The effects of age and past and present behavioral specialization on behavior of workers of the red wood ant *Formica polyctena* Först. during nestmate reunion tests

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ABSTRACT

Social insect workers usually participate first in intranidal tasks and then switch to extranidal ones. However, foragers may switch again to intranidal brood care. This process is called the behavioral reversion. We applied dyadic nestmate reunion tests to explore behavioral differences between five groups of workers of the red wood ant *Formica polyctena*: callows (newly eclosed workers), nurses, reverted nurses (foragers that switched back to intranidal brood care in response to exposure to brood in absence of nurses), and two groups of foragers. Inter-group differences between the tested ants were related both to age and past and present behavioral specialization. Callows were the least active and their behavior was characterized by the lowest tempo. Nurses usually behaved in a way intermediate in respect to behavior of callows and the ants that had already passed the transition to extranidal tasks. The behavior of reverted nurses showed both similarities and differences with respect to behavior of foragers. Some traits of behavior of reverted nurses were similar as in the case of nurses, or intermediate in respect to both nurses and foragers. Behavioral reversion of workers of *F. polyctena* has thus other behavioral correlates besides the reappearance of intranidal brood care.

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1. Introduction

Behavioral development of workers of social insects provides one of the best known examples of sequential polyphenism (appearance of different phenotypes during successive developmental stages) (Michener, 1961; Fahrbach, 1997; Herb et al., 2012). Social insect workers as a rule start their adult life from participation in intranidal tasks and then switch to extranidal activities (Forel, 1874; Otto, 1958; Dobrzańska, 1959; Wilson, 1971; Lenoir, 1987; Hölldobler and Wilson, 1990, 2009; Robinson, 2009; Mersch et al., 2013). The transition from intranidal to extranidal tasks usually involves not only changes of behavior and/or response thresholds, but also profound modifications of many other phenotype traits including dramatic changes of morphology and activity of various exocrine glands, reduction of lipid stores, regression of ovaries, modifications of neuroanatomical traits and neurochemical processes in the whole brain and in particular brain structures, changes of hemolymph titres of juvenile hormone and vitellogenin, modifications of flight metabolism and relative concentrations of various proteins in the whole-body protein profile, altered functioning of the immune system, and changes of sleep architecture (Free, 1965; McDonald and Topoff, 1988; Fénéron et al., 1996; Page et al., 2006; Page and Amdam, 2007; Eban-Rotschild and Bloch, 2008; Hölldobler and Wilson, 2009; Robinson, 2009). The transition from intranidal to extranidal tasks also involves changes in

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the expression and/or methylation levels of numerous genes (Ben-Shahar et al., 2002; Whitfield et al., 2003, 2006; Ingram et al., 2005; Herb et al., 2012; Margotta et al., 2013).

Worker behavioral development is influenced not only by worker age and attained stage of physiological maturation, but also by environmental cues including those generated by other colony members (Lenoir, 1979a, 1987; Robinson, 1992; Fénéron et al., 1996; Robinson et al., 1997; Hölldobler and Wilson, 2009; Johnson, 2010). In particular, transition from intranidal to extranidal tasks may be delayed, accelerated and even reversed in response to modifications of social context: foragers may switch again to intranidal brood care and become the so called reverted nurses (Free, 1965; Lenoir, 1979a, 1987; Robinson, 2009; Robinson et al., 1992, 1997; Hölldobler and Wilson, 2009; Amdam, 2011). The process of behavioral reversion is usually induced by exposure of foragers to brood in absence of younger workers acting as nurses (Ehrhardt, 1931; Dobrzańska, 1959; Lenoir, 1979a, 1987; Robinson et al., 1992, 1997; Page and Amdam, 2007; Hölldobler and Wilson, 2009; Robinson, 2009; Amdam, 2011; Kuszewska and Woyciechowski, 2013). However, some phenotype changes involved in behavioral reversion (including the regeneration of hypopharyngeal glands and the reversal of changes in hemolymph titers of the juvenile hormone) may be induced in honeybee foragers solely by deprivation from contacts with younger workers acting as nurses, without the exposure to brood (Huang and Robinson, 1996).

Behavioral reversion was investigated in the most detailed way in the honeybee (*Apis mellifera* L) and found to involve not only the reappearance of brood care behavior, but also other modifications of behavior including the return to arrhythmic activity (Bloch and Robinson, 2001; Bloch et al., 2001; Bloch and Grozinger, 2011), and the reversal of age-related learning deficits (Behrends et al., 2007; Baker et al., 2012). Honeybee behavioral reversion also involves the reversal of some morphological modifications associated with the transition from intranidal to extranidal tasks, including, in particular, the regeneration of the hypopharyngeal and wax glands (Rösch, 1930; Free, 1965; Huang and Robinson, 1996; Amdam et al., 2005). Recently, reverted nurses were also found to have better developed ovarioles in comparison with foragers (Kuszewska and Woyciechowski, 2013). Other phenotype modifications observed in the reverted honeybee nurses include the reversal of modifications of hemolymph titres of juvenile hormone and vitellogenin, changes of biogenic amine levels in specific brain structures, age-related decline in immunity (immunosenescence), and some (but not all) modifications of the whole-body protein profile, gene expression, and gene methylation levels (Robinson et al., 1992, 1997; Schulz and Robinson, 1999; Wagener-Hulme et al., 1999; Amdam and Page, 2005; Amdam et al., 2005; Wolschin and Amdam, 2007; Münch et al., 2008; Robinson, 2009; Amdam, 2011; Herb et al., 2012; Kuszewska and Woyciechowski, 2013; Margotta et al., 2013). Neuroanatomical changes and reduction of body lipid stores associated with the transition to extranidal tasks are, however, irreversible (Fahrbach et al., 2003; Toth and Robinson, 2005).

Behavioral reversion was also investigated in various species of ants (Ehrhardt, 1931; Weir, 1958b; Dobrzańska, 1959; Lenoir, 1979a,b; Sorensen et al., 1984; McDonald and Topoff, 1985; Wnuk et al., 2011). Phenotype modifications accompanying ant behavioral reversion are much less known than those documented in the honeybee, and several authors pointed out that honeybee and ant behavioral reversion may represent fundamentally different phenomena (Lenoir, 1979a; Sorensen et al., 1984). Whereas phenotype modifications accompanying honeybee behavioral reversion may develop in a relatively slow and gradual way (Rösch, 1930; Robinson et al., 1992; Huang and Robinson, 1996), in ants induction of intranidal brood care may occur very rapidly, within 24 h, which argues against exocrine control of that process (Lenoir, 1979a; Sorensen et al., 1984; McDonald and Topoff, 1985; Wnuk et al.,

2011). The question of expression of brood care behavior is also more complex in the case of ants than in the case of the honeybee. The phenomenon of extranidal brood care is not observed in the honeybee, but in many ant species foragers are more attracted to brood found outside the nest than nurses and show higher readiness to retrieve it to the nest (Weir, 1958b; Lenoir, 1977, 1981). Older ant workers and/or foragers may also continue to engage in intranidal brood care, and the same individuals may engage in both nursing and foraging behavior (Lenoir, 1979a, 1987; Calabi et al., 1983; Sorensen et al., 1984; Déjean and Lachaud, 1991; Retana and Cerdá, 1991; Fénéron, 1993; Godzińska et al., 1999; Seid and Traniello, 2006; Muscedere et al., 2009; Robinson et al., 2009). Therefore, some researchers studying ant behavioral development recommend to replace the notion of the transition nurse–forager by the notion of age-related expansion of behavioral repertoire (Seid et al., 2005; Seid and Traniello, 2006; Muscedere et al., 2009, 2013). Robinson et al. (2009) also confirmed that thresholds for brood transport are not lower in younger ants than in older ones, and that transport of extra brood (experimentally added) is carried out mainly by less corpulent ants, which suggests that these lean ants may have generally low thresholds to engage in work.

Honeybees and ants also show important differences in respect to neurochemical processes accompanying behavioral reversion. In the honeybee the levels of biogenic amines octopamine, dopamine and serotonin in some brain structures increase during the transition from intranidal to extranidal tasks, but are lower in reverted nurses than in foragers from the same colonies (Schulz and Robinson, 1999). In contrast, reverted nurses of the wood ant *Formica polyctena* Först. do not differ significantly from foragers in respect to brain levels of these three biogenic amines (Wnuk et al., 2011).

All these profound differences between honeybee and ant behavioral reversion strongly suggest that further research devoted to better understanding of phenotype changes accompanying ant behavioral reversion may contribute in an important way to broadening of our knowledge about sequential polyphenism, downward influences of social context on individual behavior and physiology, and reversibility of developmental and ageing processes.

Our present study belongs to a series of experiments devoted to behavioral, neurochemical and anatomical correlates of ant behavioral reversion. We were particularly interested if that process consists solely of reappearance of intranidal brood care behavior, or involves also other modifications of worker behavior. To that purpose, we applied a laboratory bioassay known as a dyadic nestmate reunion test to explore behavioral differences between five groups of workers of a common species of mound-building red wood ants (*Formica polyctena* Först.): callows (newly eclosed workers), nurses, reverted nurses (foragers that switched back to intranidal brood care in response to exposure to brood in absence of nurses), and two groups of foragers.

2. Material and methods

2.1. Collection and housing of the ants

Workers used in this experiment were collected from a colony of *F. polyctena* located in a mixed pine forest close to the village Wólka Radzymińska near Warsaw in Central Poland (52°25′57″N, 21°5′8″E) on 4th and 9th June 2009. A large colony fragment (about 10 thousands of individuals) was collected together with pupae and nest material directly from the mound. Additionally, numerous foragers (about 16 thousands) were collected from the ant trail in the vicinity of the mound (2–5 m).

The ants were transferred to laboratory and housed in artificial nests. The ants collected from the mound were housed in a large

nest composed of two open rectangular Perpex boxes connected by means of narrow tubes. The first box (38 cm × 30 cm × 15 cm) contained 20 artificial brood chambers made of large test tubes (20 cm long, 2 cm in inner diameter) filled partly with water held in by means of a cotton plug to provide humidity gradient allowing the ants to choose conditions suitable for development of brood. The tubes were covered from above by a sheet of aluminum foil to assure darkness. The second box (30 cm × 20 cm × 15 cm) served as a foraging area. Foragers collected from the trail on two different days were housed separately in two similar nests composed each of two boxes (30 cm × 20 cm × 15 cm), one containing 10 artificial nest chambers, the other acting as a foraging area. Food and water were provided on small Petri dishes. The ants had constant *at libitum* access to food exchanged for a fresh one 3 times a week. Food offered to the ants consisted of honey mixed with crushed apples and sand (added to make the mixture less sticky), and of pieces of house crickets (*Acheta domesticus* L.) killed by freezing and allowed to thaw at room temperature. Drinking water was provided on Petri dishes filled with moist cotton. The floor of the nest boxes was covered by a thin layer of dry sand. The walls of the boxes were coated with Fluon® (PTFE), a substance providing a silky smooth surface and commonly used in myrmecological research to prevent the ants from escaping from artificial nests. The nests were kept in laboratory at a natural rhythm of daylight and darkness supplemented from 7.00 to 19.00 by artificial white illumination delivered at 12:12 LD (from 7.00 to 19.00), and at stable ambient temperature (24 ± 1 °C).

2.2. Experimental groups

We tested workers of *F. polyctena* belonging to five experimental groups: callows (C) (newly eclosed intranidal workers), nurses (N) (collected from the mound and then kept together with brood and colony foragers), colony foragers (F_c) (collected from the mound and then kept together with brood and nurses), trail foragers (F_t) (collected from the trails and then kept in isolation from nurses and brood), and reverted nurses (R) (created from trail foragers by exposure to brood in absence of nurses). The last three groups of ants (R, F_c and F_t) were also labeled jointly as ants that had already passed the transition from intranidal to extranidal tasks.

Behavioral reversion was induced in foragers collected in the field from ant trails. A part of these foragers was exposed to brood in order to induce behavioral reversion, and another part continued to be kept in laboratory in isolation from other nestmates and brood. We then compared the behavior of reverted nurses with the behavior of trail foragers that had not been exposed to brood in order to identify behavioral correlates of behavioral reversion. We use the term "behavioral correlates", as behavioral differences between reverted nurses and trail foragers may involve not only behavioral consequences of behavioral reversion, but also behavioral predispositions to undergo that process.

The behavior of the reverted nurses was also compared with the behavior of nurses to check whether and in what respects the behavior of reverted nurses shows similarity to behavior of nurses, and/or is intermediate with respect to the behavior of nurses and trail foragers.

We also became aware of the fact that in our experiment absence of specific behavioral differences between reverted nurses and trail foragers does not necessarily imply that behavioral reversion has not been accompanied by the modifications of that particular behavior. As already mentioned, in the honeybees some elements of behavioral reversion may be induced in foragers solely as a consequence of deprivation from contacts with younger workers and may appear even if foragers have not been exposed to brood (Huang and Robinson, 1996). Absence of differences between reverted nurses and foragers may thus be related to the fact that

deprivation from contacts with nurses induced similar modifications of behavior in workers from both these groups. Therefore, we additionally investigated yet another worker group, the so called colony foragers (Fc): workers collected from the mound and then acting as foragers in laboratory artificial nests. Colony foragers were not deprived from contacts with younger workers, and, although they did not specialize in intranidal brood care, they were also not entirely deprived from contacts with brood. A situation in which nurses, reverted nurses and trail foragers behave in a similar way, but colony foragers differ from all these groups would thus suggest that behavioral similarities between reverted nurses and trail foragers arose as a consequence of behavioral reversion undergone by workers from both these groups rather than from absence of effects of behavioral reversion in the case of reverted nurses.

2.3. Induction of behavioral reversion

On 10th June a large group of foragers collected from the ant trail (about 5000 workers) was transferred to a new artificial nest composed of two 30 cm × 20 cm × 15 cm boxes and then a relatively large amount of homocolonial worker pupae (about 900) was gently placed close to 6 test tubes serving as artificial nest chambers. The ants readily transported all the pupae into the test tubes. After 24 h the test tubes were removed and their contents were transferred to a cylindrical glass container (10 cm high, inner diameter 23 cm) with the walls covered with Fluon®. The workers which moved away from the brood pile were put again in the nest from which they were withdrawn. All newly eclosed ants (callows) were also removed to avoid possible future difficulties in distinguishing them from the reverted nurses (callows are easy to recognize by very slightly pigmented cuticle, low activity and timid behavior). All workers staying on the brood pile and interacting with brood were then transferred together with a half of the pupae (about 450) to a new artificial nest made of a single Perspex box (27 cm × 17 cm × 12 cm) containing two tubes acting as artificial brood chambers. This procedure was repeated on four successive days. On each day we created a separate new nest with the reverted nurses (about 300–400 individuals). During the next seven successive days each nest was monitored daily. On each day we removed all newly eclosed callows and added new pupae to counterbalance brood loss occurring as a consequence of worker eclosions.

Every time when a group of reverted nurses was created, we also created two other worker groups of similar size and placed each of these ant groups in a similar nest. The first group was composed of foragers collected from the trail and then kept in isolation from other nestmates. Only workers present in the foraging area of the nest housing trail foragers were included in such groups. The second group was composed of workers collected from the mound and included both nurses and foragers, the so called colony foragers. The ants from the second group were placed in a new nest together with about 450 pupae, similarly as the reverted nurses. Experimental ants were kept in groups of similar size to reduce the effects of group size on their behavior during dyadic nestmate reunion tests. Behavior of workers of *F. polyctena* is well known to be strongly influenced by the size of group of workers kept together in a laboratory nest (Szczuka and Godzińska, 1997, 2004a,b).

2.4. Selection of workers for behavioral tests

Dyadic nestmate reunion tests were conducted on 27th–30th June 2009, always on the 8th day after the start of the process of behavioral reversion.

The ants were selected on the morning of each daily session of tests. All workers belonging to the same group (about 150–200 individuals) were placed in a cylindrical glass container (10 cm high, inner diameter 23 cm) with the walls covered with Fluon®.

Each container contained a small Petri dish filled with moist cotton to provide drinking water and a piece of aluminum foil (10 cm × 10 cm) slightly bent to form a shadowed zone.

Callows were selected from among workers at most 1 day old that had recently eclosed from pupae in the nests with the reverted nurses. We selected only the individuals which already displayed normal locomotion.

Nurses and reverted nurses were selected from among workers staying in brood chambers of laboratory nests created on the same day (the nest with reverted nurses and the nest with nurses and colony foragers). The contents of tubes serving as brood chambers were placed inside the cylindrical container in which the ants had to wait for the tests. The ants promptly transported the pupae to the shadowed zone. Only the ants that were observed to tend pupae after calming down (stayed calmly on a brood pile and/or interacted with pupae) were selected to be used in the tests.

Colony foragers and trail foragers were selected from among workers found in the foraging areas of the nests created on the same day as the nest with the reverted nurses (the nest with nurses and colony foragers and the nest with trail foragers). We selected only the individuals that responded by open-mandible threats and/or biting in response to the forceps used to capture them. The ants attempting to flee when captured were not used as subjects in the tests.

2.5. Dyadic nestmate reunion tests

Dyadic nestmate reunion tests involve a confrontation of two nestmates placed together in a small container and allow the analysis of the impact of various experimental factors on a broad spectre of behavior patterns including non-aggressive and agonistic social contacts, interactions with elements of physical environment, other forms of active behavior such as self-grooming, and various subclasses of resting behavior (Dahbi and Lenoir, 1998; Boulay et al., 1999a,b, 2000b, 2004; Cybulska et al., 2000; Korczyńska et al., 2005; Wagner-Ziemka et al., 2006, 2008). They were used in many studies devoted to nestmate recognition (Dahbi and Lenoir, 1998; Boulay et al., 2000b; Boulay and Lenoir, 2001), behavioral effects of social isolation (Boulay et al., 1999a,b, 2000b; Boulay and Lenoir, 2001; Cybulska et al., 2000; Korczyńska et al., 2005; Wagner-Ziemka et al., 2006, 2008), and effects of various pharmacological treatments (Boulay et al., 1999b, 2000b; Korczyńska et al., 2005; Wright et al., 2012). Encounters between a dyad of nestmates were also used as controls in many studies investigating ant aggressive behavior (Jutsum et al., 1979; Le Moli and Parmigiani, 1982; Le Moli et al., 1982; Le Moli and Mori, 1986; Heinze et al., 1996; Szczuka et al., 2013).

Prior to the test two workers from the same group were placed singly in small (5 cm long, 1.3 cm in inner diameter) test tubes closed with cotton plugs. The tubes were then covered with a piece of dark cloth to assure darkness and the ants were left to calm down during 15 minutes. Dyadic nestmate reunion tests were not preceded by a longer isolation period to avoid possible disruption of the process of behavioral reversion as a result of deprivation of the reverted nurses from contacts with brood.

Immediately before each test two tubes with workers belonging to the same group were opened and the open ends of the tubes were put together one against the other. Both tubes were then fixed together with a piece of transparent Scotch tape and put in front of the objective of a video camcorder (Sony Handycam Digital 8 DCR-TRV 340E) already working in the recording mode. The behavior of the interacting ants was recorded during 10 min. During the tests temperature and humidity conditions in the laboratory were fairly stable (temperature: 24 ± 0.5 °C, relative humidity of the air: 69–72%).

In total, 240 dyadic nestmate reunion tests were recorded, but finally only 40 tests were taken into account for each group (in total 200 tests). Eight tests had to be discarded as a result of worker death as a consequence of formic acid spraying (1 test with nurses, 2 tests with colony foragers and 5 tests with trail foragers), and further eight tests were discarded as a consequence of other problems (disturbed locomotion of the tested ants, technical problems with the recording). The remaining 24 tests were not analyzed.

2.6. Quantification of ant behavior

The recordings of ant behavior were analyzed by means of the software "The Observer Video-Pro" (Noldus Information Technology). The following behavioral categories were taken into account:

A. Non-social behavior (8 categories): immobility; resting with movements of the antennae and/or head; locomotion (walking or running); exploration (antennal exploration and licking of the surface of the joined test tubes and the junction between them); jerking (very short and rapid upward movement of the body of the ant, sometimes ending by a jump); self-grooming (except anal self-grooming); anal self-grooming (licking the gaster's tip).

B. Non-agonistic social social interactions (7 categories): antennal contacts with the nestmate's antennae; antennal contact with another part of the nestmate's body; licking the nestmate; being licked by the nestmate; trophallaxis (mutual contact of mouthparts involving the exchange of liquid food and/or compounds involved in nestmate recognition); offering of food in open mandibles; nestmate transport.

C. Agonistic social interactions (9 categories): dominance posture; submissive posture; open-mandible threat not accompanied by gaster flexing; open-mandible threat accompanied by gaster flexing (a more escalated form of a threat signalizing the readiness for formic acid spraying); charge (a rapid lunge toward the other ant); biting not accompanied by gaster flexing; biting accompanied by gaster flexing; dragging of the nestmate; fight (a combat of two ants).

Some of these behavioral categories were never observed (see Results, 3.1), and some were pooled for further analysis. As the values of the variables quantifying licking/being licked by the nestmate took closely similar values, finally we decided to present and discuss only the data obtained for a new behavioral category of allogrooming (licking/being licked by the nestmate pooled together).

2.7. Data analysis

The data obtained for two ants belonging to the same dyad could not be considered as statistically independent, as the behavior of one ant might have influenced the behavior of the second member of the dyad (Martin and Bateson, 2007). Therefore, we adopted one of solutions of that problem recommended by Martin and Bateson (2007) and we analyzed the behavior of only one ant from each dyad.

The rate of occurrence of particular behavioral patterns in various experimental groups (the ratio of the tests during which the behavior in question was present to those during which it was absent) was compared by means of the χ^2 test followed by post-hoc pairwise comparisons carried out by means of the two-tailed Fisher Exact Probability Test taking into account Bonferroni correction for multiple comparisons. This analysis was carried out only if the expected frequencies were sufficiently high (≥ 5).

For each behavioral category defined as a state four variables were calculated: the latency from the start of the test to the first episode of the analyzed behavior, the total number of bouts of that

behavior, the total duration of all bouts of that behavior, and the mean duration of these bouts. The latency from the start of the test to the first episode of the analyzed behavior was expressed as the percent of the duration of the whole test, as such a variable could be calculated also for the tests during which that specific behavior was absent, taking the value of 100% (Wagner-Ziemka et al., 2006). In the case of jerking (a behavioral category defined as an event, not a state) only the latency to the first episode and the total number of episodes could be analyzed, as events have no duration by definition.

Statistical analysis of these four variables was carried out by means of generalized linear models (GLMs) with an appropriate error distribution and a link function (Crawley, 2002). Experimental group was included as a fixed factor in GLM framework. Wald linear contrast was used to explore pairwise differences between experimental groups. The GLM analysis was carried out taking into account only those tests during which the behavior in question was expressed. Values are reported as means ± SEM (standard error of mean).

In all analyses we used 0.05 significance level.

3. Results

3.1. Rate of occurrence of specific behavioral categories

The rate of occurrence of specific behavioral categories observed during nestmate reunion tests with workers of *F. polyctena* usually showed significant inter-group differences (Fig. A.1, Fig. A.2). Only one behavioral category (locomotion) was observed on all tests except a single test with callows. Antennal contacts with the nestmate's body were present on all tests except about a half of the tests with callows (Fig. A.2A), and antennal contacts with the nestmate's antennae were present on all tests except about a half of the tests with callows (21 out of 40) and two tests with nurses (data not shown in a graph form, as they were closely similar to the data shown in Fig. A.2A). The rate of occurrence of these two subcategories of antennal contacts differed significantly between callows and all remaining ant groups (Fig. A.2A) and in the case of antennal contacts with the nestmate's antennae it also differed significantly between nurses and callows (two-tailed Fisher Exact Probability Test: $P < 0.0001$).

More complex patterns of inter-group differences in the rate of occurrence of specific behavioral categories were observed in the case of both subcategories of inactivity (immobility: Fig. A.1A; resting: Fig. A.1B), various subcategories of active non-social behavior (exploration: Fig. A.1C; jerking: Fig. A.1D; self-grooming: Fig. A.1E; anal self-grooming: Fig. A.1F), non-aggressive social behavior (allogrooming: Fig. A.2B; trophallaxis: Fig. A.2C; offering of food: Fig. A.2D), and agonistic behavior (both types of open-mandible threats: Fig. A2.E and A2.F).

The rate of occurrence of a yet another subcategory of agonistic behavior, charges, could not be analyzed by means of χ^2 test because the expected frequencies were too low (<5), but it also showed significant inter-group differences. Charges were absent in callows, very rarely performed by nurses and reverted nurses (observed on 2 and 3 out of 40 tests, respectively), and slightly more frequent during the tests with both groups of foragers (in each case observed on 7 out of 40 tests). The rate of occurrence of charges observed in callows and foragers represented thus two ends of a spectrum and showed a significant difference (two-tailed Fisher Exact Probability Test: $P = 0.01$). An alternative analysis of the same data in which we compared the rate of occurrence of charges observed during the tests with callows and with all remaining ant groups also yielded a significant result (two-tailed Fisher Exact Probability Test: $P = 0.02$).

Several other patterns of agonistic behavior were observed only on a few (2–3) tests throughout the whole experiment (submissive posture: 2 tests with nurses and 1 test with reverted nurses; dominance posture and biting accompanied by gaster flexing: 1 test with nurses and 1 test with reverted nurses; fight: 2 tests with trail foragers). Therefore, the rate of occurrence and other variables quantifying these behavior patterns could not be subjected to statistical analysis.

Three behavior patterns (nestmate transport, nestmate dragging and biting not accompanied by gaster flexing) were never observed during the whole experiment.

3.2. Callows

Callows were in general less active than other ants. The behavior of callows was characterized by the highest rate of occurrence (Fig. A.1A, A.1B), the most rapid onset (Fig. 1A, 1E), the longest total duration (Fig. 1C, 1G), and the longest mean duration (Fig. 1D, 1H) of both subcategories of inactivity: immobility and resting. However, callows did not differ from other ant groups with respect to the number of episodes of immobility (Fig. 1B), although the number of episodes of resting was the lowest in that group (Fig. 1F).

In the case of the majority of the variables quantifying active behavior the differences between callows and remaining ant groups had an exactly opposite character. The behavior of callows was characterized by the lowest rate of occurrence of the majority of specific patterns of active behavior [Fig. A.1C–A.2F and additionally also two variables discussed in the text (3.1): antennal contacts with the nestmate's antennae and charges]. However, callows did not differ from other ants in respect to the rate of occurrence of locomotion, which was present on all the tests except a single test with callows (3.1). They only exceptionally engaged in agonistic behavior: only 3 cases of open-mandible threats not accompanied by gaster flexing were recorded throughout the whole experiment (Fig. A.2E).

The behavior of callows was also characterized by the longest latencies from the start of the test to the first episode of locomotion (Fig. A.3A), self-grooming (Fig. 2 E), both subcategories of antennal contacts (Fig. A.4A, E), trophallaxis (Fig. 3 A), offering of food (Fig. A.5A) and open-mandible threat (Fig. 4 A). The latency to the first episode of exploration also took relatively high values in callows (Fig. 2A), but the longest latencies to the first episode of that behavior were observed in nurses. The behavior of callows was also characterized by the lowest number of episodes of locomotion (Fig. A.3B, C), exploration (Fig. 2B, C), self-grooming (Fig. 2F, G), both types of antennal contacts (Fig. A.4B, F), allogrooming (Fig. 3B), offering of food (Fig. A.5B), open-mandible threats (Fig. 4B) and jerking (Fig. A.5F). Callows also had the shortest total duration of locomotion (Fig. A.3B, C), exploration (Fig. 2B, C), self-grooming (Fig. 2F, G), anal self-grooming (Fig. A.3G), and both types of antennal contacts (Fig. A.4C, G).

Mean duration of episodes of various categories of active behavior as a rule took higher values in callows than in the remaining groups (locomotion: Fig. A.3D; self-grooming: Fig. 2H; both subcategories of antennal contacts: Fig. A.4D, H; open-mandible threats: Fig. 4D). Higher mean duration of episodes of specific behavior patterns was thus observed in callows in the case of both inactivity (Fig. 1D, H) and active behavior. However, callows did not differ from other groups in respect to the mean duration of exploration (Fig. 2D) and allogrooming (Fig. 3D).

3.3. Nurses

The values of the variables quantifying the behavior of the tested ants obtained for nurses were usually intermediate in relation to the values obtained for callows and for ants that had already passed
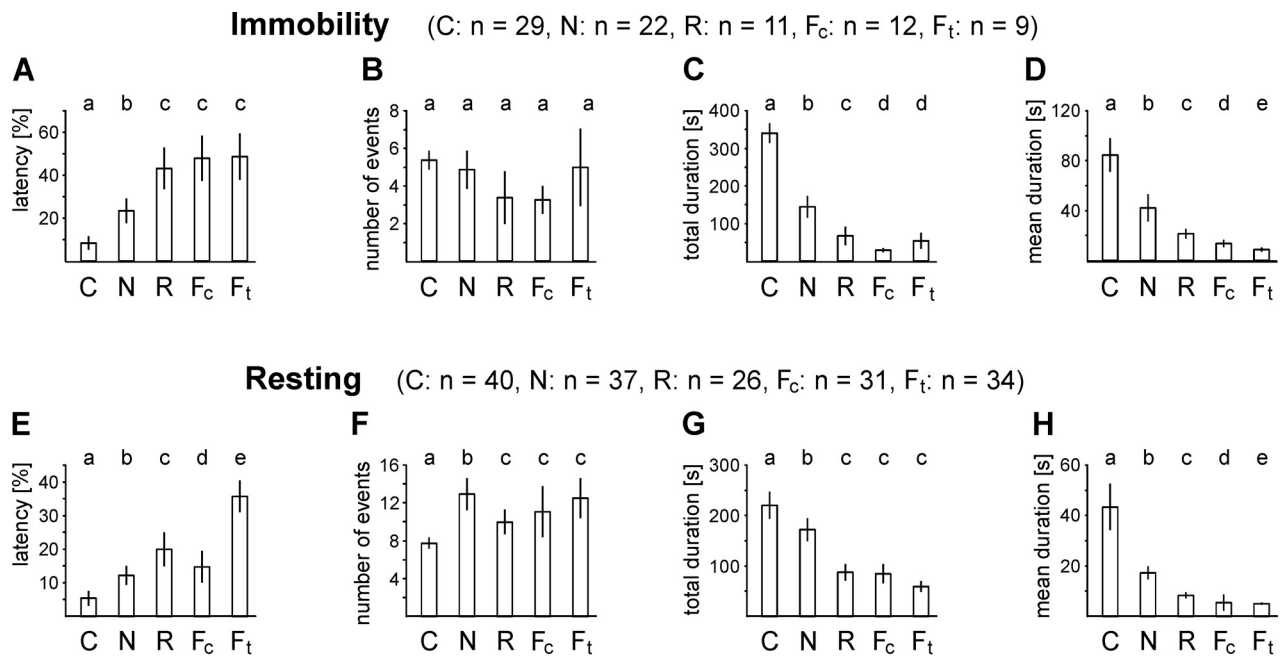
Immobility   (C: n = 29, N: n = 22, R: n = 11, F_c: n = 12, F_t: n = 9)

Resting   (C: n = 40, N: n = 37, R: n = 26, F_c: n = 31, F_t: n = 34)

Fig. 1. Inter-group comparisons of four variables quantifying two subcategories of inactivity (immobility and resting) displayed by workers of the red wood ant _Formica polyctena_ during dyadic nestmate reunion tests. A, E: latency to the first episode; B, F: number of episodes; C, G: total duration; D, H: mean duration. C: callows, N: nurses, R: reverted nurses, F_c: colony foragers, F_t: trail foragers, n: number of analyzed tests. The groups that do not differ significantly from each other are marked by the same letter. Immobility: (A) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t; (B) GLM: NS; (C) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t, R vs F_c, R vs F_t; (D) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t, R vs F_t, F_c vs F_t, $P < 0.01$: R vs F_c.
Resting: (E) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t, R vs F_c, R vs F_t, F_c vs F_t; (F) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, $P < 0.001$: N vs F_c, $P < 0.01$: C vs R, C vs F_t, N vs R, N vs F_t, $P < 0.05$: C vs F_c; (G) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t; (H) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t, R vs F_c, R vs F_t, F_c vs F_t.

Exploration   (C: n = 6, N: n = 19, R: n = 31, F_c: n = 33, F_t: n = 38)

Self-grooming   (C: n = 16, N: n = 35, R: n = 39, F_c: n = 40, F_t: n = 40)
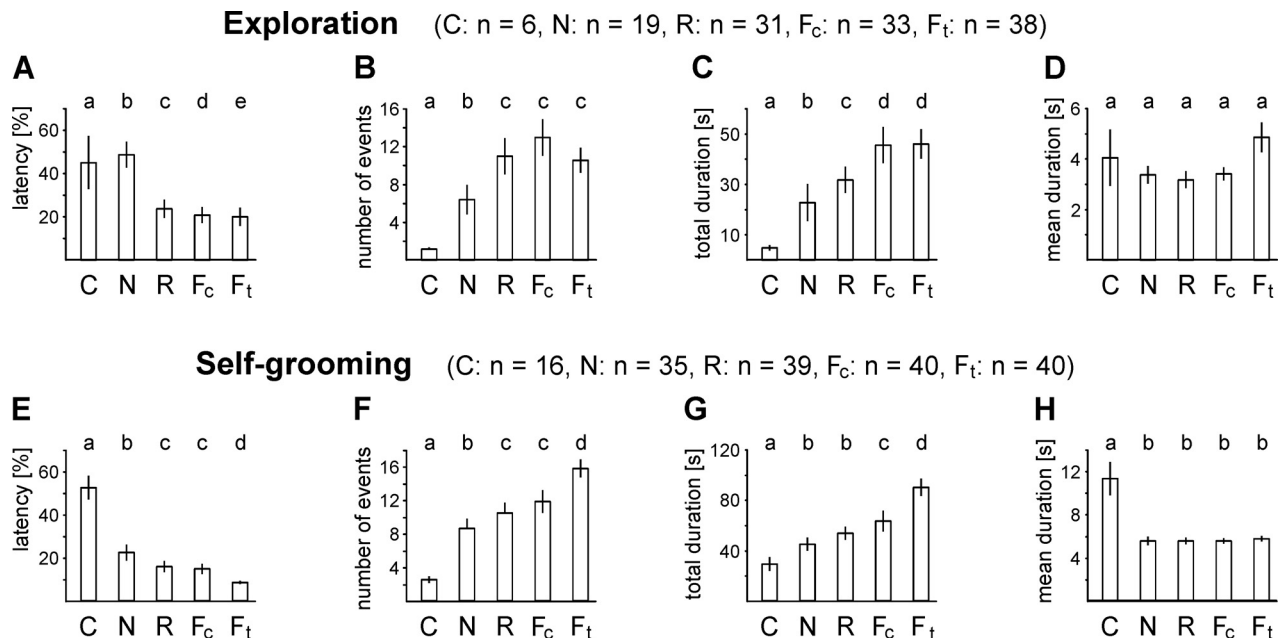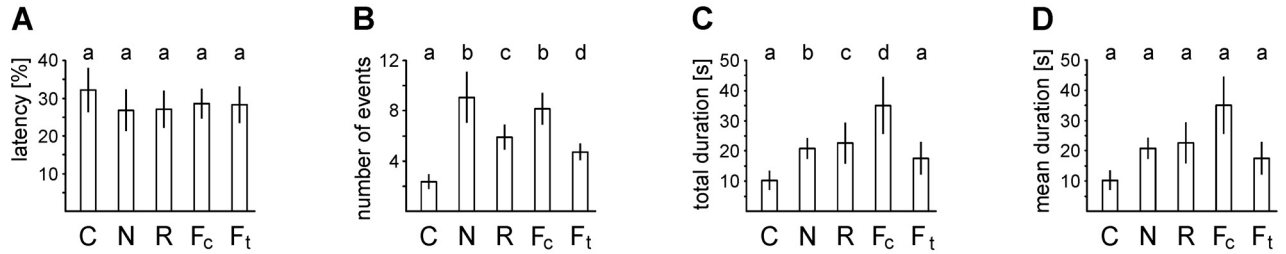

Fig. 2. Inter-group comparisons of four variables quantifying two subcategories of active non-social behavior (exploration and self-grooming) displayed by workers of the red wood ant _Formica polyctena_ during dyadic nestmate reunion tests. A, E: latency to the first episode; B, F: number of episodes; C, G: total duration; D, H: mean duration. C: callows, N: nurses, R: reverted nurses, F_c: colony foragers, F_t: trail foragers, n: number of analyzed tests. The groups that do not differ significantly from each other are marked by the same letter.
Exploration: (A) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t, R vs F_t, F_c vs F_t; $P < 0.01$: R vs F_c, (B) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, $P < 0.001$: N vs F_t; (C) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t, R vs F_c, R vs F_t; (D) GLM: NS.
Self-grooming: (E) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs R, N vs F_c, N vs F_t, R vs F_t, F_c vs F_t; (F) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t, N vs F_c, N vs F_t, R vs F_t, F_c vs F_t, $P < 0.01$: N vs R; (G) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs F_c, C vs F_t, N vs F_c, N vs F_t, R vs F_c, R vs F_t, F_c vs F_t, $P \le 0.001$: C vs N, C vs R; (H) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F_c, C vs F_t.

Allogrooming (C: n = 9, N: n = 26, R: n = 27, F$_c$: n = 28, F$_t$: n = 23)

Trophallaxis (C: n = 5, N: n = 15, R: n = 22, F$_c$: n = 22, F$_t$: n = 22)
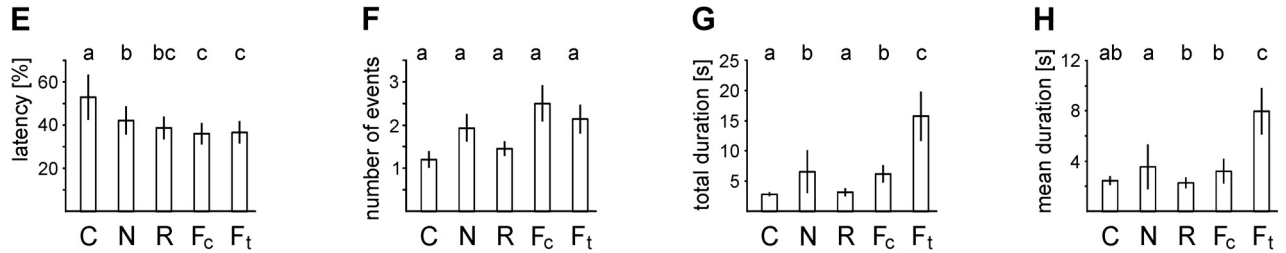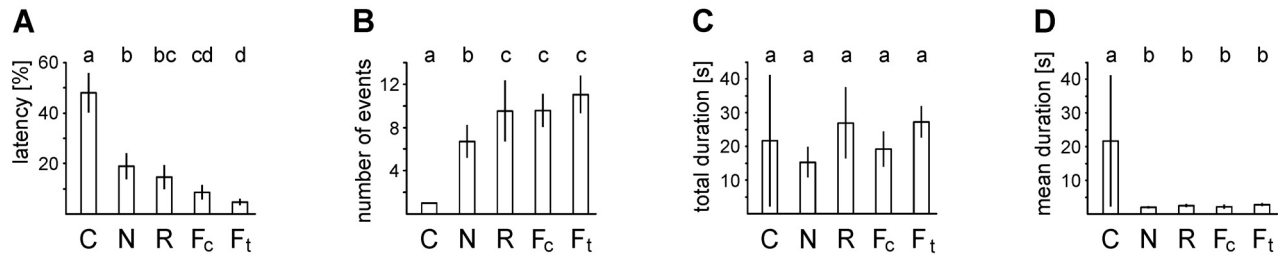

Fig. 3. Inter-group comparisons of four variables quantifying two categories of social behavior (allogrooming and trophallaxis) displayed by workers of the red wood ant *Formica polyctena* during dyadic nestmate reunion tests. A, E: latency to the first episode; B, F: number of episodes; C, G: total duration; D, H: mean duration. C: callows, N: nurses, R: reverted nurses, F$_c$: colony foragers, F$_t$: trail foragers, n: number of analyzed tests. The groups that do not differ significantly from each other are marked by the same letter.

Allogrooming: (A) GLM: NS; (B) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F$_c$, N vs R, N vs F$_t$, F$_c$ vs F$_t$; $P < 0.01$: R vs F$_t$, $P < 0.05$: C vs F$_t$, R vs F$_c$; (C) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs N, C vs R, C vs F$_c$, N vs R, N vs F$_c$, N vs F$_t$, R vs F$_c$, R vs F$_t$, F$_c$ vs F$_t$; (D) GLM: NS.

Trophallaxis: (E) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs R, C vs F$_c$, C vs F$_t$, $P < 0.01$: C vs N, N vs F$_c$, N vs F$_t$; (F) GLM: NS; (G) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs F$_t$, N vs R, N vs F$_t$, R vs F$_t$, F$_c$ vs F$_t$, $P < 0.001$: C vs N, $P < 0.01$: R vs F$_c$, $P < 0.05$: C vs F$_c$; (H) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs F$_t$, N vs F$_t$, R vs F$_t$, F$_c$ vs F$_t$; $P < 0.05$: N vs R, N vs F$_c$.

Open-mandible threat (C: n = 3, N: n = 24, R: n = 28, F$_c$: n = 29, F$_t$: n = 34)

Open-mandible threat with gaster flexing (C: n = 0, N: n = 6, R: n = 9, F$_c$: n = 11, F$_t$: n = 13)
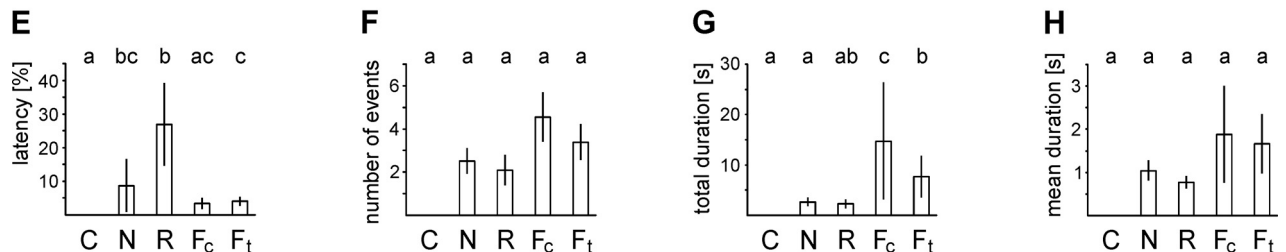

Fig. 4. Inter-group comparisons of four variables quantifying two categories of agonistic social interactions (open-mandible threat and open-mandible threat with gaster flexing) displayed by workers of the red wood ant *Formica polyctena* during dyadic nestmate reunion tests. A, E: latency to the first episode; B, F: number of episodes; C, G: total duration; D, H: mean duration. C: callows, N: nurses, R: reverted nurses, F$_c$: colony foragers, F$_t$: trail foragers, n: number of analyzed tests. The groups that do not differ significantly from each other are marked by the same letter.

Open-mandible threat: (A) GLM: $P < 0.001$, pairwise comparisons: $P < 0.001$: C vs F$_c$, C vs F$_t$, $P < 0.01$: C vs N, C vs R, N vs F$_c$, $P < 0.05$: N vs F$_t$, R vs F$_t$; (B) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs F$_c$, C vs F$_t$, N vs R, N vs F$_c$, N vs F$_t$, $P < 0.001$: C vs R, $P < 0.01$: C vs N; (C) GLM: NS; (D) GLM: $P < 0.0001$, pairwise comparisons: $P < 0.0001$: C vs R, C vs F$_c$, C vs F$_t$, $P < 0.001$: C vs N.

Open-mandible threat with gaster flexing: (E) GLM: $P < 0.05$, pairwise comparisons: $P < 0.001$: C vs R, $P < 0.01$: C vs N, R vs F$_c$, R vs F$_t$, $P < 0.05$: C vs F$_t$; (F) GLM: NS; (G) GLM: $P < 0.05$, pairwise comparisons: $P < 0.0001$: F$_c$ vs F$_t$; $P < 0.01$: C vs F$_c$, N vs F$_c$, N vs F$_t$, $P < 0.05$: C vs F$_t$, R vs F$_c$; (H) GLM: NS.

the transition to extranidal tasks (reverted nurses and both groups of foragers). Such pattern of inter-group differences (significant differences between nurses and callows and between nurses and at least one group of ants that had already passed the transition from intranidal to extranidal tasks) was observed in the case of the following 27 variables: the rate of occurrence of exploration (Fig. A.1C), the latency to the first episode of immobility (Fig. 1A), resting (Fig. 1E), locomotion (Fig. A.3A), self-grooming (Fig. 2E), both types of antennal contacts (Fig. A.4A, E), trophallaxis (Fig. 3E) and open-mandible threat (Fig. 4A), the number of episodes of locomotion (Fig. A.3.B), exploration (Fig. 2B), self-grooming (Fig. 2F), both types of antennal contacts (Fig. A.4B, F), open-mandible threats (Fig. 4B) and jerking (Fig. A.5F), total duration of locomotion (Fig. A3C), immobility (Fig. 1C), resting (Fig. 1G), exploration (Fig. 2C), self-grooming (Fig. 2G), both types of antennal contacts (Fig. A.4C, G), allogrooming (Fig. 3C) and trophallaxis (Fig. 3G), and the mean duration of immobility (Fig. 1D), resting (Fig. 1H) and trophallaxis (Fig. 3H). Additionally, in two further cases (the rate of occurrence of resting and the mean duration of an episode of offering of food; Fig. A.1B and Fig. A.5D, respectively) nurses did not differ from callows, but differed from at least one of groups of ants that had already passed the transition to extranidal tasks. Finally, in the case of further five variables the values obtained for nurses were intermediate with respect to callows and ants that had already passed the transition to extranidal tasks, but nurses did not differ significantly either from callows, or from any group of ants that had already passed the transition to extranidal tasks. Such a pattern was observed in the case of the rate of occurrence of immobility (Fig. A.1A), jerking (Fig. A.1D), anal self-grooming (Fig. A.1F), trophallaxis (Fig. A.2C) and open-mandible threat with gaster flexing (Fig. A.2F).

In further eleven cases nurses differed only from callows. Such pattern of inter-group differences was observed in the case of the rate of occurrence of self-grooming (Fig. A.1E), both types of antennal contacts (Fig. A.2.A and data not shown in graphical form), allogrooming (Fig. A.2B), offering of food (Fig. A.2D), open-mandible threats (Fig. A.2E), as well as in the case of the mean duration of locomotion (Fig. A.3D), self-grooming (Fig. 2H), both types of antennal contacts (Fig. A.4D, H) and open-mandible threats (Fig. 4D).

Much less frequently (in 5 cases only) nurses differed significantly from one or more of the remaining ant groups, but did not behave in a way intermediate in respect to callows and ants that had already passed the transition from intranidal to extranidal tasks. In the case of anal self-grooming two variables (the total duration and the mean duration) took higher values in nurses than in callows and at least one group of foragers (Fig. A.3G, H). Lastly, the latency to the first episode of exploration (Fig. 2A) and the number of episodes of resting behavior and allogrooming took the highest values in the case of nurses (Fig. 1F, Fig. 3D).

3.4. Reverted nurses

Reverted nurses behaved similarly as both classes of foragers and differently than nurses with respect to the latency to the first episode of immobility (Fig. 1A), the rate of occurrence, the number of episodes and the total duration of resting (Fig. A.1B, Fig. 1F, G), the total duration of locomotion (Fig. A.3C), the number of episodes of exploration (Fig. 2B), the total duration of all antennal contacts with the nestmate's body (Fig. A.4G), the number of bouts of open-mandible threats (Fig. 4B), and the mean duration of offering of food (Fig. A.5D).

Reverted nurses differed significantly from nurses and from both classes of foragers with respect to the total duration and the mean duration of immobility (Fig. 1C, D) and resting (Fig. 1H), the latency to the first episode of exploration (Fig. 2A), and the total duration of all episodes of exploration (Fig. 2C). In all these cases the values of the analyzed variables obtained for reverted nurses

were intermediate with respect to the values obtained for nurses and both classes of foragers. Additionally, in further two cases (the latency to resting and the number of episodes of allogrooming; Fig. 1E and Fig. 3B, respectively) reverted nurses differed significantly from nurses and both classes of foragers, but the values of the variables quantifying their behavior took intermediate values only between the values obtained for nurses and trail foragers. In yet other two cases (the total duration of allogrooming and the number of episodes of jerking; Fig. 3C, Fig. A.5F, respectively) reverted nurses differed significantly from nurses and both classes of foragers, but the values of variables quantifying their behavior took intermediate values only with respect to the values obtained for nurses and colony foragers. Furthermore, in the case of the latency and the number of episodes of self-grooming (Fig. 2E, F) and the total duration of antennal contacts with the nestmate's antennae (Fig. A.4C) reverted nurses differed significantly from nurses and trail foragers and behaved in a way intermediate with respect to these two ant groups, but did not differ from colony foragers.

In several further cases reverted nurses differed from nurses and both classes of foragers, but the values of the analyzed variables obtained for the reverted nurses did not take intermediate values with respect to those obtained for nurses and foragers. Such a pattern of significant inter-group differences was observed in the case of the number of episodes of locomotion (Fig. A.3B), the latency to antennal contacts with the nestmate's antennae (Fig. A.4A), the number of episodes of antennal contacts with the nestmate's body (Fig. A4F), and the total duration of trophallaxis (Fig. 3G). Reverted nurses differed significantly from nurses and trail foragers also with respect to the mean duration of trophallaxis (Fig. 3H), but in reverted nurses that variable took the lowest values, and not intermediate ones. Similarly, the total duration of food offering (Fig. A.5C) took lower values in the case of reverted nurses and colony foragers than in the case of nurses and trail foragers. Reverted nurses also differed from nurses and colony foragers (but not from trail foragers) with respect to the latency to locomotion (Fig. A.3A) and the first antennal contact with the nestmates's body (Fig. A.4E), and the number of episodes of antennal contacts with the nestmate's antennae (Fig. A.4B).

Most interestingly, in three cases reverted nurses did not differ from nurses, but differed from both groups of foragers. Such a pattern of inter-group differences was obtained in the case of total duration of self-grooming (Fig. 2G), mean duration of anal self-grooming (Fig. A.3H), and the latency to the first open-mandible threat with gaster flexing (Fig. 4E). In the case of yet another variable (total duration of anal self-grooming; Fig. A.3G) reverted nurses did not differ either from nurses, or from colony foragers, but differed from trail foragers, and in the case of further two variables (the latency to the first open-mandible threat and the total duration of all open-mandible threats with gaster flexing; Fig. 4A, G, respectively) reverted nurses did not differ either from nurses, or from trail foragers, but differed from colony foragers.

Lastly, reverted nurses differed only from callows in the case of several variables including the latency to trophallaxis (Fig. 3E), the number of episodes of offering of food (Fig. A.5B), and the mean duration of locomotion (Fig. A.3D), self-grooming (Fig. 2H), both types of antennal contacts (Fig. A.4D, H), and open-mandible threats (Fig. 4D).

3.5. Foragers

The behavior of both groups of foragers showed numerous significant differences. Significant differences between colony foragers and trail foragers were observed in the case of the following variables quantifying a wide spectre of behavior patterns: the mean duration of immobility (Fig. 1D), the latency to the first episode of resting and the mean duration of resting (Fig. 1E, Fig. 1H), the

latency to the first episode of locomotion (Fig. A.3A) and exploration (Fig. 2A), the latency, the number of episodes and the total duration of all episodes of self-grooming (Fig. 2E, F, G), the total duration of anal self-grooming (Fig. A.3G), the latency, the number of bouts and the total duration of all antennal contacts with the nestmate's antennae (Fig. A.4.A, B, C), the latency to the first antennal contact with the nestmate's body (Fig. A.4E), the number and the total duration of all episodes of allogrooming (Fig. 3B, C), the number and the total duration of all episodes of food offering (Fig. A.5B, C), the total duration and the mean duration of trophallaxis (Fig. 3G, H), the total duration of open-mandible threats with gaster flexing (Fig. 4D), and the number of episodes of jerking (Fig. A5F).

In comparison with other groups of ants, foragers had the most mature agonistic behavior characterized by the highest rate of occurrence of both subcategories of open-mandible threats (Fig. A.2E, F)+Fig. 4 and charges (see the text: 3.1). Fights were also noted only on 2 tests with trail foragers (see the text: 3.1). Finally, as mentioned in the description of methods (2.4), worker death as a consequence of formic acid spraying was observed on 2 tests with colony foragers, 5 tests with trail foragers and only one test with ants from another group (nurses).

4. Discussion

4.1. Dyadic nestmate reunion tests

Our experiment provides the first detailed analysis of the effects of age and past and present behavioral specialization on behavior patterns displayed by ant workers during dyadic nestmate reunion tests. So far, dyadic nestmate reunion tests were applied only to compare the behavior of two classes of nestmate ant workers: minor nurses and foragers of an African carpenter ant species *Camponotus maculatus* Fabricius) (the species name used in that paper, *Camponotus melanocnemis* Santschi, is no more valid) (Wagner-Ziemka et al., 2008).

Dyadic nestmate reunion tests are often conducted after a period of social isolation, sometimes relatively long, up to 20 days (Boulay and Lenoir, 2001; Korczyńska et al., 2005). Our present results demonstrate that dyadic nestmate reunion tests may serve as a useful bioassay to investigate ant behavior even if they are not preceded by social isolation.

4.2. Callows

A large part of behavioral differences revealed by this experiment was related to differences in worker age. In particular, callows showed many striking differences in comparison with older ants.

Callows showed the highest propensity for both subclasses of inactive behavior (total immobility and resting) and the lowest propensity for the majority of forms of active behavior. Low activity level of callows was not surprising, as it is already well documented in ants (Sudd, 1967; Lenoir, 1979b; Morel et al., 1988).

Behavior of callows was also characterized by the longest mean duration of episodes of the majority of forms of both inactive and active behavior. This implies that callows were switching from one behavior pattern to another at a lower rate than other ants. This finding can be interpreted in terms of differences in tempo, a notion coined first by Oster and Wilson (1978) to denote interspecific differences in general activity levels. Low-tempo workers were described as moving slowly and with seeming deliberation, examining objects carefully, and communicating with nestmates unexcitedly, whereas high-tempo workers were described as dashing about, skittish and excitable, and often mutually cancelling one another's actions (Oster and Wilson, 1978; Herbers, 1981, 1983; Hölldobler and Wilson, 1990). The term "tempo" was subsequently used to describe not only inter-specific, but also intra-specific and even intra-colony differences in behavior (Leonard and Herbers, 1986; Anderson and McShea, 2001; Wagner-Ziemka et al., 2006, 2008).

The behavior of callows was also characterized by a less rich repertoire of specific behavior patterns than the behavior of the remaining ant groups. This finding, too, is in concordance with the results of earlier research (Sudd, 1967; Cammaerts-Tricot, 1974, 1975; Lenoir, 1979b). In particular, callows did not engage in any forms of agonistic behavior except very infrequent open-mandible threats not accompanied by gaster flexing. The ability to display open-mandible threats was also observed in very young callows of *F. polyctena* (during the first day post eclosion) in another experiment of our team during which tethered ants were subjected to mechanical stimulation of the head (Mazurkiewicz, 2011). However, newly eclosed ants are as a rule little aggressive and avoid to participate in nest defense. For instance, callows of the fire ant *Solenopsis invicta* Buren were found to respond to alien conspecifics by death feigning, whereas older workers flee or fight back (Cassill et al., 2008). Thanks to their low propensity for aggressive behavior callows from different colonies and even from different species can be successfully used to form artificial mixed plurispecific colonies, which is not possible in the case of older workers (Jaisson, 1980; Corbara and Errard, 1991; Errard and Lenoir, 1995).

In contrast to ants from all other groups, callows very infrequently engaged in food offering. A similar result was reported by Muscedere et al. (2009) who observed that very young (0–2 days old) minor workers of the myrmicine ant *Pheidole dentata* Mayr were less active and less efficient in brood and queen care than their older nestmates. Callows also very rarely engaged in jerking. The function of that behavior is still unclear, although Wallis (1962, 1963) speculated that it is excitatory in function, rousing the ant itself and/or other individuals from inactivity. In our experiment jerking was also usually associated with a high level of arousal.

In spite of profound behavioral differences between callows and older ants, all tested groups of ants behaved in a closely similar way with respect to some variables quantifying their behavior. In particular, callows did not differ from other ants in respect to several variables quantifying agonistic and non-aggressive social contacts, such as the latency and the mean duration of allogrooming, the number of episodes of trophallaxis, the latency to the first episode of offering of food, and the total duration of open-mandible threats. In other words, although callows engaged in these forms of social contacts at a lower rate than older ants, if they performed them, some traits of their social behavior were closely similar as in the case of older workers. However, the analysis of other variables quantifying agonistic and non-aggressive social contacts of the tested workers of *F. polyctena* revealed numerous significant differences between callows and nurses, which implies that the maturation of these specific behavior patterns involves, among others, some modifications taking place relatively early, already during the transition callow-nurse. Our present findings confirm thus the conclusions of earlier research stating that trophallactic behavior of ant workers is strongly age-dependent (Wilson, 1971; Lenoir, 1979b; Meudec and Lenoir, 1982), and trophallactic behavior of callows differs from that of older ants (Heyde, 1924; Morel, 1986).

Callows did not differ from the remaining ants also with respect to the rate of occurrence of locomotion, the number of episodes of immobility, the latency to the first episode of jerking, and the mean duration of exploration. This last result is less surprising if we bear in mind that exploratory behavior of the tested ants was recorded inside a small container and, therefore, it might have corresponded to exploration of the interior of a nest chamber rather than to exploration of extranidal environment. Moreover, out of five variables quantifying exploration only one did not differ between callows and the other ants.

4.3. Nurses

Nurses as a rule behaved in a way intermediate in respect to callows and three groups of ants that had already passed the transition to extranidal tasks. This finding, too, is in concordance with the results of earlier research (Cammaerts-Tricot and Verhaeghe, 1974; Lenoir, 1979b; Morel et al., 1988). Relatively low activity level of nurses is also well documented: many authors reported that young ants and/or nurses are less mobile than older ants and/or foragers, spend much time motionless and respond to disturbances by immobility and hiding rather than by attack (Dobrzańska, 1959; Weir, 1958a, 1958b; Wilson, 1971; Cammaerts-Tricot, 1975; Lenoir, 1979b; Retana and Cerdá, 1990; Déjean and Lachaud, 1991; Fénéron et al., 1996).

In comparison with callows, behavior of nurses was also characterized by a higher number of events and a shorter mean duration of numerous behavior patterns, which can be interpreted in terms of a higher tempo. Lower tempo of nurses in comparison with foragers was in turn documented mostly by significantly higher mean duration of episodes of both forms of inactivity. Similar differences in tempo between nurses and foragers were already reported in various ant species (Dobrzańska, 1959; Bonavita-Cougourdan and Morel, 1984a,b, 1985; Wagner-Ziemka et al., 2008). In particular, striking differences with respect to the rate of switching between behavior patterns were reported in studies devoted to trophallactic behavior of workers of *Camponotus vagus* Scopoli (foragers had higher tempo than nurses) (Bonavita-Cougourdan and Morel, 1984a,b, 1985). A similar interrelationship between behavioral specialization and tempo and/or activity level was also reported by Dobrzańska (1959) in her classical study of the division of labour among the workers of *Formica sanguinea* Latreille. However, this is not a general rule. For instance, in the case of *Lasius niger* L. worker activity level and temporal polyethism were not strictly interrelated (Lenoir and Ataya, 1983).

Our present findings are only partly consistent with the results of an earlier study of our team (Wagner-Ziemka et al., 2008) carried out to compare the behavior displayed by minor nurses and foragers of *C. maculatus* during dyadic nestmate reunion tests carried out after 48 h social isolation. In that experiment nurses of *C. maculatus* differed from foragers with respect to readiness to display various forms of resting behavior (higher in nurses), and to engage in interactions with various elements of physical environment (higher in foragers). Similar results were obtained in our present experiment. However, nurses of *C. maculatus* investigated by Wagner-Ziemka et al. (2008) differed from foragers also in respect to higher propensity to engage in non-aggressive social contacts, in particular in antennal contacts and in trophallaxis. In contrast, in our present experiment nurses carried out antennal contacts with the nestmate's antennae and the nestmate's body after longer latencies than both types of foragers, and their antennal contacts with the nestmate's antennae were characterized by a lower number of episodes and a shorter total duration. Moreover, surprisingly, the highest total duration and mean duration of trophallaxis was observed in trail foragers. The differences between the results of these two experiments might have been related not only to inter-specific differences in behavior, but also to different duration of social isolation preceding the nestmate reunion test (*C. maculatus:* 48 h, *F. polyctena*: 15 min). In natural conditions nurses stay permanently in the nest chambers and remain in close contact with their nestmates. Social isolation during 48 h is very unusual for intranidal workers and leads to increasing necessity for social contacts allowing them to update and homogenize their cuticular hydrocarbons profiles acting as colony recognition cues (Bonavita-Cougourdan et al., 1987; Soroker et al., 1994; Lenoir et al., 1999, 2001; Lahav et al., 1999, 2001; Boulay et al., 2000a). The phenomenon of isolation-induced trophallaxis and its role in the homogenization of colony odor is well documented in ants, mostly thanks to experiments investigating the behavior of young intranidal workers (Soroker et al., 1995; Vienne et al., 1995; Dahbi et al., 1999; Boulay et al., 2000a, 2000b; Cybulska et al., 2000; Lenoir et al., 1999, 2001; Korczyńska et al., 2005; Wagner-Ziemka et al., 2008). As in our present experiment the tested ants were not subjected to social isolation before the test, relatively low level of trophallaxis observed in nurses might have been related to absence of isolation-induced trophallaxis.

4.4. Reverted nurses

Reverted nurses behaved similarly as both classes of foragers and differently than nurses with respect to numerous variables quantifying a wide spectrum of behavior patterns ranging from immobility up to agonistic and non-aggressive social behavior. This finding has several implications. First, it implies that behavioral reversion in *F. polyctena* does not consist in the return of a forager to fully nurse-like behavior. Second, it also implies that behavioral similarities between the reverted nurses and trail foragers are not related to the fact that ants from both these groups underwent behavioral reversion as a consequence of deprivation from contacts with younger workers acting as nurses. If it were so, both reverted nurses and trail foragers should behave in a more nurse-like way than colony foragers. However, reverted nurses differed from colony foragers and did not differ from both nurses and trail foragers only in the case of two variables quantifying two different types of open-mandible threats. Our present findings support thus also the hypothesis that honeybee and ant behavioral reversion are qualitatively different, as in the honeybees deprivation from contacts with younger workers acting as nurses is well known to induce phenotype changes characteristic for behavioral reversion (Huang and Robinson, 1996).

Our experiment also demonstrated that ant behavioral reversion has other behavioral correlates besides the reappearance of intranidal brood care. In the case of six behavioral variables quantifying two subcategories of self-grooming and two subcategories of open-mandible threats the values obtained for reverted nurses did not differ from those obtained for nurses, but differed from those obtained for both groups of foragers (three cases), trail foragers (one case), or colony foragers (two cases). Moreover, in the case of several further behavioral variables reverted nurses behaved in a way intermediate with respect to nurses and both groups of foragers, or with respect to nurses and trail foragers. Such intermediate behavior of reverted nurses was observed in the case of variables quantifying immobility, resting, exploration, self-grooming, antennal contacts with the nestmate's antennae, allogrooming and jerking.

Some behavioral differences between the reverted nurses and trail foragers are difficult to be interpreted (for instance, higher total duration and mean duration of trophallaxis observed in trail foragers in comparison with the reverted nurses), but in the majority of cases reverted nurses behaved in a more nurse-like way than trail foragers. As already stressed in the Introduction (1), at present we may, however, speak only about behavioral correlates of behavioral reversion, and not about behavioral consequences of that process, as at present we cannot exclude that at least some of behavioral differences between reverted nurses and foragers documented by our experiment were rather related to behavioral and physiological predispositions to undergo that process. This question remains to be unraveled by future research.

At present, it is also difficult to tell what factor(s) were responsible for behavioral similarities between the reverted nurses and colony foragers: contacts with brood (pupae), similar age, or perhaps both these factors. Trail foragers of *F. polyctena* and other red wood ants are known to be highly heterogenous with respect

to age (Rosengren, 1971, 1977; Rosengren and Sundström, 1987). Therefore, most probably at least a part of trail foragers investigated in this study consisted of relatively old workers. In contrast, colony foragers were taken from colony fragments composed of individuals collected from the mound, and most probably consisted mostly of relatively young foragers that had very recently passed the transition to extranidal tasks. In the honeybee the probability to undergo behavioral reversion decreases as a function of worker age and life expectancy (Page et al., 1992; Robinson et al., 1992; Kuszewska and Woyciechowski, 2013). If the same rule holds also for *F. polyctena*, our reverted nurses might have been on average younger than trail foragers not exposed to brood, and more similar with respect to age to colony foragers.

4.5. Foragers

Two groups of foragers used in this study differed in respect to several factors: origin (mound *vs* trails), present social environment (presence *vs* absence of contacts with nurses and brood), and most probably also worker age. It is therefore not surprising that colony foragers and trail foragers showed numerous behavioral differences. These findings are in concordance with numerous literature data reporting a high degree of behavioral polymorphism among the foragers of the red wood ants, both age-related and/or consisting in distinct behavioral specializations (Økland, 1930; Kiil, 1934; Otto, 1958; Mabelis, 1979; Rosengren, 1971, 1977; Horstmann, 1973; Cherix, 1987; Rosengren and Sundström, 1987).

In comparison with colony foragers, trail foragers showed higher propensity to engage in self-grooming. Extranidal activity of foragers entails frequent contacts with different elements of environment and may increase the necessity of body cleaning. Behavior of trail foragers was also characterized by the longest total duration and the mean duration of bouts of trophallaxis. As already told (4.3), in earlier experiments investigating trophallactic contacts taking place during dyadic encounters between workers of *C. vagus* the rate of switching between various behavior patterns was higher in foragers than in nurses (Bonavita-Cougourdan and Morel, 1984a). However, as only that single specific behavior pattern had the longest mean duration in trail foragers, this result should be interpreted in terms of particularly high propensity to engage in trophallaxis rather than in terms of low tempo. As red wood ant foragers may specialize in fulfilling various tasks including aphid tending and transport of the honeydew (Økland, 1930; Kiil, 1934; Otto, 1958; Mabelis, 1979; Rosengren, 1971; Horstmann, 1973; Cherix, 1987), these particularly long bouts of trophallaxis perfomed by trail foragers might have been related to their specialization as honeydew collectors.

As already mentioned (4.4), our experiment also demonstrated that deprivation from contacts with callows did not result in the induction of nurse-like behavior in trail foragers. We also did not observed suppressing effects of deprivation from contacts with younger workers on activity level of trail foragers, a phenomenon reported by Lenoir (1979a) in another formicine ant species, *Lasius niger* L. In our experiment trail foragers showed high propensity for various forms of active behavior.

Behavioral differences between three groups of the older ants (reverted nurses and both groups of foragers) were often less important than de differences between these ants and workers that had not yet passed the transition to extranidal tasks (nurses and callows). This result may be discussed in light of the repertoire expansion model proposed by Seid and Traniello (2006). As shown by these authors, minor workers of *P. dentata* increase their behavioral repertoire with age rather than shift between non-overlapping sets of tasks. Older workers retain the ability to fulfil tasks that first appeared in their repertoire and they perform both brood care and extranidal tasks with high frequency. Our present data suggest that a similar phenomenon may also occur in the case of ant behavioral reversion, and that foragers which revert to intranidal nursing behavior did not simply switch again to an earlier stage of their behavioral development, but retain behavioral modifications related to their transition to extranidal tasks, although they are unrelated to intranidal brood care that represents now their present behavioral specialization.

4.6. Conclusions

Our study confirmed that dyadic nestmate reunion test may serve as a useful tool in the study of ant behavioral ontogeny and behavioral polymorphism encountered in ant colonies. The application of that bioassay allowed us to detect numerous behavioral differences between various groups of workers of the red wood ant *Formica polyctena*. These differences were related both to age and to present and past behavioral specialization of the tested workers. In particular, we were able to identify some behavioral correlates of behavioral reversion induced in trail foragers by exposure to brood in absence of nurses, although at present it is still impossible to determine whether and to what degree these behavioral differences arose as a consequence of behavioral reversion, as they may represent behavioral predispositions to undergo that process. Our experiment also demonstrated that, in contrast to the honeybee, deprivation from contacts with young workers does not induce important elements of behavioral reversion in trail foragers, and does not exert a suppressing effect on their activity.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.beproc.2014.07.009.

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