Original Communication

Primary and secondary sex ratios in the inquiline parasitic ant *Vollenhovia nipponica*: Effects of local mate competition, timing of oviposition and host worker control

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ABSTRACT

In many social parasite ants, the worker caste is lost and host workers care for the parasite brood so that the sex ratio in reproduction is unable to be influenced by worker control. Instead, the queen is expected to bias the sex ratio to maximize her own inclusive fitness. Effects of factors other than worker control on sex ratio also can be evaluated. In this study, we examined primary and secondary sex ratios in the inquiline parasitic ant *Vollenhovia nipponica*. The following three hypotheses were proposed and tested in particular: (1) the primary sex ratio is female-biased, being determined by local mate competition among males, (2) the proportion of female eggs is higher in the early period of oviposition because females eclosed earlier are predicted to obtain higher mating and colony-founding success, and (3) the secondary sex ratio is not different from the primary sex ratio because parasite broods are not controlled by host workers. In 18 colonies, the sex ratio of eggs produced by *V. nipponica* queens was observed by counting cell chromosome numbers of eggs. Furthermore, new females and males in the colonies were collected to evaluate the secondary sex ratio. Although the primary sex ratio was female-biased over the observation period, female egg proportion was not different between the early and late periods.

Moreover, no difference was observed between the primary and secondary sex ratios, indicating that the broods were not influenced. The female-biased sex reproductive ratio was determined by queens that biased the primary sex ratio.

INTRODUCTION

Analysis of sex allocation patterns in social insects is one of the most reliable tests of the kin selection theory. In ants, relatedness asymmetries among colony members generally have an effect on the allocation pattern of sexual production [1, 2, 3, 4]. In some species, workers impose their preferred allocation pattern by preferentially raising one sex [5, 6, 7, 8]. However, in other species the queen is clearly able to control the final (secondary) sex ratio [9, 10, 11, 12]. Investigations of sexual allocation in social parasitic ants have provided important insights into the sex ratio theory [3]. Parasitic ants completely depend on host workers to rear the offspring so that parasite workers cannot directly control sex allocation. Consequently the queen can produce her offspring in accordance with the preferred sex investment ratio. In the slave-making ant *Harpagoxenus sublaevis*, the sex investment ratio of colonies was not significantly different from 0.50 (1:1 investment); however, it differed significantly from 0.75 (3:1 investment), which is in agreement with the genetic-relatedness hypothesis [13]. This suggests that parasite workers

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do not have direct influence on the sex ratio of their broods so that the parasite queen can impose her preferred sex ratio.

Inquilinism is an extremely specialized social parasitic lifestyle. In many inquiline ants, the worker caste is completely lost and queens only produce sexuals by using host workers, which would not benefit from biasing the sex ratio. Therefore, it is expected that worker influence on the sex ratio would be absent and the parasite queen would be able to impose her preferred sex ratio. As predicted by relatedness to daughters and sons, queens may evenly invest resources in female and male production. Therefore, the primary sex ratio of eggs produced will be close to 1:1. In addition, inquiline species have typically evolved characteristic traits such as wing morphology, mating in or close to the mother nest, dependent colony establishment and polygyny to suit their parasitic lifestyle [14]. Sexual allocation is expected to be affected by these traits. For example, queens of some inquiline ants are wingless, and mate with males in or near the host nests. Mating under such limiting conditions is likely to induce local mate competition (LMC) among males, leading to a heavily female-biased sex ratio [15, 16].

In this study, we examined the sex ratio of production of sexuals in an inquiline ant, *Vollenhovia nipponica*, which parasitizes the congeneric species *V. emeryi* [17]. In particular, by preliminary observations of the life history, we proposed three hypotheses for sexual allocation. First, *V. nipponica* males frequently mate with new females in the nests [18]. In late summer, new males eclose earlier, which allows them to immediately mate with late-eclosing females. Approximate 27% of the new females mate before hibernation, by intra-nest mating [19]. As mentioned above, such frequent intra-nest mating strongly suggests the existence of LMC among males. Many studies of ants demonstrate female-biased sex ratio in local situations in which males compete for mates locally [20, 21, 22, 23, 24, 25, 26]. Therefore, it can be hypothesized that the primary sex ratio of egg production is female-biased in *V. nipponica*.

Second, it is possible that the timing of egg production affects the primary sex ratio in *V. nipponica*. A new female has two alternative mating tactics, namely copulation either in the nest or during the nuptial flight [18]. Females mating in the nest form new colonies through colony budding of the host nests, whereas those mating during the nuptial flight have to find and invade new host colonies outside. However, the tactics adopted by new females is markedly affected by the timing of female eclosion. When many females had already mated in the nests, late-eclosed females could not mate in the nests and adopted mating during their nuptial flight [19]. It is possible that inter-nest mating and invasion of new host colonies is costlier than intra-nest mating and establishment of colony budding by host ants. If reproductive success is higher in females adopting intra-nest mating, mother queens should alter the sex ratio of eggs, depending on the timing of oviposition. It has been predicted that the primary sex ratio of egg production is likely to be more female-biased in the early period of oviposition.

Third, as suggested in some studies [13], broods of social parasitic ants are not controlled by host workers. In the workerless ant *Plagiolepis xene* which parasitizes on *P. pygmaea*, that the sex ratio of eggs was not significantly different from that of new sexuals [27]. In addition, production of sexuals in the parasitic ant was not affected, even when sexuals of the host species were controlled. This appears to be one of the reproductive strategies in parasitic ants; however, the proximate mechanism of avoiding host control is yet unclear. By avoiding host worker influence on the sex ratio, the parasite queen can increase her inclusive fitness. This may simultaneously result in the sex investment ratio preferred by parasite queens. In *V. nipponica*, it is also predicted that the broods would not be controlled by host workers.

According to these assumptions, we tested the following three hypotheses for both primary and secondary sex ratio in *V. nipponica*: (1) the primary sex ratio of egg production is female-biased, (2) the proportion of female eggs is higher in the early period of oviposition, and (3) the secondary sex ratio is not different from the primary sex ratio.

MATERIALS AND METHODS

Life history of *V. nipponica*

Vollenhovia nipponica is a workerless ant that parasitizes colonies of *V. emeryi*. That host ant

V. emeryi exhibits queen polymorphism with regard to wing morphology: both long- and short-winged types. *V. nipponica* frequently parasitizes colonies of short-winged queens [17]. New sexuals eclose from late August to September in host colonies, and a number of new females immediately mate with males in the nests [19]. From April to May, after hibernation, females become aggressive towards one another and fight violently. Consequently, only some dominant queens in a colony can develop ovaries and lay eggs. Other unmated females remain in the colonies until the following summer, performing nuptial and dispersal flights in July [18]. Males also eclose in late August and immediately attempt to mate with new queens in the nest. In the summer following hibernation, they perform a nuptial flight with unmated queens from the nests.

Field collection of parasitized colonies and preparation of observation colonies

From April to June 2010, *V. emeryi* colonies parasitized by *V. nipponica* were collected from a secondary forest in Kanazawa City (36º 35' N, 136º 35' E) and Takaoka City (36º 44' N, 137º 01' E). From the two sites, branches with nests of *V. emeryi* with *V. nipponica* were collected and transferred to the laboratory. Numbers of colony members and broods were counted to determine colony composition. Collected colonies were kept in plastic boxes (14.5 × 8.0 × 3.0 cm) with artificial nests at a constant temperature of 26ºC and under a natural light/dark cycle in an incubator. They were fed mealworms and cat food twice a week and were observed continuously.

To observe oviposition, 29 host colonies with 1-3 queens and 66-278 workers were prepared. *V. nipponica* queens and males already existing in the colonies were removed in advance. Only one parasite queen was introduced to 12 colonies. However, this did not provide sufficient eggs for analysis of sex ratios because the fecundity of *V. nipponica* queens varied [18]. Therefore, three queens were introduced into another 14 colonies. Introductions were performed in late May, after which the colonies were kept for 112-135 days.

Observation of primary sex ratio of egg production

To evaluate the primary sex ratio, eggs laid by *V. nipponica* queens were collected in isolation experiments. First all host queens and eggs were isolated from parasitized colonies and transferred to a small plastic box (4.3 × 5.9 × 1.6 cm) with 15-22 workers and 5-10 larvae. The colonies without host queens were kept for about two weeks, and all the eggs laid by parasite queens during this period were collected. Following this, host queens and workers were returned to their original colonies. This isolation experiment and egg collection were performed two times, once each during the initial and late season of reproduction in *V. nipponica* i.e. the middle of June (36-43 days after introducing parasite queens) and middle of August (56-87 days after introducing parasite queens), respectively.

The sex of the eggs was determined by observing the chromosome number. The collected eggs were stained to determine the karyotype following the methods [28]. The eggs were placed onto a microscope slide and 1 ml of hypotonic solution (mixed 1% sodium citrate solution mixed with 0.1% colchicine solution) was added to the slide. After 20 min, 1 ml of 60% fixative solution (60% acetic-ethanol with ethanol and glacial acetic acid) was applied and drained off slowly. The eggs were then punctured with a needle, followed by quick application of 1 ml of fixative solution and 1 ml of glacial acetic acid. After 2-3 hours, the solution was slowly drained off by inclining the slide laterally. After drying, for at least 24 h, the preparations were stained with freshly prepared Giemsa solution for 30 min and then washed with water. The stained samples were observed using a binocular microscope, and the number of chromosomes per cell was counted. For each sample, we counted the number of chromosomes in 5-15 cells (average 8.2 ± standard deviation (SD), 8.2 ± 3.1 cells) and determined whether the egg samples were diploid or haploid. The primary sex ratio of egg production was estimated from the relative proportions of diploid eggs.

Observation of secondary sex ratio of new sexual production

The secondary sex ratio was observed by counting the number of female and male sexuals produced in each experimental colony. Until October (112-135 days), 29 colonies were observed. From August to October, new sexuals that eclosed in the colonies were collected and stored in 99% ethanol. The final number was then counted.

In general, the investment ratio estimated by the dry weight of new sexuals is used as the secondary sex ratio. However, in this study, to compare the primary sex ratio (estimated as the proportion of the female egg number to total egg number), the proportion of the number of new females to total number of new sexuals was used as the secondary sex ratio.

Removal of eggs for observation of the primary sex ratio can possibly affect the secondary sex ratio. To confirm the accuracy of the secondary sex ratio, colonies parasitized in the field were collected. From these collection sites, 35 colonies were collected from August to October in both 2010 and 2011. Numbers of new *V. nipponica* sexuals were counted from these colonies, and the ratio of females to males was calculated.

Statistical analysis and effects on sex ratio of other factors

In this study, sex ratio was defined as the proportion of female offspring. Sex ratio data are characterized by binomially distributed data and most appropriately analyzed with general linear model (GLM), with binomial error structures and logit link functions [29, 30]. Statistical tests for comparison of primary and secondary sex ratios were performed using general linear model with arcsin-square root transformed sex ratios [30].

Moreover, factors effecting on primary and secondary sex ratios were also evaluated using generalized linear model (GLMs). The colony size, total number of eggs, number of parasite queens, and number of host queens were used as factors. For fixed factors, model simplification was performed to test the significance of main effects and interactions. All analyses were performed with software R 2.14.1 (http://www.r-project.org/).

RESULTS

Observation of oviposition in parasite queens

Four colonies each in the 12 single-queen and 14 multiple-queen colonies produced insufficient eggs. Our analyses were therefore based on 8 single-queen and 10 multiple-queen colonies. A total of 471 eggs were collected (single-queen 194; multiple-queen 277). In the single-queen colonies, the average number of eggs produced per day was 0.5-1.4 and 0.8-2.4 eggs in the first and second collection, respectively, whereas in the multiple-queen colonies, the average was 0.14-1.8 and 0.5-2,6 eggs/day in the first and second collection, respectively. In both the colony groups, there was no significant difference between the egg number in the first and second collection (single-queen: t=-0.59, P=0.57; multiple-queen t=-2.1, P=-0.94, paired t-test).

Primary sex ratio of egg production

In 422 of the collected eggs (single-queen: 178; multiple-queen: 244), the karyotype could be observed. We found 20 (2n=20) and 10 (n=10) chromosomes in the samples and concluded that the former were diploid (female) and the latter were haploid (male) eggs. In the single- and multiple-queen colonies, the average female egg proportion in the total eggs collected was 0.68 ± SD 0.03, and 0.71 ± 0.12, respectively. The average female egg proportions in the two colony groups were not significantly different (t=0.81, P=0.38, t-test). The ratios were female-biased and significantly different from 0.5 (P<0.0001, binomial test). Using multiple regression analysis in GLMs, no significant effects of factors and their interactions on the primary sex ratio were observed (all factors: P>0.4).

In the single-queen colonies, the female egg proportion was 0.66 ± 0.15, and 0.70 ± 0.16 in the first and second collection, respectively (Table 1). Female egg proportion in the first collection was not significantly different from that in the second collection (t=-0.67, P=0.51, paired t-test). In contrast, in the multiple-queen colonies, female egg proportion was 0.76 ± 0.23, and 0.70 ± 0.15 in the first and second collection, respectively (Table 1). However, because only 2 eggs were obtained in the first collection from No 2010603(12) colony, the data from this colony were excluded in comparing the sex ratio between first and second collections. Despite this, no significant difference in female egg proportion was observed between first and second collections in the multiple-queen colonies (t=-0.027, P=0.98, paired t-test).

Secondary sex ratio of sexual production

An average of 14.3 ± 8.3 and 10.9 ± 5.4 females and that of 6.8 ± 4.3 and 6.0 ± 4.0 males were collected from the single- and multiple-queen colonies.

Table 1. Primary and secondary sex ratio in 18 experimental colonies. Female egg proportion in collection of early (first) and late (second) period is shown as primary sex ratio. Proportion of new female sexuals produced in the colonies is also shown as secondary sex ratio.

Experimental group	Colony No	Number of observed eggs	First collection		Second collection		New sexuals	
			N	Female egg proportion	N	Female egg proportion	N	Female proportion
Single-queen	2010430(2)	31	15	0.87	16	0.56	22	0.55
	2010430(3)	12	6	0.50	6	0.83	18	0.61
	2010430(4)	22	17	0.59	5	1.00	10	0.50
	2010430(5)	11	5	0.80	6	0.50	9	0.56
	2010518(4)	14	6	0.50	8	0.75	48	0.71
	2010518(5)a	13	8	0.75	5	0.60	13	0.85
	2010518(5)b	46	19	0.74	27	0.67	26	0.62
	2010518(10)	29	6	0.50	23	0.65	22	0.91
	Average $\pm$ SD			0.66 $\pm$ 0.15		0.7 $\pm$ 0.16		0.66 $\pm$ 0.15
Multiple-queen	2010518(7)a	17	10	0.70	7	0.71	15	1.00
	2010603(12)	10	2	1.00	8	0.50	12	0.50
	2010603(13)	24	9	0.89	15	0.60	21	0.81
	2010603(16)	43	15	0.73	28	0.79	24	0.63
	2010603(17)	28	18	0.94	10	1.00	16	0.88
	2010603(20)	27	15	0.20	12	0.83	21	0.57
	2010610(12)	30	11	0.91	19	0.68	24	0.63
	2010610(14)	15	6	0.67	9	0.67	10	0.70
	2010610(15)	17	11	0.82	6	0.50	21	0.38
	2010610(16)	33	17	0.71	7	0.71	5	0
	Average $\pm$ SD			0.76 $\pm$ 0.23		0.70 $\pm$ 0.15		0.61 $\pm$ 0.28

The proportion of females in the total new sexuals was 0.67 ± 0.14 and 0.61 ± 0.28 in the single- and multiple-queen colonies, respectively. As shown in Figure 1, the secondary sex ratio was not significantly different from the primary sex ratio in both the colony groups (single-queen, t=0.049, P=0.96; multiple-queen, t=0.82, P=0.42, paired t-test). Analysis in GLMs showed that there were no significant effects of factors and interactions on the secondary sex ratio (all factors: P>0.06).

Next, the sex ratio was compared among groups in the laboratory and field. Of the 35 collected colonies, 30 produced more than 5 individuals of new sexuals (average 21.2 ± 12.7 females and 5.6 ± 6.3 males). Our analyses were based on these 30 colonies. The number of mother queens in these colonies was unclear. Therefore, in the laboratory group, the primary and secondary sex ratios in all 18 colonies were used for the analyses. As shown in Figure 2, the proportion of females was significantly different among the 3 groups, eggs and new sexuals in the laboratory and field groups ($F_{2,63}$=5.5, P=0.0061, one-way ANOVA). The female proportion in the new sexuals was higher in the field than in the laboratory group (P=0.05, Tukey-Kramer test). However, there was no difference between the eggs in the laboratory and new sexuals in the field.

DISCUSSION

The primary sex ratio of egg production is female-biased in *V. nipponica*. In Hymenoptera, the female-biased sex ratio is governed by the following factors: (1) worker control based on relatedness asymmetry [1], (2) higher male mortality caused by the haplodiploidy sex determination
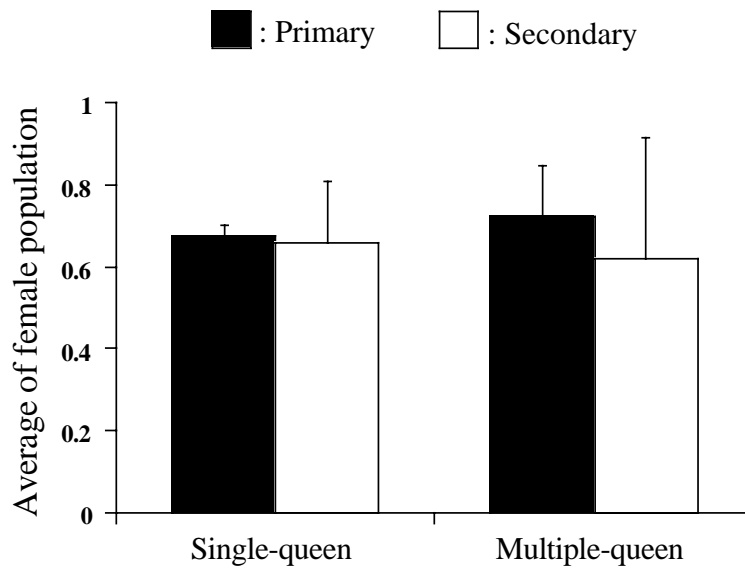
Figure 1. Comparison of the primary and secondary sex ratio in 2 experimental groups. Average of female egg and new female sexual proportion is shown as primary and secondary sex ratio. Error bars mean standard deviation.
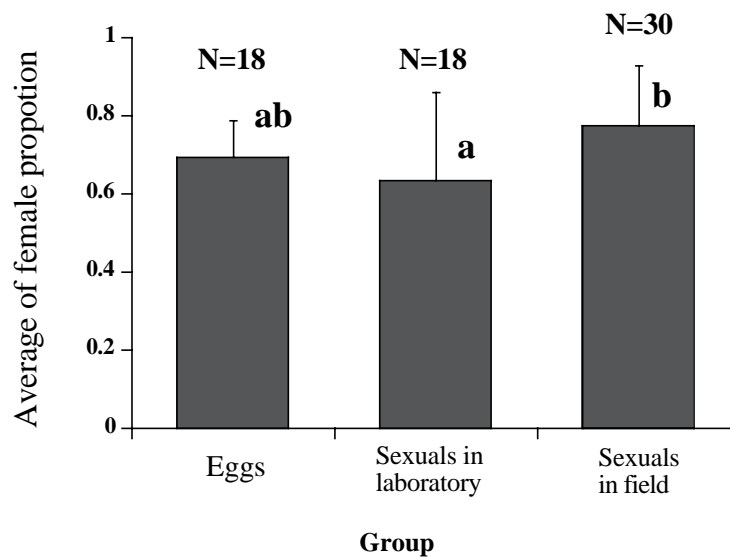

Figure 2. Comparison of sex ratio among eggs, new sexuals in laboratory, and field. Average of female proportion in eggs and new sexuals is shown. Proportion of female was significantly different among three groups ($F_{2,63}$=5.5, P=0.0061, one-way ANOVA). Different alphabets mean the significant difference among groups.

system [31] and (3) local mate competition (LMC) [15, 20]. In the workerless ant *V. nipponica*, there is no worker control. In addition, we could find no difference between primary and secondary sex ratios, suggesting that male mortality is not affected by haploidy. As suggested by some studies [7], in *V. nipponica*, LMC may be the main factor for female-biased sexual allocation. However, *V. nipponica*

males can adopt two mating tactics, namely copulation in the nest or during the nuptial flight. The tactics are not optional because many males mating in the nest perform the mating flight after hibernation [18]. Adoption of both these tactics indicates reduced intensity of mate competition among males. It is probable that the mating success in males is extremely different between these tactics and depends to a great extent on inter-nest mating success. Therefore, the female-biased sexual allocation may be induced by intensive competition among males in intra-nest mating.

The timing of oviposition during the reproductive period did not influence the primary sex ratio, although early-eclosed females can frequently adopt intra-nest mating that ensures establishment of host colonies. This means that reproductive success of new females may not be affected by the timing of eclosion. In other words, reproductive success varies little between females mating in the nests or during the nuptial flight. Compared to mating in the nests, the nuptial flight and invasion of new host colonies appear to be relatively costly for new females. However, females adopting intra-nest mating also are not always successful in securing host colonies. It is reported that the queens mating in the nest become aggressive towards one another after colony budding and that only a few dominant queens in a host colony can reproduce [18]. Reproductive success is not always higher in females adopting intra-nest mating. This correlates with the fact that the mating mode of new females is conditional, indicating that both mating tactics are beneficial to new females.

Lastly, there was no difference between the primary and secondary sex ratios in the laboratory experimental groups. The secondary sex ratio was slightly different between the laboratory and field groups and the proportion of females was lower in the laboratory group than in the field group. Laboratory conditions and egg removal probably affected the secondary sex ratio. However, the primary sex ratio did not differ from the secondary sex ratio in the field colonies. In addition, the number of host queens and colony size had no effect on the secondary sex ratio. This indicates that *V. nipponica* broods are not controlled by workers of the host ant *V. emeryi*. This finding is consistent with the observations in the parasitic

ant *P. xene* [27], strongly suggesting that the ability to produce female and male sexuals has been evolved in social parasitic ants even when the host actively controls production of its own sexuals. Although a proximate mechanism to avoid host worker control has not yet been evaluated, it is probable that parasitic ant broods may chemically mimic host worker brood [32]. In the social parasitic wasp, *Polistes sulcifer*, larvae camouflaged themselves by acquiring a chemical profile matching that of their hosts [33]. This system of avoiding host worker control may have a function in imposing the preferred sex ratio of parasite queens, and it also supports the hypothesis that a female-biased sex ratio is achieved from a queen-biased primary sex ratio, as observed in a few other studies [9, 11, 12, 21, 22,].

In conclusion, the primary and secondary sex ratios in the parasitic ant *V. nipponica* are female-biased, based on the local mate competition. However, other factors may also affect this female bias. It is argued that the mother should produce fewer offspring of one sex if the sex is more costly to produce [1]. To test this theory, we should consider the cost of producing eggs and adults of female and male sexuals. In addition, a great number of female eggs may be advantageous in life history of social parasitic ant. For instance, more female eggs may have a function in increasing the effect of mimicry of the host brood and may induce effects such as local resource enhancement (LRE). Thus, the possibility that a female-biased sex ratio represents an adaptation to the social parasitic lifestyle should also be considered.

REFERENCES

1.   Trivers, R. L. and Hare, H. 1976, Science, 191, 249-263.
2.   Nonacs, P. 1986, Q. Rev. Biol., 61, 1-21.
3.   Bourke, A. F. G. and Franks, N. R. 1995, Social evolution in ants, Princeton University Press, Princeton.

4. Meunier, J., West, S. A., and Chapuisat, M. 2008, Behav. Ecol., 19(2), 382-390.
5. Boomsma, J. J. and Grafen, A. 1990, Evolution, 44, 1026-1034.
6. Boomsma, J. J. and Grafen, A. 1991, J. Evol. Biol., 4, 383-407.
7. Aron, S., Vargo, E. L., and Passera, L. 1995, Anim. Behav., 49(3), 749-757.
8. Chapuisat, M. and Keller, L. 1999, Heredity, 82, 473-478.
9. Passera, L., Aron, S., Vargo, E. L., and Keller, L. 2001, Science, 293, 1308-1310.
10. Helms, K. R., Fewell, J. H., and Rissing, S. W. 2000, Anim. Behav., 59, 523-527.
11. De Menten, L., Fournier, D., Brent, C., Passera, L., Vargo, E. L., and Aron, S. 2005, Behav. Ecol. Sociobio., 58, 527-533.
12. Rosset, H. and Chapuisat, M. 2006, Cur. Biol., 16 (3), 328-331.
13. Bourke, A. F. G., van der Have, T. M., and Franks, N. R., 1988, Behav. Ecol. Sociobiol., 23, 233-245.
14. Hölldobler, B. and Wilson, E. O. 1990, The ants, Harvard University Press.
15. Hamilton, W. D. 1967, Science, 156, 477-488.
16. Werren, J. H. 1980, Science, 208, 1157-1159.
17. Kinomura, K. and Yamauchi, K. 1992, Jap. J. Ent., 60, 203-206.
18. Satoh, A. and Ohkawara, K. 2008, Ins. Soc., 55, 200-206.
19. Okamoto, M. and Ohkawara, K. 2009, Ethol. Ecol. Evol., 21, 137-146.
20. Alexander, R. D. and Sherman, P. W. 1977, Science, 196, 494-500.
21. Winter, U. and Buschinger, A. 1983, Entmol. Gen., 9, 1-15.
22. Pedersen, J. S. and Boomsma, J. J. 1998, Naturwissenschaften, 85, 593-595.
23. Cremer, S. and Heinze, J. 2002, Pro. Roy. Soc. Lon., 269, 417-422.
24. De Menten, L., Cremer, S., Heinze, J., and Aron, S. 2005, Anim. Behav., 69(5), 1031-1035.
25. Schrempf, A., Reber, C., Tinaut, A., and Heinze, J. 2005, Behav. Ecol. Sociobiol., 57, 502-510.
26. Foitzik. S., Kureck, I. M., Rüger, M. H., and Metzler, D. 2010, Behav. Ecol. Sociobiol., 64, 1641-1654.
27. Aron, S., Passera, L., and Keller, L. 1999, Pro. Roy. Soc. Lon., 266, 173-177.
28. Imai, H. T., Crozier, R. H., and Taylor, R. W. 1977, Chromosoma, 59, 341-393.
29. Crawley, M. J. 2002, Statistical computing. An introduction to data analysis using S-Plus. Wiely, Chichester.
30. Wilson, K. and Hardy, I. C. W. 2002, Sex ratios, Concepts and research methods, Hardy ICW (Ed.), Cambridge University Press, Cambridge.
31. Smith, R. H. and Shaw, M. R. 1980, Nature, 287, 728-729.
32. Aron, S., Passera, L., and Keller, L. 2004, Ins. Soc., 51, 395-399.
33. Cervo, R., Dani, F. R., Cotoneschi, C., Scala, C., Lotti, I., Strassmann, J. E., Queller, D. C., and Turillazzi, S. 2008, Behav. Ecol. Sociobiol., 62, 1319-1331.