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# DYADIC NESTMATE REUNION TEST IN THE RESEARCH ON ANT SOCIAL BEHAVIOR

## INTRODUCTION

### ETHOLOGY: AIMS, SCOPE AND METHODS

One of the most important behavioral sciences, ethology, can be defined as the study of mechanisms and evolution of behavior, focused both on accurate observation and description of behavior and on its causation. Ethology is concerned with four main categories of causal factors of behavior: (1) immediate causal factors, acting „here and now", (2) factors that had acted in the course of the ontogeny of the studied individuals, (3) factors that had acted in the course of the phylogeny of the studied species, and, lastly, (4) factors related to function (adaptive significance) of the studied behavioral trait. The first two categories and the second two ones are designated jointly, respectively, as proximate and ultimate (evolutionary) causal factors of behavior. Causation of behavior is studied mainly by means of experiments carried out both in laboratory conditions and in the field with the use of various behavioral bioassays. In contrast to some other behavioral sciences, general theoretical concepts of ethology arose as a result of comparative studies of many phylogenetically distant animal groups, including not only vertebrates, but also invertebrates (TINBERGEN 1963; CHMURZYŃSKI 1973; THORPE 1979; GODZIŃSKA 1996b, 1997, 2019).

## ANTS AS CONVENIENT SUBJECTS FOR COMPARATIVE RESEARCH ON BIOLOGICAL ROOTS OF SOCIAL BEHAVIOR

Comparative research devoted to biological roots of social behavior owes a lot to numerous studies using as subjects social insects, in particular the honeybee (*Apis mellifera*) and many species of ants (WILSON 1975; GODZIŃSKA 2005, 2006, 2016, 2019; GOWDY and KRALL 2016; MOFFETT 2018). The use of ants as subjects in behavioral research provides many advantages in comparison with the use of the honeybee. Ants are much more long-lived than honeybees: ant workers may live up to several years, and ant queens up to 30 years (HÖLLDOBLER and WILSON 1990; KELLER and GENOUD 1997; KELLER 1998; GODZIŃSKA *et al.* 1999; JEMIELITY *et al.* 2005). Long lifespan of ants facilitates long-term research and experiments devoted to ontogeny and acquired modifications of behavior (GODZIŃSKA 1997). Behavioral repertories of ants are very rich, and social behavior of these insects shows great complexity, plasticity and flexibility (HÖLLDOBLER and WILSON 1990, 1998, 2009; GODZIŃSKA 1996a, 2007, 2016; KORCZYŃSKA 1996; SZCZUKA 1996; WNUK 2013; MAZURKIEWICZ *et al.* 2015). Richness and complexity of ant behavior makes possible comparative analysis of social behavior of ants and humans (WILSON 1975; JAISSON 1993; GODZIŃSKA 2005, 2016; WNUK 2013; GOWDY and KRALL 2016; MOFFETT 2018). In particular, ants were used as model organ-

isms in comparative behavioral and neurobiological research on biological roots of aggression and violence (KOSTOWSKI 1994; HÖLLDOBLER and WILSON 1998; GODZIŃSKA 2005, 2007; MAZURKIEWICZ et al. 2015), and in the experiments investigating pro-social behavior, and, in particular, rescue behavior (coming to the rescue of endangered individuals) (CZECHOWSKI et al. 2002; NOWBAHARI and HOLLIS 2010; HOLLIS and NOWBAHARI 2013a). Ant behavior also shows astounding diversity, which is not surprising, as several thousands of valid extant ant species have already been described on a world scale, and many other species remain still undiscovered. More than one hundred ant species were found to inhabit Poland (HÖLLDOBLER and WILSON 1990; CZECHOWSKI et al. 2012; MAZURKIEWICZ et al. 2015). Last but not least, ant behavior can be conveniently studied in both laboratory and field conditions (HÖLLDOBLER and WILSON 1990, 1998, 2009; GODZIŃSKA 1996a, 1997, 2006, 2007; WNUK and GODZIŃSKA 2006; MAZURKIEWICZ et al. 2015). In particular, ants show interesting behavioral and physiological responses to modifications of social context (WILSON 1971; SZCZUKA 1996; GODZIŃSKA 1997, 2006, 2019; WNUK and GODZIŃSKA 2006; WNUK 2013; MAZURKIEWICZ et al. 2015; see also SZCZUKA et al. 2019, this issue of KOSMOS).

The aim of the present paper is to show how a relatively simple behavioral bioassay may provide a wealth of important findings and contribute to the extension of our knowledge about the behavior of the tested species, its causation and its variability. We choose the so called dyadic nestmate reunion test as an example of such a simple and yet very useful bioassay.

## DYADIC NESTMATE REUNION TEST

### GENERAL DESCRIPTION

Dyadic nestmate reunion test consists of a confrontation of two nestmates (members of the same colony or social group) placed together in the same container. Behavior of both individuals is recorded and then analyzed, usually in a detailed way. Analyzed behavior patterns may include various aggressive and non-aggressive contacts between the tested ants, various forms of self-focused behavior such as self-grooming, various types of resting behavior and locomotion, and various behavioral responses to elements of physical environment present during the test. Video recording of the whole test makes possible very accurate identification of all specific behavior patterns. The

test may be preceded by various treatments applied to one or both tested individuals, such as social isolation, food deprivation, and various acute or chronic pharmacological treatments (WNUK and GODZIŃSKA 2006).

### DYADIC NESTMATE REUNION TESTS PRECEDED BY SOCIAL ISOLATION AND ISOLATION-INDUCED TROPHALLAXIS

Dyadic nestmate reunion tests were used in numerous studies investigating the behavior of ants reunited with a nestmate after a period of social isolation. Ants were usually isolated singly in test tubes. At the start of each test two tubes were opened and their open ends were put together, one against the other, to allow a confrontation of a dyad of ants (BOULAY et al. 1999a, b, 2000b, 2004; CYBULSKA et al. 2000; KATZAV-GOZANSKY et al. 2004; KORCZYŃSKA et al. 2005; WAGNER-ZIEMKA et al. 2006, 2008; MAZURKIEWICZ et al. 2016).

The authors of the first studies investigating the behavior of ants during dyadic nestmate reunion tests carried out after a period of social isolation used as subjects carpenter ants from the genus Camponotus, namely Camponotus fellah (BOULAY et al. 1999a, b, 2000b, 2004; KATZAV-GOZANSKY et al. 2004), Camponotus herculeanus (KORCZYŃSKA et al. 2005), and two African species, Camponotus acvapimensis (CYBULSKA et al. 2000) and Camponotus maculatus[1] (WAGNER-ZIEMKA et al. 2008; MAZURKIEWICZ et al. 2016). These studies discovered, among others, the phenomenon of the so called isolation-induced trophallaxis. Carpenter ant workers reunited with a nestmate after a period of social isolation ranging from 24 hours (Camponotus fellah: BOULAY et al. 1999b; Camponotus acvapimensis: CYBULSKA et al. 2000) to 20 days (Camponotus fellah: BOULAY and LENOIR 2001; Camponotus herculeanus: KORCZYŃSKA et al. 2005) showed increased propensity to engage in trophallaxis, a specific category of social contacts widespread in insect societies. Trophallaxis most frequently involves mutual contacts of mouthparts of two individuals accompanied by the exchange of liquid food and/or various active compounds, in particular cuticular hydrocarbons playing a crucial role in the mediation of nestmate recognition (HÖLLDOBLER and WILSON 1990, 1998, 2009; CYBULSKA and GODZIŃSKA 1999; GODZIŃSKA 2006; MAZURKIEWICZ et al. 2015; LEONHARDT

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[1]The ants used in these two studies (WAGNER-ZIEMKA et al. 2008, MAZURKIEWICZ et al. 2016) were first identifed as Camponotus melanocnemis (Bolton, pers. comm.), and that species name was used in the paper of WAGNER-ZIEMKA et al. (2008). However, that species name is now invalid, and, therefore, the correct name (Camponotus maculatus) has been used in the paper of MAZURKIEWICZ et al. (2016).

*et al.* 2016; see also Szczuka *et al.* 2019, this issue of KOSMOS). Cuticular hydrocarbons are synthesized by each ant and then exchanged during trophallaxis, allogrooming (= licking another individual) and other social contacts. They are then stored in the post-pharyngeal glands located in the worker heads, and still later are deposited on the body surface of the ants during self-grooming. As a consequence of these exchanges ant nestmates bear similar cuticular hydrocarbon profiles on their body surface. That phenomenon plays a crucial role in nestmate recognition (Godzińska 2006; Wnuk and Godzińska 2006; Mazurkiewicz *et al.* 2015; Leonhardt *et al.* 2016; see also Szczuka *et al.* 2019, this issue of KOSMOS).

Isolation-induced trophallaxis was observed not only during dyadic nestmate reunion tests (Boulay *et al.* 1999a, b, 2000b, 2004; Cybulska *et al.* 2000; Katzav-Gozansky *et al.* 2004; Korczyńska *et al.* 2005; Wagner-Ziemka *et al.* 2008; Mazurkiewicz *et al.* 2016), but also in several other studies during which isolated ants were reintroduced into their maternal nest (Boulay *et al.* 2000a), a colony fragment created by splitting their maternal colony into smaller units (Boulay *et al.* 2004), or an experimental arena containing a large group of their nestmates (Boulay *et al.* 2003; Katzav-Gozansky *et al.* 2004).

Behavior of carpenter ants during dyadic nestmate reunion tests carried out after a period of social isolation was shown to depend in a very important way on the duration of that period. In particular, ants that had been subjected to relatively short social isolation period (1-10 days) were usually not attacked by their nestmates during dyadic nestmate reunion tests (Cybulska *et al.* 2000; Boulay *et al.* 2004; Korczyńska *et al.* 2005) or when reintroduced to their maternal nest (Boulay *et al.* 2000a). However, longer isolation periods (14-40 days) induced aggressive responses of nestmates of the tested ants as a consequence of more important divergence of cuticular hydrocarbon profiles between isolated and non-isolated ants (Boulay *et al.* 2000a, 2003, 2004; Katzav-Gozansky *et al.* 2004). However, this rule was not held unexceptionally: in the study of Korczyńska *et al.* (2005) workers of *C. herculeanus* did not engage in aggressive behavior during dyadic nestmate reunion tests irrespectively of the duration of social isolation period (5 or 20 days).

## NEUROCHEMICAL ASPECTS OF ISOLATION-INDUCED TROPHALLAXIS

The phenomenon of the isolation-induced trophallaxis was also investigated by means of neuroethological methods involving abdominal injections of various neuroactive compounds (Boulay *et al.* 1999a, 2000b; Korczyńska *et al.* 2005). These experiments revealed that isolation-induced trophallaxis can be partly suppressed by the administration of relatively high doses of the biogenic amine octopamine (Boulay *et al.* 1999a; Korczyńska *et al.* 2005; see also Szczuka *et al.* 2019, this issue of KOSMOS). Octopamine, a biogenic amine widespread in invertebrates and often considered to represent the functional equivalent of noradrenaline, acts as a neurotransmitter, neuromodulator and neurohormone, and participates in the mediation of many aspects of insect physiology and behavior (Roeder 2003; Roeder *et al.* 2005) including the phenomena of positive reinforcement and reward involved in the associative olfactory learning of the honeybees (Hammer and Menzel 1998; Farooqui *et al.* 2003; Giurfa and Sandoz 2012). It is thus possible that octopamine injections reduced isolation-induced trophallaxis by mimicking rewarding effects of trophallactic exchanges between nestmates. However, the phenomenon of octopamine-mediated social reward accompanying ant trophallactic behavior remains hypothetical (Korczyńska *et al.* 2005; see also Szczuka *et al.* 2019, this issue of KOSMOS).

An alternative hypothesis concerning causal factors responsible for inhibitory effects of octopamine administration on isolation-induced trophallaxis takes into account the results of two experiments that demonstrated that administration of octopamine may reduce not only behavioral consequences of complete social deprivation (social isolation), but also behavioral modifications induced by various forms of partial social deprivation (Szczuka and Godzińska 2008; Vander Meer *et al.* 2008; see also Szczuka *et al.* 2019, this issue of KOSMOS). In the study of Vander Meer *et al.* (2008) workers of the fire ant *Solenopsis invicta* responded to queen removal by significant decrease of both brain octopamine levels and nestmate recognition acuity that manifested itself as decreased aggressiveness towards non-nestmate conspecifics. However, chronic oral octopamine treatment counterbalanced both these effects. Chronic oral administration of octopamine was also shown to reduce modifications of responses to dead insect prey induced by dramatic reduction of worker group size in workers of the red wood ant *Formica polyctena* (Szczuka and Godzińska 2008). As demonstrated by earlier research of Szczuka and Godzińska (1997, 2000, 2004a, b), ants of that species kept in relatively small worker groups cease

to respond to dead insect prey by performing complete sequences of predatory/scavenging behavior terminated by prey retrieval to the nest. This behavioral modification is, however, reversible and may be reduced or even completely disappear if workers kept in a small group (about 25 individuals) are subjected to chronic oral administration of octopamine (Szczuka and Godzińska 2008). All these data taken together (Boulay et al. 1999b, 2000b; Korczyńska et al. 2005; Szczuka and Godzińska 2008; Vander Meer et al. 2008) suggest strongly that octopamine reduces in ants various behavioral effects of various forms of social deprivation: complete social deprivation, queen deprivation and reduction of worker group size.

However, yet another study devoted to complex interrelationships between social isolation, trophallaxis, and octopamine levels in ant brains (Wada-Katsumata et al. 2011) brought about different results. In that study workers of *Formica japonica* that had been subjected to 2 day social isolation had significantly higher brain octopamine levels than both control (non-isolated) workers and ants isolated for only 1 h. However, if ants isolated during 2 days were allowed to interact with two nestmates before the measurements of their brain octopamine levels, no significant differences were discovered between control ants, ants isolated during 1 h, and ants isolated during 2 days. In other words, brain octopamine levels of workers of *F. japonica* increased as a consequence of social isolation, but decreased again as a consequence of social interactions with nestmates involving trophallaxis and allogrooming.

Neurochemical treatments applied to carpenter ants before the start of dyadic nestmate reunion tests also included abdominal injections of three other biogenic amines: serotonin (Boulay et al. 2000b), dopamine (Korczyńska et al. 2005), and tyramine (Korczyńska et al. 2005). However, none of these compounds exerted any significant effect either on trophallactic behavior, or on other social contacts observed during dyadic nestmate reunion tests. This was surprising, as in the mediation of behavior of social insect serotonin often acts in an antagonistic way with respect to octopamine (Erber et al. 1993; Pribbenow and Erber 1996). Both serotonin and dopamine are also known to play a crucial role in the mediation of ant aggressive behavior (Kostowski 1994; Szczuka et al. 2013). Lastly, tyramine, a metabolic precursor of octopamine, also widespread in invertebrates and considered to represent a functional equivalent of adrenaline, is also known to act as a modulator of be-

havior and metabolism exerting antagonistic effects with respect to octopamine (Roeder 2003; Roeder et al. 2005). In the discussed study of Korczyńska et al. (2005) behavioral effects of octopamine and tyramine were never antagonistic, and were even strikingly similar in the case of several analyzed behavioral categories. However, only octopamine administration influenced significantly both trophallaxis and other social contacts observed during that experiment. Tyramine did not exert any significant effects on ant social behavior.

## BEHAVIOR OF NON-TROPHALLACTING ANTS DURING DYADIC NESTMATE REUNION TESTS CARRIED OUT AFTER SOCIAL ISOLATION

Trophallaxis is encountered in the majority of ant species, but not in all ants (Hölldobler and Wilson 1990; Cybulska and Godzińska 1999; Mazurkiewicz et al. 2015). In particular, trophallaxis is absent in some myrmicine ants from arid habitats, including harvester ants from the genera *Pogonomyrmex* and *Messor*, and the ants of the genus *Aphaenogaster* (Wilson and Eisner 1957; Delage 1968; Delage and Jaisson 1969; Lenoir et al. 2001a, b; Ichinose et al. 2005).

Lenoir et al. (2001a) investigated the behavior of non-trophallacting workers of *Aphaenogaster senilis* reintroduced to their mother nests after various periods of social isolation (2, 4, 6, 8, 10, 15 and 20 days), and the effects of duration of social isolation on profiles of cuticular hydrocarbons present on their body surface and in their post-pharyngeal glands. Both these categories of cuticular hydrocarbon profiles showed progressive changes as a result of social isolation. As a consequence, ants that had been subjected to relatively short isolation periods (2-10 days) induced friendly social contacts (mainly allogrooming) in their resident nestmates, but if they had been subjected to longer isolation periods (15-20 days), they were attacked. This experiment also revealed that in non-trophallacting workers of *A. senilis* allogrooming represents the major way of transfer of nestmate recognition cues. That last conclusion was fully confirmed by Lenoir et al. (2001b) who used radioactive tracers to study the transfer of radiolabeled cuticular hydrocarbons between nestmates of *A. senilis* during both dyadic nestmate reunion tests and group tests consisting of a confrontation of a donor ant with 10 recipients.

Wagner-Ziemka et al. (2006) applied dyadic nestmate reunion tests carried out after 3 days of social isolation to study in detail the behavior of workers of *A. senilis*

taken from two colonies separated by about 40 km. In this study, too, social contacts observed during the tests involved mainly various forms of allogrooming and antennal contacts. Allogrooming was, however, less frequent than expected and in the case of one of the tested colonies was observed only during a half of the tests. Moreover, behavior of the tested ants showed a surprisingly large number of significant inter-colony differences. Among others, ants from one of the tested colonies much more frequently engaged in resting behavior and licking of the inner walls of the set of two test tubes in which they were confined during the test. That last behavior might have been triggered by chemical cues left there by interacting nestmates.

DYADIC NESTMATE REUNION TESTS IN THE RESEARCH ON BEHAVIORAL POLYMORPHISM AND DIVISION OF LABOR IN ANT COLONIES

Dyadic nestmate reunion tests were also applied to study behavioral differences between nestmates from the same colony (WAGNER-ZIEMKA *et al.* 2008; MAZURKIEWICZ *et al.* 2016: *Camponotus maculatus,* a highly polymorphic African carpenter ant species; KORCZYŃSKA *et al.* 2014: *F. polyctena,* a monomorphic species belonging to the group of the red wood ants). Colonies of polymorphic ants contain minor workers (minors) with relatively small bodies and heads, major workers (majors) with large bodies and disproportionately large heads, and sometimes also intermediate forms, media workers (HÖLLDOBLER and WILSON 1990; WILSON 1971; MAZURKIEWICZ *et al.* 2015, 2016). However, in colonies of both monomorphic and polymorphic ants also exists division of labor related to worker age and/or degree of its behavioral and physiological maturation, the so called age or temporal polyethism. Young workers engage first in inside-nest (intranidal) activities and usually act as nurses taking care of the queen(s) and developing brood. As they age, they switch to outside-nest (extranidal) activities and become foragers (WILSON 1971; HÖLLDOBLER and WILSON 1990, 2009; WNUK 2013; MAZURKIEWICZ *et al.* 2015; see also SZCZUKA *et al.* 2019, this issue of KOSMOS). However, foragers may return to intranidal activities and become the so called reverted nurses in response to modifications of social context such as exposure to brood in absence of younger workers acting as nurses. This process, the so called behavioral reversion, was already extensively studied in various ants (EHRHARDT 1931; DOBRZAŃSKA 1959; LENOIR 1979a, b; SORENSEN *et al.* 1984; MC-DONALD and TOPOFF 1985; GODZIŃSKA 2006; WNUK and GODZIŃSKA 2006; WNUK *et al.*

2011; WNUK 2013; KORCZYŃSKA *et al.* 2014; BERNADOU *et al.* 2015; SYMONOWICZ *et al.* 2015; DUSSUTOUR *et al.* 2016; see also SZCZUKA *et al.* 2019, this issue of KOSMOS).

WAGNER-ZIEMKA *et al.* (2008) and MAZURKIEWICZ *et al.* (2016) compared the behavior of intranidal versus extranidal workers of the African carpenter ant species *Camponotus maculatus* during dyadic nestmate reunion tests carried out after 48 h of social isolation. Categories of workers compared in these experiments included minor nurses and foragers (WAGNER-ZIEMKA *et al.* 2008), and intranidal and extranidal major workers (MAZURKIEWICZ *et al.* 2016). All workers investigated in these two studies were taken from the same colony.

The behavior of minor nurses and minor foragers of *C. maculatus* showed numerous differences. Nurses showed higher propensity to engage in some forms of social contacts (mostly antennal contacts and trophallaxis and, to a lesser degree, also allogrooming) and in some forms of resting behavior. At the same time, they showed lower propensity to engage in behavior patterns displayed in response to various elements of their physical environment (WAGNER-ZIEMKA *et al.* 2008). Similar differences between intranidal and extranidal workers were discovered also in major workers of *C. maculatus*: intranidal majors showed higher propensity for resting behavior and lower propensity for behavioral responses to elements of physical environment than extranidal ones. However, in contrast to minor nurses and foragers of *C. maculatus*, intranidal and extranidal majors of that species did not differ with respect to propensity for locomotion, self-grooming and social behavior. In particular, longer trophallactic interactions had relatively high rate of occurrence during the tests with minor nurses and both intranidal and extranidal majors, but much lower rate of occurrence during the tests with minor foragers. In other words, the transition from intranidal to extranidal tasks seems to be accompanied by decreased propensity for trophallaxis only in the case of minors of *C. maculatus*. Majors of that species retain high propensity for trophallaxis also after having switched to extranidal tasks (MAZURKIEWICZ *et al.* 2016).

KORCZYŃSKA *et al.* (2014) compared the behavior of five categories of workers of the red wood ant *F. polyctena*: callows (newly eclosed intranidal workers), nurses (relatively young workers collected from the ant mound and then kept together with brood and colony foragers), colony foragers (ants that had been collected from the ant mound and then kept together with brood and nurses in laboratory nests in which they acted as forag-

ers), trail foragers (collected from the trails and then kept in isolation from nurses and brood), and reverted nurses (workers that had already switched to outside-nest activities, but then returned back to the role of the nurse). The reverted nurses were experimentally created from trail foragers by exposing them to brood in absence of ordinary nurses. Before the test all tested workers were subjected only to very short social isolation (15 min) to avoid possible disruption of the process of behavioral reversion in the reverted nurses as a consequence of their deprivation from contacts with brood.

Behavioral differences between five categories of ants investigated by Korczyńska et al. (2014) were related both to their age and their past and present behavioral specialization. Not surprisingly, the youngest ants, newly eclosed callows, were the least active. Nurses, relatively young workers that participated still only in intranidal tasks, usually behaved in a way intermediate with respect to behavior of callows and the ants that had already passed the transition to extranidal tasks. Reverted nurses behaved similarly as both classes of foragers and differently than nurses with respect to numerous behavior patterns ranging from immobility to agonistic behavior and non-aggressive social behavior. However, behavioral variables quantifying many other traits of behavior of reverted nurses took intermediate values with respect to both nurses and foragers. Only a few traits of behavior were closely similar in both ordinary and reverted nurses. This implies that behavioral reversion of workers of *F. polyctena* does not consist of the return of foragers to fully nurse-like behavior. Lastly, the behavior of colony and trail foragers showed both similarities and numerous differences. This is not surprising, as two groups of foragers differed with respect to origin (mound versus trails), present social environment (presence vs absence of contacts with nurses and brood), and probably also worker age (trail foragers were presumably older than colony foragers).

The results of that study also support the hypothesis that honeybee and ant behavioral reversion show qualitative differences (Lenoir 1979a; Sorensen et al. 1984; see also Szczuka et al. 2019, this issue of KOSMOS). As shown by Huang and Robinson (1996), deprivation of honeybee foragers from contacts with younger workers acting as nurses may induce behavioral and morphological phenotype changes characteristic for behavioral reversion. However, deprivation from contacts with younger workers did not result in the induction of nurse-like behavior in foragers of *F. polyctena*.

## DYADIC NESTMATE REUNION TESTS NOT PRECEDED BY SOCIAL ISOLATION IN THE RESEARCH ON ANT TROPHALLACTIC BEHAVIOR

Dyadic nestmate reunion tests were also used to study ant trophallactic exchanges not induced by social isolation. Ants used as subjects in that research belonged to the carpenter ant species *Camponotus vagus* (Bonavita-Cougourdan et al. 1979; Bonavita-Cougourdan and Gavioli 1981; Bonavita-Cougourdan 1983; Bonavita-Cougourdan and Morel 1984a, b, 1985, 1986, 1988; Morel 1986). Two interacting workers either had the same behavioral specialization (forager) (Bonavita-Cougourdan and Gavioli 1981; Bonavita-Cougourdan 1983; Bonavita-Cougourdan and Morel 1986, 1988), or differed with respect to age (callow or mature worker) (Bonavita-Cougourdan and Morel 1984a, b, 1985, 1988; Morel 1986) and/or behavioral specialization (nurse versus forager) (Bonavita-Cougourdan and Morel 1984a, b, 1985; Morel 1986). A large part of these experiments involved the analysis of antennal communication between workers of *C. vagus* during trophallactic contacts taking place during dyadic nestmate reunion tests (Bonavita-Cougourdan 1983; Bonavita-Cougourdan and Morel 1984a, b, 1985; Morel 1986). According to Lenoir (1982), antennal movements accompanying trophallactic exchanges between workers of *Myrmica rubra* transmit much less information than some other communication systems used by social insects, such as honeybee dances. Ant antennal communication cannot thus be involved in the transfer of precise, detailed information and acts rather as modulatory communication that can only mediate activity level of the partner ant. The results of the experiments with *C. vagus* supported fully that conclusion (Bonavita-Cougourdan 1983, Bonavita-Cougourdan and Morel 1984b). However, antennal movements accompanying trophallaxis showed interesting differences related to age and/or behavioral specialization of interacting workers (Bonavita-Cougourdan and Morel 1984a, b, 1985; Morel 1986). In particular, the behavior of mature donor ants differed as a function of their behavioral specialization (nurse versus forager), but only during trophallactic exchanges with mature receivers. That effect was not observed if a mature donor engaged in trophallaxis with an immature (callow) receiver. Moreover, during trophallactic exchanges with callows antennal activity of mature donors was more variable and its organization was different than during trophallaxis with mature

receivers (BONAVITA-COUGOURDAN and MOREL 1984a, 1985; MOREL 1986).

BONAVITA-COUGOURDAN *et al.* (1979) also invented an interesting method of analysis of liquid flow transmitted between two ants engaged in trophallaxis. That method involves measurements of radioactivity emitted by the radioactive isotope of gold (Au$^{198}$) added to honey offered to the donor before its dyadic encounter with the receiver. The use of that method led, among others, to a surprising discovery that the direction of food flow between two foragers of *C. vagus* may be repeatedly reversed during the same act of trophallactic behavior. Workers that practised such food flow reversals also showed higher propensity to engage in trophallaxis, and were more aggressive toward non-nestmate conspecifics (BONAVITA-COUGOURDAN and GAVIOLI 1981; BONAVITA-COUGOURDAN and MOREL 1988).

## DYADIC NESTMATE REUNION TESTS IN THE RESEARCH ON ANT AGGRESSIVE BEHAVIOR

### GENERAL REMARKS

Dyadic nestmate reunion tests were also carried out as control tests in numerous studies investigating ant aggressive behavior by means of dyadic aggression tests. These studies yielded interesting results extending our knowledge about aggressive behavior of ants from the genus *Formica* including *Formica lugubris* (LE MOLI and PARMIGIANI 1982; LE MOLI *et al.* 1983b, 1984; PARMIGIANI and LE MOLI 1987), *F. polyctena* (LE MOLI and MORI 1986; PARMIGIANI and LE MOLI 1987; BEYE *et al.* 1997; SZCZUKA *et al.* 2013), *Formica pratensis* (BEYE *et al.* 1998) and *Formica rufa* (LE MOLI *et al.* 1982, 1983b, 1984; PARMIGIANI and LE MOLI 1987; KLEINEIDAM *et al.* 2017). Other ant species tested in these studies included two other formicine species, *Polyrhachis laboriosa* (MERCIER *et al.* 1997) and *Cataglyphis iberica* (DAHBI and LENOIR 1998), and myrmicine species *Acromyrmex octospinosus* (JUTSUM *et al.* 1979), *Leptothorax nylanderi* and *Leptothorax gredleri* (HEINZE *et al.* 1996), and *A. senilis* (ICHINOSE *et al.* 2005).

### THE EFFECTS OF ADMINISTRATION OF BIOGENIC AMINES ON BEHAVIOR OF ANTS DURING DYADIC NESTMATE REUNION TESTS NOT PRECEDED BY SOCIAL ISOLATION

SZCZUKA *et al.* (2013) investigated behavioral consequences of acute administration of four biogenic amines (dopamine, serotonin, octopamine and tyramine) on behavior of workers of the red wood ant *F. polyctena*

during dyadic confrontations with four categories of opponents: a nestmate, a non-nestmate conspecific ant, an allospecific ant (a worker of *Formica fusca*), and a potential prey, a small nymph of the house cricket (*Acheta domesticus*). As revealed by that experiment, during dyadic confrontations of foragers of *F. polyctena* with nestmate or non-nestmate conspecifics the rate of occurrence of specific behavior patterns showed very little differences irrespectively of the type of the opponent and neurochemical treatment received by the tested ant. In all these situations overt aggression was almost absent, and interactions of two opponents involved mostly ritualized aggression (open-mandible threats) and friendly social behavior patterns including frequent trophallactic exchanges. Interestingly, octopamine, known to reduce isolation-induced trophallaxis (BOULAY *et al.* 1999b, 2000a; KORCZYŃSKA *et al.* 2005), did not reduce trophallactic behavior of workers of *F. polyctena* during dyadic confrontations with conspecifics that had not been preceded by social isolation.

Other biogenic amines used in that study (dopamine, serotonin and tyramine) also did not exert any influence on trophallactic behavior observed during dyadic confrontations between nestmates. However, both serotonin and dopamine exerted inhibitory effects on trophallactic contacts observed during dyadic confrontations between non-nestmate conspecifics.

Finally, all four amines used in that study exerted inhibitory effects on allo-grooming observed during dyadic confrontations with nestmates, and serotonin and tyramine exerted inhibitory effects also on antennal contacts between nestmates (SZCZUKA *et al.* 2013; see also SZCZUKA *et al.* 2019, this issue of KOSMOS).

## DYADIC NESTMATE RESCUE TESTS IN THE RESEARCH ON ANT PRO-SOCIAL BEHAVIOR

### ANT RESCUE BEHAVIOR

Pro-social behavior is defined as actions that are intended to benefit another individual/other individuals (BARTAL *et al.* 2011, 2014; UENO *et al.* 2019). One of the most interesting subcategories of risky pro-social behavior has been called the rescue behavior (CZECHOWSKI *et al.* 2002). Rescue behavior is defined as a social interaction during which one individual, the victim, is endangered, and another individual, the rescuer, places itself at risk of endangerment by engaging in rescue attempts. The behavior of the rescuer

should also be generally suited to the circumstances, and should not be inherently rewarding or beneficial to the rescuer (Nowbahari and Hollis 2010; Hollis and Nowbahari 2013a).

The research on ant rescue behavior was carried out both in the field and laboratory conditions with the use of two main bioassays: antlion larva capture bioassay, in which rescue behavior of the tested ants is elicited by stimuli emitted by a victim ant captured by a predatory antlion larva (Czechowski et al. 2002; Miler 2016; Miler et al. 2017a, b; Taylor et al. 2013), and artificial snare (entrapment) bioassay, in which rescue behavior of potential rescuers is elicited by stimuli emitted by a victim ant entrapped in an artificial snare (Nowbahari et al. 2009, 2012, 2016; Hollis and Nowbahari 2013b; Taylor et al. 2013; Cichoń 2017; Duhoo et al. 2017; Kuraszkiewicz 2017; Miler et al. 2017a, b; Miler and Kuszewska 2017). Recently, Uy et al. (2019) used a modified version of the entrapment bioassay in which victim ants, workers of the weaver ant *Oecophylla smaragdina*, were experimentally immobilized by being wrapped in spider silk. Yet another type of ant rescue behavior was reported in the termite-hunting ant *Megaponera analis*: workers of that species were observed to transport injured nestmates back to the nest and to engage in their intense allogrooming that facilitated wound healing (Frank et al. 2017, 2018). Workers of the harvester ant *Veromessor pergandeyi* were also observed to free nestmates ensnared in spider webs, transport them to the nest, and groom away their silk bindings (Kwapich and Hölldobler 2019).

Ant rescue behavior is most frequently investigated by means of a test during which five freely moving ants act as potential rescuers of a nestmate entrapped in an artificial snare (bound to a piece of filter paper that helps to capture pheromones emitted by the victim ant). Such tests were applied to study rescue behavior of workers of *Cataglyphis cursor* (Nowbahari et al. 2009, 2012, 2016; Duhoo et al. 2017) and *F. polyctena* (Cichoń 2017; Kuraszkiewicz 2017). Artificial snare bioassay was also used in the field, close to ant nests, to study rescue behavior of workers from several Mediterranean ant species (*Cataglyphis floricola, Lasius grandis, Aphaenogaster senilis, Messor barbarus* and *Messor marocanus*) (Hollis and Nowbahari 2013b), and North American ant species *Tetramorium* species E and *Prenolepis imparis* (Taylor et al. 2013).

## DYADIC NESTMATE RESCUE TESTS IN THE RESEARCH ON ANT RESCUE BEHAVIOR

Since several years dyadic nestmate rescue tests consisting of a confrontation of a single freely moving ant with a single endangered nestmate (captured by a predator or entrapped in an artificial snare) were also increasingly frequently used in the research investigating various aspects of ant rescue behavior (Taylor et al. 2013; Miler 2016; Miler et al. 2017a, b; Miler and Kuszewska 2017). These studies documented many interesting phenomena. Among others, Taylor et al. (2013) found out that a single worker of *Tetramorium* species E may successfully rescue its nestmate captured by an antlion larva. Taylor et al. (2013) also observed an interesting sequence of events during which the victim ant was released by the antlion as a consequence of a successful rescue action, but then the rescuer was in turn grabbed by the predator, and the original victim, now freed, began to engage in rescue behavior. This observation demonstrated that rescue actions may be promptly reciprocated.

A series of experiments of Miler and his coworkers explored various factors influencing nestmate rescue behavior of workers of *F. cinerea* during bioassays with antlion larvae (Miler 2016) and artificial snares (Miler and Kuszewska 2017), and during both these types of bioassays (Miler et al. 2017a, 2017b). These studies revealed, among others, that workers of *F. cinerea* with life expectancy experimentally shortened by exposure to carbon dioxide less efficiently triggered rescue behavior of their nestmates during the bioassays with antlion larvae (Miler 2016), and less readily engaged in rescue behavior in response to a nestmate entrapped in an artificial snare (Miler et al. 2017b). These findings were surprising, as in ants shortened life expectancy often leads to increased readiness to engage in risky tasks (Moroń et al. 2008, 2012). Therefore, it might have been expected that ants with shortened life expectancy will show higher propensity to engage in risky rescue behavior. However, many studies reported, that in ants shortened worker life expectancy may also lead to social withdrawal (Hölldobler and Wilson 1990; Korczyńska 2001; Ugelvig and Cremer 2007; Heinze and Walter 2010; Bos et al. 2011; Diez et al. 2015; Leclerc and Detrain 2017). The findings of Miler (2016) and Miler et al. (2017b) can thus be interpreted, too, in terms of the induction of social withdrawal. If so, these findings suggest that experimental studies of ant rescue behavior may prove to be of importance for comparative research devoted to causal factors underlying social withdraw-

al, and that they perhaps may even shed light on biological roots of autism spectrum disorders. This last possibility is very intriguing, as some autism-related genes were already found in the genome of the honey bee, a social insect closely related to the ants (Shpigler *et al.* 2017).

Dyadic nestmate rescue tests with the use of an artificial snare were also used by Miler and Kuszewska (2017) to check if rescue behavior of workers of *F. cinerea* may be released in response to secretions of mandibular glands, known to act as alarm pheromones in many ants (Wilson 1958; Bradshaw *et al.* 1975; Ali *et al.* 1990). However, their experiments brought about negative results. Rescue behavior directed by workers of *F. cinerea* towards nestmates with impaired mandibular gland communication (treated with paint over the mandibles) did not differ from responses to untreated individuals or to sham-treated ants with paint applied on the thorax. Moreover, the tested workers did not engage in rescue behavior in response to dummy ants coated with the contents of crushed mandibular glands. Interestingly, two other studies devoted to the role of mandibular gland secretions in the elicitation of ant rescue behavior brought about different results. Mandibular gland secretions proved to be involved in the elicitation of nestmate rescue behavior in the termite-hunting ant *M. analis* (Frank *et al.* 2017), and in the harvester ant *V. pergandeyi* responding to nestmates ensnared in spider silk (Kwapich and Hölldobler 2019).

In yet another study Miler *et al.* (2017a) used both types of dyadic nestmate rescue tests (with antlion larvae and with artificial snares) to investigate the impact of ecological niche on presence/absence of rescue behavior in behavioral repertoires of six ant species from both tropical and temperate regions (*Camponotus korthalsiae, Anoplolepis gracilipes* and *Iridomyrmex anceps* from Borneo, and *Myrmica ruginodis, F. polyctena* and *F. cinerea* from Poland). Rescue behavior proved to be present in behavioral repertoires of sand-dwelling ants exposed to the risk of being captured by antlion larvae and of being trapped in a collapsed chamber of their ground nest (*F. cinerea*), and ants inhabiting forest ground-cover that are also frequently exposed to various types of entrapment (*I. anceps* and *F. polyctena*). However, no rescue attempts were observed in ants from species associated with open plains, nesting in hardened soils and foraging largely on herbaceous plants (*A. gracilipes* and *M. ruginodis*), nor in ants living in close mutualistic relationship with their host plant (*C. korthalsiae*).

Absence of rescue behavior in behavioral repertoires of these three ant species was most probably related to very low risk of entrapment faced by these ants in their natural environment.

## DYADIC REUNION TESTS AND DYADIC RESCUE TESTS IN BEHAVIORAL RESEARCH ON VERTEBRATES

Tests consisting of a confrontation of two individuals were also used in the research on social and pro-social behavior of rodents. In the present paper we provide only a few examples of such studies. Thus, D'Amato and Pavone (1993, 1996) and D'Amato (1998) showed that dyadic encounters between sibling male mice taking place after a relatively long separation (about 2 months) induce the release of opioids that may act as a proximate reward mechanism for kin selection. This effect manifests itself as opioid-dependent increase in pain threshold that can be antagonized by administration of naloxone, an antagonist of opioid receptors.

Another test consisting of a confrontation of two individuals, the so called Social Proximity test, was used in the research investigating the behavior of the BTBR T+tf/J (BTBR) mice, an inbred mouse strain with low sociability phenotype resembling the first diagnostic symptoms of autism: deficits in reciprocal social interactions (Defensor *et al.* 2011; Meyza *et al.* 2015). As shown by these experiments, BTBR mice avoid reciprocal frontal orientations, among others by crawling under another mouse. That behavior resembles gaze aversion, a fundamental predictor of autism. Further research confirmed that unavoidable social interactions are highly aversive for BTBR mice: exposure to social proximity induces strong activation of periaqueductal brain regions involved in the mediation of defensive behavior.

Dyadic encounters of a freely moving potential rescuer with an entrapped victim were also used in the research investigating rescue behavior and empathy in rodents, rats (Bartal *et al.* 2011, 2014, 2016; Sato *et al.* 2015; Blystad *et al.* 2019; Carvalheiro *et al.* 2019; Yamagishi *et al.* 2019) and mice (Ueno *et al.* 2019). During the test a freely moving individual had to liberate another individual trapped in a restrainer (Bartal *et al.* 2011, 2014, 2016; Blystad *et al.* 2019; Carvalheiro *et al.* 2019), and in some cases also additionally water-soaked (Sato *et al.* 2015; Yamagishi *et al.* 2019).

## CONCLUSIONS

We hope that this review demonstrated that even relatively simple behavioral bioassays such as dyadic nestmate reunion tests may bring about interesting data. Dyadic nestmate reunion tests applied to the study of ant social behavior yielded and continue to yield a wealth of important findings extending our knowledge about behavioral effects of social isolation, causal factors involved in the mediation of various social contacts, nestmate recognition, role of biogenic amines in the mediation of ant social behavior, behavioral polymorphism and division of labor encountered in ant societies, ant behavioral ontogeny, diversity and variability of ant behavior, role of ecological factors in the evolution of ant behavior, and biological roots of aggressive and pro-social behavior.

### Summary

The main aim of this review was to show that even relatively simple behavioral bioassays may bring about important findings. Dyadic nestmate reunion test used in the research on social insect behavior consists of a confrontation of two nestmates and may be preceded by various treatments such as social isolation or administration of neuroactive compounds. Dyadic nestmate rescue test consists of a confrontation between a freely moving ant and its endangered nestmate. Dyadic nestmate reunion tests continue to yield a wealth of findings extending our knowledge on behavioral effects of social isolation, causal factors involved in the mediation of social contacts, nestmate recognition, role of biogenic amines in the mediation of ant social behavior, behavioral polymorphism and division of labor in ant societies, ant behavioral ontogeny, diversity and variability of ant behavior, role of ecological factors in the evolution of ant behavior, and biological roots of aggressive and pro-social behavior.

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TEST SPOTKANIA DWÓJKI TOWARZYSZEK W BADANIACH ZACHOWAŃ SPOŁECZNYCH MRÓWEK

Streszczenie

Głównym celem tego przeglądu było ukazanie, że nawet stosunkowo proste testy behawioralne mogą umożliwić
uzyskanie ważnych wyników. Test spotkania dwójki towarzyszek stosowany w badaniach zachowań społecznych mró-
wek polega na konfrontacji dwóch towarzyszek z tej samej kolonii i może być poprzedzany różnymi zabiegami, taki-
mi jak izolacja społeczna lub podawanie związków neuroaktywnych. Dwójkowy test badający zachowania ratunkowe
polega na konfrontacji swobodnie poruszającej się mrówki z towarzyszką znajdującą się w niebezpieczeństwie. Testy
spotkania dwójki towarzyszek umożliwiają uzyskiwanie ogromnego bogactwa wyników poszerzających naszą wiedzę o
behawioralnych efektach izolacji społecznej, uwarunkowaniach przyczynowych różnych typów kontaktów społecznych,
rozpoznawaniu towarzyszek z tej samej kolonii, roli amin biogennych w sterowaniu zachowaniami społecznymi mró-
wek, polimorfizmie behawioralnym i podziale pracy w społeczeństwach mrówek, ontogenezie behawioralnej mrówek,
różnorodności i zmienności zachowań mrówek, roli czynników ekologicznych w ewolucji zachowań mrówek, oraz bio-
logicznych korzeniach zachowań agresywnych i prospołecznych.

Słowa kluczowe: behawior, Formicidae, mrówki, test spotkania dwójki towarzyszek, zachowania społeczne