The ontogeny of immunity: Development of innate immune strength in the honey bee (*Apis mellifera*)

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A B S T R A C T

Honey bees (*Apis mellifera*) are of vital economic and ecological importance. These eusocial animals display temporal polyethism, which is an age-driven division of labor. Younger adult bees remain in the hive and tend to developing brood, while older adult bees forage for pollen and nectar to feed the colony. As honey bees mature, the types of pathogens they experience also change. As such, pathogen pressure may affect bees differently throughout their lifespan. We provide the first direct tests of honey bee innate immune strength across developmental stages. We investigated immune strength across four developmental stages: larvae, pupae, nurses (1-day-old adults), and foragers (22–30 days old adults). The immune strength of honey bees was quantified using standard immunocompetence assays: total hemocyte count, encapsulation response, fat body quantification, and phenoloxidase activity. Larvae and pupae had the highest total hemocyte counts, while there was no difference in encapsulation response between developmental stages. Nurses had more fat body mass than foragers, while phenoloxidase activity increased directly with honey bee development. Immune strength was most vigorous in older, foraging bees and weakest in young bees. Importantly, we found that adult honey bees do not abandon cellular immunocompetence as has recently been proposed. Induced shifts in behavioral roles may increase a colony's susceptibility to disease if nurses begin foraging activity prematurely.

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1. Introduction

Social insects have evolved adaptive mechanisms to decrease rates of disease transmission, including mutual grooming and removal of dead nest mates (Traniello et al., 2002). However, hygienic behavior may also increase the rate of pathogen exposure between nestmates and actually facilitate disease outbreak (Fefferman et al., 2007). Eusocial animals have a high potential risk of spreading infection among individuals from the same colony because they live in highly integrated groups with an overlap of generations. Additionally, the high level of cohesion in eusocial animals may increase the risk of disease outbreak as a result of close living quarters, high genetic relatedness between individuals, and continuous physical interactions between individuals within and across generations (Schmid-Hempel, 1998; Whiteman and Parker, 2004; Godfrey et al., 2006). In response, eusocial insects have evolved novel behavioral, physiological, and organizational adaptations to combat the increased risk of disease (Wilson, 1971, 1975; Rosengaus et al., 1999; Starks et al., 2000; Traniello et al., 2002; Hughes and Boomsma, 2004; Wilson-Rich et al., 2007, 2009; Cremer et al., 2007; Fefferman et al., 2007; Aubert and Richard, 2008).

Honey bees (*Apis mellifera*) are highly successful eusocial insects with nearly cosmopolitan distribution (Sheppard and Meixner, 2003). Honey bees defend themselves from an especially diverse range of pathogens, including bacteria, fungi, viruses, protozoa, mites, flies, beetles, and nematodes (Bailey and Ball, 1991; Schmid-Hempel, 1998; Evans and Pettis, 2005). Behavioral differences between honey bee brood and adults are likely to play an important role in disease susceptibility to different pathogens. Disease resistance capacity can be empirically tested and quantified using measures of immunocompetence (IC). We define IC as the ability of an organism to mount an immune response, either in cellular, humoral, or behavioral form (see König and Schmid-Hempel, 1995; Siva-Jothy, 1995; Schmid-Hempel and Ebert, 2003; Adamo, 2004a; Rantala and Roff, 2005; Wilson-Rich et al., 2009).

Variation in pathogen-specific selective pressure may result in IC dissimilarities across developmental stages. We hypothesize

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that selection has maximized disease resistance abilities at each developmental stage. Given that brood (larvae and pupae) are confined within a comb cell, they are limited in their ability to move away from approaching parasites or to otherwise avoid pathogens through behavioral mechanisms. As such, brood likely rely on cellular and humoral mechanisms of defense (Evans et al., 2006). Alternatively, adult honey bees display a range of hygienic and antipathogenic behaviors including grooming and removal of infected nestmates (Spivak, 1996). Adult behavior is influenced by age, through an ontogenetic process termed temporal polyethism (Winston, 1987; Starks et al., 2005). During this progression, young adults ("nurse bees") feed larvae until they develop into pupae, which are capped with wax and isolated until eclosion as nurse bees. Nurses typically remain in the hive and perform hygienic activities and tend to the brood, while older adult bees (i.e. foragers) leave the hive to collect pollen and nectar (reviewed by Winston, 1987).

The majority of disease phenotypes are expressed either in brood or in adults, but seldom in both (Bailey, 1968a), although many pathological microorganisms may commonly be present at tolerable levels in the hive (Bailey, 1968b). Honey bee brood are infected by different pathogens than are adults. Brood are susceptible to bacterial disease (e.g. American and European foulbrood, caused by Paenibacillus larvae and Streptococcus pluton, respectively; Govan et al., 1998, 1999), fungal disease (e.g. chalk brood, caused by Ascophaera apis; Gilliam et al., 1983; Johnson et al., 2005), and viral disease (e.g. sacbrood, caused by the SBV virus; Ghosh et al., 1999). Adults are affected by protozoan disease (e.g. nosema, caused by Nosema apis; Gatehouse and Malone, 1998), hemophilic mite parasitism (e.g. the tracheal mite, Acarapis woodi and the Varroa mite, Varroa destructor (formerly jacobsoni); Sammataro et al., 2000; Evans et al., 2007), and viral disease (e.g. acute and chronic bee-paralysis viruses and Israeli acute paralysis virus; Bakonyi et al., 2002; Ribière et al., 2002; Cox-Foster et al., 2007). Of note, at least one adult parasite, the Varroa mite, also affects brood. Pathogens are likely present at multiple developmental stages, yet the disease phenotype is most clearly observed at only one.

Here, we detail our findings from a study of honey bee cellular and humoral IC over four developmental stages: larvae, pupae, nurses (1-day-old bees), and foragers (22–30 days old adults). To examine if honey bee IC varies with developmental stage, we assayed physiological IC across honey bee developmental stages: total hemocyte concentration, encapsulation response, fat body mass, and phenoloxidase activity. Due to differences in pathogen pressure and behavioral capacity, we predicted that larvae and pupae would have higher physiological IC than nurses and foragers. Because disease resistance is difficult to measure (Luster et al., 1993; Keil et al., 2001; Adamo, 2004a; Rantala and Roff, 2005), we took multiple measures of immune strength to achieve a broad spectrum analysis of honey bee IC.

2. Methods

2.1. Specimen collection

One frame of brood and 40 foraging adult honey bees (A. mellifera) were collected from 10 colonies among three field sites in Massachusetts (N = 1200 bees total collected, although not all were used due to challenges specific to each particular assay; see below for details). All source colonies were briefly inspected for symptoms of bacterial, fungal, or viral disease; only healthy bees were collected. Specimens were collected from four colonies of Italian bees at the International Social Insect Research Facility (ISIRF) at the Tufts University Cummings School of Veterinary Medicine in North Grafton, Massachusetts, four colonies of Carnolian bees at the Bee-Cause Apiary in Dunsboro, Massachusetts, and two colonies of Italian–Carnolian mixed breed at the Bee-Cause Apiary in Tyngsboro, Massachusetts. All specimens were transported to Tufts University in Medford, Massachusetts. At the laboratory, larvae and pupae were excised from the brood frame. Each brood frame was then immediately isolated and incubated for 24 h at 32–33 °C. One-day-old nurse bees were collected after the incubation period (for similar methods see Starks et al., 2005). Because stress may influence some measures of immunocompetence (see Braude et al., 1999; Adamo and Parsons, 2006), animals were exposed to similar conditions within each developmental stage so as to minimize differences in stressful handling within groups.

2.2. Hemolymph collection

Hemolymph was collected immediately from larvae and pupae by puncturing the soft cuticle with fine forceps sterilized in 95% ethanol. Nurses and foragers were ice anesthetized before hemolymph was collected by severing the abdomen and collecting samples from the proximal abdominal opening. Five microliters of hemolymph was collected from the resulting bubble of hemolymph, transferred to a 96-well plate with individual cells containing 95 μl PBS (Sigma, pH 7.4), and frozen at −20 °C to disrupt hemocytes for later analyses of phenoloxidase activity (Gilliam and Shimanuki, 1970; Wilson et al., 2001; Chan et al., 2006). One additional microliter of hemolymph was collected with a micropipette and transferred to a microcentrifuge tube (0.5 ml, BD Falcon) containing 9 μl of sterile deionized water and used for total hemocyte count. Any fluid which appeared yellow or brown was avoided as this was likely not hemolymph but gastric fluid (Chan et al., 2006).

2.3. Total hemocyte count

A total hemocyte count was performed as an indirect measurement of baseline cellular immunocompetence (Wilson et al., 2001, 2002; Lee et al., 2006). Hemocyte counts have been shown to correlate positively with encapsulation response (Rantala et al., 2000; but see Doums et al., 2002), phenoloxidase activity (Cotter et al., 2004) and parasitoid resistance (Eslin and Prévost, 1998; Kraaijeveld et al., 2001a), and also to correlate negatively with aging (Amdam et al., 2004, 2005; Schmid et al., 2008). To perform this test, we added the diluted hemolymph solution to an improved Neubauer hemocytometer (Fisher Scientific), where all hemocytes were counted under a light microscope. Hemocytes counts in sterile, deionized water were reliable and repeatable (personal observation).

2.4. Encapsulation response

A standard encapsulation response assay (König and Schmid-Hempel, 1995; Schmid-Hempel and Schmid-Hempel, 1998; Rantala et al., 2000; Vainio et al., 2004; Rantala and Roff, 2005; Lee et al., 2006; Kapari et al., 2006; Haviola et al., 2007) was used as a direct measurement of an insect's ability to neutralize a foreign body that cannot be ingested by phagocytosis. On the one hand, the ability to encapsulate a novel foreign body correlates positively with resistance to virally infected cells (Washburn et al., 1996; Trudeau et al., 2001), parasitoids (Carton and David, 1983; Kraaijeveld et al., 2001b) and parasites (Doums and Schmid-Hempel, 2000), as well as with male dominance (Rantala and Kortet, 2004) and female mate choice (Rantala et al., 2002; Rantala and Kortet, 2003; but see Rantala et al., 2003). On the other hand, encapsulation correlates negatively with the total number of

hemocytes (Doums et al., 2002), suggesting the encapsulation process requires hemocytes be removed from circulation. Cellular encapsulation may occur when the cuticle is punctured by a foreign body, as first noted in Metchnikoff's Nobel Prize winning (1908) experiments, and may be induced when a parasite invades the host hemocoel (Wilson et al., 2002). We induced an encapsulation response in honey bees by mimicking the behavior of the common Varroa mite (Sammataro et al., 2000) with a nylon 'pseudoparasite' (Cox-Foster and Stehr, 1994). Nylon line (0.004 mm diameter, Scientific Anglers Tippet, 3 M) was cut by hand with a razor blade into approximately 2 mm long segments and sterilized in 95% ethanol. Cuts were implanted individually inside larvae and pupae medially on the ventral side so that approximately 1 mm of the nylon cut remained outside the body wall. Nurses and foragers were first ice anesthetized and then immobilized using a 'threading technique', whereby curved forceps press down on all six legs to expose the ventral side. Each specimen was implanted with a nylon cut through the medial ventral intersegmental membrane between the 3rd and 4th sternites (Allander and Schmid-Hempel, 2000).

After implantation, adult bees (i.e. nurses and foragers) were moved to a 1.5-ml microcentrifuge tube with holes poked through cap to isolate bees from grooming activity so that the implant remains in place, while maintaining access to air. Specimens were left at room temperature for 4 h, after which a glass slide was created of thread in glycerol medium (see Kapari et al., 2006; Calleri et al., 2006, 2007). Explanted threads were photographed at 400× magnification using an Olympus VX40 fluorescence detecting microscope and image capturing software (Optronics Magna Fire-SP, v1.0 ×5). Three pictures were taken of each explant to accurately quantify a three-dimensional process using two-dimensional tools (Rantala et al., 2000; Rantala and Kortet, 2003; Contreras-Garduño et al., 2006; Kapari et al., 2006; Haviola et al., 2007). Each image was captured through a multi-wavelength filter (emittance range approximately 400–600 nm) to detect melanin, an autofluorescent protein produced within encapsulating cells (Calleri et al., 2007). The excitation wavelength of melanin has been previously reported at 488 nm (Kozikowski et al., 1984; Meredith and Sarna, 2006). This allowed for the control of any non-melanized debris which may have accumulated on the monofilament. The mean gray value was calculated for the inserted portion of the thread and all capsules on it using image analysis software (ImageJ 1.34s, National Institutes of Health, USA; Rasband, 2007), and compared to an unimplanted control thread (Allander and Schmid-Hempel, 2000; Rantala et al., 2000; Haviola et al., 2007).

2.5. Phenoloxidase activity

The phenoloxidase (PO) pathway is a central component to invertebrate immune reactions occurring in the hemolymph (Söderhäll and Cerenius, 1998; Lourenço et al., 2005). PO activity assays are a commonly used to quantify immune enzyme activity via melanin production in the absence of cells (Wilson et al., 2001, 2002; Rolff and Siva-Jothy, 2002; Cotter et al., 2004; Lee et al., 2006; Mullen and Goldsworthy, 2006). Although PO plays an important role in cellular IC (among other roles in cuticular sclerotization and quinone production; Lavine and Strand, 2002), PO may work solely as an independent humoral immune protein during melanotic encapsulation (Gillespie et al., 1997). Moreover, PO activity and the ability to encapsulate a novel foreign body are heritable (Cotter and Wilson, 2002), and so natural selection should favor those with the most effective immune response.

PO is produced when its zymogen prophenoloxidase (proPO) is activated in response to any of many triggers, including wounding, mechanical agitation, and various chemicals (Ashida and Brey,

1997). PO acts by oxidizing tyrosine derivatives to form toxic quinones, which are then polymerized into melanin. Measurements of immunity using PO have provided valuable insights into invertebrate immunology (Nigam et al., 1997). There is a well-documented relationship between PO activity and resistance to viruses (Wilson et al., 2001; Beck and Strand, 2007), bacteria (Pye, 1974; Ashida and Brey, 1997), fungi (Ochiai and Ashida, 1988), parasites (Leonard et al., 1985; Paskewitz and Riehle, 1994; Gorman et al., 1996; Siva-Jothy, 2000), and parasitoids (Wilson et al., 2001). It is important to note that recent evidence from mutant, PO-deficient *Drosophila* have raised important questions about the exact role of PO in defense, as these flies were able to survive a microbial infection as well as wild-type flies (see Leclerc et al., 2006).

The protein concentration of each diluted hemolymph sample was determined by a standard protein quantification assay (Bradford, 1976; Wilson et al., 2001; Lee et al., 2006). To control for variation in hydration state of individuals, hemolymph protein concentration was controlled at 0.02 mg/ml and added in varying volumes to the reaction mixture (Parkinson and Weaver, 1999). The enzymatic substrate L-dopa, a tyrosine derivative, was added to each solution to reach a final concentration of 0.03 M. Absorbance measurements were recorded at 492 nm before and immediately after L-dopa was added. Data were recorded using a Bio-Rad Benchmark Plus microplate spectrophotometer and Microplate Manager software (Bio-Rad, version 5.2.1). L-Dopa is a chromogenic substrate and appears colorless when dissolved in water; however, in the presence of PO, melanin is produced and the solution turns brown.

PO analysis for each bee was repeated three times and a mean v_{max} was calculated. PO activity was quantified by recording the change in sample absorbance at 492 nm every 30 s for 9 min. Phenolthiocarbamide was then added to each well to inhibit PO activity and ensure melanin production was a result of PO activity alone (Eshete and LoVerde, 1993; Parkinson and Weaver, 1999; Wilson et al., 2001; Adamo, 2004b; Zettervall et al., 2004; Richards et al., 2005). Absorbance readings then continued for 9 more minutes. Within the spectrophotometer, all temperatures were maintained at a set range between 32 and 34 °C to mimic brood comb conditions (Winston, 1987). PO activity was quantified as the slope of the linear phase of reaction (Rolff and Siva-Jothy, 2002).

2.6. Fat body quantification

Fat body quantification is an indirect measurement of induced humoral immunocompetence (Ellers, 1996; Doums et al., 2002). The fat body is functionally analogous to the vertebrate liver and produces antipathogenic proteins (Faye and Wyatt, 1980; de Verno et al., 1984; Ellers, 1996; Gillespie et al., 1997; Lavine and Strand, 2002; Brown et al., 2003). As such, a relative comparison of fat body size serves as an indirect assessment of induced humoral immune strength and of overall condition. Adult abdomens were severed from thoraces and dried for 3 days at room temperature. Abdomens were weighed and washed in ethyl ether for 24 h to dissolve fat. Larvae and pupae were not included in this assay because of their lack of hardened cuticle. Abdomens were then dried for 3 days and weighed again. The fat body was calculated as the percent change in abdominal weight after the ethyl ether wash (Ellers, 1996; Doums et al., 2002).

2.7. Morphometric analysis

Measurements were taken of head width (McMullan and Brown, 2006), forewing length (Lobo et al., 1989; Diniz-Filho and Malaspina, 1995; Mostajeran et al., 2006), femur length and tibia

length (Mostajeran et al., 2006) for both the nurses and foragers in order to detect any size differences between colonies or developmental stage. Digital images of body parts were captured using a Nikon SMZ 1500 dissection microscope and the image capturing software Spot (Diagnostic Instruments, v. 4.5.9). Morphometric data were collected using ImageJ (Rasband, 2007).

2.8. Statistical methods

IC measures (total hemocyte count, encapsulation response, total hemolymph protein, phenoloxidase activity, and fat body mass) were not normally distributed and non-parametric statistics were used. Each IC measure was compared between developmental stage (larvae, pupae, nurses, foragers) and races (Italian, Carnolian, Italian/Carnolian mix) using Kruskal–Wallis tests followed by pairwise comparisons with Mann–Whitney U tests. η^2 values were also calculated to determine the proportion of variance in each IC measure that may be explained by developmental stage or race. All statistical tests were run with the computer program SPSS for Windows (v. 11).

## 3. Results

3.1. Total hemocyte count

The median total hemocyte count for each honey bee developmental stage was significantly different from the other three life stages (Fig. 1). In general brood displayed more hemocytes than adults. Pupae had significantly greater cell density than all other developmental cohorts (Mann–Whitney U, $p < 0.001$). Larvae had the second highest density of hemocytes (Mann–Whitney U, $p < 0.001$). Foragers had the second lowest density of hemocytes (Mann–Whitney U, foragers versus nurses: $p < 0.05$). Hemocyte counts were not normally distributed (Shapiro–Wilk test: $p < 0.001$). Of note, there was a large amount of intercolonial variation with regards to total hemocyte counts (data not shown). No colony or race effect was found.

3.2. Encapsulation response

Each developmental stage displayed a very similar ability to encapsulate a foreign body. There were no obvious trends in any of
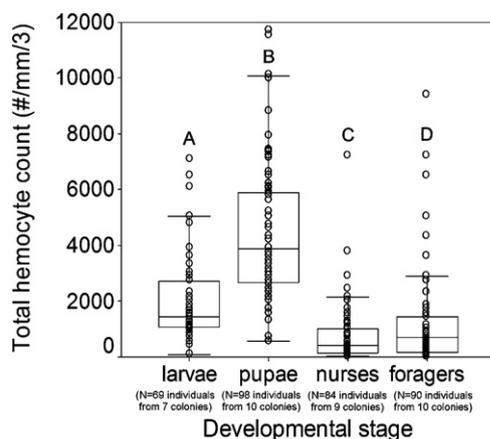
Fig. 1. Hemocyte number varies with honey bee (*A. mellifera*) ontogeny. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. Statistics were calculated using SPSS v.11 Kruskal–Wallace with Mann–Whitney U pairwise comparisons. Significant differences indicated with letters.
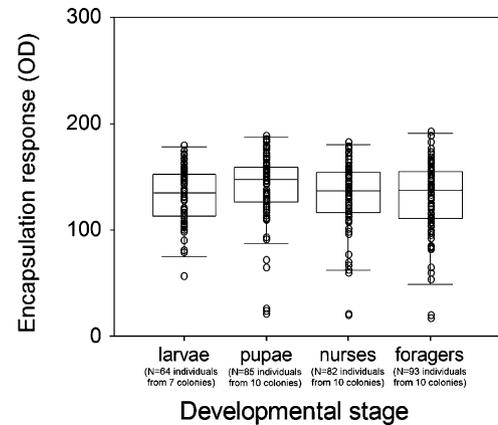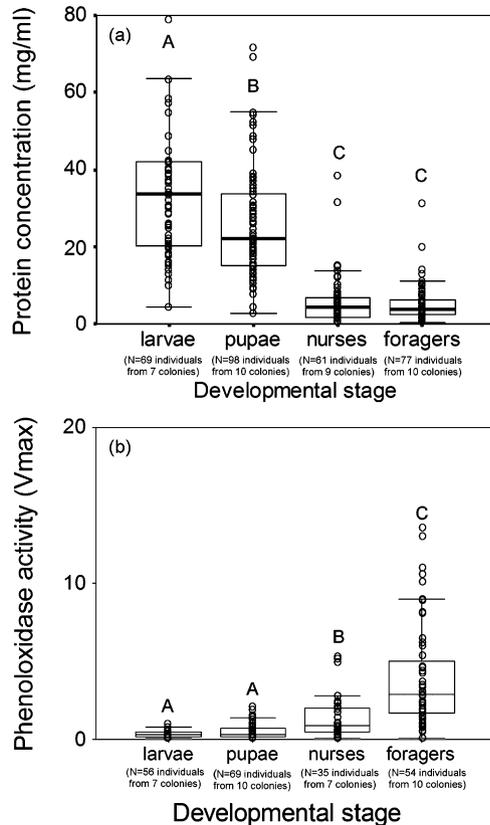

Fig. 2. Encapsulation response does not vary with honey bee (*A. mellifera*) ontogeny. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. There were no significant differences in encapsulation rate between developmental stages. Statistics were calculated using SPSS v.11 Kruskal–Wallace test with Mann–Whitney U pairwise comparisons.

the colonies sampled ($N = 10$ colonies, 324 individuals). When the data were combined across colonies, there was strikingly little difference in encapsulation ability between developmental stages (Fig. 2; Kruskal–Wallis, df = 3, $p > 0.05$). The encapsulation response of individuals within each developmental stage was not normally distributed (Shapiro–Wilk test, $p < 0.05$). Although there was a large amount of intercolonial variation with regards to the ability to encapsulate a foreign body, variation in encapsulation response was not explained by developmental stage ($\eta^2 = 0.013$) or by race ($\eta^2 = 0.003$).

3.3. Phenoloxidase activity

Hemolymph protein concentration was quantified as a preliminary step of the PO assay to control for differences in hydration state between specimens. A general decline in protein concentration was noted from juvenile to adult (Fig. 3a; Kruskal–Wallis, df =3, $p < 0.001$; Mann–Whitney U, $p < 0.001$ between all developmental stages). Nurses and foragers were the only cohort pair that showed no significant difference in hemolymph protein concentration. The concentration of total protein in the hemolymph of individuals within each developmental stage was not normally distributed (Shapiro–Wilk test, $p < 0.001$).

While juvenile bees had greater protein density, foragers has the highest PO activity. PO activity followed the opposite trend from the protein concentration, whereas PO activity increased with ontogeny (Fig. 3b; Kruskal–Wallis, df = 3, $p < 0.001$). A common trend was seen across 90% of colonies sampled ($N = 9$ out of 10 colonies, 214 individuals total). One colony displayed slightly decreased PO activity in foragers compared to nurses, though both cohorts remained higher than juvenile bees within that colony. The median maximum reaction velocity (v_{max}) of PO was greatest in foragers (compared to larvae and pupae, $9.7\times$ greater activity, Mann–Whitney U, $p < 0.001$; compared to nurses, $3.2\times$ greater activity, $p < 0.001$). Nurses had the second highest PO activity (compared to larvae and pupae, $3.0\times$ greater activity, $p < 0.001$). There was no significant difference between larvae and pupae with regards to PO activity. The PO activity of individuals within each developmental stage was not normally distributed (Shapiro–Wilk test, $p < 0.01$). About a third of the variation seen in PO activity could be explained by developmental stage ($\eta^2 = 0.297$), though race made a minimal contribution to variation ($\eta^2 = 0.016$).

Fig. 3. Hemolymph protein concentration (a) and phenoloxidase (PO) activity (b) vary with honey bee (*A. mellifera*) senescence. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. Statistics were calculated using SPSS v.11 Kruskal–Wallace test with Mann–Whitney *U* pairwise comparisons. Significant differences indicated with letters.

3.4. Fat body quantification

The difference between nurses and foragers differed across colonies, with all but one colony showing nurses with more fat mass than foragers (Fig. 4; Mann–Whitney *U*: *p* < 0.001). The fat body mass of individuals within each developmental stage were not normally distributed (Shapiro–Wilk test, *p* < 0.001).

3.5. Morphometric analysis

Morphometric measurements showed no significant differences between colonies within races with regards to any quantified body parts (head width, femur length, tibia length, and forewing length). As such, the data between colonies of the same race were combined. All morphometric data were normally distributed, so parametric statistics (1-way ANOVAs followed by Tukey's HSD post-hoc tests) were used for analysis. Racial differences were noted with regards to size (head width: $F(2, 266) = 3.73$, $p = 0.025$; femur length: $F(2, 266) = 11.81$, $p < 0.001$; tibia length: $F(2, 266) = 20.87$, $p < 0.001$; forewing length: $F(2, 266) = 5.09$, $p = 0.007$). Head width of Italians was larger than Carnolians (mean difference = 0.029 mm, SE = 0.012, Tukey's HSD $p = 0.038$). Femur length of Italians was larger than both Carnolians (mean difference = 0.0745 mm, S.E. = 0.0175, Tukey's HSD $p < 0.001$) and mixed breeds (mean difference = 0.0885 mm, S.E. = 0.0224, Tukey's HSD $p < 0.001$). Tibia length of Italians was larger than both Carnolians (mean difference = 0.0588 mm, S.E. = 0.0153, Tukey's HSD $p < 0.001$) and mixed breeds (mean difference =
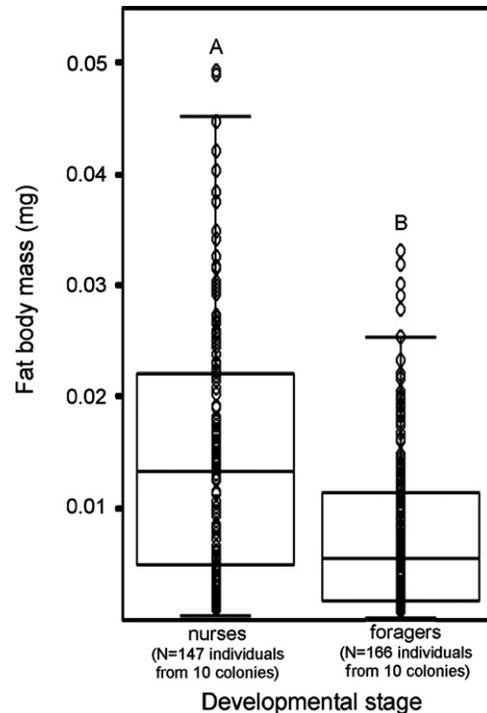
Fig. 4. Fat body mass decreases as adult honey bees (*A. mellifera*) age. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. Statistics were calculated using SPSS v.11 Kruskal–Wallace test with Mann–Whitney *U* pairwise comparisons. Significant differences indicated with letters.

0.1243 mm, S.E. = 0.0224, Tukey's HSD $p < 0.001$). Tibia length also differed between Carnolians and mixed breeds, with Carnolians being larger (mean difference = 0.0655 mm, S.E. = 0.0190, $p = 0.002$). Forewing length of Italians was larger than Carnolians (mean difference = 0.1225 mm, S.E. = 0.0405, $p = 0.008$). However, there was no correlation between race or size and encapsulation response or PO activity (Pearson Correlation: race and encapsulation response, $r = 0.018$, df = 324, $p = 0.74$; race and PO activity, $r = -0.093$, df = 212, $p = 0.175$; head width and encapsulation response, $r = 0.071$, df = 176, $p = 0.348$; head width and PO activity, $r = 0.055$, df = 89, $p = 0.611$). Any difference was controlled for (e.g. concentration of protein used for PO assay) or irrelevant (e.g. size differences did not correlate with IC measures).

4. Discussion

In this paper we report how the immune system of honey bees varies between four distinct life stages: larvae, pupae, nurses, and foragers. We hypothesized that there would be a difference in IC with developmental stage, based on differences in host behavior and pathogen pressure between developmental stages (Winston, 1987; Bailey and Ball, 1991; Schmid-Hempel, 1998). Results from total hemocyte count, fat body quantification, and PO activity (Fig. 3a and b) support this hypothesis. However, these data contrast with our expectation that larvae and pupae would have higher cellular (vis-à-vis total hemocyte count and encapsulation response) and humoral IC (vis-à-vis fat body mass and PO activity) than nurses and foragers. Instead, larvae and pupae had low cell counts and PO activity (although hemolymph protein concentration decreased). Encapsulation response did not change with development. Nurse bees had greater fat body mass than foragers, indicating greater ability of antipathogenic peptide production in

young adult bees compared to older bees (Brown et al., 2003). Our results are in line with previous studies investigating honey bee immunity. For example, Schmid et al. (2008) reported an increase in PO activity and a decrease in hemocyte count as adult bees develop from nurses to foragers.

The decline in hemocytes has been shown to be age-dependent and not task-dependent (Schmid et al., 2008), so the immunodeficiency of foraging nurses remains independent of hormonal control. However, our results contrast with Schmid et al. (2008), as we show the average encapsulation response of honey bees remains stable across developmental stages. The similarity between the ability of each developmental stage to encapsulate a novel foreign object was not significantly influenced by size, race, or nest of origin. Adult bees were immobilized and isolated from other bees during this procedure, and it may be argued that this procedure stressed the bees and therefore indirectly influenced their immune response; however, stress is expected to weaken immunocompetence (Adamo and Parsons, 2006). If stress does influence encapsulation ability, then our results are a conservative estimate of adult bee cellular immunocompetence.

As such, it appears that adult honey bees do not abandon cellular IC as previously suggested (Bedick et al., 2001; Amdam et al., 2004, 2005; Schmid et al., 2008). Although our study and Schmid et al. (2008) study found a significant decrease in total hemocytes with development, neither study limited cell counts to those with immune function. If non-immune hemocytes (prohemocytes) are reduced while hemocytes with immune activity (granulocytes and plasmatocytes) are conserved, then this would support our interpretation that cellular IC remains as a viable mode of immunity. This hypothesis may be tested by counting each type of hemocyte (see Manfredini et al., 2008).

The non-cellular component of the honey bee immune system also changes with development. However, a fairly uniform humoral response might be predicted given evidence from the honey bee genome. Honey bees possess fewer immune sequences than found in other insect genomes, including the fruit fly, *Drosophila melanogaster* and the mosquito, *Anopheles gambii* (Evans et al., 2006). The noted small number of immune alleles impacts every step of the immune response, from pathogen recognition to the production of immune proteins (The Honeybee Genome Sequencing Consortium, 2006). This finding implies a reduced flexibility in the abilities of honey bees to recognize and resist pathogens (The Honeybee Genome Sequencing Consortium, 2006), yet does not explain the high intercolonial variability seen in our encapsulation response data. Likewise, honey bees possess only one proPO (PO precursor) gene compared to three *Drosophila melanogaster* proPO genes and nine *Anopheles gambiae* proPO genes, which may contribute to the consistent direct relationship between ontogeny and PO activity between colonies (Evans et al., 2006). The proPO gene is expressed more strongly in adults and older pupae than in younger pupae and larvae (Lourenço et al., 2005). This is also in line with our findings of PO activity, which were taken from diluted hemolymph samples and so actual PO activity may be higher than noted in our results. Moreover, Chan et al. (2006) found that the actual proPO zymogen was 50-fold more prevalent in the hemolymph of adult honey bee workers compared to larvae, which also agrees with our results. Additional proteomic study of honey bee hemolymph showed the antibacterial peptide hymenoptaecin is expressed in lower baseline amounts in larvae versus adults (Chan et al., 2006), a trend reflected in the PO data reported here.

Although the encapsulation response is maintained across developmental stages, there was a noticeable amount of intercolonial variation. Larvae and pupae had high encapsulation in some colonies, but very low in others. Explanations for this variation could not be found in morphometric or race analyses. This assay was the clearly the most variable of all IC tests performed. We feel encapsulation response would not be adequate by itself to sufficiently document the ontogeny of honey bee immunity. This finding is potentially alarming considering the high number of papers that draw conclusions from this measurement of IC alone (including but not limited to König and Schmid-Hempel, 1995; Schmid-Hempel and Schmid-Hempel, 1998; Allander and Schmid-Hempel, 2000; Zuk et al., 2004; Civantos et al., 2005; Kapari et al., 2006; Haviola et al., 2007; Sorvari et al., 2007).

Our results have potential application as standard IC parameters to evaluate colony health. While we collected data from only three field sites, additional data from multiple populations of honey bee colonies will enable a larger geographic mosaic overview (Thompson, 1994). Our results show that older adult bees have the greatest PO activity per unit of hemolymph protein. As such, if foraging bees are lost, then the disease resistance capacity of the colony is reduced. Because nurse bees are not as immunologically competent as foraging bees (Fig. 3), they are not as well equipped to combat the increased pathogen exposure that older foragers encounter. In the absence of foragers, younger nurse bees prematurely transition to precocious foragers (Huang and Robinson, 1996). This behavioral shift is likely to negatively impact colony fitness, regardless of the pathogen pressure. Examples of when foragers may be lost include the recent onset of Colony Collapse Disorder (Oldroyd, 2007) or through present U.S. apicultural practices involving the transport of honey bee colonies for seasonal pollination, although this has not yet been empirically shown. In this light, foraging bees may play a similar role as vaccinated individuals in a population by providing a type of herd immunity, and in their absence the disease resistance capacity of the group is likely compromised.

In all, these results elucidate the patterns of cellular and molecular organization in the eusocial superorganism, *A. mellifera* (Page and Erber, 2002; Amdam and Seehuus, 2006). Further investigation should explore the relationship between genetic diversity, behavior, IC, and intercolonial variation. High genetic diversity has been shown to decrease variation in disease resistance across honey bee colonies (Tarpy, 2003). Because disease susceptibility should increase with the number of mates (Schmid-Hempel, 1998), there is a conflict between queen mating behavior (i.e. polyandry) and colony-level infection. However, the benefit of increased genetic diversity at disease resistance loci may provide an even greater benefit to the colony (Schmid-Hempel, 1998; Tarpy and Seeley, 2006; Seeley and Tarpy, 2007; Reber et al., 2008), and warrants additional study.

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