

RESEARCH ARTICLE

Social dominance and reproductive differentiation mediated by dopaminergic signaling in a queenless ant

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ABSTRACT

In social Hymenoptera with no morphological caste, a dominant female becomes an egg layer, whereas subordinates become sterile helpers. The physiological mechanism that links dominance rank and fecundity is an essential part of the emergence of sterile females, which reflects the primitive phase of eusociality. Recent studies suggest that brain biogenic amines are correlated with the ranks in dominance hierarchy. However, the actual causality between aminergic systems and phenotype (i.e. fecundity and aggressiveness) is largely unknown due to the pleiotropic functions of amines (e.g. age-dependent polyethism) and the scarcity of manipulation experiments. To clarify the causality among dominance ranks, amine levels and phenotypes, we examined the dynamics of the aminergic system during the ontogeny of dominance hierarchy in the queenless ant *Diacamma* sp., which undergoes rapid physiological differentiation based on dominance interactions. Brain dopamine levels differed between dominants and subordinates at day 7 after eclosion, although they did not differ at day 1, reflecting fecundity but not aggressiveness. Topical applications of dopamine to the subordinate workers induced oocyte growth but did not induce aggressiveness, suggesting the gonadotropic effect of dopamine. Additionally, dopamine receptor transcripts (*dopr1* and *dopr2*) were elevated in the gaster fat body of dominant females, suggesting that the fat body is a potential target of neurohormonal dopamine. Based on this evidence, we suggest that brain dopamine levels are elevated in dominants as a result of hierarchy formation, and differences in dopamine levels cause the reproductive differentiation, probably via stimulation of the fat body.

KEY WORDS: Biogenic amine, Reproductive dominance, Primitively eusocial, Ponerine ant, *Diacamma*

INTRODUCTION

Many animal societies consist of a few breeders and many helpers; thus, individuals often struggle for reproductive opportunities (Sherman et al., 1995). The repeated agonistic interactions among group members can result in the appearance of consistent winners (i.e. dominants or high-rankers) and losers (i.e. subordinates or low-rankers). In this dominance hierarchy, the reproductive opportunity is often skewed to high-rankers (Drews, 1993; Monnin and Ratnieks, 1999). Such a dominance-mediated reproductive skew

(i.e. the unequal sharing of reproductive opportunity) is widespread in various animal taxa (Sherman et al., 1995; birds, Emlen and Wrege, 1992; mammals, Jarvis, 1981; Keane et al., 1994; Nievergelt et al., 2000). In extreme cases, it can result in the reproductive division of labor, such as in social insects and naked mole rats (Wilson, 1971; Sherman et al., 1995; Reeve and Keller, 2001).

In highly eusocial insects (honeybees, most ants and termites), developmental differentiation of morphological caste is the basis of social organization (Wilson, 1971). By contrast, there are morphologically casteless social insects (some wasps, bumblebees and queenless ants) in which the dominance hierarchy plays a central role in division of labor. In these species, physical aggression among nestmates results in the production of a dominant female that retains aggressiveness towards nest mates and of subordinates that lose aggressiveness (Premnath et al., 1996; Peeters and Tsuji, 1993; Monnin and Peeters, 1999; Liebig et al., 2000). Thereafter, the dominant becomes an egg-layer, whereas the subordinates become sterile workers (Fletcher and Ross, 1985; Ito and Higashi, 1991; Peeters, 1991; Turillazzi and West-Eberhard, 1996). This means that dominance ranks that are defined by aggressiveness coincide with reproductive roles (Drews, 1993). Such reproductive differentiation based on a dominance hierarchy is thought to reflect a primitive phase of eusociality (West-Eberhard, 1979, 1981; Gadagkar, 1991; Turillazzi and West-Eberhard, 1996). Despite evolutionary importance, the physiological mechanisms regulating the aggressiveness and fecundity during hierarchy formation remain largely unknown.

Biogenic amines are thought to be involved in dominance hierarchy formation by affecting various behaviors and physiology in both vertebrates and invertebrates (vertebrates, Winberg and Nilssen, 1993; Nelson, 2006; invertebrates, Kravitz and Huber, 2003; Roeder, 2005). Several studies have shown the correlation between brain amine levels and dominant status in social insects. In *Polistes* paper wasps and the worker-totipotent ant *Harpegnathos saltator*, brain dopamine levels are elevated in dominant individuals (Sasaki et al., 2007; Penick et al., 2014), whereas brain octopamine levels are known to be elevated in dominants in bumblebee (Bloch et al., 2000) and the queenless ant *Streblognathus* (Cuvillier-Hot and Lenoir, 2006). In addition to aggressiveness, the positive correlation between dopamine levels and ovarian activity is observed in bees, wasps and ants (Sasaki et al., 2007; Bloch et al., 2000; Harris and Woodring, 1995; Penick et al., 2014). Besides dominance hierarchy, biogenic amines are also an important mediator of the age-dependent division of labor (Shultz and Robinson, 1999; Kahmi and Traniello, 2013). For example, brain octopamine levels are elevated in old honeybee workers to trigger foraging behavior (Shultz and Robinson, 2001). The age-dependent changes in brain amine levels are also observed in ants, implying that age-dependent transition of amine levels commonly occur in social Hymenoptera (Seid and Traniello, 2005; Seid et al., 2008; Wnuk et al., 2011).

Although correlational evidence is accumulating, the causal relationship between amines and the dominance-dependent phenotypes (i.e. fecundity and aggressiveness) has yet to be elucidated. One

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reason for this is a scarcity of empirical studies that show actual physiological effects of amines on fecundity and aggressiveness. Examples are limited to a few studies: oral administration of dopamine stimulates reproduction in honeybee queenless workers (Dombroski et al., 2003) and paper wasp workers (Sasaki et al., 2009). Another difficulty comes from the pleiotropic functions of biogenic amines. Brain amine levels are also associated with worker division of labor and amine levels tend to be age dependent (Schultz and Robinson, 1999, 2001; Seid and Traniello, 2005; Seid et al., 2008; Wnuk et al., 2011). As age is also a major determinant of dominance rank (Strassmann and Meyer, 1983; Higashi et al., 1994; Monnin and Ratnieks, 1999; Tsuji and Tsuji, 2005), brain amine levels may be under complex regulation by age and dominance rank (Cuvillier-Hot and Lenoir, 2006). For these reasons, the causality of aminergic signaling and dominance-based physiological changes remain to be tested. Here, we have used the queenless ants *Diacamma* sp. (the only *Diacamma* species from Japan) as a model system to clarify the aminergic basis of reproductive differentiation, by controlling age and dominant status altogether, and by testing the amine functions through topical application.

The ant genus *Diacamma* is a morphologically casteless ‘queenless’ ant. Extant ants generally have distinct morphological queen castes as a synapomorphy, but queenless ponerine ants have secondarily lost the morphological queen to recapitulate the primitively eusocial state (Peeters, 1993; Monnin and Ratnieks, 1999). *Diacamma* have a clear reproductive division of labor that arises from a unique dominance interaction. All enclosed females have wing vestiges called ‘gemmae’ and they possess reproductive potential at eclosion. In each colony, the only female that retains her gemmae mates and reproduces as a functional queen known as a ‘gamergate’ (Fukumoto et al., 1989; Peeters and Higashi, 1989). In this genus, the gemmae-possessing female mutilates the gemmae of newly eclosed females, and this mutilation is a dominance interaction that leads the eclosed callows to become subordinate sterile workers (Fukumoto et al., 1989; Peeters and Higashi, 1989). The callow females violently resist the mutilation, but as long as the gamergate is vigorous, the gamergate mutilates the gemmae of callows and the mutilated females become timid (Peeters and Higashi, 1989). During this event, nestmate workers support the gamergate by immobilizing the victim female (see Fukumoto et al., 1989; Peeters and Higashi, 1989), but the most active female is always the gamergate. If a gamergate is not present in a colony, the callow becomes a new dominant, i.e. a gamergate, and her ovaries clearly develop within 7 days of eclosion (Okada et al., 2010a). Irrespective of the colony status (i.e. the presence or absence of gamergate), the unmutated callow females always behave aggressively to secure dominant possession. Therefore, the aggressiveness of callows can be viewed as a rank-securing dominance behavior in a broad sense. The rapid changes in aggressiveness and fecundity of *Diacamma* provide a model in which physiological changes during dominance hierarchy formation can be precisely examined (Okada et al., 2010a; Fuchikawa et al., 2014). Here, we have investigated the ontogenic changes of aggressiveness, brain amine levels and amine receptor expression during dominance hierarchy formation. Additionally, topical applications of dopamine and octopamine were performed to test the functions of amines in aggressiveness and fecundity.

RESULTS

Behavioral ontogeny

Future gamergates (FGs, prospective dominant egg-layers) showed strong aggressiveness at eclosion, which lasted for about 7 days (Fig. 1A). To quantify the effects of age on aggressiveness, Poisson

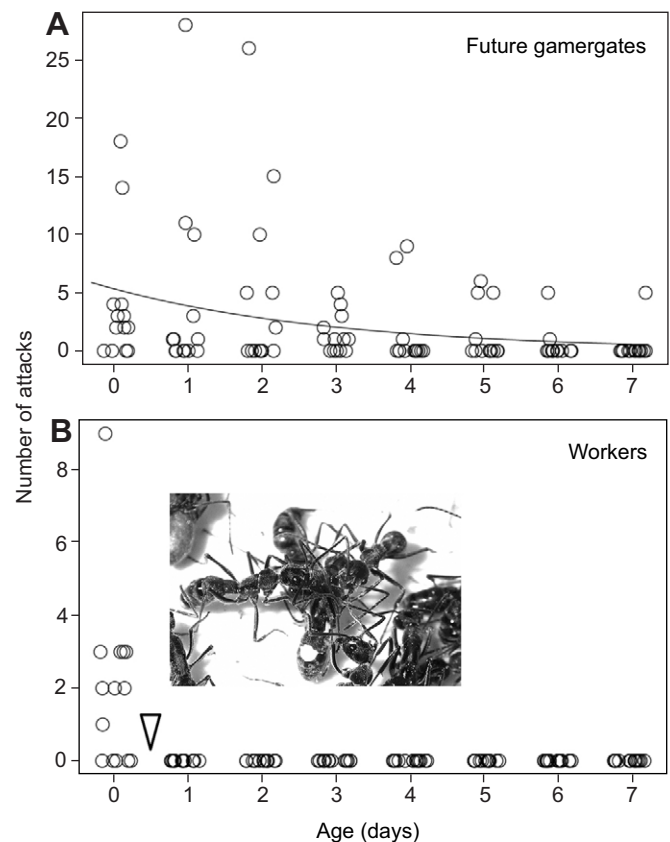


Fig. 1. Ontogenic changes of aggressive behavior. Total number of aggressive attacks per 10 min. (A) Future gamergates ($N=13$). Fitted line is depicted by Poisson regression [Number of attacks = $\exp(1.68 - 0.32 \times \text{day})$]. (B) Workers ($N=14$). An arrowhead indicates the timing of gemmae mutilation. Photo shows a newly eclosed female (a marked ant in the center) having her gemmae mutilated by nestmates. The photo was taken by Y.O.

regression, a subset of the generalized linear model (GLM), was performed with age (x) as an explanatory variable and aggressiveness (y) as a dependent variable. The model was defined as $y = \exp(a + bx)$. GLM revealed a significant negative effect of age on aggressiveness ($b = -0.32$, $\chi^2 = 108.4$, $P < 0.001$). By contrast, eclosed females in gamergate-right colonies exhibited a strong aggressiveness at day 0, but this aggressiveness suddenly disappeared after the mutilation of gemmae (day 1 to day 7, Fig. 1B).

Ontogeny of brain biogenic amine level

In general, levels of all biogenic amines exhibited age-dependent increases [ANOVA, dopamine: $F_{3,81} = 60.8$, $P < 0.0001$; octopamine: $F_{3,72} = 17.9$, $P < 0.0001$; serotonin (5-HT): $F_{3,59} = 35.0$, $P < 0.0001$]. Thus, the different age categories were analyzed separately by pairwise comparisons of FGs and workers. Dopamine did not differ between day-1 workers and FGs (1W and 1FG), but a significant difference was found between day-7 workers and FGs (7W and 7W, Fig. 2A). Octopamine and 5-HT levels exhibited no significant differences between workers and FGs at day 1 and day 7.

Ontogeny of amine receptor expression in brain, ovary and fat body

All examined receptors in the brain showed age-dependent expression dynamics (ANOVA, *dopr1*: $F_{2,33} = 4.6$, $P < 0.017$; *dopr2*: $F_{2,33} = 3.4$, $P = 0.047$; *oar*: $F_{2,35} = 21.7$, $P < 0.0001$; *5ht*:

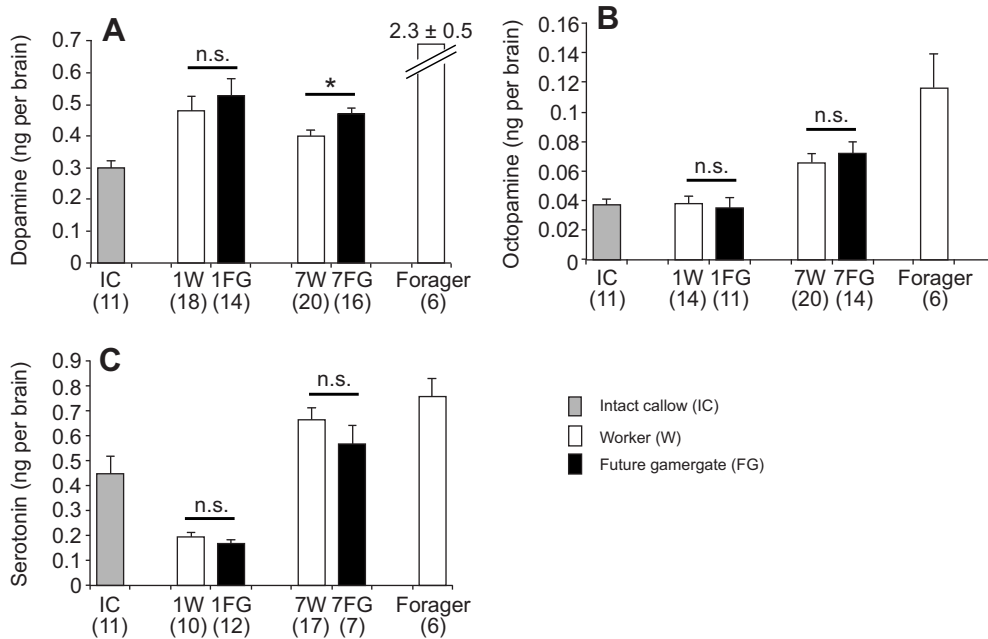


Fig. 2. Brain biogenic amine levels. (A) Dopamine (DA), (B) octopamine (OA) and (C) serotonin (5-hydroxytryptamine, 5-HT) levels (means ± s.e.m.). IC, intact callow; 1W, day-1 worker; 1FG, day-1 future gamergate; 7W, day-7 worker; 7FG, day-7 future gamergate. Asterisk indicates a significant difference (*t*-test, **P* < 0.05). Levels of significance for day-1 and day-7 pairwise comparisons were (A) *P* = 0.510 and 0.0262, (B) *P* = 0.732 and 0.510 and (C) *P* = 0.226 and 0.293, respectively. Sample sizes are provided in parentheses. n.s., not significant.

$F_{2,33}=69.4$, $P<0.0001$), and therefore the different age categories were analyzed separately as above. The examined amine receptors did not show differential expression patterns between FGs and workers (Fig. 3A–D).

As dopamine was upregulated in FGs at day 7 (Fig. 2) and dopamine had gonadotropic effects (see Fig. 5A), we focused on fat bodies and ovaries as target tissues of dopamine. The two transcripts of dopamine receptors (*dopr1* and *dopr2*) in these tissues were quantified in day-7 females. In the fat body, *dopr1* and *dopr2* were highly expressed in FGs (Fig. 4A), whereas

dopr1 and *dopr2* were highly expressed in worker ovaries (Fig. 4B).

Effect of dopamine and octopamine on ovarian development and aggressiveness

Topical application of dopamine had significant effects on oocyte development in young mutilated workers (Fig. 5A). This effect exhibited a counter dose-dependency (Fig. 5A). In contrast to dopamine, octopamine did not have a gonadotropic effect (Fig. 5B). We also evaluated the effects of dopamine and octopamine on

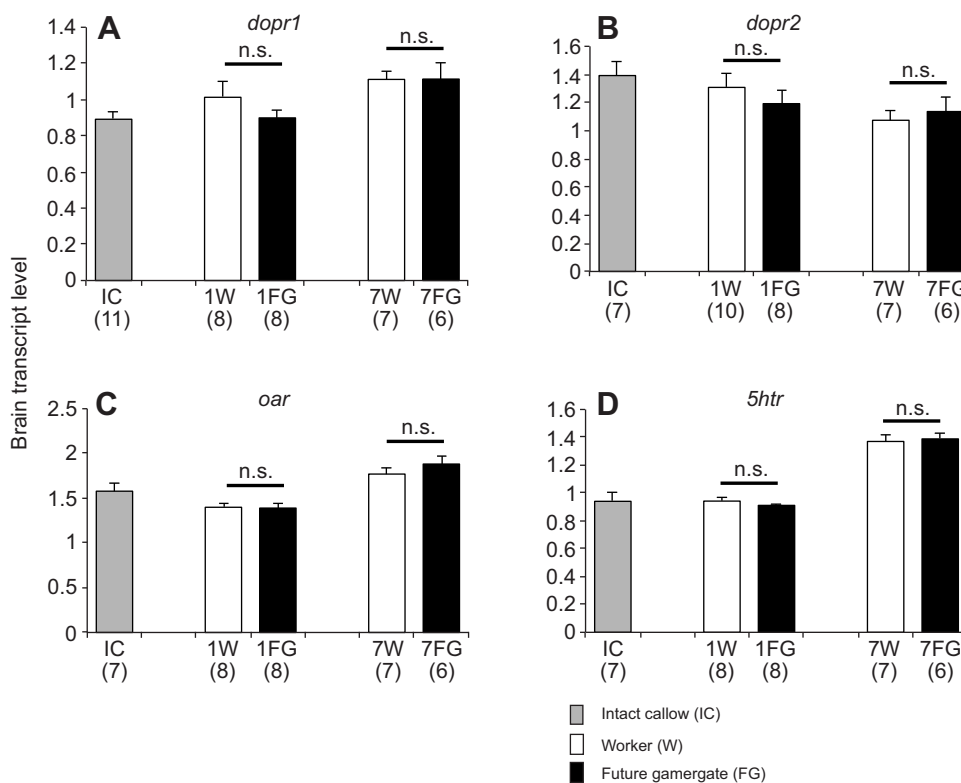


Fig. 3. Brain biogenic amine receptor levels. (A) Dopamine receptor 1 (*dopr1*), (B) dopamine receptor 2 (*dopr2*), (C) octopamine receptor (*oar*) and (D) serotonin receptor (*5htr*) transcript levels relative to *gapdh* (reference gene) are shown (mean ± s.e.m.). IC, intact callow; 1W, day-1 worker; 1FG, day-1 future gamergate; 7W, day-7 worker; 7FG, day-7 future gamergate. Levels of significance for day-1 and day-7 pairwise comparisons were (A) *P* = 0.267 and 0.985, (B) *P* = 0.399 and 0.620, (C) *P* = 0.872 and 0.372, (D) *P* = 0.344 and 0.764, respectively. Sample sizes are provided in parentheses. n.s., not significant.

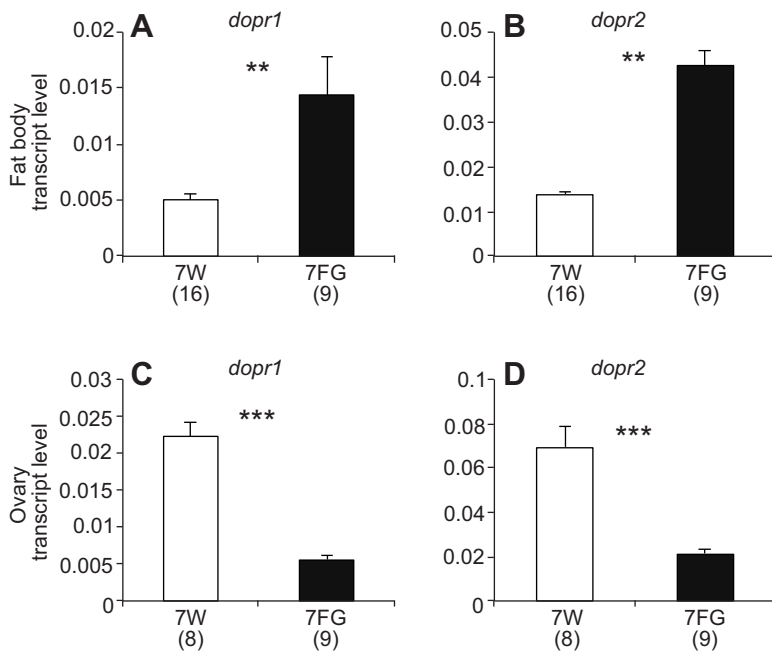


Fig. 4. Dopamine receptor levels in fat body and ovary. *dopr1* (A,C) and *dopr2* (B,D) levels in fat body (A,B) and ovary (C,D). Transcript levels relative to *gapdh* (reference gene) are shown (means \pm s.e.m.). 7W, day-7 worker; 7FG, day-7 future gamergate. Asterisks indicate significant differences (*t*-test, ** $P < 0.01$, *** $P < 0.001$, (A) $P = 0.0018$, (B) $P = 0.0061$, (C) $P < 0.0001$, (D) $P = 0.0001$). Sample sizes are provided in parentheses.

behavior, but the topical application of dopamine and octopamine did not increase the aggressiveness in mutilated workers (Fig. 5C,D).

DISCUSSION

Formation of dominant status

The callow females of *Diacamma* sp. rapidly lost aggressiveness after mutilation (Fig. 1; Peeters and Higashi, 1989), and this is interpreted as the completion of subordination. By contrast, the non-mutilated FGs retained aggressiveness that then declined at day 7 (Fig. 1A). In parallel with the loss of aggressiveness, FGs clearly developed their ovaries at day 7 (Okada et al., 2010a). In the congeneric species, *Diacamma ceylonense*, a similar decline of FG aggressiveness was also observed after ovarian development

(Cuvillier-Hot et al., 2002). This has been interpreted as indicating that the chemical signals specific to the egg-layer (FG-specific cuticular hydrocarbon profiles; Cuvillier-Hot et al., 2002) have replaced the physical aggression by chemical communication after attaining the egg-laying phase (Cuvillier-Hot et al., 2001, 2002; Liebig et al., 2000; Monnin, 2006). Instead of physical aggression, a mature gamergate actively antennates nestmates and the nestmate workers respond to the gamergate's contact by a submissive 'crouching' behavior (Tsuji et al., 1998, 1999; Kikuchi et al., 2008, 2010). At day 7, FGs showed antennation behaviors and workers responded by crouching (Y.O., personal observations). These features suggest that the physiology of day-7 FGs may be similar to that of mature gamergates, except for mating status.

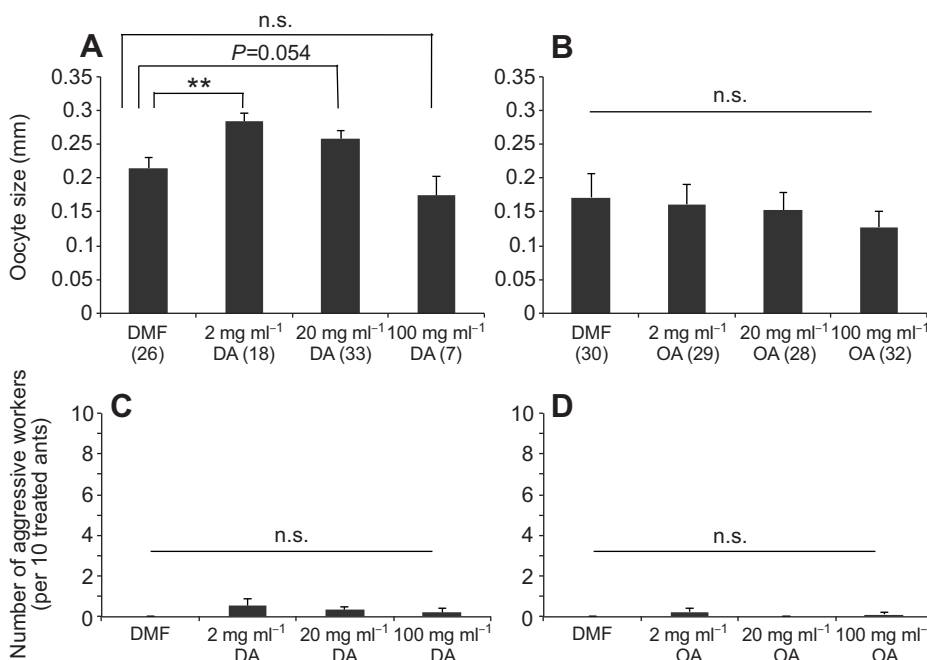


Fig. 5. Effect of biogenic amines on fecundity and aggressiveness. Effects of dopamine (DA) and octopamine (OA) on fecundity (A,B) and aggressiveness (C,D). Mean number of aggressive workers per 10 treated workers in each focal colony are shown (\pm s.e.m.) after (C) dopamine treatment ($N = 9$ colonies) and (D) octopamine treatment ($N = 8$ colonies). Asterisks indicate a significant difference (Dunnett's test for multiple comparisons, ** $P < 0.01$). Levels of significances against DMF controls of 2, 20 and 100 mg ml⁻¹ treatments were (A) $P = 0.0057$, $P = 0.0538$ and $P = 0.402$, (B) $P = 0.989$, $P = 0.936$ and $P = 0.548$, (C) $P = 0.195$, $P = 0.577$ and $P = 0.814$, (D) $P = 0.227$, $P > 0.99$ and $P = 0.726$, respectively. Sample sizes are provided in parentheses. n.s., not significant.

Dominance-dependent changes of aminergic signaling

Brain dopamine levels were found to be higher in FGs than in workers at day 7 but not at day 1 (Fig. 2A). At day 7, ovarian size differed clearly between workers and FGs (Okada et al., 2010a), and aggressiveness calmed down in FGs (Fig. 1A). Eclosed females were highly aggressive at day 0 and this aggressiveness declined at day 1 and later (Fig. 1B). Despite the strong aggressiveness of day-0 females (intact callows, ICs) and the distinct difference of aggressiveness between FGs and workers at day 1, ICs had low dopamine levels and day-1 FGs and workers had similar levels of dopamine (Fig. 2A). Considering the temporal dynamics of aggressiveness, ovarian development and dopamine levels altogether, the dopamine levels seemed to correspond with ovarian activity but not with aggressiveness. Together with the fact that the topical application of dopamine did not induce aggressiveness (Fig. 5C), we conclude that the elevated brain dopamine levels at day-7 FGs are a result of the established egg-laying status, not because of the aggressive dominance behavior. Similar dominance-dependent changes of dopamine were also observed in the ponerine ant *Harpegnathos saltator*; the brain dopamine levels of the prospective high-rankers are reduced by an aggression towards them (i.e. policing, Penick et al., 2014). These studies indicate that brain dopamine is modified as a result of the dominance interaction.

Although biogenic amines often cause aggressiveness in invertebrates (Huber et al., 1997; Adamo et al., 1995; Hoyer et al., 2008) and vertebrates (Winberg and Nilsson, 1992, 1993; Miczek et al., 2002), our analyses of amine levels, receptor expression and topical application did not support the amine-mediated aggression in *Diacamma* sp. The rapid decline of aggressiveness in mutilated workers may be mediated by a non-aminergic system. Alternatively, it is also possible that the potential behavioral effects dopamine were not detected in this study due to the small sample sizes or unknown factors. These issues are open to debate in future studies.

Gonadotropic effect of dopamine

Topical application of dopamine had a significant effect on oocyte growth, whereas octopamine did not have such effect (Fig. 5A,B). Together with the brain dopamine difference at day 7, we suggest that brain dopamine somehow has a gonadotropic function. Brain biogenic amines can be transferred to distant tissues via hemolymph ('neurohormones', Evans, 1980; Blenau and Baumann, 2001). In honeybees, it has been shown that dopamine synthesis mainly occurs in the brain, and levels of both hemolymphal and brain dopamine are higher in queens than in workers (Sasaki et al., 2012). Because insect ovarian development is caused by activity of the ovary itself (Okada et al., 2010a) and by vitellogenin synthesis by the fat body (Heming, 2003), the ovary and fat body can be candidate targets of dopamine. Receptor expression analysis revealed that *dopr1* and *dopr2* were highly expressed in the fat body of day-7 FGs (Fig. 4), suggesting that the fat body is a potential target of dopamine. A plausible scenario in *Diacamma* is that synthesis of dopamine is elevated in the brain of future gamergate, and dopamine is delivered to the fat body via hemolymph as a neurohormone to cause oocyte development. In contrast to the fat body, *dopr1* and *dopr2* were elevated in worker ovaries. The reason for worker-specific dopamine receptor transcription in ovaries remains unknown, but the worker-specific elevation of dopamine receptor transcripts is also observed in the ponerine ant *H. saltator* (*dop1* and *dop3*, Penick et al., 2014) and in the honeybee (*dop3*, Vergoz et al., 2012), suggesting a shared mechanism in social

Hymenoptera. One hypothetical mechanism is that dopamine has a suppressive effect on ovarian growth, probably via induction of apoptosis (Vergoz et al., 2012) or via inhibition of vitellogenin uptake. The gonadotropic effects of dopamine exhibited counter dose-dependence (Fig. 5). The brain dopamine level showed a strong age-dependent increase, resulting in the highest levels in foragers (Fig. 2A). We speculate that an excessive dose of dopamine may cause a forager-like physiology that suppresses oocyte growth.

Age dependence and pleiotropic roles of biogenic amines

Age-dependent increases of brain amine levels have been reported in workers of various social insects, implying strong links between biogenic amine and age-dependent worker polyethism (Schultz and Robinson, 1999, 2001; Seid and Traniello, 2005; Seid et al., 2008; but see Wnuk et al., 2011). In *Diacamma*, workers specialize in inside-nest tasks for 1–2 months after eclosion (Nakata, 1995); thus, day-7 workers are still nurses. Nevertheless, the fact that the levels of brain amines (dopamine, octopamine and 5-HT) and their receptors (*dop1*, *oar* and *5ht*) increase only within 7 days is noteworthy, emphasizing the importance of age in the interpretation of amine dynamics. In social Hymenoptera, the individual age is a major determinant of dominant status (Strassmann and Meyer, 1983; Higashi et al., 1994; Monnin and Ratnieks, 1999; Tsuji and Tsuji, 2005). Thus, age- and dominance-dependent changes should be combined to result in complex amine dynamics that may potentially provide pseudo-correlations. By controlling age, aggressiveness and fecundity, our study in *Diacamma* concludes that dominance interactions cause a difference in dopamine levels and lead to reproductive differentiation. Unfortunately, the ontogeny of the amine levels of established gamergates were not investigated in this study. The lower dopamine levels in FGs compared with sterile foragers imply that heightened dopamine may function during the early stages of reproductive differentiation rather than in its maintenance at older stages. How gamergate physiology follows the age-dependent dynamics should be addressed in future studies in order to understand the combined effects of amines on age- and dominance-based polyethism.

Conserved function of dopamine in reproductive differentiation

In solitary adult insects, juvenile hormone (JH) generally stimulates sexual maturity and reproduction (Nijhout, 1998; Hartfelder, 2000; Raikhel et al., 2005). In adult social Hymenoptera, however, gonadotropic effects of JH are only supported in ancestral lineages (bumblebee, Bloch et al., 1996; paper wasp, Barth et al., 1975; Bohm, 1972) and not in the derived lineages such as honeybees (Robinson et al., 1991, 1992) and several ant species (*Diacamma*, Sommer et al., 1993; *Streblognathus*, Brent et al., 2006; *Harpegnathos*, Penick et al., 2011; *Solenopsis*, Brent and Vargo, 2003), implying that the gonadotropic function of JH is lost in derived lineages (Hartfelder, 2000). In contrast to JH, data are accumulating to support the commonness of positive associations between brain dopamine and reproductive status across the lineages (paper wasp, Sasaki et al., 2007; bumblebee, Bloch et al., 2000; honeybee, Harris and Woodring, 1995; ants, Penick et al., 2014 and this study). As gonadotropic effects of dopamine are also found in solitary insects (fruit fly, Neckameyer, 1996; Pendleton et al., 1996; cockroach, Pastor et al., 1991), we speculate that the gonadotropic function of dopamine is ancestral. The gonadotropic function of dopamine may have been recruited to the dominance-based physiological regulation at the primitive phase of eusocial evolution, and is now conserved in primitive and derived species to complement the reproductive function of JH (Sasaki et al., 2007).

Future comparative studies may reveal the involvement of dopamine during the social evolution.

MATERIALS AND METHODS

Insects

Colonies of *Diacamma* sp. (the only species of *Diacamma* in Japan) were excavated in Nakijin (Nakijin), Kenmin-no mori (Onna), Hantagawa Park (Naha) and Sueyoshi Park (Naha) in Okinawa, Japan. Gamergate-right colonies (the colonies containing a mated gemmae-possessing female) containing 30–200 workers were kept in plastic artificial nests (9 cm diameter × 1.5 cm height) filled with moistened plaster. The artificial nests were placed in a plastic arena. The nests were maintained at 25°C under 16 h light:8 h dark conditions (light: 08:00 h–24:00 h). Reared colonies were fed chopped mealworms and crickets three times a week.

Induction of reproductives and sterile workers

A newly eclosed female without interaction with nestmates is defined as an intact callow (IC). An IC can differentiate into a gamergate or a worker, depending on the colony status. For the brain amine measurements and the receptor level quantifications of ICs, ICs were obtained by artificially opening the female cocoons to subsequently obtain the eclosed adults, because naturally eclosed callows cannot avoid social interactions. Eclosed day-0 females were used as ICs. In an orphan colony (i.e. gamergate-absent colony), a newly eclosed female retains gemmae and becomes a prospective egg-layer [future gamergate (FG), Okada et al., 2010a]. The FGs were kept unmated to equalize the mating status with workers. We created satellite orphan colonies that consisted of 15 mutilated workers each. A new female naturally eclosed from the cocoon in each orphan colony was used as the FG. In gamergate-right colonies, the gemmae of eclosed females were subjected to natural mutilation, so that they become workers (Fukumoto et al., 1989; Fuchikawa et al., 2014). Eclosed ants were individually marked, and day-1 and day-7 mutilated workers (1W, 7W) and future gamergates (1FG, 7FG) were collected for the following experiments (between 09:00 h and 14:00 h). Mutilated workers aged more than 2 months that were walking around the arena were defined as foragers.

Observation of aggressiveness

The behavioral ontogenies of newly eclosed females in gamergate-right and orphan colonies were quantified. Biting and jerking of the appendages of nestmates ('bite and jerk', Peeters and Tsuji, 1993) and stinging behavior were regarded as aggressive attacks. Total numbers of aggressive attacks were counted for 10 min every day from day 0 to day 7. This observation is performed during the daytime (09:00 h–14:00 h) in which their dominance behavior is known to be active (Fuchikawa et al., 2014). In gamergate-right colonies, mutilation is completed within a few hours of eclosion (Fuchikawa et al., 2014). Therefore, to observe the behavior of non-mutilated females (i.e. day-0 worker), ICs were artificially returned to the original gamergate-right colony and the behavior before the mutilation was quantified.

Measurement of biogenic amines

Day-0 IC (IC), day-1 W (1W), day-1 FG (1FG), day-7 W (7W), day-7 FG (7FG) and foragers were obtained as above, and were frozen and stored in liquid nitrogen immediately after the collection. Each sample corresponded to an individual ant. Brains (including subesophageal ganglia) were dissected in ice-cold bee saline (Bailey, 1952) immediately after removal from the liquid nitrogen and homogenized in 50 µl of ice-cold 0.1 mol l⁻¹ perchloric acid containing 12.5 ng ml⁻¹ 3,4-dihydroxyphenylacetic acid (DHBA) as an internal standard. Then, samples were centrifuged at 20,000 g for 30 min at 0°C and supernatants were used in HPLC-ECD analysis according to Mezawa et al. (2013) (see below).

The HPLC-ECD system consisted of a solvent-delivery pump, a refrigerated automatic injector and a C₁₈ reverse-phase column (UG 120, Shiseido, Japan) maintained at 35°C in a column oven. An electrochemical detector (ECD-300, EICOM, Japan) with a glassy carbon electrode was set at 0.82 V against an Ag/AgCl reference electrode. Signals from the electrochemical detector were recorded and integrated using analysis software PowerChrom (ADInstrument, Australia). The mobile phase contained 0.18 mol l⁻¹ of monochloroacetic acid and 40 µmol l⁻¹ of

Na₂EDTA (Wako) adjusted to pH 3.6 with NaOH (Wako). Sodium-1-octanesulfonate (1.62 mmol l⁻¹) (Nacalai Tesque, Kyoto, Japan) and CH₃CN (final concentration 7.4%, v/v, Nacalai) were added to this solution. A constant flow rate of 0.7 ml min⁻¹ was employed. External standards [octopamine, dopamine, serotonin and DHBA (Sigma, St Louis, USA)] were used for the chemical identification and quantification. Each biogenic amine peak was identified by comparing the retention time and hydrodynamic voltammogram with those of the standards, while uncertain peaks were eliminated from the analysis. Measurements based on the peak area of the chromatograms were obtained by calculating the ratio of the peak area of a substance to the peak area of the external standard.

Receptor gene expression

To quantify the transcript levels of amine receptors, the partial gene sequences for two dopamine receptors (*dopr1* and *dopr2*), one octopamine receptor (*oar*) and one serotonin receptor (*5htr*) were sequenced after PCR amplification by gene-specific degenerated primers (supplementary material Table S1). Brain-derived cDNA was used as template and PCR was performed with Ex taq polymerase (Takara, Shiga, Otsu, Japan) as follows: 94°C for 30 s, 58.4°C for 30 s and 72°C for 3 min for 35 cycles. Cloned fragments were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA; see Okada et al., 2010b, for cloning details) and confirmed their homologies to the annotated sequences in honeybees (*Apis mellifera*) and jumping ants [*Harpegnathos saltator*, the closest relative of *Diacamma* whose genome has been sequenced (Bonasio et al., 2010) by blastx (supplementary material Table S1)]. Two dopamine receptors of *Diacamma* (*dopr1* and *dopr2*) were both categorized into D1-like excitatory receptors.

For RNA extraction, IC, 1W, 1FG, 7W and 7FG were obtained as above, anesthetized on ice and dissected in ice-cold PBS. Brain, ovaries and fat bodies from gasters were isolated and mashed in the lysis buffer of the RNA extraction kit (RNAqueous micro, Life Technologies, Tokyo, Japan) and stored at -80°C until RNA extraction. Tissues from three individuals were combined as one sample. Extracted RNA was subjected to DNase treatment (DNase I, Life Technologies) and reverse transcription (High-capacity cDNA Reverse Transcription kit, Life Technologies). Kapa SYBR Fast qPCR kit (KAPA, Wilmington, USA) and Thermal Cycler Dice Real Time System II (Takara, Shiga, Japan) were used in real-time qPCR with the gene-specific primers (supplementary material Table S1). We used *gapdh* (glyceraldehyde 3-phosphate dehydrogenase) as an internal control gene because Normfinder (Andersen et al., 2004) indicated *gapdh* was the most stable of the five genes examined (28S ribosomal RNA, 28S ribosomal protein, *gapdh* and two *actin* genes) across the tissues and castes in our samples.

Topical application of biogenic amine

Based on the results showing brain dopamine in FG (see result) and the potential effect of octopamine on aggressiveness (Adamo et al., 1995; Hoyer et al., 2008), topical applications of dopamine and octopamine were performed to confirm the functions of these biogenic amines. We followed the method described by Barron et al. (2007) in which thoracic topical applications of biogenic amines dissolved in a carrier solvent, dimethylformamide (DMF), effectively altered honeybee brain amine levels within 15–60 min. Dopamine and octopamine were diluted in DMF at concentrations of 2, 20 and 100 (µg µl⁻¹) and 2 µl of solution was topically applied on the thorax and gaster of ants. We used 2 µg µl⁻¹ as the lowest dose due to its effectiveness in honeybees (Barron et al., 2007).

For the evaluation of gonadotropic effects, mutilated workers, less than 2 months old, were individually isolated from gamergate-right colonies. Workers originated from three different colonies were randomly allocated to the above treatments. Ants were isolated in small petri dishes (4 cm diameter and 1 cm height) and had the amine solution or DMF (control) applied topically every other day. Isolated ants were made to have free access to food (chopped mealworms and sugar water). After 9 days of individual rearing, their ovaries were dissected and oocyte sizes (represented by largest oocyte) were measured by dissection microscope (Olympus, SZX7, Tokyo, Japan). Although most mutilated workers are not aggressive (Peeters and Higashi, 1989), gamergate-right colonies sometimes contain a few aggressive

mutilated workers that contrive to lay eggs (beta, gamma and delta, Nakata and Tsuji, 1996; Shimoji et al., 2014). These potential high rankers were identified by 30 min of observation for two consecutive days before the experiment and were not used.

In the analysis of aggressiveness, 40 mutilated workers (younger than 2 months old and inside-nest workers) were randomly selected from gamergate-right colonies that consisted of 80–150 workers (high-rankers were not used as described above). Ten out of 40 workers were treated once with a 2 µl solution of either of 2, 20 or 100 (mg ml⁻¹) dopamine or DMF, as described above. Treated workers were returned to the colony. After 1 h of settlement, the behaviors of treated workers were observed for 30 min (*N*=9 colonies). octopamine treatment (*N*=8 colonies) was also performed, similar to the dopamine treatment.

Acknowledgements

We thank two referees and the editor for improving our manuscript. We also thank T. Kikuchi, M. K. Hojo, S. Dobata, H. Fujioka, S. Yonamine, M. Okamoto-Miyakawa and A. S. Mikheyev for supporting the collection and rearing of *Diacamma* colonies. Our thanks go to T. Kubo and S. Akasaka for kind statistical and technical support.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.O., K.S. and T.M. conceived and designed the experiments; Y.O., K.S. and S.M. performed the experiments; Y.O., K.S., S.M., H.S., K.T. and T.M. interpreted the data and wrote the manuscript.

Funding

This work was supported by Kakenhi [26870121 to Y.O., 26440181 to K.S.] and by Research and Education Platform for Dynamic Living States to Y.O. H.S. is supported by Grant-in-Aid for JSPS Fellows [11J03715].

Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.118414/-/DC1>

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