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Senescence of immune defence in *Bombus* workers

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Abstract. 1. Senescence in workers of social insects is a particularly intriguing life-history trait as the future fitness of workers relies primarily on age-dependent survival rate. The pattern of senescence of immune defence traits was investigated under laboratory conditions in workers of two bumble bees: *Bombus terrestris* and *B. lucorum*.

2. In both species, there was a significant decrease with age in the ability to encapsulate a foreign object (a global measure of the efficiency of immune systems). This pattern of senescence was observed in all colonies in *B. terrestris* (seven) and *B. lucorum* (eight) assayed, even though, for the latter, there was some heterogeneity among colonies.

3. In *B. terrestris*, two other measures of immune defence were taken: the relative percentage of fat body in the abdomen and the concentration of haemocytes (the immune defence cells). The quantity of fat body increased only slightly with age and there was no effect for the concentration of haemocytes. Interestingly, the concentration of haemocytes decreased strongly after an encapsulation response, regardless of the age of workers.

4. The importance of the senescence pattern observed for the immune defence traits is discussed in the context of the social biology of workers.

Key words. Ageing, *Bombus terrestris*, *Bombus lucorum*, bumble bees, encapsulation, haemocytes, immune defences, senescence, social insects.

Introduction

Senescence is the continuous deterioration of fitness components with age. According to theory, the pattern of senescence should evolve in response to externally imposed schedules of survival and reproduction (Rose, 1991; Stearns, 1992). Therefore, senescence in workers of social insects, such as bees or ants, is a particularly intriguing life-history trait as these individuals typically do not reproduce but gain fitness by helping their kin to have offspring (Hamilton, 1964; Wilson, 1971). At the same time, worker mortality rates change strikingly with age. Future fitness is therefore determined primarily by age-dependent survival rates whereas a worker's own fertility is negligible. Survival rates are given by the characteristic age-related schedule of activities that a worker follows throughout its life.

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Typically, young workers care for brood or the construction and maintenance of the nest itself. These activities take place inside a sheltered nest (as in most ants and bees) or on well-defended brood combs (as in some bees and many wasps). Mortality rate is therefore comparably low. Later in life, the workers show a marked shift to tasks such as guarding the nest entrance or foraging. These activities are associated with considerably higher mortality rates. Workers of honey bees, for example, show a characteristic profile of low mortality over the first 20 days of life but a large increase thereafter (Seeley, 1985). A corresponding increase in the rate of senescence would be expected, if the hypothesised relationship exists. Senescence in honey bee workers has been inferred from systematic changes in food choice (Barker & Lehner, 1974), flight physiology (Harrison, 1986), or physiological and morphological changes in the brain (Rockstein, 1950) with increasing age. In contrast, workers of bumble bees have a much less pronounced age-related activity schedule. Most workers will start to work both inside and outside the nest a few days after hatching and continue to perform such activities throughout their life, even though a very small percentage of workers, usually

the smaller ones, can stay within the nest all their life (e.g. Alford, 1975). As a consequence, the average age-dependent survival rates are roughly constant over a large part of a worker's lifespan (e.g. Goldblatt & Fell, 1987; Müller & Schmid-Hempel, 1992).

Although social insects offer a wide range of different life histories, it is often difficult to find traits that are easy to measure and are expected to show senescence. Here, the age-related performance of the immune system of workers of bumble bees, *Bombus terrestris* L. and *Bombus lucorum* L., was investigated. Immune defence is an important trait that ensures the future of the organism. It should therefore be a major fitness component, especially in social insects where survival is the main parameter affecting the workers' inclusive fitness. Moreover, such defences are costly in terms of negative covariance with other fitness components (e.g. Sutter *et al*., 1968; König & Schmid-Hempel, 1995; Kraaijeveld & Godfray, 1997). Hence, traits linked to the immune system should be under selection and, as a consequence, show senescence.

The immune defence system of invertebrates is far less complex than the vertebrate immune system even though many components are homologous (Beck & Habicht, 1996). In particular, most insects do not show an immunological memory and therefore rely primarily on innate immunity (e.g. Faye & Hultmark, 1993; Gillespie *et al*., 1997; but see Rosengaus *et al*., 1999). Humoral factors are based on, for example, anti-microbial peptides that can kill the parasites directly (Gillespie *et al*., 1997). Cellular mechanisms involve phagocytosis and encapsulation of an immunogen by haemocytes. With the encapsulation process, haemocytes are recruited and form multiple layers around the object. Eventually, the capsule is melanised (Götz, 1986). The immune response in insects has been shown to vary with temperature, the presence of parasites, age, sex, condition, and genotype (e.g. Carton *et al*., 1992).

The responses of the insect immune system are nevertheless manifold. In the work reported here, only three of many possible expressions of the immune system in workers of *B. terrestris* were analysed: encapsulation/melanisation of a large object, the total number of circulating haemocytes, and the size of the fat body. Even though this is only a limited view of the immune system, such measures were considered to be a reasonable first attempt to investigate whether and how its responses vary with age. The fat body of workers is considered to be an important storage organ, the size of which often decreases with age. Moreover the fat body is also important for the insect immune system as the site of synthesis of immunoproteins (e.g. Zachary & Hoffmann, 1984; Boman, 1986; Hultmark, 1993; Hoffmann & Reichhart, 1997; Levashina *et al*., 1999). Although the fat body is clearly a major site of production of antimicrobial peptides, there are as yet no data showing how the size of the fat body correlates with immune response. The working hypothesis adopted here, that a large fat body not only provides energy but also contributes directly to the immune system, is therefore likely. Similarly, the number of haemocytes circulating in the body cavity reflects the capacity of the immune system to cope with an immunogenic challenge at that particular time. Although the different types of haemocyte differ in their precise role and function within the defence system (e.g. Drif & Brehélin, 1993), current understanding assumes that most of the recruitable prophenoloxidase enzyme that catalyses crucial steps in the melanisation response appears to reside in the haemocytes circulating in the blood stream (Söderhall, 1998). The total haemocyte concentration in the haemolymph thus serves as an approximation to this capacity and is expected to decrease with age, if senescence occurs. A novel immunogen is quickly encapsulated by haemocytes and melanised. This capacity can be estimated by measuring the degree of melanisation of an experimental implant. A first experiment on *B. lucorum* analysed the variation of melanisation with age of workers, while all three aspects were measured in a second experiment with workers of *B. terrestris* to check whether the same pattern may hold across other *Bombus* species and can be observed on the different measurements of the immune system.

Materials and methods

Bumble bees

Bumble bees are primitive eusocial insects with an annual life cycle. Only the fertilised queens overwinter to start their own colony the next spring. *Bombus terrestris* and *B. lucorum* queens are generally singly mated so workers within colonies are closely related full sisters (Schmid-Hempel & Schmid-Hempel, 2000). Colonies grow in numbers over the season. Reproduction takes place when males and gynes (daughter queens) are produced.

Collection and culturing

In spring 1995, queens of *Bombus lucorum* L. were caught in two regions of Switzerland: the lowlands, i.e. areas around Zurich and Basle (elevation ≈ 400 m; leading to $n = 3$ used colonies) and the western Swiss Alps, i.e. areas in the Valais (elevation ≈ 1600 m; $n = 5$ colonies). The queens were allowed to start a colony in the laboratory. The established colonies were then kept at a standard temperature of $28 \pm 2\,^{\circ}$C (60% RH, LD 12:12 h cycle) for their entire life cycle, with food (pollen and sugar water) provided *ad libitum*.

Bombus terrestris occurs only in the lowlands, and queens were caught in spring 1997 in areas around Zurich. Some of these queens started a colony that was kept in the laboratory for one generation for a different purpose. The daughter queens were mated with unrelated males (not from the same family, as known from their sampling locations and pedigree) in the laboratory and only queens originating from different mother queens were selected in order to avoid replicate colonies from the same family. These queens were allowed to start a colony that was then reared in a

climate chamber under the same conditions as *B. lucorum*. Seven colonies were finally used in the experiment.

Experimental design

In the first experiment on *B. lucorum*, melanisation only was measured. For each of the eight colonies used in the experiment, all the workers were marked individually at hatching. For the experiment, workers were selected at random until all age classes had sufficient sample sizes, and were implanted with a nylon monofilament (see below) immediately after removal from the colonies.

In the second experiment on *B. terrestris*, three aspects of the immune system were measured. The experimental design was a little more complex in order to have better control of the age investigated, ranging from 1 to 27 days. This age range was shorter than that investigated in *B. lucorum* but mimics the age range that can be observed in the field, 20 days being the average lifespan of *Bombus* workers in the field (Rodd *et al.*, 1980; Schmid-Hempel & Heeb, 1991; Schmid-Hempel, 1998). The presence of callow was checked twice a day until at least 26 callow had been found in each of the seven colonies. Each callow was marked individually and immediately put back in its native colony. For each colony, workers born on the same day were paired for reasons explained below. This requirement and natural worker mortality reduced the number of workers that could be used in the experiment to an average of 33 workers per colony (range 27–43). The day (age) on which the three immune system parameters had to be measured for a given pair was assigned randomly. Note that the number of callows was not sufficient to yield a pair of callows for each age in each colony so even if, overall, all ages were represented, some were missing in each colony. When the pairs of workers reached the assigned age, they were removed from the colony for the tests.

One worker of each pair was implanted with a nylon monofilament (see below). The other worker was submitted to the same treatment (sham-operated) but no implant was inserted in the abdomen. A comparison of these two treatments allowed investigation of the effect of building an immune response on the number of circulating haemocytes and on the fat body.

Measuring the immune response

Encapsulation/melanisation. This measure was taken in both species. The encapsulation response was tested against a novel, standardised immunogen, consisting of a nylon monofilament, similar in size to an egg of conopid flies endoparasitic to bumble bees (≈ 0.18 mm diameter $\times$ 0.8 mm length). The immunogen was implanted into CO_2-anaesthetised bumble bees (see Schmid-Hempel & Schmid-Hempel, 1998). The workers' immune system was allowed to react to this object for 10–12 h in both species. During this time, the animals were kept in wooden boxes at 28 °C to control for temperature effects during the actual encapsulation process (Blumberg & DeBach, 1981; Blumberg, 1988). The bumble bees were freeze-killed and the implant was then removed and embedded into Eukitt® (O. Kindler GmbH & Co., Freiburg, Germany). The encapsulation reaction darkens the implants. The corresponding reduction in light transmission was measured on a light table using a video camera mounted on a stereo-microscope and connected to a Macintosh computer using the public domain NIH image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The resulting transmission values were standardised with the background of the image of each measurement to control for variation in light transmission between different sets of measurements.

Total haemocyte concentration. This measure was only taken in the experiments with *B. terrestris* just before removal of the implant. Two to 4 µl of haemolymph were extracted from the abdomen of the CO_2-anaesthetised worker using a microcapillary and diluted to 50% with Toisson's fluid (1 g NaCl, 8 g Na_2SO_4, 30 ml glycerine, 15 ml crystal violet, 160 ml distilled water). The number of haemocytes was counted directly in a Neubauer chamber under the microscope.

Fat body and body size. These measures were taken only in the experiments with *B. terrestris*. The size of the fat body in the abdomen was estimated using the ether extraction method described by David *et al.* (1975) and Ellers (1996). Percentage values are given as dry mass of fat body/dry mass of the abdomen. For each worker, body size was measured as the length of the radial cell in the right forewing. This measure was chosen as it is easy to make and correlates highly (usually with $r^2 > 0.9$) with other body size measures, such as head width and tibia length. In addition, previous measurements had never revealed any useful pattern of fluctuating asymmetry in these measures (P. Schmid-Hempel, unpublished) so only one side was measured.

Statistical analyses

In *B. lucorum*, the melanisation response in workers was analysed using a general linear model with population of origin (lowlands, Alps) and colony (the individual colony) as factors nested within the population (Zar, 1984), age as a covariate, and the interactions between age and population or colony.

In *B. terrestris*, a possible confounding correlation between each immune defence measurement and body size of workers was first tested. A significant correlation was observed between total haemocyte concentration and body size ($r = 0.29$, $n = 211$, $P < 0.001$). No significant correlations were detected between body size and size of the fat body ($r = -0.14$, $n = 211$, NS) or degree of melanisation ($r = -0.09$, $n = 104$, NS). To remove the effect of body size on total haemocyte concentration, the residuals of the regression of haemocytes on body size were entered in the further analyses. To test for the effect of age, colony, and

treatment (with or without implant) on each trait studied (melanisation, haemocyte concentration, fat body), a general linear model was performed with age, fat body, and haemocyte concentration as covariates. For the melanisation response, only implanted workers contributed to the data set. The full model included colony and treatment as main effects and the interaction between colony and treatment as well as all the covariates (except the dependent variable) and their interactions with the main factors. Colony was considered as a fixed factor given that the colonies studied were not a random sample but were chosen to be unrelated to one another. Haemocyte concentration and fat body were $\log_{10}$-transformed to fulfil the assumptions of the general linear model.

In both species, the analyses were performed using Proc GLM of SAS 6.12 for Windows (SAS Institute, 1990) with type III sums of squares. The regression line between the measurements of immune system and the age of workers was calculated using a weighted regression (mean value per age weighted by the number of individuals of this age) as advised when there is more than one value of Y per X (Sokal & Rohlf, 1995).

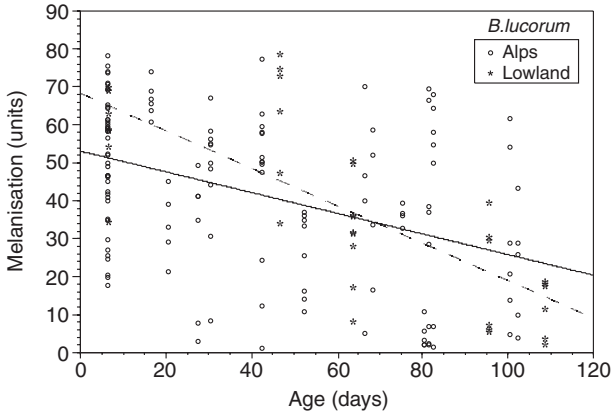

Fig. 1. Degree of melanisation, Y, as a function of age for *Bombus lucorum* workers from colonies of Alpine origin (five colonies, 134 workers) (solid line: weighted regression $Y = 50.45 - 0.254 \times$ age, $r = 0.375$, $F_{1,132} = 21.629$, $P < 0.001$) and of lowland origin (three colonies, 34 workers) (dashed line: weighted regression $Y = 67.79 - 0.489 \times$ age, $r = 0.764$, $F_{1,32} = 44.798$, $P < 0.001$) (comparing slopes: t-test = 0.6, d.f. = 20, NS). Each dot is a measurement on one individual.

Results

Melanisation in Bombus lucorum

A total of eight colonies could be used for this experiment, with five colonies from the Alpine population and three from the lowlands. A total of 168 animals was included in the analysis. There was significant variation among colonies in the degree of melanisation, while population of origin did not have an effect on the degree of melanisation of the workers (Table 1). Age had a strong effect on the melanisation response in workers, with old workers showing lower response than younger workers (Fig. 1). Over the tested range, the decline seemed to be linear and no different for Alpine and lowland populations (Table 1, Fig. 1), whereas the interaction between colony and age was significant (Table 1). This interaction is more likely to reflect an experimental artefact than a different senescence pattern among colonies as the age range investigated within each colony differed between colonies. The correlation between melanisation and age was negative in all colonies (two-tailed sign test, $P = 0.016$).

Table 1. Full general linear model for degree of melanisation in *Bombus lucorum* workers.

Source[a]	SS	d.f.	F	P
Age (covariate)	10799.32	1	36.26	< 0.001
Population	216.46	1	0.12	0.75
Colony within population	5230.19	3	5.85	< 0.001
Age × population	5.03	1	0.02	0.90
Age × colony	6650.68	3	7.44	< 0.001
Error	46163.84	155		

[a]Population as fixed effect, colony as random effect nested within population.

Immune responses in Bombus terrestris

Seven colonies could be used for this experiment, and 211 individuals for which all three parameters were measured successfully were included in the analysis. In this experiment, two workers of the same colony and age were paired, with one worker receiving an implant and the other being sham-operated in order to evaluate the pattern of senescence on haemocyte number with and without an immune challenge. For the melanisation response, only data for the implanted workers of the pairs are available. For the other two measures, however, the design also allowed testing of the effect of the implantation. The results of the general linear model for each immune defence trait with age as covariate are summarised in Table 2.

Similar to the results with *B. lucorum*, there was a highly significant effect of age for the degree of melanisation ($P < 0.001$) and for the fat body ($P < 0.05$) while total haemocyte concentration did not vary significantly with the age of workers (Table 2, Fig. 2). There was no significant difference among colonies in the rate of decrease in melanisation with age (general linear model, age–colony interaction term: $F_{6,90} = 1.01$, $P =$ NS). The correlation between melanisation and age was negative in all colonies (two-tailed sign-test, $P = 0.008$).

The treatment (being implanted or sham-operated) had no effect on the fat body but a significant effect on total haemocyte count (Table 2). The total haemocyte count was lower for implanted animals (overall mean: $x = 2363.46 \pm 153.32$ SE cells per µl, $n = 107$ workers) than for their non-implanted counterparts ($x = 3179.07 \pm 192.48$

Table 2. Reduced general linear model for the different traits related to immune defences. The full model included all the covariates, i.e. haemocyte concentration and fat body for the degree of melanisation, fat body for the haemocyte concentration, and haemocyte concentration for fat body, and the age of workers for each dependent variable and their interactions with the main factors. Except for the age of workers, which was of main interest in the study, the non-significant covariates and their interactions were removed from the full model[a].

	SS	d.f.	*F*	*P*
Degree of melanisation				
Age (covariate)	12881.93	1	25.37	< 0.001
Colony	1877.75	6	0.62	0.71
Error	48750.54	96		
Haemocyte concentration[b]				
Age (covariate)	0.03606	1	0.46	0.49
Colony	1.18149	6	2.54	0.02
Treatment	0.83769	1	10.72	0.001
Colony × treatment	0.17585	6	0.38	0.89
Error	15.3114	196		
Fat body				
Age (covariate)	0.08271	1	4.90	0.03
Colony	0.13071	6	1.29	0.26
Treatment	0.00404	1	0.24	0.62
Colony × treatment	0.02537	6	0.25	0.96
Error	3.31139	196		

[a]The covariates were non-significant for the degree of melanisation (fat body, $F_{1,76} = 1.15$, $P > 0.05$; haemocyte concentration, $F_{1,76} = 0.56$, $P > 0.05$), haemocyte concentration (fat body, $F_{1,188} = 0.27$, $P > 0.05$), and fat body (haemocyte concentration, $F_{1,188} = 0.01$, $P > 0.05$). The interaction terms between the factors and the covariates were never significant at $P = 0.05$ (data not shown).
[b]Analysis of residuals of the regression between haemocyte concentration and body size to remove the effect of body size.

cells per µl, $n = 104$ workers). The activation of the immune system following implantation therefore decreased the circulating number of haemocytes. Note that these conclusions refer to the window of 10–12 h post-implantation, suggesting that over this time period the demand for melanisation exceeds the recruitment of new cells.

Another interesting aspect that could be investigated from the data is the occurrence of a trade-off between the three traits measured. Such a trade-off could not be tested directly at the individual level given that the concentration of haemocytes and fat body was estimated after implantation only. This trade-off could only be tested at the colony level, i.e. by correlating the colony average degree of melanisation in implanted bees with the average haemocyte concentration in their non-implanted counterparts. When this was done, there was a significant negative correlation (Spearman rank correlation, $r = -0.89$, $n = 7$ colonies, $P < 0.01$), indicating that colonies characterised by a higher degree of melanisation had a lower concentration of haemocytes. The correlations between the degree of melanisation and the percentage fat body in the non-implanted counterparts and between the percentage fat body and haemocyte concentration in the non-implanted counterparts were not significant at the colony level ($P > 0.05$).

Discussion

A decline in immune response with age is arguably a crucial component of senescence. The results showed that the ability to encapsulate and melanise an immunogenic challenge declines with age (Figs 1 and 2). In *B. terrestris*, this decline is observed before the average age of death of workers from field colonies (≈ 20 days, see Introduction) and is therefore relevant for wild populations. The relative size of the fat body, which acts both as a storage organ for energy and as a site of biosynthesis for defence components, decreases only marginally, especially during the first 10 days of life (Table 2, Fig. 2). Similarly, the concentration of circulating haemocytes remains virtually unchanged with age (Fig. 2). The decline of melanisation with age is therefore not due directly to a decrease in the number of circulating haemocytes. The fact that 10–12 h after implantation, fewer haemocytes circulate in the haemolymph (cf. Table 2), regardless of age, suggests that these are somehow *used up* in mounting the response against the implant; however this *usage* is perhaps not as efficient in old workers as in young workers. Studies with blood cell-deficient *Drosophila* lines have shown that haemocytes are not essential for antimicrobial peptide production in the fat body whereas they seem to be more important although not indispensable for the melanisation response (Braun *et al.*, 1998). On the other hand, Allander and Schmid-Hempel (2000) could not find hard evidence for a real decline in the strength of the melanisation response when two challenges (implants) followed each other at intervals of 1–48 h. Adding the finding here that melanisation response and haemocyte count correlate negatively across colonies, suggests that there is no easy relationship between haemocyte numbers and the strength of the melanisation response such as suggested by Kraaijeveld *et al.* (2001) and Wilson (2001). These results therefore also provide some, although limited, insight into the how the immune response is regulated.

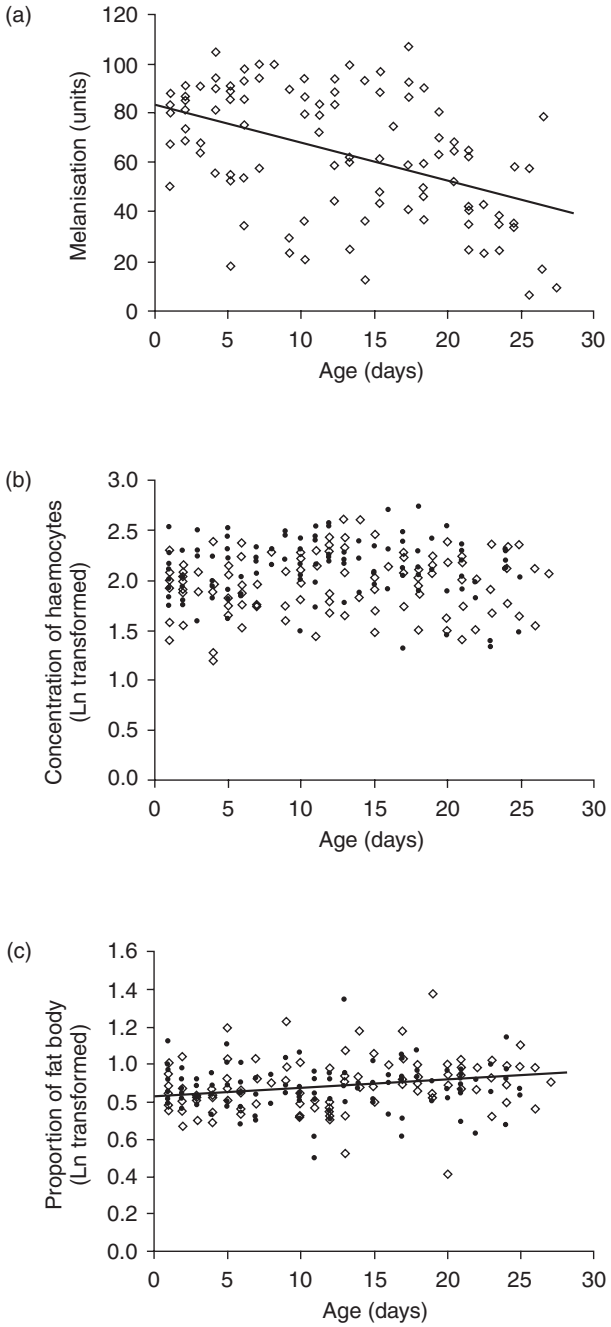

Fig. 2. Relationship between measures of immune defence and age in workers of *Bombus terrestris* that have either been implanted (◇) or not implanted (•). (a) Degree of melanisation for implanted workers (weighted regression $Y = 83.32 - 1.55 \times \text{age}$, $F_{1,23} = 29.65$, $P < 0.001$). (b) Haemocyte concentration per µl (weighted regression for implanted: $F_{1,23} = 1.93$, $P > 0.05$; for not implanted: $F_{1,23} = 0.005$, $P > 0.05$; comparing slopes: $F_{1,46} = 0.68$, $P > 0.05$). (c) Relative size of fat body as proportion of dry abdomen mass (weighted regression for implanted: $Y = 0.83 + 0.005 \times \text{age}$, $F_{1,23} = 5.9$, $P < 0.05$; for not implanted: $F_{1,23} = 0.6$, $P > 0.05$; comparing slopes: $F_{1,46} = 2.07$, $P > 0.05$). Each dot is a measurement on one individual. Only significant regression lines are represented.

The decline in immune responses with age could, in principle, also be due to a trade-off between immune defence and other important traits such as foraging. A direct cost of foraging on encapsulation/melanisation has indeed been demonstrated in *Bombus terrestris* (König & Schmid-Hempel, 1995; Doums & Schmid-Hempel, 2000), however the experiments presented here were run under laboratory conditions, which prevent the workers from flying during foraging and therefore limit the cost of foraging. Moreover, laboratory conditions are typically more benign than field conditions. It therefore seems that the results reflect a genuine process of senescence.

Given the biology of social insects, the decrease in the ability to encapsulate and melanise an invading parasite with age is interesting for at least two reasons. Firstly, older workers are more exposed to and more likely to already have encountered a parasite such as a conopid fly (Müller, 1993; Müller & Schmid-Hempel, 1993). Their decreasing ability to react to such a challenge reduces their age-related value to the colony still further. Moreover, even though there is a turnover of workers during the colony lifespan, older colonies are more likely to be composed of older workers than are young colonies. The decline of immune response in older workers could also permit a higher susceptibility of older colonies to parasites (Schmid-Hempel, 1998). Secondly, senescence in social insects is intriguing, because its consequences must affect both workers and their mother queen in different ways. Typically, queens live longer than workers (e.g. Wilson, 1971; Keller, 1997). As the inclusive fitness of workers depends on senescence in the queen, too, individual worker senescence could also reflect selection on the queen, depending on their relative longevities (Keller, 1997). So far, no attempt has been made to relate rates of senescence to differences in life history or social structure.

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