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The effects of serotonin, dopamine, octopamine and tyramine on behavior of workers of the ant *Formica polyctena* during dyadic aggression tests

Anna Szczuka[1], Julita Korczyńska[1], Andrzej Wnuk[1], Beata Symonowicz[1], Anna Gonzalez Szwacka[1], Paweł Mazurkiewicz[1,2], Wojciech Kostowski[3], and Ewa Joanna Godzińska[1]*

[1]Laboratory of Ethology, Department of Neurophysiology, Nencki Institute of Experimental Biology, Warsaw, Poland, *Email: e.godzinska@nencki.gov.pl; [2]College of Inter-Faculty Individual Studies in Mathematics and Natural Sciences, University of Warsaw, Warsaw, Poland; [3]Department of Pharmacology and Physiology of the Nervous System, Institute of Psychiatry and Neurology, Warsaw, Poland

We investigated the effect of injections of four biogenic amines (serotonin, dopamine, octopamine and tyramine) on behavior patterns displayed by workers of the red wood ant *Formica polyctena* during dyadic confrontations with four types of opponents: a nestmate, an alien conspecific, an allospecific ant (*Formica fusca),* and a potential prey, a nymph of the house cricket (*Acheta domesticus*). Significant effects of biogenic amine administration were observed almost exclusively in the case of confrontations with allospecific opponents. Serotonin treatment exerted stimulatory effects on behavior patterns involving physical aggression (biting accompanied by gaster flexing, dragging and formic acid spraying), but these effects were relatively weak and/or documented by indirect evidence. Dopamine administration exerted a stimulatory effect on open-mandible threats directed by *F. polyctena* to *F. fusca* and to cricket nymphs, and on biting behavior directed to cricket nymphs. Surprisingly, octopamine treatment did not exert significant effects on aggressive behavior of the tested ants. Tyramine administration exerted a suppressing effect on threatening behavior directed to *F. fusca*, but led to shortening of the latencies to the first open-mandible threat during the tests with cricket nymphs. Biogenic amine administration also influenced non-aggressive behavior of the tested ants. Our findings confirmed the role of serotonin and dopamine in the mediation of ant aggressive behavior and documented for the first time significant effects of tyramine treatment on ant aggressive behavior. We also demonstrated that not only specific patterns of ant aggressive behavior, but also behavioral effects of biogenic amine treatments are as a rule strongly context-dependent.

Key words: serotonin, dopamine, octopamine, tyramine, aggressive behavior, *Formica polyctena*, *Formica fusca*, *Acheta domesticus*

INTRODUCTION

Biogenic amines, neuroactive compounds acting as neurotransmitters, neuromodulators and neurohormones, play a very important role in the mediation of insect physiology and behavior, including both aggressive and non-aggressive inter-individual interactions (Roeder et al. 2003, Roeder 2005, Scheiner et al. 2006, Farooqui 2007, Gauthier and Grünewald 2012). The involvement of biogenic amines in the mediation of various forms of insect aggressive behavior was first documented in the ants (Kostowski and Tarchalska 1972, Kostowski et al. 1972) and then was also extensively studied in many other solitary and social insect species including the fruit flies of the genus *Drosophila,* the cricket *Gryllus bimaculatus*, and the honeybee (*Apis mellifera*) (Adamo et al. 1995, Robinson et al. 1999, Baier et al. 2002, Stevenson et al. 2005, Zwarts et al. 2012).

Ants (Formicidae, Hymenoptera) are particularly suitable as model organisms for the research on neurobiological basis of aggressive behavior, as they are highly social and show a rich repertoire of behavior patterns expressed in a wide range of contexts includ-

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Correspondence should be addressed to E.J. Godzińska
Email: e.godzinska@nencki.gov.pl

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ing confrontations with nestmates, conspecific ants from different nests, and various allospecific enemies, competitors, intruders, potential slaves and potential prey (Mabelis 1979, Hölldobler and Wilson 1990). Neurochemical processes underlying ant aggressive behavior were extensively studied by Kostowski and his team in workers of the common species of mound-building red wood ants, *Formica rufa* (Kostowski 1966, 1968, Kostowski and Tarchalska 1972, Kostowski et al. 1966, 1972, 1975a,b, Tarchalska et al. 1975). The main bioassay used in these experiments consisted of a short (3–15 min) confrontation between a small group of ants (10–15 individuals) and a large beetle (*Geotrupes* sp.). Aggressive responses directed by the tested ants to the beetle and to other nestmates participating in that group confrontation were considered to represent the expression of, respectively, inter-specific and intra-specific (intra-colony) aggressive behavior (Kostowski 1966, 1968, Kostowski and Tarchalska 1972, Kostowski et al. 1972, 1975a,b, Tarchalska et al. 1975). Intra-colony aggressive interactions were also investigated by means of another bioassay consisting of a group confrontation between 10 nestmates subjected to antennalectomy to induce mutual aggressiveness (Kostowski et al. 1975a, Tarchalska et al. 1975). The relationships between neurochemical and behavioral processes taking place during aggressive confrontations of the tested ants with nestmates and allospecific opponents were investigated by means of a wide spectrum of research techniques including the administration of neuroactive compounds in food (Kostowski 1966, 1968, Kostowski et al. 1966) and by means of injections into abdominal cavity (Kostowski 1966, 1968, Kostowski et al. 1966, 1972, 1975b, Kostowski and Tarchalska 1972), and the measurements of brain contents of specific neurochemicals (Kostowski et al. 1972, 1975a,b, Tarchalska et al. 1975).

As revealed by these experiments, neurochemical processes underlying ant aggressive behavior show both differences and similarities in respect to vertebrates. In particular, biogenic amines serotonin (5-HT) and dopamine (DA) known to play an important role in the mediation of aggressive behavior of vertebrates (Olivier 2004, De Almeida et al. 2005, Miczek and Fish 2005a,b, Ryding et al. 2008) were also found to be critically involved in the mediation of ant aggressive behavior (Kostowski and Tarchalska 1972, Kostowski et al. 1972, 1975a,b, Tarchalska et al. 1975). Participation of workers of *F. rufa* in a group confrontation with a beetle and a group confrontation between antennalectomized nestmates was followed by a similar significant increase of brain levels of serotonin (Kostowski et al. 1975a, Tarchalska et al. 1975). However, abdominal injections of serotonin and its metabolic precursor 5-hydroxytryptophan (5-HTP) carried out before a confrontation of a group of workers of *F. rufa* with a *Geotrupes* beetle increased the number of the ants fighting between themselves, but decreased the number of the ants attacking the beetle (Kostowski and Tarchalska 1972, Kostowski et al. 1972). Abdominal injections of dopamine (DA) and drugs increasing DA levels in the brain such as DOPA (an immediate precursor of dopamine) and diethyldithiocarbamate (DDTC) (a potent inhibitor of dopamine hydroxylase) also exerted a strong stimulatory effect on aggressive responses of workers to *F. rufa* to their nestmates during a group confrontation with a beetle, but had no significant effect on their aggressive responses to the beetle or even reduced their level (Kostowski et al. 1975b). It was therefore not surprising that abdominal injections of a DA antagonist haloperidol brought about opposite effects: an increase of attacks directed to the beetle with a concomitant decrease of attacks directed to other ants (Kostowski et al. 1975b).

Further research devoted to neurochemical processes underlying ant aggressive behavior confirmed that dopamine plays an important role in the mediation of ant aggressive behavior. In the study of Rioux (1983) injections of apomorphine, an agonist of DA receptors, enhanced aggressive responses of resident workers of the carpenter ant *Camponotus aethiops* to allospecific intruders, workers of *Camponotus vagus*. In particular, apomorphine exerted a strong stimulatory effect on biting behavior.

Several studies also revealed important involvement of yet another biogenic amine, octopamine (OA), in the mediation of various forms of ant aggressive behavior (Ozaki et al. 2004, Cuvillier-Hot and Lenoir 2006, Kaita et al. 2006, Szczuka and Godzińska 2008, Vander Meer et al. 2008, Aonuma and Watanabe 2012). Octopamine, a biogenic amine widespread in insects and many other invertebrates, is commonly assumed to act as a functional analogue of noradrenaline, whereas its metabolic precursor tyramine is assumed to act as a functional analogue of adrenaline (Roeder et al. 2003, Roeder 2005). In ants octopamine plays an important role among others in the mediation of social interactions between nestmates (Boulay et al. 1999, 2000, Korczyńska et al. 2005, Wada-Katsumata et al. 2011) and of various forms of aggressive behavior (Ozaki et al. 2004, Cuvillier-Hot and Lenoir 2006, Kaita et al. 2006, Szczuka and Godzińska 2008, Vander Meer et al. 2008, Aonuma and Watanabe 2012). As shown by Cuvillier-Hot and Lenoir (2006), dominant (alpha) workers of the queenless ponerine ant *Streblognathus peetersi* have higher brain OA levels than other high-ranking workers and subordinate workers. In the red imported fire ant (*Solenopsis invicta*) queen absence leads to dramatic decrease of both worker aggressiveness towards non-nestmates and worker brain OA levels. Both these effects of queen absence can be counterbalanced by chronic oral administration of octopamine (Vander Meer et al. 2008). Chronic oral octopamine treatment was also found to exert a stimulatory effect on responses of the red wood ants (*Formica polyctena*) to potential insect prey (dead houseflies) (Szczuka and Godzińska 2008). Most recently, Aonuma and Watanabe (2012) also interpreted significant differences between the levels of N-acetyloctopamine (NAcOA) in the brains of queens and workers of the Japanese wood ant *Formica japonica* Motschoulsky as an evidence for the involvement of octopaminergic system in the mediation of aggressive motivation in these ants.

However, it should also be noted that some studies devoted to unraveling of interrelationships between various biogenic amines and various forms of ant aggressive behavior brought about negative results. Thus, Hoyer and coauthors (2005) did not find any marked modifications of serotonergic and dopaminergic neurons in the central nervous system of workers of the ponerine ant *Harpegnathos saltator* as a result of participation in intra-colony aggressive interactions. Similarly, Cuvillier-Hot and Lenoir (2006) did not find significant differences between 5-HT contents in the brains of dominant (alpha), high ranking and subordinate workers of *Streblognathus peetersi.* Moreover, surprisingly, higher DA levels were detected in the brains of subordinate workers than in the brains of more aggressive dominant and high ranking workers. Lastly, octopamine treatment was found to exert a suppressing effect on aggressive behavior of active foragers of *Camponotus japonicus* responding to non-nestmates (Ozaki et al. 2004) and in workers of that species responding to non-nestmate cuticular hydrocarbons (Kaita et al. 2006). Complex interrelationships between various biogenic amines and various forms of ant aggressive behavior are thus still far from being ultimately unraveled.

The present study was carried out in order to revisit in more detail the question of the involvement of various biogenic amines in the mediation of ant aggressive behavior. We used as subjects ants of the common species of mound-building red wood ants, *Formica polyctena* Först., closely akin to *F. rufa,* a species investigated earlier by Kostowski and his coworkers (Kostowski 1966, 1968, Kostowski and Tarchalska 1972, Kostowski et al. 1966, 1972, 1975a,b, Tarchalska et al. 1975). *F. polyctena* was also used as a model species in extensive research investigating various forms of ant aggressive behavior including intra-specific aggression, aggressive interactions with other ant species and predatory aggression (among others De Bruyn and Mabelis 1972, Mabelis 1979, 1984a,b, Le Moli and Mori 1986, Savolainen and Vepsäläinen 1989, Savolainen 1990, 1991, Beye et al. 1997, Heuts et al. 2003, Czechowski and Markó 2006, Czechowski et al. 2013). Moreover, since several years we focused our research among others on proximate causal factors underlying the responses of these ants to insect prey (Szczuka and Godzińska 1997, 2000, 2004a,b, 2008) and on the role of octopamine in the mediation of their behavior (Szczuka and Godzińska 2008, Wnuk et al. 2011).

As in the experiments of Kostowski and his colleagues (Kostowski 1966, 1968, Kostowski and Tarchalska 1972, Kostowski et al. 1966, 1972, 1975b) main stress was laid on the analysis of neurochemical aspects of the investigated phenomena and behavior of the tested ants was recorded and analyzed in a less detailed way, in the present study we wanted to throw more light on the effects of biogenic amine administration on various patterns of aggressive and non-aggressive behavior displayed by workers of *F. polyctena.* Moreover, during a group confrontation of several workers with a beetle aggressive responses directed by the ants to their nestmates might have been also influenced by the stimuli related to the presence of the beetle. Therefore, this time we decided to use a different type of aggression test: a dyadic aggression test consisting of a confrontation of a single ant with a single opponent. Various types of dyadic aggression tests were used in numerous studies investigating interactions between both conspecific and allospecific ants (among others Le Moli and Parmigiani 1981, 1982, Le Moli et al. 1982, 1983, 1984, Le Moli and Mori 1986, Mori and Le Moli 1993, Dahbi et al. 1996, Beye et al. 1997, Mercier et al. 1997, Boulay and Lenoir 2001, Heuts et al. 2003, Roulston et al. 2003, Newey et al. 2010). We also decided to record the behavior of the tested ants by means of a video camcorder to facilitate its subsequent detailed analysis.

In his experiments with *F. rufa* Kostowski and his coworkers (Kostowski and Tarchalska 1972, Kostowski et al. 1972, 1975a,b, Tarchalska et al. 1975) investigated two types of aggressive behavior: intra-specific intra-colony aggression (attacks directed to nestmates) and inter-specific aggression (attacks directed to a beetle). However, it was not clear whether and to what degree that allospecific opponent represented for the ants an intruder/competitor, or a potential prey. In the present study we wanted to investigate the involvement of biogenic amines in the mediation of ant aggressive behavior in a yet more wide range of contexts. Therefore, workers of *F. polyctena* were subjected to confrontations with four categories of opponents: a nestmate, a non-nestmate conspecific, an allospecific ant (*Formica fusca* L.), and a small potential prey (a small nymph of the house cricket, *Acheta domesticus* L.). This allowed us to study aggressive behavior expressed in four main contexts: intra-specific intra-colony aggression, intra-specific between-colony aggression, inter-specific aggression triggered by a potential competitor, and inter-specific aggression triggered by a potential prey. We prefer to speak about “aggressive responses to potential prey” rather than about “predatory behavior”, as it is difficult to tell whether and to what degree the responses of *F. polyctena* to cricket nymphs encountered during dyadic aggression tests were indeed driven by internal factors underlying predatory behavior, as they might at least partly represent defensive responses triggered by the close contact with another insect taking place within a limited closed space. Moreover, the distinction between predatory behavior and other forms of aggressive behavior is particularly difficult in the case of *F. polyctena*, as during the periods of shortage of food workers of that species may attack alien conspecifics by employing exactly the same behavior patterns as when hunting other insect prey (De Bruyn and Mabelis 1972, Mabelis 1979, 1984a).

The tested ants received abdominal injections of saline solution (in the case of the control group) and of four biogenic amines (serotonin, dopamine, octopamine and tyramine) dissolved in saline solution. Whereas the first three of these biogenic amines were already known to influence ant aggressive behavior, the possible role of tyramine in the mediation of ant aggressive behavior was investigated for the first time. We decided to study the effects of the administration of this amine, too, as it is assumed to act as the functional equivalent of adrenaline in the insect nervous system, and, therefore, is increasingly frequently used in the research devoted to the role of various biogenic amines in the mediation of behavior and physiology of various insects (Roeder et al. 2003, Roeder 2005, Sasaki and Harano 2007), including ants (Korczyńska et al. 2005).

METHODS

Subjects

The experiment consisted of two identical series of tests carried out during the same season (November – beginning of December) on two successive years. During both series of tests we used as subjects workers of the red wood ant *Formica polyctena* Först. collected in the field and then kept in laboratory conditions. Ants tested during the first series of tests were collected in a mixed pine forest situated close to the village Rzeszotków (near Siedlce in central-eastern Poland; 52º16’24” N, 22º20’39” E). Ants tested during the second series of tests were collected in a similar forest situated close to the village Wólka Radzymińska (near Warsaw in central Poland; 52º25’57” N, 21º5’8” E). The collected colony fragments consisted each of a few thousands of workers taken directly from the mound together with the nest material. The colony fragment collected during the first year of the experiment contained 14 queens, but only seven of them were retained together with the collected workers. The colony fragment collected during the second year of the experiment contained no queen, but we supplemented it, too, with seven queens collected from the same nest several weeks earlier.

In the laboratory the ants were housed in artificial nests made each of three open rectangular Perspex containers (29 cm × 19 cm × 15 cm) with the walls coated with Fluon® (PTFE), a substance providing a silky smooth surface and, hence, commonly used in myrmecological research to prevent the ants from escaping from artificial nests. The containers were connected together by means of narrow plastic tubes. Two out of three interconnected boxes contained numerous large glass test tubes serving as artificial nest chambers. Each tube was filled partly with water trapped in by means of a cotton plug to provide a humidity gradient allowing the ants to choose their preferred humidity conditions. The tubes were covered from above with a sheet of aluminum foil to assure darkness. The third box served as a foraging area. The ants had constant access to water provided in small Petri dishes filled with moist cotton and to carbohydrate and protein food (honey mixed with crushed apple and sand added to make the mixture less sticky and pieces of house crickets killed by freezing and allowed to thaw at room temperature). Food was exchanged for fresh one three times a week. The nests were kept at a stable ambient temperature (22 ± 2°C) and relative humidity of the air (40–45%) and exposed to a natural rhythm of daylight and darkness supplemented with artificial white light illumination delivered at 12:12 LD.

As revealed by earlier research of our team (Szczuka and Godzińska 2000, 2004a), workers of *F. polyctena* collected in the field started to behave in a predictable way during behavioral tests employed to investigate their responses to insect prey after about 3–4 weeks of habituation to laboratory conditions. As we wanted to avoid the effects of insufficient habituation to laboratory conditions, we started to carry out our behavioral tests after a several week delay from the collection of the ants (about 7 weeks during the first part of experiment, about 4 weeks during its second part).

As demonstrated by many classical and recent studies (among others, Dobrzańska 1959, Hölldobler 1983, Nowbahari and Lenoir 1989, Sturgis and Gordon 2012), ant aggressive behavior depends strongly on worker age and/or behavioral specialization: older workers are as a rule more aggressive than younger ones and foragers are more aggressive than nurses. Therefore, we used as subjects only a subclass of foragers selected by means of the so called “fishing rod technique” used in earlier experiments of our team to identify ants responding persistently to insect prey (Szczuka and Godzińska 1997, 2004a,b, 2008). A dead housefly fixed to a thread was repeatedly lowered into the foraging area of the nest, allowing us to collect foragers responding to it by at least such persistent biting that they could be lifted up from the nest together with the fly when it was being removed.

Alien conspecifics used as opponents of the tested workers of *F. polyctena* were taken from queenless colony fragments collected in the field at a distant location from the colonies of *F. polyctena* used as a source of workers subjected to biogenic amine treatments. Each colony fragment consisted of several thousands of workers. During the first year of the experiment alien conspecifics of *F. polyctena* were collected close to Wólka Radzymińska (52º25’57” N, 21º5’8” E), at about 100 km from Rzeszotków, the site of origin of *F. polyctena* with which they were subsequently paired. During the second part of the experiment they were collected close to Dziekanów Leśny (52º20’31” N, 20º51’33” E), at about 30 km from the site Wólka Radzymińska, the site of origin of their opponents used in this part of the experiment. The sites Dziekanów Leśny and Wólka Radzymińska were also separated by a large river (Vistula). Colony fragments providing workers of *F. fusca* used as allospecific opponents in the dyadic aggression tests with *F. polyctena* were collected near Wólka Radzymińska (52º25’57” N, 21º5’8” E) on both years of the experiment. Both colony fragments were queenless and each of them consisted of several hundreds of workers. All the ants to be used as opponents during dyadic aggression tests were housed in the same laboratory in similar artificial nests, received the same food and were exposed to the same laboratory conditions as the tested workers of *F. polyctena.*

Nymphs of the house cricket (*Acheta domesticus*) used in dyadic aggression tests were also reared in the same laboratory. They were kept in large groups consisting of individuals of similar age and size. Cricket nymphs used in the tests were carefully selected to match closely the size of workers of *F. polyctena* with which they were paired during dyadic aggression tests.

Biogenic amine administration

The ants were anaesthetized on ice immediately after being withdrawn from the foraging area of their nests and received abdominal injections of 0.5 μl of 0.6% saline (control group) or of 0.5 μl of 0.08 M serotonin, dopamine, octopamine or tyramine hydrochlorides dissolved in 0.6% saline. Biogenic amines were purchased at Sigma-Aldrich Poland. The doses of biogenic amines (an equal number of mM/mg of the average worker body weight) corresponded to the dose of octopamine that provoked the most strongly expressed behavioral effects during dyadic confrontations between nestmates of the carpenter ant *Camponotus fellah* Della Torre taking place after a period of social isolation (Boulay et al. 2000). Average body weight of a worker of *F. polyctena* was estimated as 15 mg on the basis of measurements carried out by means of a microtorsion balance. The injections were carried out by means of a glass capillary tube connected with a Hamilton microsyringue. After the injection the ant was placed in a test tube (5 cm long, 13 mm in diameter) closed by a cotton plug. The interval of 1 hour between the injection and the beginning of a dyadic aggression test was chosen on the basis of literature data (Kostowski and Tarchalska 1972, Kostowski et al. 1972, 1975b, Boulay et al. 1999, 2000, Korczyńska et al. 2005). In the case of abdominal injections of dopamine the most important modifications of aggressive behavior were observed even more rapidly, after 30 min post injection, and after 1 h post injection they were already less well expressed (Kostowski et al. 1975b). However, significant modifications of aggressive behavior of workers of *F. rufa* following abdominal injections of serotonin were observed after 1 h (Kostowski and Tarchalska 1972, Kostowski et al. 1972). Similarly, Boulay and others (1999) demonstrated that abdominal injections of octopamine lead to significant increase of octopamine contents in brains of workers of the carpenter ant species *C. fellah* and that the maximum effect is attained after 1 h. Consequently, the delay of 1 h was used in two experiments investigating behavioral consequences of abdominal injections of octopamine in workers of two carpenter ant species, *C. fellah* and *C. herculeanus* L. and in both cases octopamine administration brought about significant modifications of ant behavior: partial suppression of isolation-induced trophallaxis, a specific social interaction consisting of a mutual contact of mouthparts accompanied by the exchange of liquid food and/or signal compounds involved in the nestmate recognition (Boulay et al. 2000, Korczyńska et al. 2005).

Ants acting as the opponents of the tested workers of *F. polyctena* were collected in the foraging areas of their artificial nests and placed singly in identical test tubes an hour before the test. Conspecific opponents (nestmates and non-nestmates) were marked with color paint on the previous day and returned to their nest for night. Cricket nymphs were collected from the containers housing their laboratory cultures.

Dyadic aggression tests

As already stated, the tested workers were isolated singly in small test tubes during one hour before the test. They were observed and individuals showing impaired locomotion or any other behavioral disorder were discarded.

At the start of the test the cotton plugs closing the tubes containing a worker of *F. polyctena* and its opponent were withdrawn 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 the set of two tubes containing a dyad of ants was fixed on a shelf of a vertical stand put before a digital video camcorder Sony DCR-TRV 340E to record the lateral view of the interacting ants. This method of recording of ant behavior facilitates the identification of various forms of interactions between the tested ants which are sometimes difficult to tell apart if the ants are observed from above (Cybulska et al. 2000, Korczyńska et al. 2005, Wagner-Ziemka et al. 2006, 2008). After a 10 min test the two tubes were separated and their interior parts were checked for the presence of the smell of the formic acid which would provide the evidence of the occurrence of formic acid ejection by one or both interacting ants.

In the main analysis of our data we decided to take into account the data obtained for 40 dyadic aggression tests recorded for each combination of neurochemical treatment and opponent type (5 × 4 = 20 combinations). For each such combination 20 tests were recorded during the first year of the experiment and 20 tests were recorded during its second year. However, during some tests formic acid released by the tested ants led to strongly disturbed behavior, impairment of locomotion and sometimes even to death of one or both opponents. Such tests (0–26 per experimental group) were not included in the main analysis. As during such tests the behavior of the ants was analyzed only until the appearance of the disturbed behavior, these data could be used only in a separate additional analysis (see below).

Quantification and analysis 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:

Aggressive behavior: open-mandible threat; open-mandible threat accompanied by gaster flexing; charge (a rapid lunge toward the opponent); biting; biting accompanied by gaster flexing; dragging of the opponent; fight (a combat of two opponents). Presence/absence of gaster flexing was noted because in the formicine ants gaster flexing signalizes the readiness to display formic acid spraying. Therefore, open-mandible threats and biting accompanied by gaster flexing represent more escalated forms of, respectively, threatening behavior and overt aggressive behavior.

Non-aggressive social interactions between the opponents: indifferent behavior (absence of any evident behavioral response) in respect to the opponent staying in the close vicinity of the observed ant (at a lesser distance than its approximate body length); antennal contacts with the antenna of the opponent; antennal contacts with other parts of its body; allogrooming; trophallaxis. The last two behavioral categories were not observed during the tests with cricket nymphs.

Other behavior patterns: self-grooming; locomotion; resting.

Some of these behavioral categories were also pooled for further statistical analysis (e.g. open-mandible threats accompanied and not accompanied by gaster flexing, biting accompanied and not accompanied by gaster flexing, antennal contacts with the antennae and with other parts of the body of the opponent). The results of the tests were analyzed in several alternative ways.

The overall result of each test was first quantified as an aggression score denoting the most escalated pattern of aggressive behavior observed during the test. We adopted finally the following ranking order (1–6): (1) no aggression; (2) threatening behavior (open-mandible threats accompanied or not accompanied by gaster flexing); (3) charge(s); (4) biting behavior (accompanied or not accompanied by gaster flexing); (5) dragging behavior; (6) fight (see also Fig. 1A). Scores obtained for various experimental groups were compared by means of Kruskal-Wallis ANOVA followed by Siegel and Castellan *post-hoc* tests for pairwise comparisons of independent data taking into account the inherent error rates accompanying multiple comparisons (Siegel and Castellan 1988). Further in the text these tests will be denoted as Siegel-Castellan *post-hoc* tests.

In a similar way we also quantified the overall result of each test by assigning to it a score denoting the most advanced form of non-aggressive social interactions (1–5) : (1) only aggressive contacts; (2) no interactions; (3) antennal contacts; (4) allogrooming; (5) trophallaxis (see also Fig. 1B). The data obtained for various experimental groups were then compared in the same way as in the case of aggression scores.

Finally each behavioral category taken into account in the main analysis of behavior displayed during the aggression test was quantified by the following variables: (1) the rate of occurrence in the experimental group (the ratio of the tests during which the behavior in question was present to those during which it was absent), (2) the latency from the start of the test to the first episode of the behavior in question, (3) the total number of bouts of that behavior observed during the test, and (4) the total duration of all bouts of that behavior observed during the test [s]. The latency from the start of the test to the first episode of the analyzed behavior was expressed as the per cent of the total test time until the onset of the first bout of that behavior, as such a variable could be calculated also for the tests during which that behavior was absent, taking the value of 100% (Wagner-Ziemka et al. 2006). As our data distributions were often not normal or even approximately normal, we applied non-parametric tests to carry out inter-group comparisons of the values of variables quantifying the analyzed behavior. As we wanted to focus attention on the effects of biogenic amines observed in different contexts, and not on comparisons of responses of ants to various opponents, the data obtained for the tests with each of the four opponents were analyzed separately. This also made possible a considerable reduction of the number of multiple comparisons, and, as a consequence, facilitated the detection of some effects of biogenic amine treatments which might have remained undetected as a consequence of the application of corrections for numerous multiple comparisons.

The rate of occurrence of the analyzed behavior pattern in various experimental groups 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) and was carried out in two ways, with and without taking into account the tests involving the release of the formic acid.

The values of the remaining three variables quantifying a given behavioral category (the latency from the start of the test to the first episode of analyzed behavior, the total number of bouts of that behavior, and the total duration of all bouts of that behavior) obtained for different experimental groups were compared by means of the Kruskal-Wallis ANOVA followed by Siegel and Castellan *post-hoc* tests. For each analyzed variable, two inter-group comparisons were carried out: (1) an analysis taking into account all the tests (*n*=40 in each group), and (2) an analysis taking into account only the tests during which the behavior in question was expressed. This second analysis was not carried out if the sample size of any of the compared groups was lower than *n*=4. Siegel-Castellan tests were also calculated in two ways: the groups of ants treated with various biogenic amines were compared only with the control group, or also with each other.

The latencies from the start of the test to the first episode of a given behavior pattern were also analyzed by means of an additional Kruskal-Wallis ANOVA analysis followed by Siegel-Castellan *post-hoc* tests including also the tests involving formic acid release. As formic acid spraying belongs to the most escalated patterns of aggressive behavior in formicine ants, the information about the tests during which it was displayed should not have been entirely discarded. However, the remaining two variables quantifying various behavior patterns displayed during the tests – the total number and the total duration of their episodes during the test – could not be analyzed in this additional way, because the behavior displayed by the ants during such tests was analyzed only up to the moment of appearance of disturbed behavior and, hence, these initial parts of the tests had different length.

In all analyses we considered as significant the differences at the level of at least $P \leq 0.05$. However, in the description of the results and in the figures we also mentioned non-significant trends with significance level taking values in the interval between 0.05 and 0.1.

RESULTS

Responses of *F. polyctena* to various opponents

Irrespectively of the treatment, workers of *F. polyctena* responded in a strikingly different way to their conspecifics (both nestmates and non-nestmates) and allospecifics (workers of *F. fusca* and nymphs of the house cricket). In the case of both aggressive behavior (Fig. 1A) and non-aggressive social interactions (Fig. 1B) the data obtained for any experimental group in which the ants were paired with an conspecific opponent differed in a highly significant way from the data obtained for any experimental group in which the ants were paired with an allospecific opponent. Whereas on the majority of the tests with conspecific opponents aggressive behavior of *F. polyctena* was absent or limited to threatening behavior, during their confrontations with allospecifics *F. polyctena* displayed as a rule two most escalated patterns of physical aggression: dragging of the opponent and fights (Fig. 1A). Dragging of the opponent as the most escalated form of aggressive behavior occurred more frequently during the confrontations of *F. polyctena* with cricket nymphs than during their confrontations with workers of *F. fusca* simply because crickets fought back at a lower rate than allospecific ants (Fig. 1A).

Similar striking differences between the responses of *F. polyctena* to conspecific and allospecific opponents were also discovered by the analysis of non-aggressive social interactions between the opponents taking place during dyadic aggression tests. The confrontations of *F. polyctena* with allospecifics usually involved only antennal contacts. Trophallaxis and allogrooming appeared only during the tests with *F. fusca* and were very infrequent. In contrast, the confrontations of *F. polyctena* with conspecifics – both nestmates and alien conspecifics from distant colonies – as a rule involved trophallaxis (Fig. 1B).

Effects of biogenic amines on aggressive behavior of *F. polyctena*

The analysis of scores quantifying aggressive and non-aggressive social interactions did not discover any significant effects of any of the tested biogenic amines on behavior of *F. polyctena* confronted with the opponents belonging to the same class (conspecifics vs. allospecifics) (Fig. 1A,B). However, the analysis of other variables quantifying aggressive behavior displayed by *F. polyctena* during various types of dyadic aggression tests discovered numerous significant effects of biogenic amine treatments. With a single exception, biogenic amine treatments influenced in a significant way only aggressive behavior displayed by workers of *F. polyctena* during the confrontations with allospecific opponents: workers of *F. fusca* and nymphs of the house cricket.

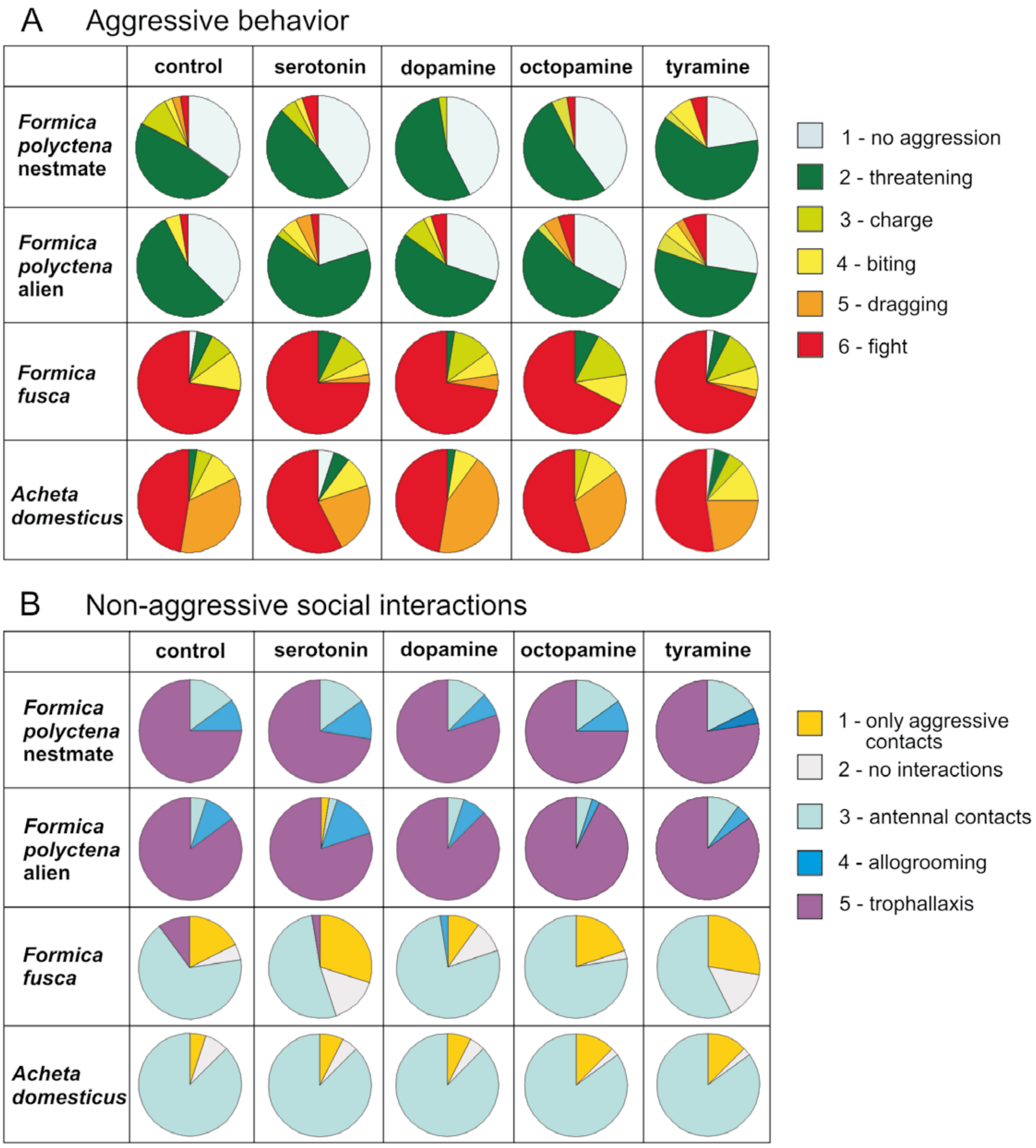

Fig.1. Occurrence of different types of (A) aggressive behavior and (B) non-aggressive social interactions during 10 min dyadic confrontations of workers of *F. polyctena* with four types of opponents: a nestmate (*Formica polyctena* nestmate), an alien conspecific (*Formica polyctena* alien), an allospecific ant (*Formica fusca*), or a nymph of the house cricket (*Acheta domesticus*). The tests were carried out 1 h after abdominal injection of 0.6% saline (control) or 0.08 M of serotonin, dopamine, octopamine or tyramine dissolved in 0.6% saline. n=40 in each experimental group. The overall result of each test was quantified in two alternative ways: (A) as a score (1–6) denoting the most escalated pattern of aggressive behavior observed during the test, and (B) as a score (1–5) denoting the most advanced form of non-aggressive social interactions observed during the test. Statistics: Kruskal-Wallis ANOVA (H_{19}=505.12, P<0.00001 for aggressive behavior, H_{19}=582.96, P<0.00001 for non-aggressive social interactions) followed by Siegel and Castellan *post-hoc* tests which revealed highly significant differences (P<0.00001) in the case of all pairwise comparisons of the data obtained for experimental groups involving the confrontations with opponents belonging to different classes (conspecific vs. allospecific), and no significant differences in the case of all other pairwise comparisons. The results are shown in form of circle charts as such presentation reveals more information than a bar graph showing medians and quartiles of scores quantifying aggressive and non-aggressive interactions. χ^2 test could not be applied to the analysis of these data because of too low expected frequencies for some behavioral categories.

Serotonin

Administration of serotonin exerted stimulatory effects on some aspects of aggressive behavior of workers of *F. polyctena* displayed during dyadic confrontations with allospecific opponents. Significant stimulatory effects of serotonin on aggressive behavior of workers of *F. polyctena* were revealed mainly by the analyses of the data including the results of the tests involving the release of the formic acid. Such tests occurred relatively infrequently during the confrontations of *F. polyctena* with their nestmates (6 cases), alien conspecifics (7 cases) and nymphs of the house cricket (9 cases), but were much more numerous during the confrontations between *F. polyctena* and *F. fusca* (72 cases) and the highest rate of occurrence of such tests (36%), significantly higher than in the case of the control ants (8.3%), was observed in the ants treated with serotonin (Fig. 2). *F. fusca* as a rule do not use formic acid spraying during aggressive encounters (Wallis 1962) and they were never observed to display that behavior during pilot dyadic confrontations with *F. polyctena.* Formic acid released during these tests was thus most probably produced exclusively by *F. polyctena.* This finding suggests thus strongly that during dyadic confrontations with *F. fusca* the highest ratio of the most aggressive workers of *F. polyctena* displaying formic acid spraying behavior was observed in the group treated with serotonin.

During the confrontations with *F. fusca* the latencies from the start of the test to the first episode of dragging of the opponent were also significantly shorter in the ants treated with serotonin than in the control group (Fig. 3). This effect, too, was significant only if the analysis of the data took into account the tests involving the release of the formic acid.

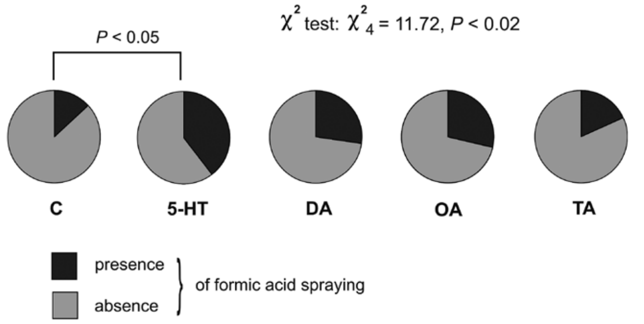

Fig. 2. The rate of occurrence of the tests involving the release of formic acid observed during 10 min dyadic confrontations of workers of *F. polyctena* with workers of *F. fusca* carried out 1 h after abdominal injection of 0.6% saline (C; *n*=46) or 0.08 M of serotonin (5-HT; *n*=66), dopamine (DA; *n*=55), octopamine (OA; *n*=56), or tyramine (TA; *n*=49) dissolved in 0.6% saline. Statistics: 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.

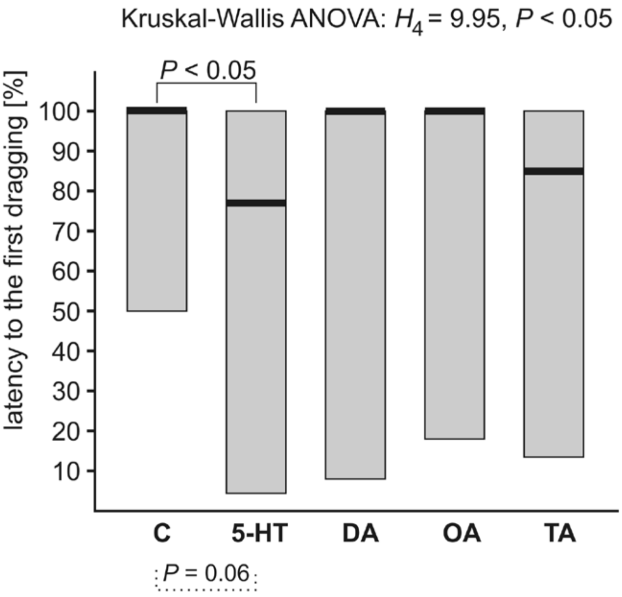

Fig. 3. Medians and quartiles of the latency from the start of the test to the first episode of dragging of the opponent performed by workers of *F. polyctena* during 10 min dyadic confrontations with workers of *F. fusca* carried out 1 h after abdominal injection of 0.6% saline (C; *n*=46) or 0.08 M of serotonin (5-HT; *n*=66), dopamine (DA; *n*=55), octopamine (OA; *n*=56), or tyramine (TA; *n*=49) dissolved in 0.6% saline. The latencies are expressed as the per cent of the total test time until the onset of the first bout of dragging. The analysis included also the tests involving the release of formic acid. Statistics: Kruskal-Wallis ANOVA followed by Siegel-Castellan *post-hoc* tests calculated in two ways: comparisons of each group subjected to biogenic amine treatment only with the control (the results shown above the graph) and all possible comparisons (the results shown below the graph).

During the confrontations of *F. polyctena* with cricket nymphs ants treated with serotonin were displaying the highest number of bouts of biting accompanied by gaster flexing. However, that effect was observed only when the tests during which that behavior was absent had been discarded from the analysis and even then it was only close to be significant (Fig. 4A). Interestingly, the ants treated with serotonin did not differ from the control ants in respect to the total duration of bouts of that behavior (Fig. 4B). If the tests during which biting accompanied by gaster flexing

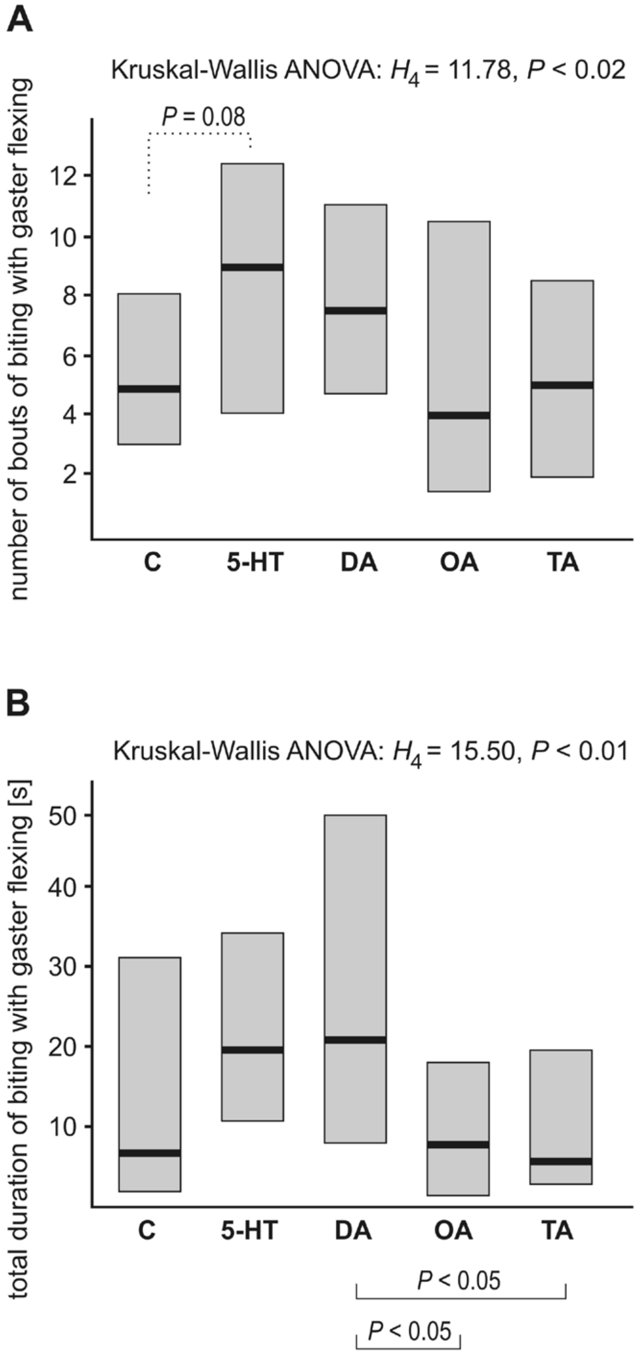

Fig. 4. Medians and quartiles of the total duration of all bouts of biting accompanied by gaster flexing performed by workers of *F. polyctena* during 10 min dyadic confrontations with nymphs of the house cricket (*A. domesticus*) carried out 1 h after abdominal injection of 0.6% saline (C; n=32) or 0.08 M of serotonin (5-HT; n=32), dopamine (DA; n=32), octopamine (OA; n=31), or tyramine (TA; n=23) dissolved in 0.6% saline. The analysis included only the tests during which biting accompanied by gaster flexing was observed. Statistics: see Figure 3.

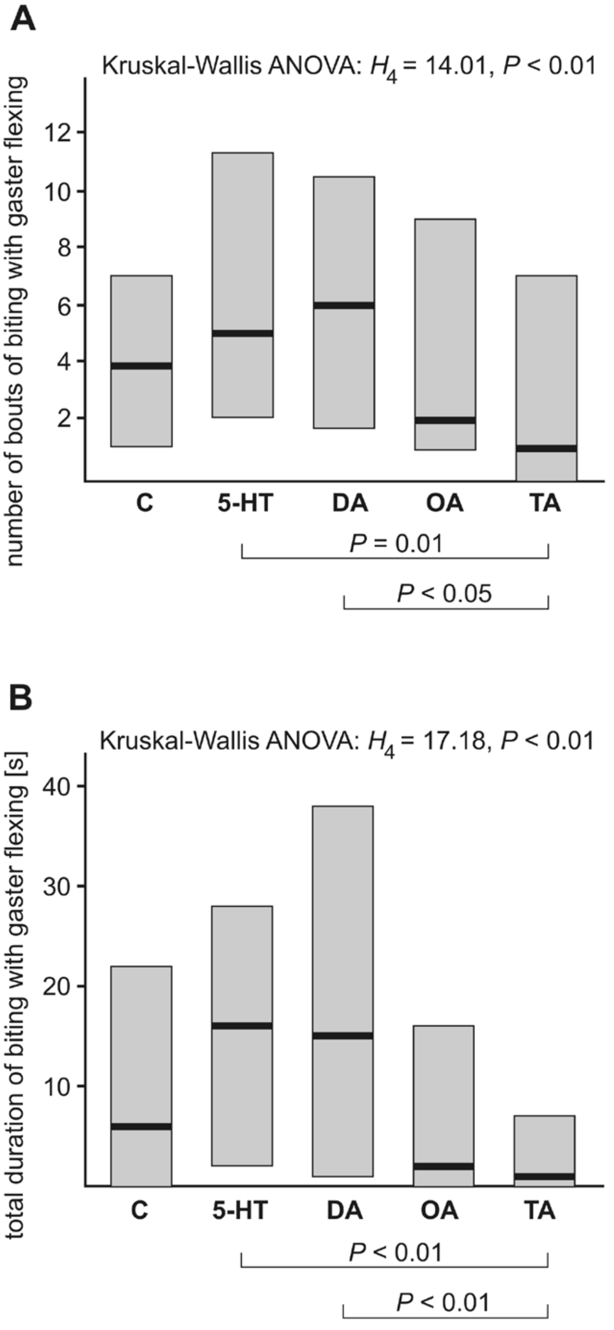

Fig. 5. Medians and quartiles of (A) the total number and (B) the total duration of all bouts of biting accompanied by gaster flexing performed by workers of *F. polyctena* during 10 min dyadic confrontations with nymphs of the house cricket (*A. domesticus*) carried out 1 h after abdominal injection of 0.6% saline (C) or 0.08 M of serotonin (5-HT), dopamine (DA), octopamine (OA), or tyramine (TA) dissolved in 0.6% saline. The analysis included the tests during which biting accompanied by gaster flexing was absent. n=40 in each experimental group. Statistics: see Figure 3.

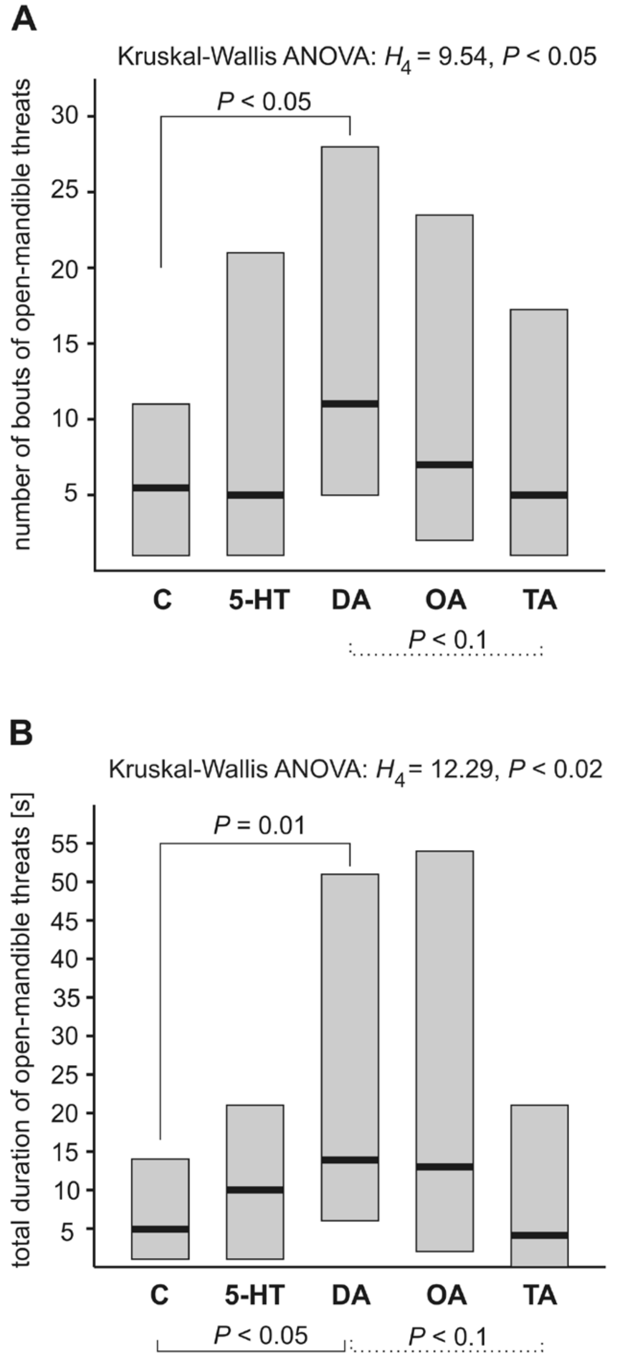

Fig. 6. Medians and quartiles of (A) the total number and (B) the total duration of all bouts of open-mandible threats not accompanied by gaster flexing performed by workers of *F. polyctena* during 10 min dyadic confrontations with workers of *F. fusca* carried out 1 h after abdominal injection of 0.6% saline (C) or 0.08 M of serotonin (5-HT), dopamine (DA), octopamine (OA), or tyramine (TA) dissolved in 0.6% saline. *n*=40 in each experimental group. Statistics: see Figure 3.

was not observed were not discarded from the analysis, the total number of episodes of that behavior and their total duration were significantly higher in the ants treated with serotonin than in the ants treated with tyramine (Fig. 5A,B), but the comparison with the control did no more yield significant results.

Dopamine

Dopamine treatment exerted a stimulatory effect on threatening behavior displayed by workers of *F. polyctena* during their confrontations with *F. fusca* and on both threatening and overt aggressive behavior displayed by *F. polyctena* during the confrontations with cricket nymphs.

During the confrontations between *F. polyctena* and *F. fusca* dopamine treatment was followed by a significant increase of both the total number (Fig. 6A) and the total duration (Fig. 6B) of open-mandible threats (not accompanied by gaster flexing). The total duration of all forms of open-mandible threats (accompanied and not accompanied by gaster flexing) was also significantly higher in ants treated with dopamine (Kruskal-Wallis ANOVA: H_4=10.28, P<0.05; Siegel-Castellan *post-hoc* test: P<0.05).

Dopamine treatment also led to increased level of both threatening behavior and overt aggression during the confrontations of workers of *F. polyctena* with nymphs of the house cricket. The latency from the start of the test to the first episode of any form of open-mandible threat (either accompanied or not accompanied by gaster flexing) was significantly shorter in the ants treated with dopamine than in the control group and in the group treated with serotonin (Fig. 7). These effects were also observed when the tests during which open-mandible threats were absent were discarded from the analysis (Kruskal-Wallis ANOVA: H_4=15.07, P<0.01; Siegel-Castellan *post-hoc* test: P< 0.01 for the comparison DA vs. control and P<0.05 for the comparison DA vs. 5-HT). Similar effects were also observed when we took into account the tests involving the release of the formic acid. When compared with the controls, the ants treated with dopamine showed then significantly shorter latencies to the first episode of any form of open-mandible threat (Kruskal-Wallis ANOVA: H_4=14.99, P<0.005; Siegel-Castellan *post-hoc* test: P<0.01), and to the first episode of open-mandible threat not accompanied by gaster flexing (Kruskal-Wallis ANOVA: H_4=9.51, P<0.05; Siegel-Castellan *post-hoc* test: P<0.05).

Dopamine treatment also significantly increased the total number of bouts of biting directed to cricket nymphs in comparison with the data obtained for the control ants and for the ants treated with tyramine (Fig. 8). A similar effect was also observed in the case of the total duration of biting, but this time only the comparison with the group treated with tyramine revealed a significant difference (Kruskal-Wallis ANOVA: H_4=13.10, P<0.01; Siegel-Castellan *post-hoc* test: P<0.01). In the case of biting accompanied by gaster flexing dopamine treatment also did not bring about significant effects in comparison with the control, but both the total number of episodes of that behavior and their total duration were significantly higher in the ants treated with dopamine than in the ants treated with tyramine (Fig. 5A,B). The latencies from the start of the test to the first episode of biting accompanied by gaster flexing also tended to be shorter (Kruskal-Wallis ANOVA: H_4=10.42, P<0.05; Siegel-Castellan *post-hoc* test: P<0.1) in the ants treated with dopamine than in those treated with tyramine. A similar stimulatory effect (but this time significant; Kruskal-Wallis ANOVA: H_4=14.54, P<0.01; Siegel-Castellan *post-hoc* test: P<0.01) was also observed in the case of the latencies from the start of the test to the first act of dragging of the cricket nymph, which were shorter in the ants treated with dopamine than in those treated with tyramine.

Similar effects were also observed when the tests during which the behavior in question was absent were discarded from the analysis of the results. This time, the total duration of biting accompanied by gaster flexing was significantly higher in the ants treated with dopamine than in the ants treated with tyramine and with octopamine (Fig. 4B). Interestingly, neither of these effects was significant in the case of the total number of episodes of that behavior (Fig. 4A). However, when biting accompanied and not accompanied by gaster flexing have been pooled into a single category, the total number of episodes of that behavior also proved to be significantly higher in the ants treated with dopamine than in the ants treated with tyramine (Kruskal-Wallis ANOVA: H_4=11.13, P<0.05; Siegel-Castellan *post-hoc* test: P<0.05). Inter-group differences in the total duration of that behavior were now also slightly more strongly expressed than in the case of biting accompanied by gaster flexing (Kruskal-Wallis ANOVA: H_4=16.35, P<0.01; Siegel-Castellan *post-hoc* test: P<0.01 for the comparison DA vs. TA and P<0.05 for the comparison DA vs. OA; compare with Fig. 4B).

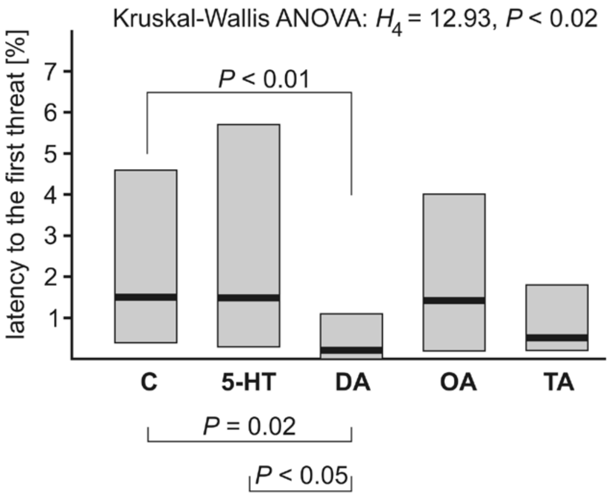

Fig. 7. Medians and quartiles of the latency from the start of the test to the first open-mandible threat (accompanied or not accompanied by gaster flexing) performed by workers of *F. polyctena* during 10 min dyadic confrontations with nymphs of the house cricket (*A. domesticus*) carried out 1 h after abdominal injection of 0.6% saline (C) or 0.08 M of serotonin (5-HT), dopamine (DA), octopamine (OA), or tyramine (TA) dissolved in 0.6% saline. The latencies are expressed as the per cent of the total test time until the first open-mandible threat. n=40 in each experimental group. Statistics: see Figure 3.

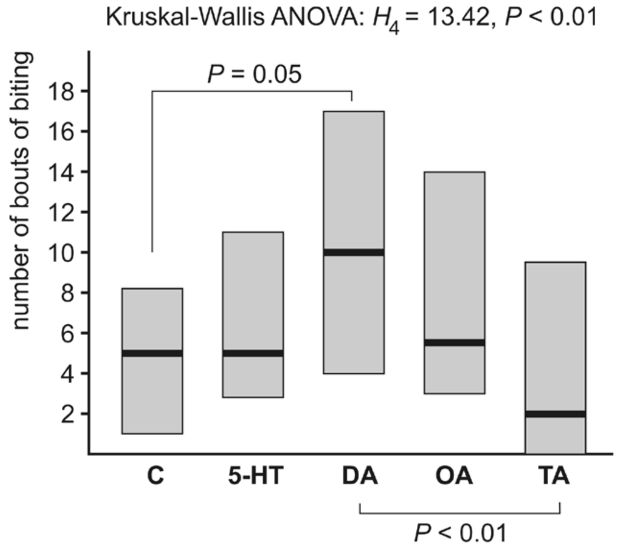

Fig. 8. Medians and quartiles of the total number of all bouts of biting not accompanied by gaster flexing performed by workers of *F. polyctena* during 10 min dyadic confrontations with nymphs of the house cricket (*A. domesticus*) carried out 1 h after abdominal injection of 0.6% saline (C) or 0.08 M of serotonin (5-HT), dopamine (DA), octopamine (OA), or tyramine (TA) dissolved in 0.6% saline. n=40 in each experimental group. Statistics: see Figure 3.

Octopamine

Surprisingly, octopamine treatment did not exert any significant effects on aggressive behavior of the tested workers of *F. polyctena*. Although medians and quartiles of variables quantifying open-mandible threats directed by *F. polyctena* to *F. fusca* took very similar values in the case of the ants treated with dopamine and with octopamine (Fig. 6A,B), statistical analysis did not reveal any significant or even nearly significant differences between the ants treated with octopamine and the control ants.

The analysis of responses of *F. polyctena* to conspecific workers from an alien colony revealed a decrease in the total duration of all forms of open-mandible threats in the ants treated with octopamine, but only in relation to the ants treated with tyramine and serotonin and only when the tests during which open-mandible threats were absent were discarded from the analysis (Fig. 9). Similar but weaker effects were also observed in the case of the total number of that behavior (Kruskal-Wallis ANOVA: H_4=10.40, P<0.05; Siegel-Castellan *post-hoc* test*:* P=0.05 for the comparison OA vs. TA). The comparisons of the ants treated with octopamine with the control ants did not, however, yield significant results.

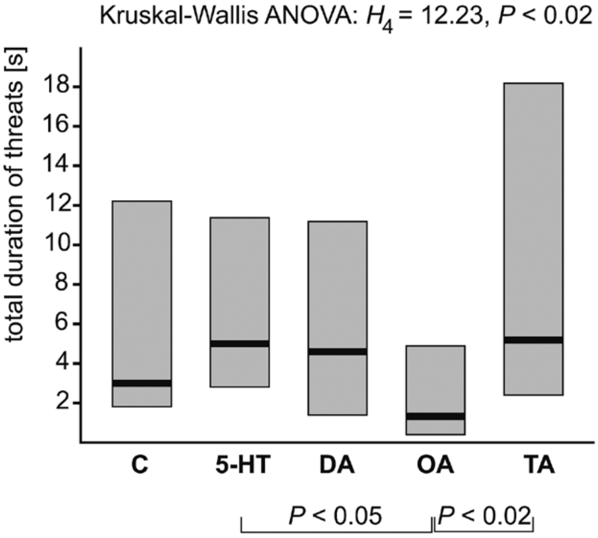

Fig. 9. Medians and quartiles of the total duration of all bouts of open-mandible threats (accompanied or not accompanied by gaster flexing) performed by workers of *F. polyctena* during 10 min dyadic confrontations with alien conspecifics carried out 1 h after abdominal injection of 0.6% saline (C; n=24) or 0.08 M of serotonin (5-HT; n=29), dopamine (DA; n=26), octopamine (OA; n=25), or tyramine (TA; n=28) dissolved in 0.6% saline. The analysis included only the tests during which any form of open-mandible threat was observed. Statistics: see Figure 3.

Tyramine

Significant effects of tyramine administration were revealed only when the tests during which the analyzed behavior was not observed were discarded from the analysis. During the confrontations with *F. fusca* workers of *F. polyctena* subjected to tyramine treatment showed decreased number of open-mandible threats accompanied by gaster flexing as compared to the control ants and to the workers treated with dopa-mine (Fig. 10A). The total duration of that behavior was also significantly shorter in the ants treated with tyramine than in the ants treated with dopamine (Kruskal-Wallis ANOVA: H_4=14.02, P<0.01; Siegel-Castellan *post-hoc* test: P<0.01). However, during the encounters with cricket nymphs the latencies from the start of the test to the first open-mandible threat accompanied by gaster flexing were shorter in the ants treated with tyramine than in the ants from the control group and tended to be shorter than in the ants treated with serotonin (Fig. 10B).

Effects of biogenic amines on non-aggressive behavior of *F. polyctena* observed during dyadic aggression tests

In contrast to aggressive behavior, which was influenced by biogenic amine treatments almost exclusively in the context of confrontations with allospecific opponents, significant effects of biogenic amine treatments on various patterns of non-aggressive behavior of workers of *F. polyctena* were discovered during their confrontations with all types of opponents.

Confrontations with nestmates

During the confrontations of workers of *F. polyctena* with their nestmates biogenic amine treatments exerted inhibitory effects on some forms of social contacts between the interacting ants, namely, antennal contacts and allogrooming. Serotonin and tyramine administration was followed by the increase of the latency from the start of the test to the first antennal contact with the nestmate (Kruskal-Wallis ANOVA: H_4=13.74, P<0.01; Siegel-Castellan *post-hoc* tests*:* P=0.01 and P<0.05, respectively) and to the first act of allogrooming (Kruskal-Wallis ANOVA: H_4=16.54, P<0.01; Siegel--Castellan *post-hoc* test*:* P<0.01 for the effects of both serotonin and tyramine). Closely similar results were also obtained when the tests during which the behavior in question was not expressed were discarded from the analysis (the latency from the start of the test to the first antennal contact: Kruskal-Wallis ANOVA: H_4=12.90, P<0.02; Siegel-Castellan *post-hoc* tests: P<0.02 for the effect of serotonin and P<0.05 for the effect of tyramine; the latency from the start of the test to the first act of allogrooming: Kruskal-Wallis ANOVA: H_4=22.0, P<0.001; Siegel-Castellan *post-hoc* tests: P<0.001 for the effects of both serotonin and tyramine).

Serotonin, dopamine and tyramine treatment also exerted suppressing effects on the total number of bouts of allogrooming of a nestmate (serotonin; Fig. 11A) and on the total duration of that behavior (all three amines; Fig. 11B). In both cases the ants treated with serotonin differed significantly not only from the control ants, but also from the ants treated with octopamine (Fig. 11A, B). However, octopamine also exerted a significant inhibitory effect on allogrooming, but it involved another variable quantifying that behavior, the latency from the start of the test to the first episode of allogrooming, which was significantly longer in ants treated with octopamine (Kruskal-Wallis ANOVA: H_4=16.54, P<0.01; Siegel-Castellan *post-hoc* test: P<0.05).

The values of the variables quantifying trophallaxis with the nestmates were not influenced by biogenic amine treatments.

Confrontations with alien conspecifics

Both serotonin and dopamine treatment decreased the number of bouts (Fig. 12A) and the total duration of trophallaxis with an alien conspecific (Fig. 12B). These effects were discovered only when the tests during which trophallaxis was absent were not included in the analysis.

Tyramine treatment reduced the total number of episodes of self-grooming (Kruskal-Wallis ANOVA: H_4=13.53, P<0.01; Siegel-Castellan *post-hoc* test: P<0.01) and the total duration of all episodes of that behavior (Kruskal-Wallis ANOVA: H_4=16.70, P<0.01; Siegel-Castellan *post-hoc* test: P<0.001).

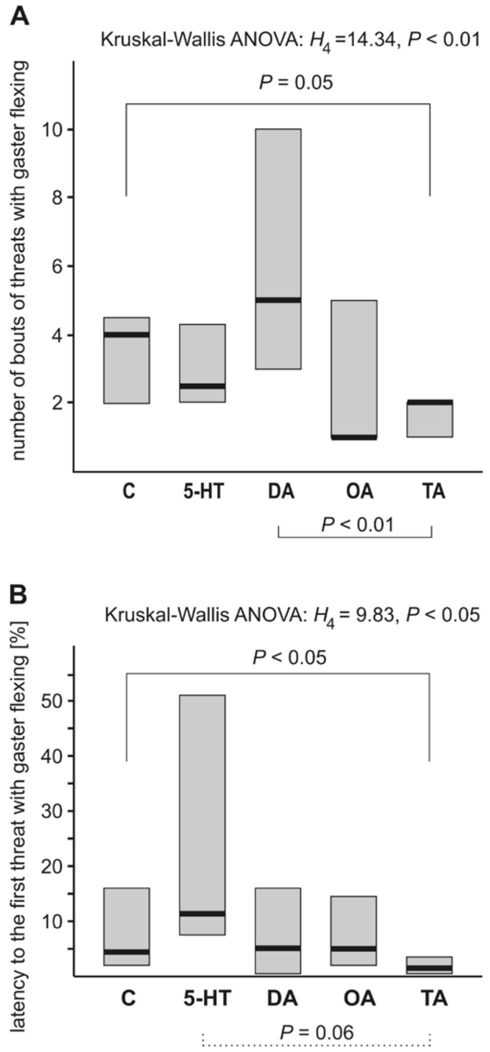

Fig. 10. (A) Medians and quartiles of the total number of all bouts of open-mandible threats accompanied by gaster flexing performed by workers of *F. polyctena* during 10 min dyadic confrontations with workers of *F. fusca* carried out 1 h after abdominal injection of 0.6% saline (C; n=19) or 0.08 M of serotonin (5-HT; n=22), dopamine (DA; n=17), octopamine (OA; n=18), or tyramine (TA; n=23) dissolved in 0.6% saline. (B) Medians and quartiles of the latency from the start of the tests to the first open-mandible threat accompanied by gaster flexing performed by workers of *F. polyctena* during 10 min dyadic confrontations with nymphs of the house cricket (*A. domesticus*) carried out 1 h after abdominal injection of 0.6% saline (C; n=27) or 0.08 M of serotonin (5-HT; n=20), dopamine (DA; n=29), octopamine (OA; n=25), or tyramine (TA; n=21) dissolved in 0.6% saline. The latencies are expressed as the per cent of the total test time until the first open-mandible threat accompanied by gaster flexing. In both cases (A and B) the analysis included only the tests during which any form of open-mandible threat was observed. Statistics: see Figure 3.

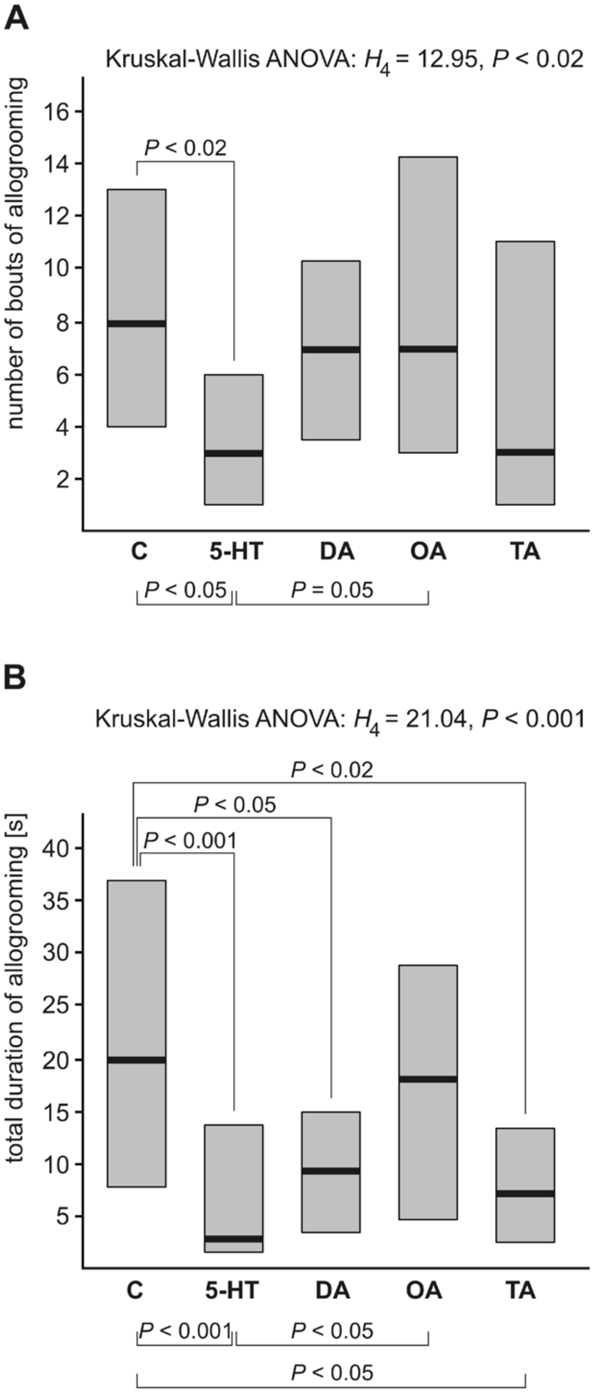

Fig. 11. Medians and quartiles of (A) the total number and (B) the total duration of all bouts of allogrooming performed by workers of *F. polyctena* during 10 min dyadic confrontations with nestmates carried out 1 h after abdominal injection of 0.6% saline (C; *n*=31) or 0.08 M of serotonin (5-HT; *n*=25), dopamine (DA; *n*=28), octopamine (OA; *n*=24), or tyramine (TA; *n*=25) dissolved in 0.6% saline. The analysis included only the tests during which biting accompanied by gaster flexing was observed. Statistics: see Figure 3.

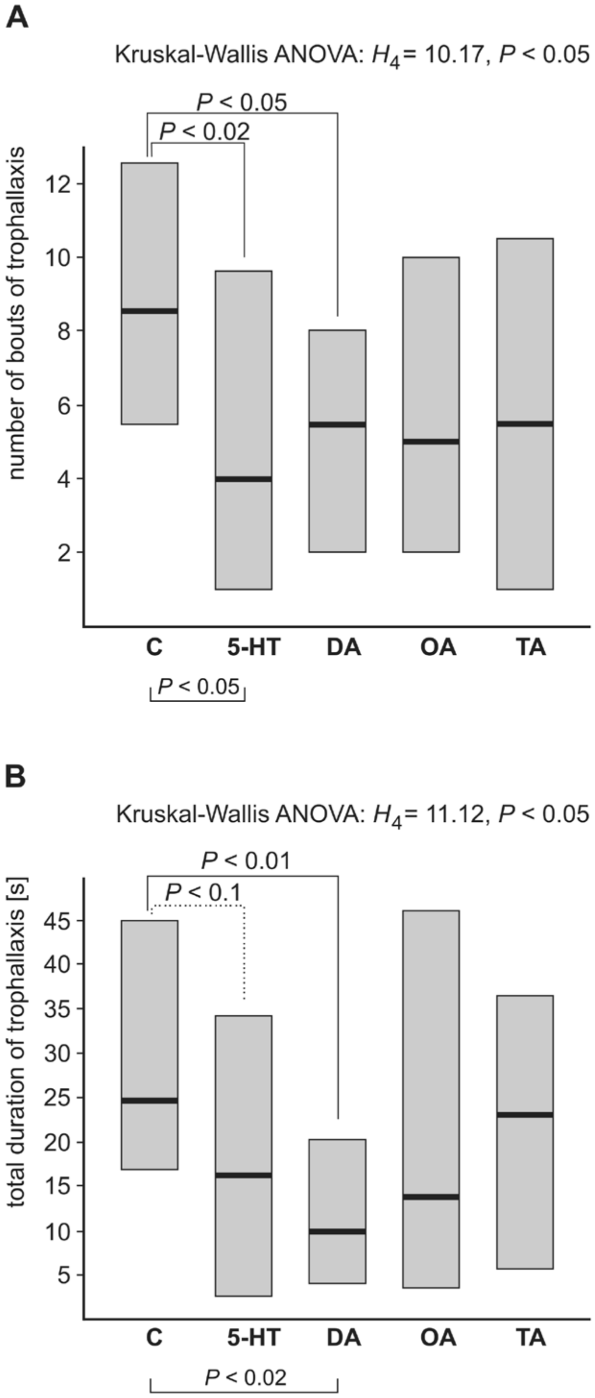

Fig. 12. Medians and quartiles of (A) the total number and (B) the total duration of all bouts of trophallaxis performed by workers of *F. polyctena* during 10 min dyadic confrontations with alien conspecifics carried out 1 h after abdominal injection of 0.6% saline (C; *n*=32) or 0.08 M of serotonin (5-HT; *n*=32), dopamine (DA; *n*=34), octopamine (OA; *n*=36), or tyramine (TA; *n*=32) dissolved in 0.6% saline. The analysis included only the tests during which trophallaxis was observed. Statistics: see Figure 3.

Confrontations with allospecific ants (*F. fusca*)

Serotonin administration was followed by a significant decrease of the ratio of the tests during which the ants engaged in self-grooming [χ^2_4=13.83, *P*<0.01; Fisher Exact Probability Test: *P*=0.03 (with the Bonferroni adjustment for multiple comparisons)].

Confrontations with cricket nymphs

Octopamine treatment exerted a stimulatory effect on the number of bouts of locomotion shown by workers of *F. polyctena* (Fig. 13). Tyramine treatment decreased the number of episodes of locomotion, but that effect was significant only for the comparison with the ants treated with octopamine (Fig. 13).

Several further effects of biogenic amine treatments on non-aggressive behavior patterns displayed by *F. polyctena* during the confrontations with the cricket nymphs were revealed only when the tests during which the behavior in question was absent were discarded from the analysis. These effects included significantly or nearly significantly longer latencies from the start of the test to the first episode of resting behavior observed in ants treated with serotonin, dopamine and octopamine (Kruskal-Wallis ANOVA: H_4=10.35, *P*<0.05; Siegel-Castellan *post-hoc* tests: *P*=0.07, *P*<0.02, *P*=0.07, respectively). Tyramine treatment brought about a yet different effect: it was followed by shortening of the latency from the start of the test to the first episode of indifferent behavior shown in response to the opponent (Kruskal-Wallis ANOVA: H_4=13.46, *P*<0.01; Siegel-Castellan *post-hoc* test: *P*<0.05).

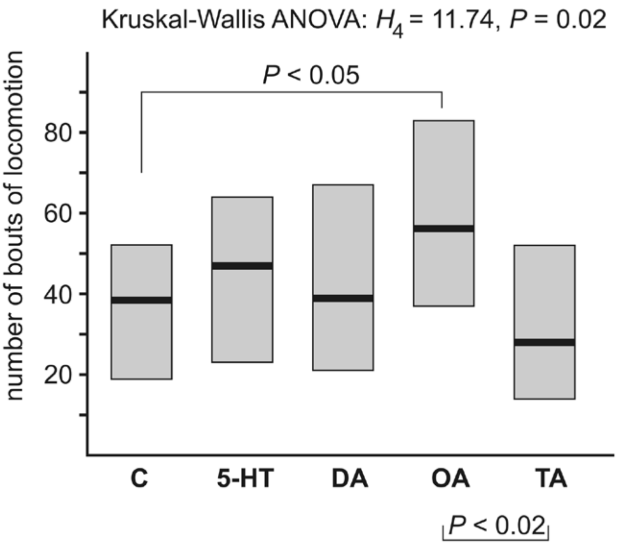

Fig. 13. Medians and quartiles of (A) the total number and (B) the total duration of all bouts of locomotion performed by workers of *F. polyctena* during 10 min dyadic confrontations with with nymphs of the house cricket (*A. domesticus*) carried out 1 h after abdominal injection of 0.6% saline (C) or 0.08 M of serotonin (5-HT), dopamine (DA), octopamine (OA), or tyramine (TA) dissolved in 0.6% saline. *n*=40 in each experimental group. Statistics: see Figure 3.

DISCUSSION

The results of our experiment throw further light on two important questions: diversity and context-dependence of aggressive responses of the ants to various opponents, and the effects of biogenic amine treatments on behavior patterns displayed by the ants during these confrontations.

Our experiment revealed profound and highly significant differences between the behavior of workers of the red wood ant *F. polyctena* during dyadic confrontations with various opponents. Interestingly, the frontier line between the responses involving no aggression or only threatening behavior and the responses involving acts of escalated physical aggression was related to the distinction between conspecific and allospecific opponents, and not to the distinction between nestmates and non-nestmates. Responses of *F. polyctena* to both types of conspecific opponents involved as a rule no aggression or only threatening behavior and a similarly high rate of non-aggressive social contacts, in particular trophallaxis. However, similar relatively mild responses of various red wood ants (*F. polyctena*, *F. rufa* and *Formica lugubris* Zett) to alien conspecifics are already documented in literature. Initially, dyadic laboratory confrontations between nestmates of various red wood ant species were found to involve no aggression (*F. polyctena*: Le Moli and Mori 1986; *F. lugubris*: Le Moli and Parmigiani 1982, Le Moli et al. 1984; *F. rufa:* Le Moli et al. 1982, 1983, 1984), and similar dyadic confrontations between conspecific non-nestmates were found to involve mostly various forms of threatening behavior (*F. polyctena*: Le Moli and Mori 1986; *F. lugubris*: Le Moli and Parmigiani 1982; *F. rufa*: Le Moli et al. 1982). However, further research revealed that behavior displayed by the red wood ants during dyadic confrontations with alien conspecifics is highly variable and may even involve fights, sometimes at a relatively high rate (*F. rufa*: Mori and Le Moli 1993; *F. polyctena*: Beye et al. 1997). Moreover, during the periods of shortage of food in field conditions *F. polyctena* may respond to conspecifics from neighbor colonies by very escalated aggression, the so called ant wars (De Bruyn and Mabelis 1972, Mabelis 1979, 1984a,b).

Presence of highly escalated aggressive behavior during dyadic confrontations between red wood ants (including *F. polyctena*) and allospecific ants of the subgenus *Serviformica* (including *F. fusca*) is also well documented (Le Moli and Parmigiani 1981, Le Moli et al. 1983, Le Moli and Mori 1986, Heuts et al. 2003). In the study of Le Moli and coauthors (1983) attack behavior displayed by the red wood ant workers was observed during the majority of the dyadic confrontations between *F. rufa* and *F. cunicularia*, although it never led to death of the attacked ant. Dyadic confrontations of *F. cunicularia* with *F. polyctena* and *F. lugubris* involved even more escalated aggressive behavior: attack behavior was displayed by all tested red wood ant workers and led to death of the attacked workers of *F. cunicularia* during 35% of tests with *F. polyctena* (Le Moli and Mori 1986) and 75% of the tests with *F. lugubris* (Le Moli and Parmigiani 1981). Occurrence of highly escalated aggressive behavior leading to serious injuries and/or worker death during dyadic confrontations between *F. polyctena* and *F. fusca* was also reported by Heuts and colleagues (2003). In field conditions *F. polyctena* and other red wood ants act as top dominants (Vepsäläinen and Pisarski 1982, Savolainen and Vepsäläinen 1988, 1989, Pisarski and Vepsäläinen 1989, Czechowski et al. 2013). Workers of *F. fusca* may nest within their territories and usually avoid their aggressive attacks thanks to evasive behavior characteristic for submissive species, observed both in the field (Vepsäläinen and Pisarski 1982, Savolainen and Vepsäläinen 1989, Czechowski and Markó 2005) and in laboratory (Wallis 1963). However, *F. polyctena* were observed to chase them away from food sources by threats and aggressive attacks (Savolainen 1991).

Similarity of responses of *F. polyctena* to both types of allospecific opponents, workers of *F. fusca* (potential competitors) and nymphs of the house cricket (potential prey) is not surprising in light of overall similarity of behavior patterns displayed by these ants in the context of predation and during aggressive confrontations with alien conspecifics (De Bruyn and Mabelis 1972, Mabelis 1979, 1984a). However, although *F. polyctena* behaved similarly during the confrontations with *F. fusca* and with cricket nymphs, their behavior in these two contexts was not identical. Cricket nymphs fought back less readily than workers of *F. fusca*, and, therefore, escalated aggression displayed by *F. polyctena* during the confrontations with these opponents was often limited to dragging behavior. Moreover, workers of *F. polyctena* sometimes engaged in trophallaxis and allogrooming with workers of *F. fusca*, but these two forms of social contacts were entirely absent during their confrontations with cricket nymphs.

Context-dependence of ant aggressive behavior is already documented by numerous studies (among others Dobrzański 1959, Beye et al. 1997, Buczkowski and Silverman 2005, Barth et al. 2010, Tanner and Adler 2009, Scharf et al. 2011). In particular, patterns of aggressive behavior were repeatedly found to depend in a crucial way on the type of the bioassay used in the experiment (Le Moli and Parmigiani 1981, Le Moli and Mori 1986, Le Moli et al. 1984, Mercier et al. 1997, Roulston et al. 2003, Buczkowski and Bennett 2008, Martin et al. 2008, Newey et al. 2010). Dyadic aggression tests involve a confrontation of two opponents within a limited closed space and, therefore, in comparison with field conditions the tested ants have only a limited possibility to avoid the attacks by evasive behavior. However, as a rule ants tested in dyadic aggression tests display much less escalated aggressive behavior than during group aggression tests, as aggressive motivation is stimulated by the increasing group size. Stimulatory effects of group size on the expression on various forms of ant aggressive behavior have been reported in the context of responses to alien conspecifics (Skibińska 1982), allospecifics (Tanner 2006, 2008) and potential prey (Szczuka and Godzińska 1997, 2000, 2004a,b). Although dyadic aggression tests are less reliable than group tests in triggering ant aggressive behavior, they allow a more precise analysis of various behavior patterns displayed during the test. During group aggression tests it is also difficult (if not impossible) to determine whether and to what extent aggressive responses of the tested ants are triggered/influenced by internal and external factors related to presence and behavior of the opponent(s) and of other nestmates participating jointly in the test.

In our experiment significant effects of biogenic amine treatments on ant aggressive behavior were discovered solely in the context of the confrontations of the tested workers of *F. polyctena* with allospecific opponents, workers of *F. fusca* and cricket nymphs

Table I

Summary of the main effects of administration of various biogenic amines on behavior of workers of the red wood ant (*Formica polyctena*) during 10 min dyadic aggression tests consisting of a confrontation with a nestmate, an alien conspecific, an allospecific ant (*F. fusca*), or a nymph of the house cricket (*Acheta domesticus*)

	F. polyctena nestmate	*F. polyctena* alien	*F. fusca*	Cricket nymph
1. Aggressive behavior				
Threats			▲DA ▼TA	▲DA ▲TA
Charges				
Biting				▲DA ↑5-HT
Dragging			▲5-HT	
Fight				
Formic acid spraying			▲5-HT	
2. Non-aggressive social interactions				
Indifferent behavior				▲TA
Antennal contacts	▼5-HT ▼TA			
Allogrooming	▼5-HT ▼DA ▼OA ▼TA			
Trophallaxis		▼5-HT▼DA		
3. Other behavior patterns				
Selfgrooming		▼TA	▼5-HT	
Locomotion				▲OA
Resting				▼DA ↓OA ↓ 5- HT

(DA) dopamine; (5-HT) serotonin; (OA) octopamine; (TA) tyramine. Only statistically significant ($P \leq 0.05$) and nearly significant ($0.05 < P < 0.1$) differences with control group are taken into account. (▲) a significant stimulatory effect; (▼) a significant suppressing effect; (↑) a nearly significant stimulatory effect; (↓) a nearly significant suppressing effect.

(Table I). Administration of biogenic amines influenced only non-aggressive behavior shown by the tested ants during confrontations with conspecific opponents. These findings are surprising, as in the majority of earlier studies significant effects of biogenic amine administration were expressed above all in the context of interactions with nestmates (Kostowski et al. 1972, 1975b, Kostowski and Tarchalska 1972) and non-nestmate conspecifics (Vander Meer et al. 2008, Ozaki et al. 2004, Kaita et al. 2006).

Our present results confirmed the main conclusions of earlier research documenting the involvement of biogenic amines serotonin (Kostowski and Tarchalska 1972, Kostowski et al. 1972, 1975a, Tarchalska et al. 1975) and dopamine (Kostowski et al. 1975b) in the mediation of aggressive behavior of the red wood ants. Similarly as in these earlier studies, in our present study the most important effects of biogenic amine administration were also documented in the ants treated with dopamine. However, in contrast to the study of Kostowski and coworkers (1975b) in which stimulatory effects of dopamine and drugs enhancing dopamine contents in ant brains modified mostly aggressive responses directed by workers of *F. rufa* to their nestmates, in the present experiment significant effects of dopamine administration were observed solely in the context of aggressive confrontations with both types of allospecific opponents (Table I). Dopamine administration exerted stimulatory effects on both threatening behavior and escalated physical aggressive behavior of *F. polyctena* (Table I). However, both these main types of aggressive behavior patterns were influenced by dopamine treatment only in the case of the confrontations of *F. polyctena* with cricket nymphs. During the tests with *F. fusca* only threatening behavior of *F. polyctena* was influenced by dopamine administration (Table I).

Serotonin administration also exerted stimulatory effects on some patterns of aggressive behavior of the tested workers of *F. polyctena* (Table I). However, the effects of serotonin treatment were less strongly expressed and less general than in the case of dopamine. Whereas dopamine treatment influenced both threatening behavior and displays of physical aggression, serotonin administration enhanced only some highly escalated forms of aggressive behavior such as biting accompanied by gaster flexing, dragging of the opponent and formic acid spraying (Table I). It should also be stressed that significant effects of serotonin treatment influenced solely the responses of *F. polyctena* to *F. fusca,* whereas during earlier experiments of Kostowski and his coworkers (Kostowski and Tarchalska 1972, Kostowski et al. 1972) serotonin administration influenced aggressive responses of *F. rufa* directed both to nestmates and to an allospecific intruder (a beetle), increasing mutual aggression between the ants, but decreasing the level of their aggression directed to the beetle. The differences between the results of earlier research of Kostowski and his coworkers and our present experiment are most probably related to the differences between the experimental methods applied in these studies, and, in particular, different bioassays.

Octopamine treatment did not influence aggressive behavior of the tested ants in any of the experimental contexts applied in this study. This result was surprising, as the involvement of octopaminergic system in the mediation of aggressive behavior of invertebrates is very well documented. Octopamine and/or drugs enhancing brain octopamine levels are known to exert stimulatory effects on various forms of aggressive behavior in a wide range of invertebrates including crustaceans (Kravitz and Huber 2003), the fruit fly *Drosophila* (Baier et al. 2002, Zwarts et al. 2012), crickets (Adamo et al. 1995, Stevenson et al. 2005), the honey bee (Robinson et al. 1999), and the ants (Vander Meer et al. 2008). However, as already told, absence of effects and/or inhibitory effects of OA treatment on ant aggressive behavior have also been signaled (Ozaki et al. 2004, Kaita et al. 2006).

Absence of effects of octopamine treatment on responses of workers of *F. polyctena* to nymphs of the house cricket was particularly surprising, as in an earlier study of our team (Szczuka and Godzińska 2008) octopamine treatment was shown to enhance responses of workers of *F. polyctena* to another species of potential prey, adult houseflies (*Musca domestica* L.). However, in that study the ants were not tested by means of a dyadic aggression test, but by means of a group test during which a group of workers had to respond to a dead prey item offered to them within the foraging area of their nest. Our present findings and our earlier data taken together demonstrate thus that the effects of octopamine treatment on responses of ants to potential prey are context-dependent, and, in particular, depend on the bioassay used in the experiment.

As far as we know, the effects of tyramine administration on ant aggressive behavior are reported for the first time. Tyramine treatment influenced significantly aggressive responses of workers of *F. polyctena* to both allospecific opponents, but – in contrast to serotonin - significant effects of tyramine administration were limited to threatening behavior (Table I) and had different character in the case of confrontations with *F. fusca* and with cricket nymphs (inhibitory and stimulatory influence, respectively) (Table I).

Our study also revealed several effects of administration of biogenic amines on non-aggressive behavior patterns displayed by workers of *F. polyctena* during dyadic aggression tests with various opponents. In particular, serotonin and tyramine exerted a suppressing effect on antennal contacts between the opponents (Table I), and all four biogenic amines tested in our experiment exerted a suppressing effect on allogrooming (Table I). Interestingly, all these effects were observed only in the context of a dyadic confrontation between two nestmates and were absent during all other types of aggression tests. It may also be noted that an earlier study of our team (Korczyńska et al. 2005) revealed both similarities and important differences between the effects of administration of octopamine and tyramine on various behavior patterns displayed by workers of the carpenter ant *Camponotus herculeanus* L. during dyadic confrontations between nestmates taking place after a period of social isolation. In particular, differently than in the present experiment, social contacts between the nestmates (antennal contacts, allogrooming and trophallaxis) were influenced solely by octopamine. Tyramine and dopamine injections did not exert any significant effects on that group of behavior patterns.

In contrast to antennal contacts and allogrooming, trophallaxis between nestmates was not influenced by any biogenic amine treatment (Table I). However, trophallaxis between non-nestmates was partly suppressed by serotonin and dopamine (Table I). These findings are surprising, as the responses of *F. polyctena* to nestmates and non-nestmate conspecifics proved to be in general surprisingly similar in respect to both aggressive behavior (prevalence of non-aggressive interactions and threatening behavior, almost complete absence of physical aggression) and non-aggressive social behavior (high rate of trophallaxis). It was also surprising that trophallaxis between non-nestmates was partly suppressed by serotonin and dopamine and not by octopamine, as suppressing effects of octopamine treatment on the so called isolation-induced trophallaxis (trophallaxis observed during dyadic confrontations of nestmates reunited after a period of social isolation) are well documented in two species of formicine ants (*Camponotus fellah* – Boulay et al. 1999, 2000; *Camponotus herculeanus* – Korczyńska et al. 2005).

Biogenic amine administration influenced also behavior patterns not related to the interactions of the tested ants with their opponents. Both tyramine and serotonin exerted suppressing effects on self-grooming, but these two effects were observed in different contexts (tyramine: confrontations with alien conspecifics, serotonin: confrontations with allospecific ants; Table I). It may be noted that tyramine injections also influenced self-grooming observed during dyadic confrontations between the nestmates of the carpenter ant *C. herculeanus* taking place after a period of social isolation, but their effect was stimulatory (Korczyńska et al. 2005).

Stimulatory effect of octopamine on locomotion of workers of *F. polyctena* during the confrontations with cricket nymphs (Table I) was not surprising, as such relationships were already reported in the ants (David and Verron 1982). However, this question, too, is not unequivocally resolved, as Boulay and others (2000) did not discover significant effects of octopamine administration on locomotory behavior of workers of *C. fellah*. Moreover, it cannot be easily explained why stimulatory effects of octopamine on locomotion of *F. polyctena* were present only in the context of their confrontations with cricket nymphs, but were absent during their confrontations with all other opponents.

Finally, suppressing effects of serotonin, dopamine and octopamine on resting behavior observed during the confrontations of *F. polyctena* with cricket larvae (Table I) are most probably related simply to stimulatory effects of these amines on active behavior patterns of the tested ants.

Taken together, our present findings demonstrate that not only specific patterns of ant aggressive behavior, but also behavioral effects of biogenic amine treatments are strongly context-dependent. Stimulatory effect of dopamine treatment on threatening behavior, observed during the confrontations with both allospecific opponents (Table I), provides the only major exception from that general rule.

A question arises if some of the effects revealed by the analysis of the results of our experiment do not represent only artifacts generated by the analysis of multiple variables characterizing the behavior of the same individuals. Only future research will be able to solve definitely that question. Such an eventuality is, however, little probable in situations in which more than one type of biogenic amine treatment exerts significant effects of a similar type in the same context (for instance in the case of suppressing effects of all four biogenic amines on various variables quantifying allogrooming between nestmates; Table I) .

The results of our experiment also show that the conclusions drawn on the basis of the results of experimental research on ant aggressive behavior depend strongly not only on the bioassay(s) used in the experiment, but also on the method of data quantification and analysis. Similar remarks on the effects of application of various alternative methods of quantification and analysis of ant aggressive behavior were already provided by Ichinose and colleagues (2005). In our study simple quantification of aggressive behavior in form of aggression scores did not reveal any significant effects of biogenic amine treatments which were discovered only when the behavior of the tested ants was quantified in a much more detailed way. Moreover, as repeatedly shown during the presentation of the results of our experiment, presence/absence and significance level of specific effects as a rule depends on many additional factors including, in particular, (1) the choice of behavioral categories used to quantify the behavior in question (some effects were revealed only when ant behavior was quantified in a very detailed way, with fine distinctions between behavioral categories, but others became significant only when similar behavioral categories were pooled), (2) the choice of variables used to quantify the same behavioral category (compare Fig. 4A with 4B), (3) the selection of the tests taken into account in the analysis of the data (all tests, only the tests without the release of formic acid leading to disturbed behavior and/or worker(s) death, or only the tests during which the behavior in question was actually observed), and, lastly, (4) the selection of groups subjected to *post-hoc* pairwise comparisons (all possible comparisons vs. comparisons of each group subjected to biogenic amine treatment only with the control).

CONCLUSIONS

Dyadic aggression tests consisting of confrontations of workers of the red wood ant *Formica polyctena* with four types of opponents (a nestmate, an alien conspecific, an allospecific ant and a nymph of the house cricket) were applied to investigate the effects of administration of four biogenic amines – serotonin, dopamine, octopamine and tyramine – on various patterns of ant aggressive behavior. Responses of the tested ants to various opponents showed profound differences. Interestingly, the frontier line between the responses involving either no aggression or only threatening behavior and the responses involving acts of escalated physical aggression was related to the distinction between conspecific and allospecific opponents, and not to the distinction between nestmates and non-nestmates. Significant effects of biogenic amine treatments on ant aggressive behavior were discovered only in the context of confrontations with allospecific opponents. The results of our study confirmed that dopamine and serotonin are involved in the mediation of ant aggressive behavior. However, whereas dopamine treatment exerted stimulatory effects on both threatening behavior and displays of physical aggression, serotonin administration enhanced only some highly escalated forms of aggressive behavior. In contrast to serotonin, significant effects of tyramine administration were limited to threatening behavior and had both inhibitory and stimulatory character. The effects of tyramine administration on ant aggressive behavior are reported for the first time. Surprisingly, octopamine treatment did not influence aggressive behavior of the tested ants in any of the experimental contexts applied in this study. Our study also revealed several effects of administration of biogenic amines on non-aggressive behavior patterns displayed by workers of *F. polyctena* during dyadic aggression tests, all of them highly specific for a particular context. Our present findings demonstrate that not only specific patterns of ant aggressive behavior, but also behavioral effects of biogenic amine treatments are as a rule strongly context-dependent. They also provide numerous examples of crucial importance of both the bioassay(s) and the adopted method of data quantification and analysis for the conclusions drawn from the data obtained in the experiments investigating ant aggressive behavior.

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