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Pteridine levels and head weights are correlated with age and colony task in the honey bee, *Apis mellifera*

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**Background.** The age of an insect strongly influences many aspects of behavior and reproduction. This interaction is epitomized in the temporal polyethism of honey bees in which young adult bees perform nurse and maintenance duties within the colony, while older bees forage for nectar and pollen. Task transition is dynamic and is driven by colony needs. However, an abundance of precocious foragers or overage nurses may have detrimental effects on the colony. Additionally, honey bee age affects insecticide sensitivity. Therefore, determining the age of an individual honey bee would be important to provide a measurement of colony health. Pteridines are purine-based pigment molecules found in many insect body parts. Pteridine levels correlate well with age, and wild caught insects may be accurately aged by measuring pteridine levels. The relationship between pteridines and age varies with a number of internal and external factors among many species. Thus far, no studies have investigated the relationship of pteridines with age in honey bees. **Methods.** We established single-cohort colonies to obtain age-matched nurse and forager bees. Nurses and foragers were collected every 3-5 days for up to 42 days. Heads were removed and weighed before pteridines were purified and analyzed using previously established fluorometric methods. **Results.** Our analysis showed that pteridine levels were higher in foragers than nurses of the same age, and pteridine levels significantly increased with age in a linear manner. Head weight significantly varied with age increasing until approximately 28 days of age, then declining thereafter for both nurse and forager bees. **Discussion.** Although the relationship between pteridine levels and age was significant, a large amount of variation in the data yielded an 8-day window in age estimation. This allows an unambiguous method to determine whether a bee may be a young nurse or old forager. Pteridine levels in bees do not correlate with age as well as in other insects. However, most studies used insects reared under tightly controlled laboratory conditions, while we used free-living bees. The dynamics of head weight change with age is likely to be due to growth and atrophy of the hypopharyngeal glands. Taken together, these methods represent a useful tool for assessing colony demography after a colony experiences a stress event. Future studies utilizing these methods will provide a more holistic view of colony health.
Pteridine Levels and Head Weights are Correlated with Age and Colony Task in the Honey Bee, *Apis mellifera*

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ABSTRACT

Background. The age of an insect strongly influences many aspects of behavior and reproduction. The interaction of age and behavior is epitomized in the temporal polyethism of honey bees in which young adult bees perform nurse and maintenance duties within the colony, while older bees forage for nectar and pollen. Task transition is dynamic and is driven by colony needs. However, an abundance of precocious foragers or overage nurses may have detrimental effects on the colony. Additionally, honey bee age affects insecticide sensitivity. Therefore, determining the age of an individual honey bee would be important to provide a measurement of colony health. Pteridines are purine-based pigment molecules found in many insect body parts. Pteridine levels correlate well with age, and wild caught insects may be accurately aged by measuring pteridine levels. The relationship between pteridines and age varies with a number of internal and external factors among many species. Thus far, no studies have investigated the relationship of pteridines with age in honey bees.

Methods. We established single-cohort colonies to obtain age-matched nurse and forager bees. Nurses and foragers were collected every 3-5 days for up to 42 days. Heads were removed and weighed before pteridines were purified and analyzed using previously established fluorometric methods.

Results. Our analysis showed that pteridine levels were higher in foragers than nurses of the same age, and pteridine levels significantly increased with age in a linear manner. Head weight significantly varied with age increasing until approximately 28 days of age, then declining thereafter for both nurse and forager bees.

Discussion. Although the relationship between pteridine levels and age was significant, a large amount of variation in the data yielded an 8-day window in age estimation. This allows an
unambiguous method to determine whether a bee may be a young nurse or old forager. Pteridine
levels in bees do not correlate with age as well as in other insects. However, most studies used
insects reared under tightly controlled laboratory conditions, while we used free-living bees. The
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hypopharyngeal glands. Taken together, these methods represent a useful tool for assessing
colony demography after a colony experiences a stress event. Future studies utilizing these
methods will provide a more holistic view of colony health.
INTRODUCTION

Insect age directly affects behavior, vectorial capacity, reproductive output, as well as insecticide detoxification and susceptibility. Under normal circumstances, age largely dictates the progression of tasks a honey bee worker performs for the colony by way of a juvenile hormone regulatory mechanism (Huang & Robinson 1996; Robinson 1992). As bees age, they transition from cleaning brood cells and building wax comb, to storing pollen and nectar, defending the entrance, and ultimately foraging for nectar and pollen outside of the colony. This stereotyped progression of behaviors is highly plastic, and disturbances to colony demography, food availability, and seasonal changes may induce reversions between behavioral states (Robinson 1992). The transition from nurse to forager accompanies an extensive physiological rearrangement and dramatic increases metabolic rate (Harrison 1986; Harrison & Fewell 2002).

From a toxicological perspective, honey bee age affects insecticide sensitivity (Rinkevich et al. 2015). Therefore, insecticide exposure may induce a demographic shift in the colony. Detecting changing demography in the colony provides a new way to interpret field-based studies on honey bee health and insecticide sensitivity.

Many methods exist for age determination in insects such as measuring ovarian follicle characteristics, counting cuticular bands, grading amount of fat body, and cuticular degradation (Hayes & Wall 1999). However, most of these methods are tedious, do not allow processing many samples, based upon a single sex, and have limits of age determination. The use of pteridines to determine the age of an insect drastically reduces these limitations. Pteridines consist of fused pyrimidine and pyrazine ring byproducts of purine metabolism, which affect excretion, body coloration, and eye pigmentation (Zeigler & Harmsen 1969). These pteridines accumulate with age, allowing accurate age determination by measuring pteridine levels. Using pteridines to
determine insect age has been studied in a number of Dipteran species in which light intensity, sex, and temperature may affect the relationship of pteridines with age (Robson et al. 2006). However, an attempt to use pteridines to determine the age of the eusocial Hymenopteran ant, *Polyrachis sexpinosa* showed that only head weight, but not pteridine levels were correlated with age (Robson & Crozier 2009). To date, no published methods use pteridines to estimate bee age. Here, we report on using pteridine levels and head weight to determine the age of nurse and forager bees.
MATERIALS AND METHODS

Honey Bees

Frames of emerging brood were removed from colonies of Italian bees, cleared of all bees, then placed in wooden enclosures (60 cm L x 15 cm W x 45 cm H) with wire mesh on the wide faces of the box. Frames were stored at 33 °C with >70% RH in continuous darkness. The next morning, bees were brushed off of the frames into a plastic tub (50 cm L x 40 cm W x 15 cm H) with a thin layer of petroleum jelly around the rim. Individual bees were marked with a dot of enamel paint (Testors, Vernon Hill, IL) on the thoracic dorsum. Single-cohort colonies (SCCs) were created by housing more than 2000 marked newly emerged bees marked in a 5-frame nucleus box with 1 frame of honey and pollen, 1 frame of brood, and 3 frames of empty drawn out comb. Each SCC was headed by an Italian queen (Wooten’s Golden Queens, Palo Cedro, CA). The SCCs were stored at the environmental conditions mentioned above for 5 days, then placed in an apiary at the USDA Honey Bee Breeding, Genetics and Physiology Research Unit in Baton Rouge, LA. Four SCCs were constructed from four independent batches of emerging brood from 6 source hives. A sample of three nurses and three foragers were collected every 3-5 days up to 42 days from each SCC. Nurse bees were collected as they were actively nursing larvae on brood comb. The entrances to the SCCs were blocked to collected returning foragers. A total of 871 bees were evaluated in this study. Bees were frozen at -80 °C until pigment extraction later that same day.

Pteridine Extraction

Pteridines were extracted according to previously published methods (Robson & Crozier 2009). Frozen bee heads were removed with a scalpel. Individual heads were weighed to the nearest mg and placed in a 1.5 mL tube on ice. A 0.5 mL volume of chloroform:methanol (2:1
(v/v) was added to each tube. Heads were ground with a plastic pestle for 30s. Samples were sonicated using a Q125 Sonicator (QSonica, Newtown, CT) at 5 W for 15s. Samples were placed on ice for >1min, then sonicated for another 15s. Tubes received 0.75 mL of 0.1 N NaOH (adjusted to pH 10 with 11.5 g/L glycine). All tubes were vortexed for 10s then centrifuged for 5 min at 5000xg at 4°C. The 0.75 mL supernatant was saved and used for fluorescence determination. Pteridine fluorescence was measured with excitation at 355 nm (5 nm slit) and scanned in the emission spectra from 365 to 500 nm based on methods published by Robson and Crozier (Robson & Crozier 2009). The peak absorbance was recorded. The ng of pteridines in the sample were calculated based on a standard curve of 6-biopterin (Mail & Lehane 1988). The ng of pteridines was standardized by head weight (ng pteridines/mg head weight).

**Statistical Analysis**

All statistics were performed with R or Minitab. In order to determine the correlation between age and pteridine level, a linear regression analysis was performed. Total pteridine levels were compared between nurse and forager bees using Chi Square analysis. The rate of increase was compared between nurse and forager bees using linear regression analysis. A curve estimation test was performed to evaluate the relationship between bee head weight and age.
RESULTS

Pteridine levels significantly increased in a linear manner with age for all bees regardless of behavioral status (Figure 1A, B, C). Comparison of pteridine levels between nurses and foragers showed that foragers possessed significantly higher pteridine levels than nurses (General Linear Model df=1, SS=12654.5, F Ratio= 16.1, p<<0.0001). The differences in pteridine levels between nurses and foragers became significantly wider with age (R²=0.37, F=15.01, p=0.001). However, there was no difference in the rate of increase in pteridine levels by comparison of the slopes for both nurses and foragers (t=0.16, df=71, p=0.87), most likely due to the large error in the slopes.

Head weight for all bees was significantly correlated with age, but the relationship was best fit with a quadratic equation. Head weight increased until 28 days of age, then declined though day 45 (Figure 2A). The relationship between age and head weight was highly correlated (R²=0.70) and highly significant (F=39.11; p<0.001) for all bees (Figure 2A). The relationship between age and head weight varied between nurses (R²=0.54) and foragers (R²=0.25), but remained highly significant in both cases (F=16.18, p<0.001; F=5.71, p=0.007, respectively). Nurses had significantly higher head weight than foragers on days 8-16 (Figure 2B and 2C, F = 10.09, p <<0.001). While the slope of the curve of the relationship between age and head weight was more pronounced for nurses than it was for foragers, the difference was not significant (t=0.012, df=60, p=0.99).
In 1891, Hopkins first discovered pteridine pigments in butterfly wings (Hopkins 1891), and their use in aging would later be exploited for multiple invertebrate groups. Because pteridines are so widespread in insects, and can be easily detected through their fluorescence, they are considered one of the most important pigments in insects (Wigglesworth 1964). Although primarily used in insects of medical and forensic importance, the use of pteridines in aging has recently been evaluated in other insects, such as the ant, *Polyrhachis sexpinosa* (Robson & Crozier 2009), and pink-spotted bollworm, *Pectinophora scutigera* (Noble & Walker 1990). However, few studies go beyond using pteridines as a tool to determine age. This is the first study to identify pteridine levels that may be employed as a method to determine demographic shifts among a population used to extrapolate potential health effects of honey bee colonies.

Results of our study showed a linear increase in pteridine concentration for both nurse and forager bees over time. While the size of the bee varied, it is unlikely this size variation contributed to our results. Studies simply reporting fluorescence values correlated to age fail to take into account the size variation of the insect. Therefore, determining the concentration of pigment in relation to body size is critical. In this study, we used 6-biopterin as our standard in order to calculate concentration of pteridines from fluorescence. Wu & Lehane (1999) conducted HPLC recordings of fluorescent compounds in mosquitoes of different ages. Although they identified several pteridine compounds, 6-biopterin varied the most with age. Noble and Walker (Noble & Walker 1990) also isolated 6-biopterin from pink bollworm moths at increasing ages. However, their results did not show a correlation with age. Although their use in age has not been evaluated in bees, biopterin has been isolated from bees (Hanser & Rembold 1960; Haydak...
Therefore, we are confident that use of 6-biopterin as our standard was an appropriate technique to estimate pteridine concentration in bees. However, future studies to isolate and quantify specific pteridines in bees via HPLC analysis should be considered.

Bee age significantly correlated with pteridine concentration, and our results suggest this method can be used to compare nurse and forager bees. The variation in the confidence interval for the slope of pteridines for all bees as related to age (m = 1.049, 95%CI = 0.883-1.215) produces an 8 day window in predicting the age of the bee. While this level of precision yields a rough estimate of bee age, it can be used to confidently distinguish an old nurse bee from an old forager bee. After a stress event perturbs the demography of a hive, precocious foraging likely initiates, and this method may easily detect precocious foraging and demographic shifts in a colony.

In precocious foraging, younger honey bees, which typically nurse inside of the hive, begin foraging at an earlier age. This type of response usually results because of stressors such as pests, pathogens, poor nutrition, or environmental parameters (Perry et al. 2015). Given the recent importance of colony declines to domestic bee keepers, determining precocious foraging in the field would be a beneficial tool to better understand the impact of these stressors.

Sex of the insect, ambient light intensity and temperature may affect pteridine levels (Robson et al. 2006). Since all worker bees are female, as well as the low light conditions and relatively constant temperature within the colony (Seeley & Heinrich 1981), these factors are largely controlled in this study. The exception is when foragers leave the colony. We found that foragers have significantly higher pteridine levels than nurses. This may be the result of the much higher light intensity encountered by foragers outside of the colony as increased light.
intensity has been shown to increase pteridine accumulation in *Drosophila serrata* (Robson et al. 2006).

The relationship of head weight with age varies less than the relationship of pteridine levels with age by comparison of the $R^2$. The range in age estimate using head weight is 4 days. However the quadratic nature of the weight/age relationship yields two date outcomes. In order to overcome some of these potential conflicts, any value that produces an age of $<0$ can be ignored and the older date should be considered as the true age. The age as determined by head weight would be more accurate if other characteristics are considered such as amount of hair on the body, wing wear, body pigmentation, and the behavioral state of the bee when it was collected.

The pattern of the increase in head weight is likely due to the fluctuation in the size of the hypopharyngeal gland (Hrassnigg & Crailsheim 1998). The hypopharyngeal gland grows in size upon emergence to allow nurses to produce brood food. The hypopharyngeal gland atrophies at the transition from nurse to other colony tasks (Johnson 2010). The precocious foraging in our SCC set up showed that the behavioral state affects the head weight of bees (Figure 2B, 2C), suggesting that age alone is not the only factor affecting head weight. Head weight decreases in older nurse bees. Despite the fact the older nurse bees (>21-days old) with lighter head weight (and presumably smaller hypopharyngeal glands) were actively nursing at the time of collection, it is likely these overage nurses are less effective at producing brood food with atrophied hypopharyngeal glands. Smaller hypopharyngeal glands have reduced rates of protein synthesis (Huang et al. 1989). While overaged nurses (>15 days-old) rear smaller adults with developed ovaries (Wegener et al. 2009), they have been associated with rearing queen larvae (Jung-Hoffman 1966).
To date, this is the first study to evaluate the use of pteridine pigments in the aging of bees. While this study did not find a highly accurate correlation of pteridines with age that was comparable to studies in medically and forensically important insects, we do see the value in being able to evaluate the age structure of a colony, as well as the likelihood of precocious foraging. We also feel that future studies that compare various treatment and control groups in the field could utilize this type of analysis to compare overall age structures as an additional measurement of colony health.
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Figure Legend:

Figure 1: Linear relationship between bee age and pteridine concentration for all bees (1a), nurse bees (1b), and forager bees (1c).

Figure 2: Quadratic relationship between bee age and head weight for all bees (2a), nurse bees (2b), and forager bees (2c).
Figure 1A.
Figure 1B
Figure 1C
Figure 2A
Figure 2B
Figure 2C
234 Literature Cited

235 Hanser G, and Rembold UH. 1960. About the queen cells jelly of the honey bee, VI. The metabolism of biopterin in the honeybee. *Hoppe-Seyler's J of Physiol Chem* 319:213-219.

237 Harrison JF. 1986. Caste-specific changes in honeybee flight capacity. *Physiol Zool* 59:175-187.

238 Harrison JF, and Fewell JH. 2002. Environmental and genetic influences on metabolic rate in the honey bee, *Apis mellifera*. *Comp Biochem Physiol Part A: Molec Integ Physiol* 133:323-333.

241 Haydak MH, and Vlivo AE. 1950. The changes in the thiamine, riboflavin, niacin and pantothenic acid content in the food of female honeybees during growth with a note on the vitamin K activity of royal jelly and bee bread. *Annals Entom Soc Am* 43:361-367.

244 Hayes EJ, and Wall R. 1999. Age-grading adult insects: a review of techniques. *Physiol Entomol* 24:1-10.

246 Hopkins FG. 1891. Pigments in yellow butterflies. *Nature* 45:197-198.

249 Huang ZY, Otis GW, and Teal PEA. 1989. Nature of brood signal activating the protein synthesis of hypopharyngeal gland in honey bees, *Apis mellifera* (Apidae: Hymenoptera). *Apidologie* 20:455-464.

252 Huang ZY, and Robinson GE. 1996. Regulation of honey bee division of labor by colony age demography. *Behav Ecol Sociobiol* 39:147-158.

254 Johnson BR. 2010. Division of labor in honey bees: form, function, and proximate mechanisms. *Behav Ecol Sociobiol* 64:305-316.

256 Jung-Hoffman I. 1966. Die determination von konigin und arbeiterin der honigbiene. *Z Bienenforsch* 8:296-322.

258 Lingens F, and Rembold UH. 1959. Über den Weiselzellenfuttersaft der Honigbiene, III. Vitamingehalt von Königinnen- und Arbeiterinnefuttersaft. *Hoppe-Seyler's Z physiol Chem* 314:141-146.

261 Mail TS, and Lehane MJ. 1988. Characterisation of pigments in the head capsule of the adult stable fly *Stomoxys calcitrans*. *Entomologia Experimentalis et Applicata* 46.

263 Noble RM, and Walker PW. 1990. Pteridine compounds in adults of the pink spotted bollworm, *Pectinophora scutigera*. *Entomol Exp Appl* 57:77-83.

265 Perry CJ, Sovik E, Myerscough MR, and Barron AB. 2015. Rapid behavioral maturation accelerates failure of stressed honey bee colonies. *PNAS* 112:3427-3432.
Rinkevich FD, Margotta JW, Pittman JM, Danka RG, Tarver MR, Ottea JA, and Healy KB. 2015. Genetics, synergists, and age affect insecticide sensitivity in the honey bee, Apis mellifera. *PLoS One* 10:e0139841.

Robinson GE. 1992. Regulation of division of labor in insect societies. *Annu Rev Entomol* 37:637-665.

Robson SKA, and Crozier RH. 2009. An evaluation of two biochemical methods of age determination in insects (pteridines and lipfuscin) using the ant Polyrachis sexpinosa Latrielle (Hymenoptera: Formicidae). *Australian J Entomol* 48:102-106.

Robson SKA, Vickers M, Blows MW, and Crozier RH. 2006. Age determination in individual wild-caught Drosophila serrata using pteridine concentration. *J Exp Biol* 209:3155-3163.

Seeley TD, and Heinrich B. 1981. Regulation of temperature in the nests of social insects. In: Heinrich B, ed. *Insect thermoregulation*. New York: Wiley.

Wegener J, Lorenz MW, and Bienefeld K. 2009. Physiological consequences of prolonged nursing in the honey bee. *Insectes Sociaux* 56:85-93.

Wigglesworth VB. 1964. *The life of insects*. Minneapolis, MN: Weidenfeld and Nicolson.

Wu D, and Lehane MJ. 1999. Pteridine fluorescence for age determination of Anopheles mosquitoes. *Med Vet Entomol* 13:48-52.

Zeigler I, and Harmsen R. 1969. The biology of pteridines in insects. *Adv Insect Physiol* 6:139-203.