The effect of age on non-reproductive division of labour in the tropical primitively eusocial wasp, *Ropalidia cyathiformis*

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ABSTRACT Division of labour among workers (non-reproductive division of labour), a characteristic feature of eusocial insects enables the efficient functioning of their colonies. In many advanced insect societies division of labour is based on age (age polyethism). Primitively eusocial insects however are believed to have a weak age polyethism. Here we investigated the role of age in non-reproductive division of labour in the tropical primitively eusocial wasp, *Ropalidia cyathiformis* and compared it with that in *Ropalidia marginata*, a congeneric species that exhibits relatively strong age polyethism. Age had a significant effect on the first performance of the four tasks studied; tasks were initiated in the sequence feed larva, build, bring food and bring building material. We measured task performance as the absolute frequency of tasks performed (FTP) and the probability of performing a task relative to other tasks (PTP) and age as absolute age in days since eclosion as well as relative age compared to nestmates. FTP varied significantly with both absolute and relative age, although absolute age explained more variance. PTP varied significantly with absolute age but not always with relative age. This is contrary to *R. marginata*, where more variation is explained by relative age than by absolute age. There was no trade-off between intranidal and extranidal tasks in *R. cyathiformis* unlike in *R. marginata* where the frequency of intranidal tasks decreased and that of extranidal tasks increased with age. We conclude that age polyethism is weak and less flexible in *R. cyathiformis* compared to that in *R. marginata*.

KEY WORDS: age polyethism, social evolution, worker polyethism, highly eusocial, *Ropalidia cyathiformis*

Introduction

Division of labour resulting in different tasks being performed by different specialized groups of individuals, is the hallmark of all eusocial insects. In eusocial species, a minority of individuals reproduce while the majority are non-reproductive workers (Wilson, 1971; Jeanne 1991). This is referred to as reproductive division of labour. In addition there may also be division of non-reproductive tasks among workers. Among eusocial insects, highly eusocial insects are those which have morphological differentiation between reproductive and non-reproductive individuals, whereas primitively eusocial insects do not have this morphological differentiation. In many highly eusocial insect societies different non-reproductive tasks are performed by subsets of workers differing in their morphology and indeed, they are morphologically adapted to the tasks they perform (Hölldobler and Wilson, 1990; Noirot, 1989). For example, in the *Atta* ants workers with the largest body mass specialize in colony defense, while the medium sized ones perform the tasks of cutting and carrying leaves and the smallest ones process the leaves and tend to the fungus garden (Helanterä and Ratnieks, 2008). Morphology-based division of labour are strongly developed in termites (Noirot, 1989) and some species of ants (Hölldobler and Wilson, 1990).

In many highly eusocial species different tasks are performed by individuals of different ages (Hölldobler and Wilson, 1990; Johnson, 2010; Lindauer and Watkin, 1953; Michener, 1974; Seeley, 1982; Seeley and Kolmes, 1991; Sommeijer, 1984). Such age-based division of labour is termed age polyethism (Wilson, 1971) and is more strongly developed in honeybees (Seeley, 1995), stingless bees (Sommeijer, 1984), swarm-founding wasps (Jeanne et al., 1988; O’Donnell and Jeanne, 1992) and some species of ants. The honey bee *Apis mellifera*, for example, exhibits an extraordinarily well-developed age polyethism. There are about 3-4 age castes within the worker caste known as cell-cleaning caste, brood...
nester caste, food storage caste and forager caste (Seeley, 1982). Similarly, in the case of the swarm founding wasp, Polybia occidentalis, as well there is clear age based division of labour present with off-nest tasks being performed at older ages. P. occidentalis also shows quite a lot individual variation among the wasps and hence the transition from the on-nest tasks to the off nest tasks are more abrupt at the individual wasp level as compared to the group level, where the progression is more gradual and smooth (Jeanne et al., 1998; O’Donnell and Jeanne, 1992). In addition, the age polyethism in Apis mellifera as well as in Polybia occidentalis is flexible to cope with changes in its worker composition. Or in other words, when individuals of an age class are removed from the colony, other individuals take over the activities of these individuals thereby allowing the colony to adjust to changes in worker composition that might arise due to predation or natural disasters. For example, in the absence of older workers in the colony, young workers become precocious foragers while in the absence of young workers old workers become over-aged nurses in the case of A. mellifera (Huang and Robinson, 1996; Page et al., 1995; Robinson et al., 1992).

Division of labour based on morphological castes is absent in primitively eusocial species (in most ant species and in all flying social Hymenoptera), where there is no strong morphological differentiation between the different individuals. In these species age polyethism is weak or non-existent (Cameron, 1989; Cecílio et al., 2016; Jeanne, 1991; Torres et al., 2012; Tsuchida, 1991), though it must be noted that these species have not been so well studied in this regard. The tropical primitively eusocial wasp Ropalidia marginata appears to be an exception to this because it has relatively well-developed age polyethism reminiscent of highly eusocial species (Naug and Gadagkar 1998a). It also exhibits a clear trade-off between intranidal and extranidal tasks, with young individuals performing the intranidal tasks such as feed larva and build nest while older individuals perform the extranidal task of foraging. In addition, age polyethism is based on relative age and hence is flexible, with young individuals becoming precocious foragers on the removal of older individuals (Naug and Gadagkar, 1998b). It is not clear whether this is because R. marginata has been better investigated from this point of view or whether it is a feature of the genus Ropalidia. In order to distinguish between these possibilities, here we have studied the congeneric Ropalidia cyathiformis using methods as closely resembling those used for R. marginata as possible. R. cyathiformis is very well suited for such a comparison. While R. marginata also has many other features reminiscent of highly eusocial species such as docile queens (Bhadra et al., 2010; Sumana and Gadagkar, 2003) who produce pheromones (Bhadra et al., 2010; Mitra et al., 2011), R. cyathiformis appears to be more typical of primitively eusocial species with queen succession involving overt dominance behaviour and display (Unnikrishnan and Gadagkar, 2017). In addition, both species nest throughout the year and have no differences in their nesting cycles. The average number of females and males in a colony in R. cyathiformis is 11.9±3.9 (range: 1-22) and 0.8±3.3 (range: 0-21) respectively (Kardille and Gadagkar 2005), and that for R. marginata is 21.9±22.3 (range: 1-200) and 2.4±5.4 (range: 0-33) (Gadagkar 2001).

### Results

We investigated the effect of age on non-reproductive division of labour in the primitively eusocial wasp, Ropalidia cyathiformis by focusing on two intranidal tasks, feed larva (FL) and build (BU) as well as two extranidal tasks, bring food (BF) and bring building material (BB) of a total of 179 uniquely marked wasps of known age in four naturally occurring colonies. There was a clear effect of age on the first performance of each behaviour (Fig.1) – wasps

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**Fig. 1. Kruskal Wallis for both Absolute and Relative ages, absolute age – χ² = 35.4, df = 3 P < 0.001; relative age – χ² = 15.5, df = 3, P = 0.001.** Mann Whitney U test showed significant difference between mean ages for successive tasks in absolute age (FL vs BU - U = 576, P = 0.001; BU vs BF – U = 332, P < 0.03; BF vs BB – U = 219, P = 0.03). Relative age showed no difference between mean ages for successive tasks (Mann Whitney U test, FL vs BU – U = 435, P = 0.14; BU vs BF – U = 298, P = 0.19; BF vs BB – U = 202, P = 0.12), but showed significant difference between, FL and BF and FL and BB and Build and BB (Mann Whitney U test, FL vs BF – U = 520, P = 0.003; BU vs BB – U = 228, P = 0.016). N = 100 (FL), 91 (Build), 44 (BF) and 26 (BB).

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**Fig. 2. Proportions of individuals performing tasks at specific sequence.** The numbers above each box is the observed proportion of wasps who did the task in that position, independent of what they did at any other position. Each of these proportions was significantly different from the expected proportion (1/4). (G = 143.4, 107.8, 73.8 and 54.5 respectively, P < 0.0001). The numbers below each box at the head of the arrow represent the observed proportions of wasps that followed the particular sequence corresponding to the arrow. Since there are four tasks to choose from, the expected probability of a sequence with two tasks is 1/12 and that of a sequence with three or four tasks is 1/24. The observed proportions were significantly different from expected. (G = 35, 17, 28.64 and 46.63 respectively, P < 0.0001).
performing FL for the first time in their lives were $5.2 \pm 2.9$ days old, those performing BU for the first time were $8.1 \pm 3.9$ days old, those performing BF for the first time were $10.6 \pm 5$ days old and those performing BB for the first time were $15.1 \pm 6.9$ days old. The successive ages of first performance of the four tasks were significantly different from each other, when the absolute ages of the wasps were considered (Fig. 1). When the relative ages of the wasps were considered instead, the trend was similar although the ages of first performance of successive tasks were not significantly different (Fig. 1). Nevertheless, there was a significant difference between the relative age of the first performance of FL as compared to BF and of BU as compared to BB (Fig. 1).

Because of considerable variation in the ages of the wasps at the time of the first performance of the different tasks, we more explicitly determined the sequence of task performance for each wasp. Of the 44 wasps that performed both intranidal (FL + BU) and extranidal (BF + BB) tasks, all wasps performed intranidal tasks before they performed any extranidal tasks. Among the wasps that performed at least one task (100), FL was the most commonly performed first task (82) after eclosion (Fig. 2). Among those that performed two or more tasks (91), the most common sequence of task initiation was FL followed by BU (40/47) (Fig. 2). Among those that performed three or more tasks (44), the most common sequence of task initiation was, FL, BU followed by BF (13/18) (Fig. 2). Among the wasps that performed all four tasks (26), the most common sequence of task initiation was FL, BU, BF followed by BB (21/26) (Fig. 2). These patterns are all statistically significant and could not have been obtained by chance alone (Fig. 2).

To understand how the age of the wasps affected their rates of performing different tasks, we regressed the frequency of task performance against both their absolute as well as relative ages (Figs. 3 and 4 respectively). We found that the basic qualitative pattern was the same for all four tasks whether we used absolute or relative age, with the frequency of each task increasing, reaching a maximum in the middle ages and then declining. Thus, it appears that individual wasps perform all the tasks at almost all ages although at different frequencies. This pattern is even more striking when we compare the combined intranidal tasks (FL+BU) and combined extranidal tasks (BF+BB) (Figs. 3 and 4). Hence, the wasps do not appear to compensate for increased FTP of extranidal tasks by reducing FTP of intranidal tasks. Because of the considerable variation in the activity levels of different wasps, we also regressed the probability of task performance (PTP, defined as the probability that a particular task will be performed by an age class as opposed to the other tasks) against both absolute as well as relative ages. Now some of the patterns change, especially for intranidal tasks (Fig. 5) or become noisy especially in relation to relative age (Fig. 6). PTP of intranidal tasks as a function of age is almost an inverse of the pattern in FTP – the probability of performance of intranidal tasks in relation to extranidal tasks is high in young and old wasps while it dips in middle age wasps (Fig. 6). As in the case of age of first performance, absolute age

![Fig. 3. Frequency per hour of task performance (FTP) as a function of absolute age fitted with second order polynomial regression lines. Data points represent the mean value (in frequency/hour) for all individuals in that age class in 4 colonies. Intranidal tasks are the sum of the two intranidal tasks Feed Larva (FL) and Build and extranidal constitute the two extranidal tasks Bring Food (BF) and Bring Building material (BB).](image1)

![Fig. 4. Frequency per hour of task performance (FTP) as a function of relative age fitted with second order polynomial regression lines. Data points represent the mean value (in frequency/hour) for all individuals in that age class in 4 colonies. Intranidal tasks are the sum of the two intranidal tasks Feed Larva (FL) and Build and extranidal constitute the two extranidal tasks Bring Food (BF) and Bring Building material (BB).](image2)
fared better than the relative age in describing the effect of age on behaviour. In the regression models, the $r^2$ values obtained for each task for absolute age were significantly higher than that of the corresponding values for relative age for both FTP and PTP (Wilcoxon matched pairs signed ranks test, $Z = -2.42, P < 0.05$).

**Discussion**

There is an effect of age on the division of labour in *R. cyathiformis* as can be seen from the age of first performance of tasks. The two intranidal tasks are initiated before the two extranidal tasks and there is a specific sequence of task initiation. The trend of performing intranidal tasks and then progressing to extranidal task as they age is a common theme in other primitively eusocial species (Cameron, 1989; Cecílio et al., 2016; Dew and Michener, 1981; Jeanne, 1991; Murakami et al., 2013; Torres et al., 2012, 2013; Tsuchida, 1991; Yoshikawa, 1963). It is intuitive to understand why the intranidal tasks might be initiated before the extranidal tasks, as it is riskier and costlier to perform tasks outside the nest where they might be exposed to predators. In *Polistes jokahamae* (Tsuchida, 1991), a primitively eusocial wasp, “young workers” of less than 10 days old were predominantly involved in intranidal activities, while “middle-aged workers” (between 11-25 days old) were involved mostly in the extranidal activities of the colony. Similarly, in *Polistes fuscatus* (Post et al., 1988) the oldest worker was the primary forager and in the case of *Polistes dominula* (Jeanne 1991), workers in the “juvenile phase” (1-5 days old) remained in the nest and workers belonging to the second stage or “construction phase” (6-20 days old) were involved in construction activities and foraging. In *Mischocyttarus consimilis* (Torres et al., 2012) two groups of workers were identified, with young workers preferentially performing intranidal activities and older workers performing extranidal activities in the colony. Hence, the general trend is for young individuals to perform relatively “safe” and less costly intranidal activities while the older individuals perform the riskier and costlier extranidal activities in eusocial insects. This seems to be the case even in species where age polyethism is not as well developed as in highly eusocial systems. But whether this is due to explicit selection for this pattern or whether it is a carry-over from ancient hormonal and ovarian cycles of solitary ancestors, remains to be ascertained (O’Donnell and Jeanne, 1993; West-Eberhard, 1996).

In *R. cyathiformis* the difference between various tasks seems to be mainly in the initiation of tasks. Once all the tasks are initiated the wasps continue to perform all the tasks at all ages so that the intranidal tasks are not completely stopped with an increase in the age of the workers. Hence there is no trade-off between the intra- and extranidal tasks, making their age polyethism less striking or weaker than is seen in highly eusocial species. The fact that *R. cyathiformis* has such a weak age polyethism seems to be in keeping with what we already know in other primitively eusocial wasps where there is either a rudimentary age polyethism.

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**Fig. 5 (left).** Probability of task performance (PTP) as a function of absolute age fitted with second order polynomial regression lines. Data points represent the mean value for all individuals in that age class in 4 colonies. Intranidal tasks are the sum of the two intranidal tasks Feed Larva (FL) and Build and extranidal constitute the two extranidal tasks Bring Food (BF) and Bring Building material (BB).

**Fig. 6 (right).** Probability of task performance (PTP) as a function of relative age fitted with second order polynomial regression lines. Data points represent the mean value for all individuals in that age class in 4 colonies. Intranidal tasks are the sum of the two intranidal tasks Feed Larva (FL) and Build and extranidal constitute the two extranidal tasks Bring Food (BF) and Bring Building material (BB).
It is especially instructive to compare age polyethism in *R. cyathiformis* with that in *R. marginata* (Naug and Gadagkar 1998a), for two reasons. One reason is that *R. marginata* is the only other species where the exact methods we have used for *R. cyathiformis* have been used to study age polyethism. The second reason is that age polyethism in *R. marginata* is quite a contrast from that seen in *R. cyathiformis*. As in *R. cyathiformis*, there is a clear effect of age on the initiation of different tasks in *R. marginata* resulting in a specific sequence of task initiation, the only minor difference being that the initiation of BF precedes initiation of BB while it is the reverse in *R. marginata*. An interesting difference between the two species is that while absolute age explains more variance in the data in *R. cyathiformis*, relative age explains more variance in *R. marginata* (Naug and Gadagkar 1998a). Thus, division of labour seems to be based on absolute age in *R. cyathiformis* and based on relative age in *R. marginata*. We conclude that absolute-age-based polyethism in *R. cyathiformis* is somewhat rigid, preventing colonies from rapidly responding to changes in their age composition. On the other hand, relative-age-based polyethism in *R. marginata* is expected to be more flexible and hence help the colony adjust better to changes in age distribution that might arise due to ecological or demographic factors. A removal experiment should be able to give us a better idea about this, but unfortunately the age group that can be studied with such a removal is a very narrow one. The only option is to create and maintain a colony with only very young individuals before they have initiated extranidal tasks, and then see if individuals initiate extranidal tasks earlier than before. But this might be very difficult considering how sensitive *R. cyathiformis* colonies are to any external disturbances or manipulations.

When we say that age polyethism is weak and less flexible in *R. cyathiformis* we are not denying the day to day fluctuation that might arise with respect to the presence of brood in the colony. In those species, where there is a well-developed age polyethism, like in the case of *R. marginata*, division of labour is more flexible, with individuals performing tasks based on relative age rather than absolute age and this allows them to face the changes in the colony whether it be the demands of the brood or the loss of individuals due to predation or other natural calamities. In *R. cyathiformis* on the other hand, the effect of age on division of labour is not very strong, and hence we say it is a weak age polyethism. Moreover, because task performance is more a function of absolute rather than relative age, this species cannot cope with changes in brood, worker composition etc, by the same mechanism as *R. marginata* can and therefore we label its age polyethism as more rigid.

Another difference between the two species is that, unlike in *R. cyathiformis*, there is a clear trade-off between intranidal tasks and extranidal tasks in *R. marginata* so that intranidal tasks which are initiated early decrease gradually as extranidal tasks are initiated and increase in frequency (Naug and Gadagkar 1989a). In *R. cyathiformis*, once the extranidal tasks are initiated there is no decrease in the intranidal tasks in response to this initiation of extranidal tasks. In fact, in the middle ages both intra and extranidal tasks are performed at their highest frequencies. This difference between the two species is clearly evident when the age-dependent frequencies of the performance of intranidal and extranidal tasks are compared between the two species (Fig. 7). We argue that in *R. marginata* the trade-off between intranidal and extranidal tasks allows for more efficient work organisation because it permits individuals to specialise in one of the two tasks.

Another interesting difference between the two species is that unlike in *R. marginata*, the patterns of age polyethism based on FTP and PTP are approximately mirror images of each other in *R. cyathiformis*. This is because unlike in *R. marginata*, *R. cyathiformis* workers perform intranidal tasks more or less throughout their lives whereas they perform extranidal tasks more in middle ages. This results in a higher probability of performing intranidal tasks (relative to extranidal tasks) (PTP) both in young and old ages. The relative absence of extranidal tasks in old wasps which may be due to their lack of energy seems to be compensated by their having a longer work day. *R. cyathiformis* individuals have a longer working day as compared to *R. marginata* individuals (*R. cyathiformis* workers initiate extranidal tasks in the morning about a couple of hours before and continue for about an hour after *R. marginata* does so; S. Unnikrishnan and R. Gadagkar unpublished independent observations).

Taken together this comparison of the two species suggests that age polyethism in *R. cyathiformis* is more simple and perhaps more primitive and that in *R. marginata* is somewhat more elaborate and perhaps more advanced. In fact we see an evolution of age polyethism when we compare the two species. The pattern of age polyethism seen in *R. marginata* resembles the pattern seen in highly eusocial species (Akre et al., 1976; Seeley 1982; Matsuura 1984; Sommeijer 1984; Robinson 1987; Jeane et al., 1988; O’Donnell and Jeane 1992; Johnson 2010). Based on the behaviour of their queens (Chandraseshekar and Gadagkar, 1989; Cecilio et al., 2016; Dew and Michener, 1981; Jeanne, 1991; Murakami et al., 2013; Torres et al., 2012; Torres et al., 2013; Tsuchida, 1991; Yoshikawa, 1963) or no age polyethism present as in the case of Belonogaster petiolata (Keeping, 1992) and Mischocyttarus latior (Cecilio et al., 2016).
1991; Gadagkar and Joshi, 1983, 1984; Kardile and Gadagkar, 2002; Sumana and Gadagkar, 2003) and modes of reproductive regulation (Bang and Gadagkar, 2012; Bhadora et al., 2010; Mitra et al., 2011; Unnikrishnan and Gadagkar, 2017), we have previously argued that R. marginata is relatively more advanced among the primordially eusocial species, and that R. cyathiformis is more similar to what is expected of a primordially eusocial species (Unnikrishnan and Gadagkar, 2017). Comparison of age polyethism in the two species reinforces this argument and suggests that such comparison may be useful for understanding the origin and evolution if not just age polyethism but of eusociality itself in social insects.

Materials and Methods

This study was conducted on 4 colonies of Ropalidia cyathiformis in the campus of the Indian Institute of Science, Bangalore (13°00’N and 77°32’E) during the period of December 2014 to June 2016. We identified healthy nests with 10 or more pupae and the individuals present were uniquely marked using Testors® non-toxic enamel paints. We took daily census and individuals eclosing on each day were marked. This was continued until all the unknown-age individuals had disappeared and the nest consisted of only those wasps with known ages. Hence there were individuals of various ages when the experiment was started and some of these wasps (older than day 1 when observations were started) remained till the end of the observations for the particular nests and would be much older than the total number of days the nest was observed for.

We then commenced observations for 6 hours each day. The observation schedule alternated between 0630 – 0830 hrs, 1030 – 1230 hrs and 1430 – 1630 hrs on one day and 0830 – 1030 hrs, 1230 – 1430 hrs and 1630 – 1830 hrs on the other day and continued until the nest got destroyed by predators or other natural calamities. This observation schedule ensured that observations were made throughout the activity period of the wasps namely 0630 to 1830 hrs. The 4 nests were observed for 15, 20, 27 and 47 days respectively. Observations consisted of equal number of scans and all occurrences randomly intermingled with each other, so that on each day there were 30 scans and 30 all occurrences giving a total of 300 minutes of observation per day. Scans were used to estimate the proportions of time spent by each wasp in different behaviours. All occurrence sessions were used to estimate the frequency per hour with which different wasps performed different behaviours. Each scan and all occurrence lasted for 5 minutes. Each observation session consisted of 2 hours with 10 scans and 10 all occurrences randomly intermingled with each other within this 2 hour of observations, with a one minute gap between these 5 minute scans and/or all occurrences. Each day had 30 scans and 30 AOS which equalled to 150 minutes of scans and 150 minutes of AOS each day. In one hour we had 5 scans and 5 AOS, which equals to 25 minutes per hour of scans and AOS each. Although all the behaviours shown by the wasps were recorded during the observation sessions, only four well-defined behaviours which have the most functional significance were used for the analysis to understand the effect of age on division of labour. The four behaviours chosen were food larva (FL), build (BU), bring food (BF) and bring building material (BB). Feed larva is the behaviour of feeding solid food to the larva in different stages of growth by the adult wasps in the colony. Build is the task of building or constructing and includes building of new cells, extension of old cell walls, shaping and mouthing old cell walls and breaking of old cell walls. In build construction and includes building of new cells, extension of old cell walls, shaping and mouthing old cell walls and breaking of old cell walls. The youngest wasp in the colony was given a rank of one and the older wasps given successively higher ranks with the oldest wasp receiving the highest rank. In case of wasps with same age, tied ranks were allocated. The range was then scaled between 0 and 100 by using the following equation (Naug and Gadagkar, 1998a):

\[ SR_i = \frac{(R_i - 1)}{(N - 1)} \times 100, \]

where \( SR_i \) = Scaled rank of wasp i, \( R_i \) = rank of a wasp i, \( N \) = number of wasps present in the colony.

We calculated the age at which each task was observed to be performed for the first time by each wasp. Based on this we assessed the sequence in which the wasps performed each of the four tasks for the first time in their lives. We designated the rate (frequency per hour) at which a wasp of a particular age performs a particular task as the frequency of task performance (FTP). We designated the probability that a wasp of particular age will perform a particular task as opposed to other tasks as the probability of task performance (PTP). PTP was calculated by the following equation (Naug and Gadagkar, 1998a; Seeley, 1982):

\[ R_{ij} = \frac{N_i}{\sum n_{ij}}, \]

where \( P_i \) = probability of performance of task i by wasps of age class j, \( N_i = number of times task i was performed by members of age j, K = number of tasks considered.

We used Kruskal-Wallis and Mann Whitney U (post-hoc) tests to examine if the ages of the first performance of different tasks were significantly different from each other. We used G test to examine whether the observed sequences of initiating different tasks were significantly different from what might be expected by chance. We used polymorphism to examine the relationship between FTP and PTP on the one hand and absolute and relative age on the other hand. After performing the regression analyses separately for each colony, we pooled data from all four colonies after asserting that there was no significant effect of colony on age dependant task performance (linear mixed effects models used with nest as random effect) and that the same equations described the effect of age on the performance of each task in all four colonies. We used Wilcoxon’s matched pair signed-ranks test to compare the variance explained (r²) by relative age vs absolute age for both FTP and PTP. We did all the analysis using R Studio 3.4.3 using the Im function of the “MASS” package for the regression analysis (RStudio Team 2016).

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