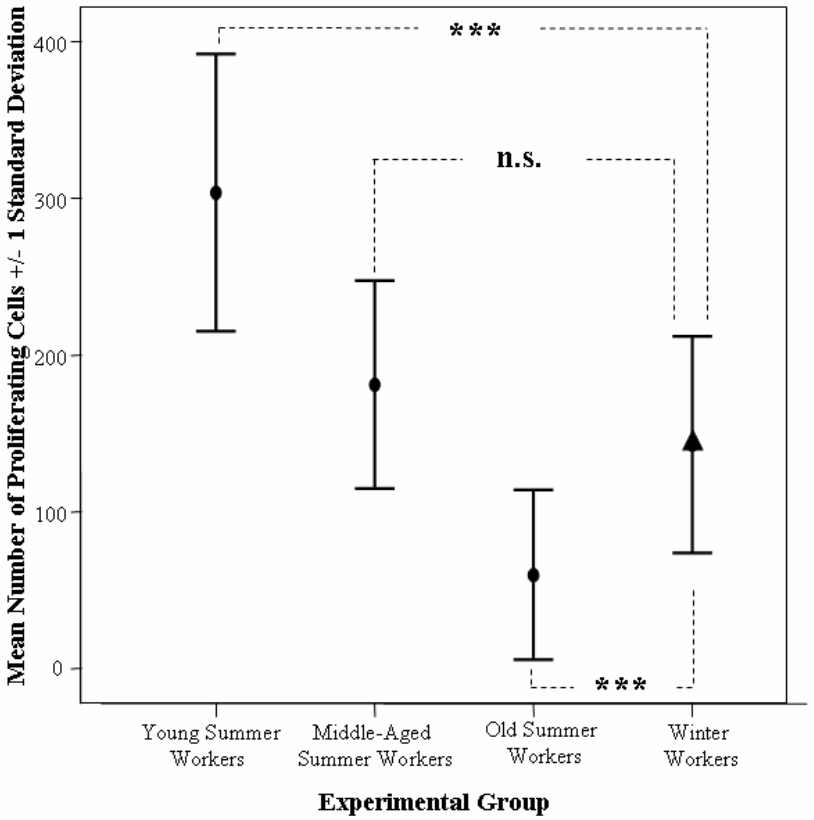

Figure 10. Comparison of the amount of cellular proliferation between winter workers and the three age groups of summer workers (mean and standard deviation are given). Statistical differences are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

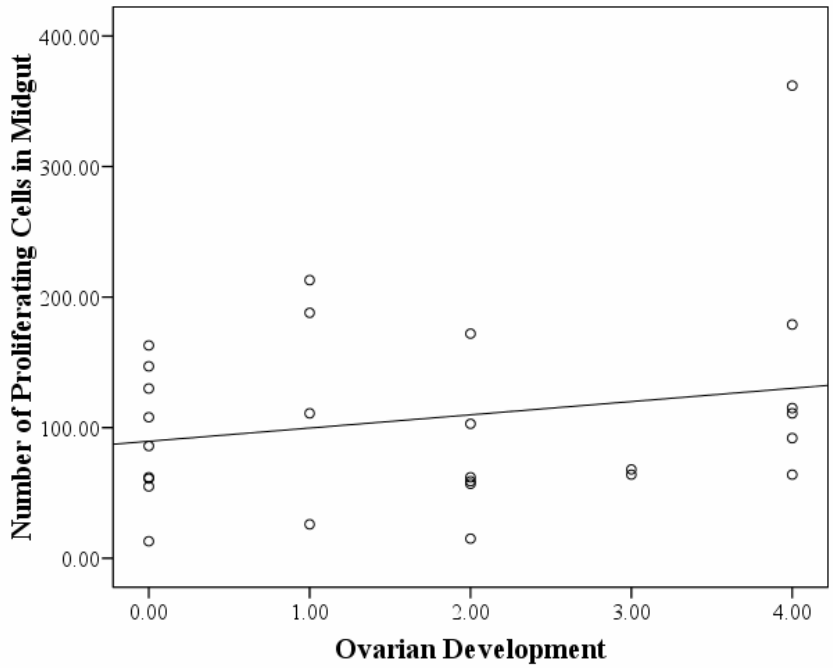

Figure 11. No relation between ovarian development and the amount of cellular proliferation in the midgut in workers under queen-less conditions.

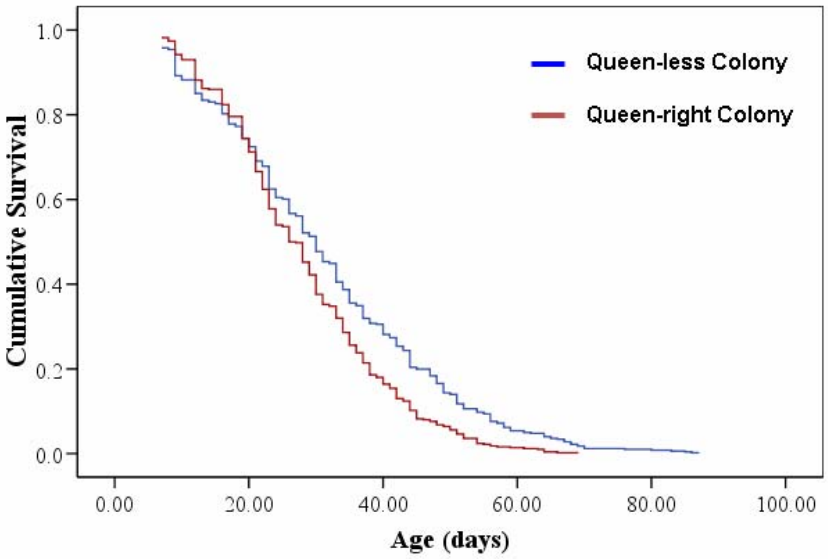

Figure 12. Cumulative survival curve of workers in queen-less hive and workers in queen-right hive.

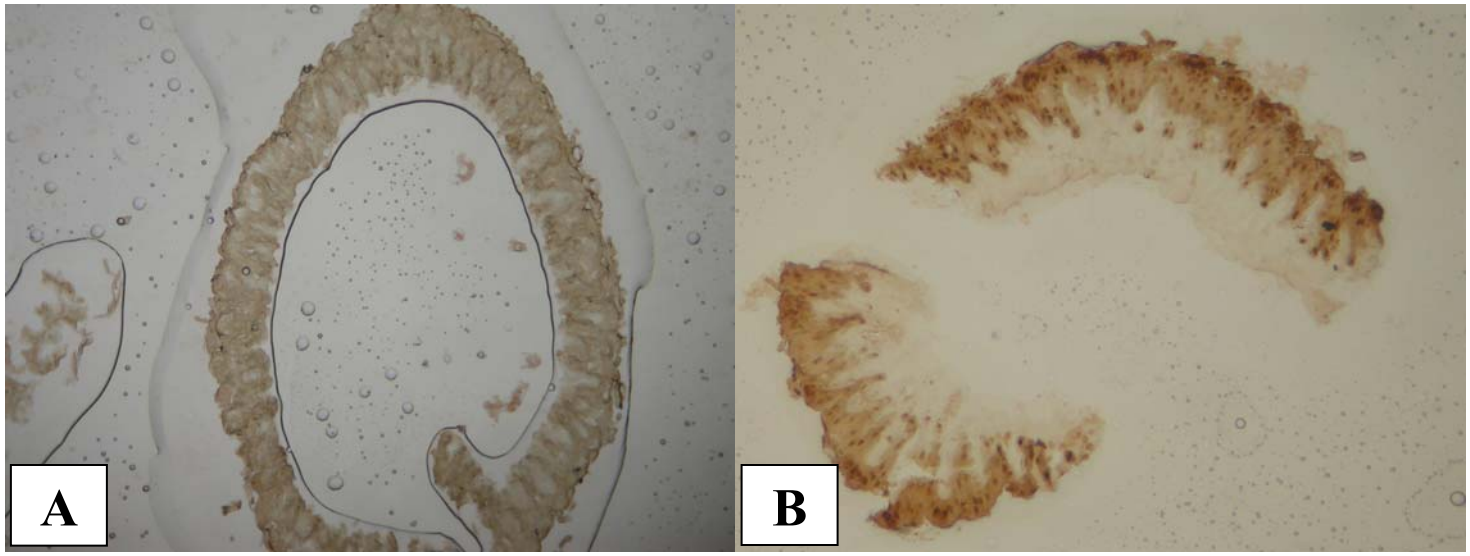

Figure 13. Pictures of apoptosis assays in honey bee midgut. A: Cross section of honey bee midgut. Absence of staining indicates no DNA fragmentation. B: Labeled DNA fragmentation in honey bee midgut tissue which was exposed to DNase (positive control).