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Age-dependent changes in cuticular color and pteridine levels in a clonal ant

Clara Hartmann*, Jürgen Heinze, Abel Bernadou

Zoology / Evolutionary Biology, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany

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ABSTRACT

Social insects are emerging models for studying aging and the longevity/fecundity trade-off. Research on the demography of colonies and populations are hampered by the lack of reliable age markers. Here we investigate the suitability of cuticular pigmentation and pteridine fluorescence for age grading individuals of the clonal ant *Platythyrea punctata.* We found that both traits varied with age. Cuticular color darkened with individual's age until 25–30 days after hatching. For pteridine fluorescence, we found that *P. punctata* workers show a decrease in head pteridine levels over time until 70–80 days of age. Together with other markers, such as age-based behavior, cuticular coloration and pteridine fluorescence may help to estimate the age structure of colonies.

1. Introduction

Aging is the most universal and unavoidable phenomenon in biology. More than 300 theories have been proposed to explain why organisms age and why they do so at different rates (Medvedev, 1990; Viña et al., 2007), but we still do not fully understand the causes of inter-individual variation in longevity and the tempo of senescence. Expanding research to non-model organisms with a large range of life histories and life spans might therefore help to determine the causes of commonalities and differences in aging patterns across taxa (Cohen, 2018; Fletcher and Selman, 2015; Jemielity et al., 2005; Jones et al., 2014). Unfortunately, demographic studies are often constrained by the absence of reliable information on the age structure of populations. Research on aging and senescence therefore typically relies on experimental cohorts of individuals of known age or on physiological, morphological, or behavioral correlates assumed to vary with age.

In insects, the most common age grading methods involve changes in characteristics related to physiological processes, of a somatic, reproductive, or mechanical nature (Hayes and Wall, 1999; Southwood, 1978; Tyndale-Biscoe, 1984). However, most of these changes are not completely reliable. For example, progressive cuticular degradation and the degree of wing fray, often collectively referred to as "wear and tear," have been found to be too imprecise for accurate age grading (Michener et al., 1955; Southwood, 1978; Tyndale-Biscoe, 1984). Cuticular growth layers allow determining the chronological age of individuals and have been used, for instance, to gage the age structure of both laboratory and field populations of *Drosophila* (Johnston and Ellison, 1982). This method, however, is only applicable for a short time after eclosion, species-specific, and can be influenced by temperature and nutrition (Neville 1963, 1970; Schlein and Gratz, 1972; Southwood, 1978).

Cuticular darkening and pteridine accumulation are also common methods of age grading in insect studies (e.g. Armitage and Boomsma, 2010; Rinkevich et al., 2016; Robson and Crozier, 2009; Thompson et al., 2002). Cuticular pigmentation darkens after the hatching of adult insects over several days or weeks. Pteridines are degradation products of purine metabolism present in most insects and appear to accumulate over time (Robson et al., 2006; Silver, 2008; Wu and Lehane, 1999; Ziegler and Harmsen, 1970). After Mail et al. (1983) established a protocol to quantify the accumulation of a specific pteridine, 6-biopterin, with age in the stable fly *Stomoxys calcitrans* (L.) using spectrofluorometric measurements, numerous studies have investigated the relationship of pteridine concentration not only with age, but also sex, temperature, nutrition and light intensity in various species (e.g., Robson et al., 2006).

Social insects are currently emerging as promising models for the study of aging and more specifically the longevity/fecundity trade-off (Heinze and Schrempf, 2008; Keller and Genoud, 1997; Korb, 2016). Cuticular coloration has been shown to be a useful chronological age marker of workers in the early days after eclosion (e.g. Armitage and Boomsma, 2010; Csata et al., 2017). However, the suitability of pteridine fluorometry as an age marker is more elusive. Pteridines have previously been studied only in two taxa, the honey bee and the weaver ant *Polyrhachis sexspinosa*. Similar to what has been found in many dipterans (e.g., Bernhardt et al., 2017; Robson et al., 2006), pteridine levels increased with age in honeybees (Rinkevich et al., 2016). However, in the ant *Polyrhachis sexspinosa* they seemed to be influenced only by head weight (Robson and Crozier, 2009).

* Corresponding author.
E-mail address: Clara.Hartmann@stud.uni-regensburg.de (C. Hartmann).

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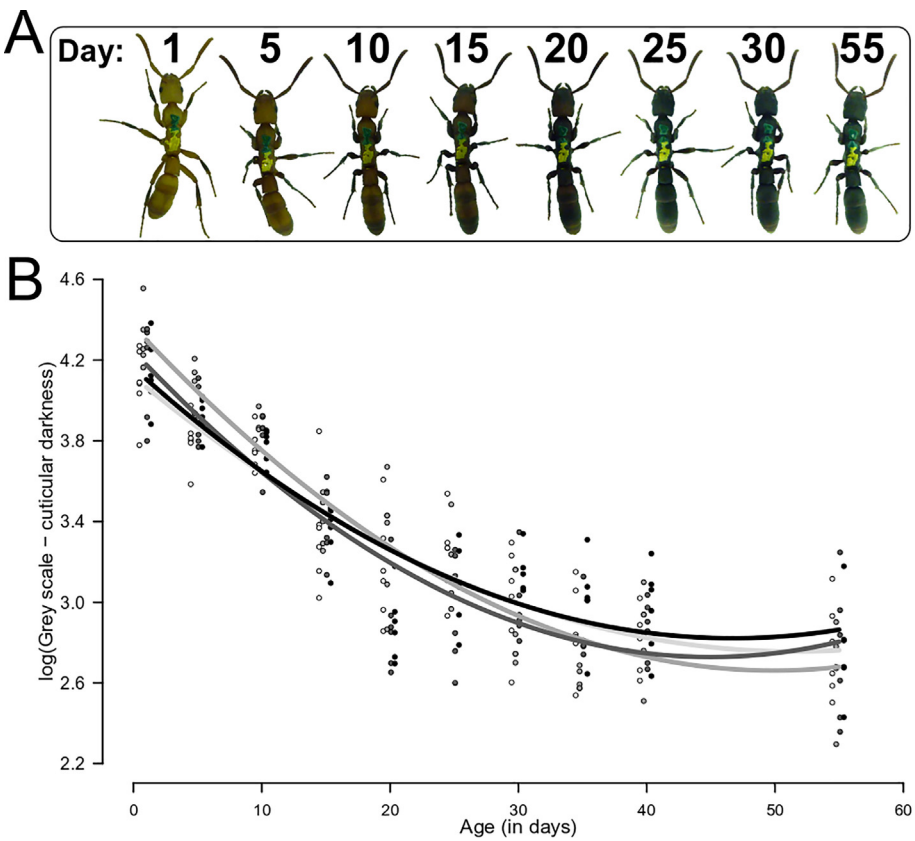

Fig. 1. (A) Examples of changes in cuticular coloration over time (in days) in a *P. punctata* worker since hatching until day 55. To allow recognition of the individual worker it was marked with a dot of green-yellow enamel paint (B) Quantification of changes in cuticular coloration over time (in days) in *P. punctata* workers since hatching until day 55 (transformed data: log(X)). Six individuals from four different colonies (the colors white, light grey, dark grey and black represent the colonies PR03, PR26, PR34 and PR42 respectively) were used. Original pictures were converted to weighted RGB grey-scale (highest values, light coloration; lowest values, darkest coloration).

In this study, we determined the applicability of cuticular pigmentation and the quantification of 6-biopterin in individual ant heads following the method developed by Robson and Crozier (2009) and Rinkevich et al. (2016) for age grading in the clonal ant *Platythyrea punctata*. This ant is particularly suitable for studies on the longevity/fecundity trade-off and aging in social insects: because of thelytokous parthenogenesis, in which diploid females develop from unfertilized eggs, reproductives and non-reproductives are fully identical in genotype, ontogeny, and morphology (Heinze and Hölldobler, 1995). Nevertheless, as in standard social insects (Keller and Genoud, 1997), reproductives outlive their non-reproductive clonemates, leading to a positive rather than the typical negative association between fecundity and lifespan (Hartmann and Heinze, 2003). We expected that cuticular pigmentation can be useful over a short period of time after hatching to determine chronological age while pteridines would accumulate with age over several weeks as in honeybees and therefore would be a valuable age marker over longer time spans.

2. Material and methods

2.1. Study species and colony maintenance

Colonies of *Platythyrea punctata* were collected in 2012 in Puerto Rico (PR03: Parcelas Vieques; PR26, PR34, PR42: Yuquiyu). Since that time, colonies were maintained in the laboratory in climatic chambers (22–26 °C and a 12 h light: 12 h dark cycle) in plastic boxes (L × W × H: 20 × 20 × 9 cm) with a plaster floor. Cavities in the plaster, covered by glass plates and black plastic film, served as nesting site. Humidity in the nest was controlled by moistening the plaster regularly. Colonies were fed with diluted honey, small cockroaches or fruit flies three times per week and a plastic tube plugged with cotton wool provided water *ad libitum* (Bernadou et al., 2018).

2.2. Age-dependent changes in cuticular color

To test whether cuticular coloration changed consistently with age in *P. punctata*, we individually marked six newly hatched workers from each colony on the thorax using Edding® paint markers. Workers were photographed on days 1, 5, 10, 15, 20, 25, 30, 35, 40, and 55 using a Panasonic Lumix DMC-TZ7 digital camera. Ants were collected from their mother colonies, placed on a white background and surrounded by a plastic ring (diameter: 2.5 cm) coated with Fluon® to prevent the ants from escaping. Once immobile, the plastic ring was gently moved to position the ants in its center. Individuals were photographed dorsally three times. Images were always taken in the same set-up and inside the same climatic chamber to ensure similar ambient light conditions across days. Pictures were processed using the GNU Image Manipulation Program (GIMP 2.8) (colors: levels and auto) and subsequently converted to weighted RGB grey-scale with ImageJ (Schneider et al., 2012). The greyscale varied between 0 and 255 (0 darkest and 255 lightest cuticle). A similar method was previously used to quantify age-dependent changes in cuticular color in other insects (e.g. in beetles: Thompson et al., 2002; ants: Armitage and Boomsma, 2010). Cuticular darkness was measured by drawing a 100 × 100 pixel square on the center of the head, the first and the second gastral segments (ants were marked at the thorax level). The grey-scale value for each body part was calculated as a mean of the three pictures. All body part measurements correlated positively and significantly with each other (Spearman's rank correlation: head vs. 1st segment: $r_S = 0.83$, $n = 240$, $P < 0.001$; head vs. 2nd segment: $r_S = 0.80$, $n = 240$, $P < 0.001$; and 1st segment vs. 2nd segment: $r_S = 0.86$, $n = 240$, $P < 0.001$). The head values were chosen for the following analysis because they had the largest grey range (head: 9.97–95.52, 1st segment: 4.93–66.72 and 2nd segment: 5.10–78.97). A subset of pictures from the head were analyzed by two of the authors (C.H. and A.B.) independently and yielded similar results (Spearman's rank correlation: $r_S = 0.98$, $n = 27$, $P < 0.001$).

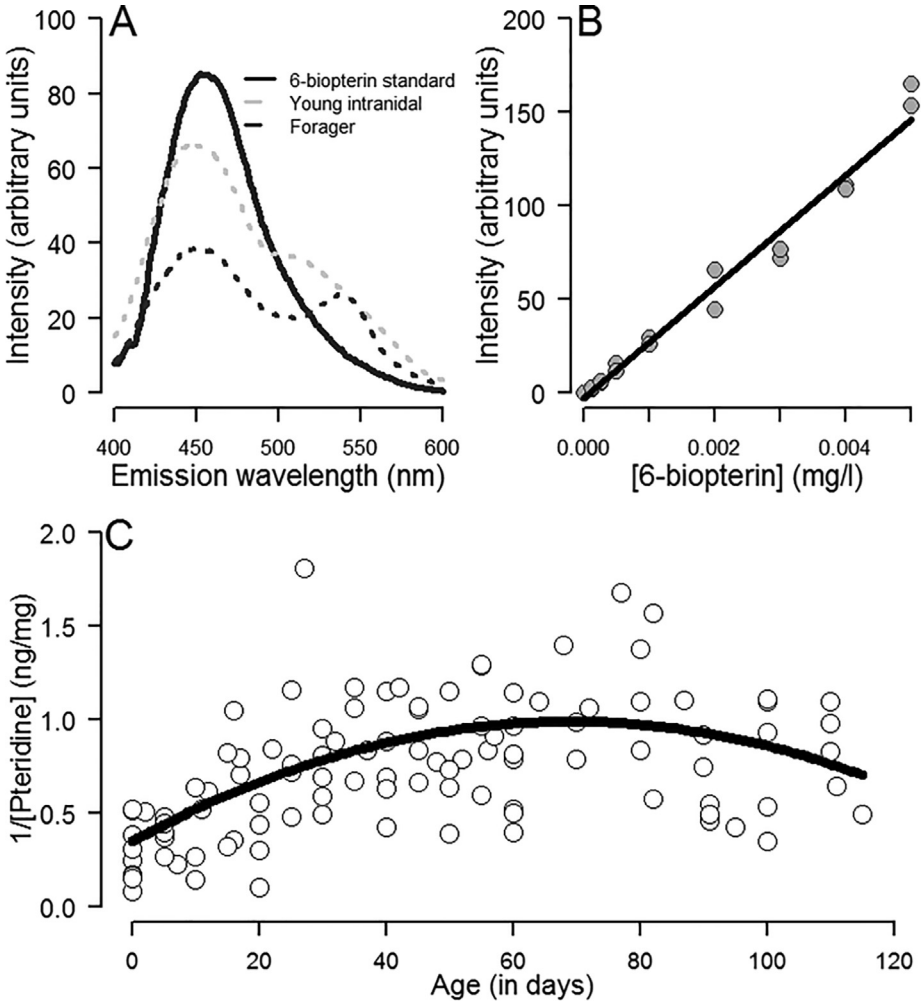

Fig. 2. (A) Emission spectra for the 6-biopterin standard solution (concentration: 0.002 mg/L) and for the extracts of one young intranidal and one forager at an excitation wavelength of 371 nm. (B) Calibration curves from 2016 and 2018 describing the relationship between 6-biopterin concentration and emission intensity at 371 nm. The lowest concentration measured was 0.000125 mg/L, which corresponded to an intensity of 2.3 arbitrary units. The lowest pteridine concentration extracted from a single ant head was 0.0008 mg/L, which corresponded to an intensity of 21.25 arbitrary units. (C) The relationship between age and pteridine concentration (transformed data: 1/X) in individual *Platythyrea punctata* workers. Pteridine concentration decreases significantly with both the linear (LME: $X_1^2 = 43.79$, $P < 0.001$) and the quadratic (LME: $X_1^2 = 26.93$, $P < 0.001$) term of worker's age.

2.3. Pteridines as an age marker

2.3.1. Pteridine detection and quantification

We first used 6-biopterin (Sigma-Aldrich: Munich, Germany), one of the major pteridines found in head extracts of insects (flies: Mail and Lehane, 1988; Robson et al., 2006; ants: Robson and Crozier, 2009, bees: Rinkevich et al., 2016), to determine whether our protocol was able to detect and reliably quantify the levels of pteridines found in *P. punctata*. Following the procedures used in previous studies (Mail and Lehane, 1988; Rinkevich et al., 2016; Robson et al., 2006; Robson and Crozier, 2009), 6-biopterin was diluted in 0.1 N NaOH (adjusted to pH 10 with 11.5 mg/L glycine) to create a stock solution (concentration: 10 mg/L). We then treated and extracted 0.75 mL of this stock solution as we did with the tissue samples (see below).

The maximum excitation and emission wavelengths of the extracted 6-biopterin stock solution were determined by successively scanning the solution at increasing activating wavelengths (10 nm increments from 300 to 500 nm and 1 nm increments from 350 to 380) using a Jasco FP-6500 spectrofluorometer.

To ensure that pteridine levels within individual ant heads were accurately measured by the spectrofluorometer (Robson et al., 2006), we prepared serial dilutions from the 6-biopterin stock solution from 0.005 to 0.000125 mg/L as well as blank samples (see below). In addition, we used the spectral intensity recorded for each dilution to construct calibration curves. Two serial dilutions replicates were prepared from the same 6-biopterin stock solution for each calibration curve.

2.3.2. Establishment of colonies of known-aged individuals

To determine how pteridine levels vary with age and whether they can be used as reliable age markers, we set up colonies of workers of known age. Stock colonies of *P. punctata* were checked daily for newly hatched workers. All newly emerging workers (callows) were marked with a color code on the day of eclosion and returned to their mother colony. Workers were then collected regularly from the day they hatched until 115 days of age, which is approximately 60% of the mean lifespan of non-reproductive workers of *P. punctata* in the laboratory (Hartmann and Heinze, 2003). After collection, workers were placed individually in labelled Eppendorf cups, killed and stored by freezing at −20 °C until pteridine extraction. A total of 104 workers from four different stock colonies were used (PR03: 25; PR26: 37; PR34: 24 and PR42: 18 workers). We quantified pteridines contained in 30 and 74 heads of workers in 2016 and 2018 respectively. To control for variation in machine efficiency and to enable comparability of the sample sets, separate calibration curves with 6-biopterin stock solution (see above) were established in the two measurement periods.

2.3.3. Pteridine extraction

Prior to extraction ant heads were separated from the thorax with a scissor, weighed to the nearest 0.0001 mg with a Sartorius SC2 ultramicrobalance and placed in clean 1.5 mL Eppendorf cups. Pteridines were extracted following methods described for flies (Robson et al., 2006), ants (Robson and Crozier, 2009), and bees (Rinkevich et al., 2016). Frozen ant heads were ground in liquid nitrogen with a plastic pestle for 60 s. A solution of 0.5 mL chloroform:methanol (2:1 v/v) was then added to each sample and the tubes were placed on ice for 60 s.

After 3 min of sonication, all tubes received a volume of 0.75 mL of 0.1 N NaOH (adjusted to pH 10 with 11.5 g/L glycine). The samples were then vortexed for 10 s and centrifuged for 5 min at 5000*g* at 4 °C. A volume of 0.7 mL of the resulting supernatant of each sample was transferred into new Eppendorf cups and analyzed with a spectrofluorometer for pteridine quantification. All samples were excited at 371 nm and the emission spectra between 400 and 600 nm were recorded (Rinkevich et al., 2016; Robson and Crozier, 2009). The greatest intensity of the spectra at an emission wavelength of 455 nm was registered in arbitrary units (see results). We calculated the quantity of pteridines in each sample based on the standard curves of 6-biopterin and standardized by head weight (Rinkevich et al., 2016).

Pteridines were extracted in batches of 10–20 ant heads. Worker ages and colonies were balanced within every batch of extraction. With each batch we prepared two control solutions (blank, extract solutions treated as a tissue sample) and measured them before and after each series of sample measurements to control for daily fluctuations of the spectrofluorometer. The blank emission values were subtracted from the emission spectra of the pteridine measurements.

2.4. Statistical analyses

The changes in cuticular coloration with age (in days) were analyzed by a linear mixed model (LME, lmer function, lme4 package: Bates et al., 2015). The factors "days^2" (quadratic term), "days", "colony" and their interaction were introduced in the model as explanatory variables. We included a quadratic term to model for non-linear changes in the cuticular color with age. As we repeatedly measured the same workers over time, we included the term "worker identity" as a random factor.

The linearity of the calibration curves (for 2016 and 2018) were tested by LMEs. We ran the models with the spectral intensity as a response variable and concentration as explanatory variable. The batches of dilution were entered as random factor.

We used a LME to test the relationship between the quantity of pteridine found in worker's head and worker's age (linear and quadratic term), colony and their interactions as independent variables. The factor "year (2016/2018)" was entered as random factor.

All analyses were performed and graphs generated with the statistical software R 3.3.2 (R Development Core Team, 2016). We obtained minimal models by successively removing the least non-significant terms ($P > 0.05$) and by comparing the nested models by likelihood ratio tests. The P values for the final models were obtained by the function Anova from the "car" package (Fox and Weisberg, 2011). Model residuals were checked for the assumptions of normality (Shapiro–Wilk test of normality) and homoscedasticity (Levene's test for homogeneity of variance). Data were transformed when necessary.

3. Results

3.1. Changes in cuticular color

The color of the cuticle changed with age and remained almost constant after 25–30 days after hatching. Workers had a yellowish coloration in the early days after hatching, passed through dark brown and eventually became dark brown to black (Fig. 1A and B; changes in cuticular coloration are plotted as transformed data). The differences in the changes of cuticular coloration between days for the colonies are shown in Fig. S1 (Supplementary material): though coloration changed with age, it was not a precise marker for determining a worker's exact age. The minimal model for the cuticular color included the linear (LME: $X_1^2 = 348.85$, $P < 0.001$) and the quadratic (LME: $X_1^2 = 160.79$, $P < 0.001$) effect of worker's age (Fig. 1B). There was a significant effect of colony origin on cuticular color: callows hatched with a lighter coloration in some colonies than other (LME: $X_3^2 = 8.89$, $P = 0.03$). There was a significant interaction between age and colony of origin, which means that individuals darkened slower or faster depending on their colony origin (LME: $X_3^2 = 10.59$, $P = 0.01$). The interaction between days (quadratic) and colony (LME: $X_3^2 = 2.98$, $P = 0.39$) was not significant and removed from the initial model.

3.2. Pteridine as an age marker

The spectral intensity of the extracted 6-biopterin stock solution (concentration: 10 mg/L) was highest at an activating wavelength of 371 nm and corresponded to an emission wavelength of 445 nm (Fig. 2A). The emission spectrum of the 6-biopterin standard at a concentration of 0.002 mg/L was similar in terms of spectral range, shape and maximum emission wavelength to pteridine spectra of extracts from a young worker and a forager (Fig. 2A).

Spectral intensity at a wavelength of 371 nm was significantly and linearly correlated with 6-biopterin concentration (LME: $X_1^2 = 1053.2$, $P < 0.001$ and $X_1^2 = 724.54$, $P < 0.001$ for 2016 and 2018 respectively; Fig. 2B). The lowest emission intensity recorded from a single ant head (0.0008 mg/L corresponded to an intensity of 21.25 arbitrary units) was at least ten times higher than the minimum level tested (0.000125 mg/L corresponding to 2.3 a.u.).

The minimal model for the pteridine concentration in individual ant heads included the linear (LME: $X_1^2 = 43.79$, $P < 0.001$) and the quadratic (LME: $X_1^2 = 26.93$, $P < 0.001$) effect of worker's age (Fig. 2C). Pteridine levels decreased until 70–80 days and then seemed to increase until 115 days (Fig. 2C; pteridine concentration are plotted as transformed data). The interactions between age (quadratic or linear term) and colony (LME: $X_3^2 = 4.55$, $P = 0.21$ and $X_3^2 = 7.23$, $P = 0.06$) or the factor colony alone (LME: $X_3^2 = 3.75$, $P = 0.29$) were not significant and successively removed from the initial model.

4. Discussion

Accurate methods to obtain reliable information on the age structure of insect populations are of importance to better understand ecology and aging patterns across taxa (Hayes and Wall, 1999). In this study, we used two methods, i.e. cuticular color and pteridine concentration, for age grading workers of the ant *Platythyrea punctata*. Both traits varied with age but none of them were accurate markers for determining a worker's exact age. Cuticular darkening gave a rough information on age for young workers until 25–30 days after hatching, and pteridine levels offer only an indication for age grading. Both methods need therefore to be used in combination with other age markers.

We found that cuticular color of callows gradually turned from yellow to light-brown, to dark-brown, and finally to black within 25–30 days after hatching. After three to four weeks, callows reached their final cuticular pigmentation and became indistinguishable from older workers. Our results are within the ranges reported in other ant studies, e.g., 16 days after eclosion in *Pheidole dentata* (Wilson, 1976), around 21 days for *Myrmica scabrinodis* (Csata et al., 2017), 10 weeks for *Acromyrmex octospinosus* (Armitage and Boomsma, 2010) or up to six months for *Myrmica rubra* (Cammaerts-Tricot, 1974). Moreover, it should be noted that cuticular darkening proceeded in a colony-specific manner. In some colonies, callows hatched with a lighter coloration than in others and darkened more slowly or faster depending on their colony origin. Environmental effects can be excluded as colonies were maintained under controlled conditions. Genetic differences or colony size likely influence the rate of darkening but this needs to be verified in future studies.

In a previous study, we found that among 23 observed callows, 13 individuals were seen outside their nest for the first time around three to six weeks after hatching (Bernadou et al., 2015), which is at the end of the cuticular pigmentation phase (this study). Cuticular changes seem to be linked to age-related tasks for non-reproductive individuals, as suggested by Armitage and Boomsma (2010). Workers need a

sclerotized cuticle when they start performing tasks outside the nest in order to be protected from environmental factors (e.g. pathogens, Armitage and Boomsma, 2010). Although we did not directly assess cuticular resistance, cuticular darkening was associated with cuticle hardening, i.e., yellowish callows had a softer cuticle than brownish or black workers.

Pteridine levels in workers of the ant *Platythyrea punctata* decreased from hatching to a chronological age of 70–80 days. Thereafter, they appeared to stabilize and even to increase from 80 to 115 days. Though pteridine levels change with age this method cannot be used alone as it was not a reliable and precise marker for determining an individual's age. Hence, the age-trajectories of pteridine levels in the hitherto studied social insects are equivocal. In the ants *Polyrhachis sexspinosa* (Robson and Crozier, 2009) and possibly also in *Cardiocondyla obscurior* (Königseder et al., unpublished results), pteridine levels were independent of age while they significantly increased with age in the honeybee (Rinkevich et al., 2016). We are convinced that the decrease of pteridine levels with age in *P. punctata* is not caused by inappropriate detection techniques. The emission spectra of extracted pteridines were similar to the 6-biopterin standard. Moreover, the lowest emission intensity recorded in our samples was well above the minimum level detected in the serial dilutions. Furthermore, *P. punctata* is not the only insect in which pteridine levels decrease with age: a similar decrease has been reported for mosquitoes (for *Anopheles gambiae* and *A. stephensi*: Wu and Lehane, 1999; *A. albimanus*: Penilla et al., 2002). In general, pteridine levels appear to be also influenced by many factors in addition to age, such as, e.g. temperature (Lehane and Mail, 1985; Robson et al., 2006), light intensity (Robson et al., 2006), or nutrition (Penilla et al., 2002). While all our ants were kept under an identical temperature and light regime and the clonality of individuals minimizes the influence of genotypic variation, we cannot exclude that their diet has changed with age, in particular, as young individuals stay in the nest and take care of the young while older individuals leave and forage for food (age polyethism; Bernadou et al., 2015; Hartmann and Heinze, 2003; Hartmann et al., unpublished data). Whether the difference between our data and those from other ants and the honeybee (Rinkevich et al., 2016) are taxon-specific idiosyncrasies, depend on rearing conditions or on other factors remains to be tested.

In conclusion, cuticular pigmentation and pteridine fluorescence varied with age but, rather than precise age determination, both methods offered only a rough estimate of an individual's age. They were not reliable markers and therefore have to be used in combination with the age polyethism mentioned above to provide a more reliable estimate of chronological age.

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Appendix A. Supplementary data

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