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Prevention of parasite infection in birds: adaptive advantages of anting behaviour

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Abstract

Anting, the application of ants to plumage, has been reported in more than 200 bird species worldwide. The advantage of the peculiar behaviour is hypothesized to be self-treatment against parasites and bacteria because many ant species eject pungent fluids from their exocrine glands, which might help remove ecto- and microparasites from bird's body. However, this hypothesis has not been well explored. In this study, by using thrush *Turdus cardis* and two ant species, *Lasius spathepus* and *L. nipponensis*, it was tested whether any chemical components on bird's body surface were altered by anting and whether any ectoparasites were excluded or repelled from the bird's body. Artificial ant treatments and GC analysis revealed the chemical components on body surface of *T. cardis* were remarkably altered by anting. Specifically, dendrolasin, a species-specific volatile chemical in the two ants, was added to the body. The anting treatments were significantly effective in removing ectoparasites, louse flies and dipteran insects. Insecticidal effects also were confirmed. Additional experiments showed dendrolasin and some chemical components had directly repellent effects on dipteran insects. It suggests the advantages of anting include the exclusion of harmful dipteran insects and preventive medication for the diseases they introduce, such as avian malaria.

Introduction

In the wild, organisms are often threatened by infections with harmful viruses, bacteria, and parasites that use them as a medium. The impacts of infection have encouraged the evolution of self- and preventive medications in organisms. Therefore, various properties for infection avoidance have evolved in many animal groups, from the immune system at the physiological level to health care activities at the behavioural level[1]. However, finding evidence for self- and preventive medications in the field is difficult, because it is hard to confirm whether diseased animals seek out and consume substance having healing and preventive effects, which is not part of its normal diet and whether its health should improve within a reasonable timescale after the performance[2]. Although some cases have been strongly suggested, few observations satisfy these conditions [2, 3, 4, 5].

Self- and preventive medications have been recognised when wild animals eat or use soils, rocks, mineral-rich waters, sunlight, charcoal, bark and toxic organisms in ways that can only be described as medicinal[1]. For instance, some animals consume bioactive compounds or rub them on their bodies. Anting, the application of ants or ant substitutes to plumage, is a specialised behaviour in birds[6]. It was first recorded in the second quarter of the 18th century. Since then, it has been described in more than 200 avian species belonging to several orders, mostly in passerines[7, 8, 9, 10, 11, 12]. Many scientists have shown an interest in this peculiar phenomenon. Although several hypotheses have been proposed, the adaptive advantage is unknown. The hypothesis of self-treatment against parasites and bacteria is most convincing, because many ant species eject any poisonous materials and pungent fluids from poison glands[13], which might help remove mites and lice, other arthropods as ectoparasites and fungal or bacterial microparasites. Ohkawara et al.[14] provided detailed observation data of anting behaviour in

some birds at forest in central Japan. It was commonly practised by juveniles with immature plumage, that are frequently infested by ectoparasites in nests during the brooding period. Furthermore, anting is a behavioural practice, different from foraging, meaning that ants are not a necessary diet for birds. However, as of yet, there is no evidence that anything reduces the number of ectoparasites or lowers bacterial infections[11, 15, 16, 17].

To test the hypothesis, several factors should be considered. First, changes in the chemical components on the surface of bird bodies should be investigated. No study has compared the components of chemical substances on bird body surfaces before and after anting sessions. Second, if any changes in the chemical components on the body surface are confirmed, the effects of the changes and their potential beneficial effects on birds should be identified. Specifically, it must be quantitatively confirmed whether the frequency of infection by any ectoparasites or microparasites is reduced. Ohkawara et al.[14] reported that Japanese gray thrush *Turdus cardis* frequently practised anting by using two ant species, *Lasius spathepus* and *L. nipponensis* (Fig. S1). Moreover, *T. cardis* exhibits stamping behaviour as specific patterns of anting: birds rapidly stamp their feet on the swarm of ants. It suggests that specific interactions among them exist. In the present study, we conducted field observations and experiments to clarify the effects of anting behaviour of *T. cardis* using the two ant species, *L. spathepus* and *L. nipponensis*. Specifically, we investigated whether any chemical component on the bird's body surface was altered by anting, whether ectoparasites were excluded or repelled from the bird's body after anting, and whether chemical substances emitted by the ants have repellent effects on ectoparasites.

Results

Comparison of chemical components on the body surface before and after anting

To investigate changes of chemical components on bird's body surface by anting, a total of 18 individuals of *T. cardis* were captured in 2019 and nine individuals were used in the artificial anting treatments with *L. spathepus* and *L. nipponensis*, respectively (Fig. 1a, 1b). The GC analysis confirmed the presence of volatile chemical substances in the plumages of the treatment groups (Fig. 1c, 1d). In the treatments with *L. spathepus* and *L. nipponensis*, three and nine peaks were integrated respectively. More chemical components were included in the substance of *L. nipponensis* than that of *L. spathepus*. The chemical structures were identified by GC-MS analysis (Table S1). In both treatments, the amount of dendrolasin, 3-(4,8-dimethyl-3,7-nonadienyl)furan, a species-specific volatile chemical, was the largest. Beta-citronellal and farnesol were identified as the other main components. Figure 2e and 2f shows the plots of the first and second principal components in the PCA analyses and the vectors of the factors based on the chemical components in anting treatments. The plots before and after the treatments were classified by both principal components. Specifically, dendrolasin contributed to the classification as a main factor.

Effects on the removal of ectoparasites

In 2020 and 2023, 129 and 36 individuals of *T. cardis* were captured (male: 99, female: 66) and the effects of anting on exclusion of ectoparasites was tested by some experiments. The captured individuals were almost juveniles and their reproductive status did not differ. Fifty-seven individuals were used in each of the three experimental treatments. The bodies of *T. cardis* placed in cloth bags with ants smelled distinctive. From all bags, 45 louse flies of the family Hippoboscidae, eight black flies of the family Simuliidae and 208 blood-sucking mites of the family Ixodidae were collected as ectoparasites. Of the flies (dipteran insects), 86.7% were *Ornithoica momiyamai* (Table S2, Fig. S2). The dipteran insects and mites were also collected from the control group, probably because they were collected by disturbance caused by putting birds into the cloth bags. The collection rate of dipteran insects was significantly higher in the anting treatments than that in the control (*L. spathepus* – control: $p = 0.014$, *L. nipponensis* – control: $p = 0.038$, GLM, Fig. 3a). The average collection number was also significantly different among the experimental groups ($F_{2,168} = 3.6$, $p = 0.03$, GLM anova, Fig. 3b). Specifically, the difference between the two anting treatments and control was significant (*L. spathepus* – control: $p = 0.01$, *L. nipponensis* – control: $p = 0.05$). In addition, GLMM showed that only anting treatments had significant effects on the collection frequency (*L. spathepus*: $p = 0.015$, *L. nipponensis*: $p = 0.044$) and collection number (*L. spathepus*: $p = 0.008$, *L. nipponensis*: $p = 0.05$). Other factors, the year of the experiments and the sex of individuals had no effects on them. However, the collection frequency of mites and the collection number were not significantly different among the two anting treatments and control groups (collection frequency: $p = 0.8$; collected number: $p = 0.33$, Fig. 3c and 3d, Fig. S2). Other factors also did not affect the frequency and number of collections.

A total of 220 ectoparasites were collected from *Turdus* species. As the group of louse flies, the experiments of insecticidal effects were performed for 24 individuals of louse flies *O. momiyamai.* Eight flies were respectively used for three treatments, control, ant treatments of *L. spathepus* and *L. nipponensis.* As a result, four (50.0%) and five (62.5%) were dead in the experimental treatments of the two ants (Table S3). They were dead within 5 min after the start of the treatments. The mortality rate was significantly higher in the ant treatment groups than in the control group (*L. spathepus* – control: $p = 0.008$, *L. nipponensis* – control: $p = 0.002$, GLM anova). However, in other ectoparasite groups, blood-sucking mites, feather mites, and lice individuals for the ant treatments mostly survived (Table S3). There was no difference in the mortality rate among the ant treatments and control groups.

Repellent effects on mosquitoes

In 2019, laboratory experiments were performed to confirm whether the chemical substances of the two ant species prevented mosquitoes, *Aedes albopictus* and *Ae. flavopictus* as dipteran insects from accessing bird feathers. The behaviour of mosquitoes was remarkably different between the cages in which treated and non-treated feather pieces were placed. When the non-treated pieces were placed, many mosquitoes frequently remained still on the wall and pieces for more than 10 s. In contrast, in the

cages with treated pieces, the mosquitoes flew into the cages and frequently stayed on the walls distant from the pieces. The average number of mosquitoes remaining still on the non-treated pieces was significantly larger than on the treated pieces (*L. spathepus*: $F_{1, 38} = 57.2$, $p < 0.0001$; *L. nipponensis*: $F_{1, 38} = 25.2$, $p < 0.0001$, GLM anova, Fig. 4c). It strongly suggests that the volatile substances emitted by the two ant species have repellent effects on dipteran insects, including mosquitoes.

In the experiments using the chemical fractions divided in GC-MS analyses, the four main components were used: dendrolasin, beta-citronellal, citral, and farnesol (Fig. 1c, 1d). Average number of mosquitoes exhibiting avoidance was significantly different among the chemical components (*L. spathepus*: $F_{4.30} = 72.0$, $p < 0.0001$; *L. nipponensis*: $F_{5.36} = 28.3$, $p < 0.0001$, GLM anova, Fig. S3a and S3b). In *L. spathepus*, the number of mosquitoes that avoided dendrolasin was significantly greater than those of other components ($p < 0.0001$, Tukey-Kramer HSD test). In *L. nipponensis*, the number was greater in citral and dendrolasin, whereas beta-citronellal and farnesol were also avoided ($p < 0.05$).

Discussion

Our findings provided an explanation for the functions of peculiar anting behaviour. The chemical substances emitted by *L. spathepus* and *L. nipponensis* workers frequently excluded dipteran insects, particularly louse flies from the bodies of *T. cardis*. At a minimum, anting may function to decrease the parasitic rates by the dipteran insects. Probably, the health of birds practising the specific behaviour may improve within a reasonable timescale after consuming the substance. Although it is necessary to test for health improvement, our results can support the hypothesis that the advantage of anting is self-treatment against ectoparasites. In the forest of central Japan, various bird species visited nests of *L. spathepus* and *L. nipponensis* for anting: 102 cases by eight bird species were confirmed during the three years[14]. It suggests the self-treatment behaviour using *Lasius* ant species has been established as a general characteristic in the bird species.

This study revealed the proximate factors in the exclusion of dipteran insects as ectoparasites. Three components of chemical substances emitted by *L. spathepus* and *L. nipponensis* had repellent and insecticidal effects for dipteran insects: dendrolasin, citral and beta-citronellal. Specifically, dendrolasin and citral were more effective, though the effect of beta-citronellal was slightly lower. It has been reported that some plants had citral and citronellal functioning as repellents for mosquitoes[18, 19, 20, 21]. It is also suggested that ant species of the subgenus *Dendrolasius* in the genus *Lasius* have volatile chemical substances to protect from egg-laying by any parasitic flies[22, 23]. However, the composition of chemical substances was more complicated in *L. spathepus* than in *L. nipponensis*, indicating the subjects and mechanisms for defence are different between these species. Probably, the chemical substances have evolved as defence substances for any dipteran parasites and pests in various organisms. It appears that anting behaviour using the two *Lasius* species has evolved to exclude selectively harmful dipteran insect groups, based on the special selectivity properties of these chemical components.

However, why do bird species exclude dipteran insects by practising anting? Louse flies belonging to the family Hippoboscidae are obligate blood-feeding ectoparasites of birds and mammals[24, 25]. They specialise in a parasitic lifestyle and are harmful to hosts by sucking their blood. Furthermore, they can serve as vectors for introducing many diseases. Some studies show that the flies are important vectors of blood parasites such as *Haemoproteus* spp. and *Plasmodium* spp. that cause avian malaria[26, 27, 28]. Avian malaria has heavily impacted many populations and communities in the world. The significant devastation of bird populations on the Hawaiian Islands is a representative case[29, 30, 31, 32, 33, 34]. Other bird diseases also can be introduced by the hippoboscid flies[35, 36. 37]. Additionally, other dipteran insects such as *Simulium* black flies and *Culicoides* biting midges play a role in the introduction of bird diseases[38, 39, 40]. Therefore, the exclusion of dipteran insects through anting is beneficial for birds as a self-treatment. Anting may have repellent effects on some mosquito species. Birds are one of the hosts of mosquitoes for blood meals[41]. The mosquitoes are also important vectors of avian malaria and other blood parasites[27, 42, 43, 44]. It has been reported that mosquitoes distributed in Japan are vectors of multiple lineages of *Plasmodium* protozoa[45, 46, 47]. Bloodmeals of the two species *Ae. albopictus* and *Ae. flavopictus* observed in this study were biased toward mammals[48, 49]. However, it was indicated that they are opportunist[50, 51, 52] and feed on wild birds. Moreover, the repellent effect of ant's chemical substances may be not specific to only *Aedes* species. Probably, it is effective on mosquito species of other genera *Culex* and *Anopheles* as feeders for birds. Thus, anting seems an effective prevention of some bird diseases introduced by dipteran insects.

As shown by previous studies[11, 16, 17], anting had no exclusion effects on other ectoparasites, mites, feather mites and lice. Many species of mites and lice subsist on feathers and skin and suck blood. Large numbers of blood-sucking mites and lice can cause anaemia and death in young birds[53]. Nests heavily infested with feather mites may cause the abandonment of eggs and young birds. It has been also reported some mite species are involved in the transmission of several pathogenic agents responsible for serious diseases in birds and animals[54, 55, 56]. Birds likely exclude the ectoparasites with various body maintenance behaviours: self-preening, allo-preening, scratching, water bathing, and dusting[11, 15]. Probably, direct exclusion may be more effective for them. Moreover, other ant's chemical substances are possibly effective in exclusion of the ectoparasites. As a preliminary observation, it was confirmed that some bird species frequently practised anting using *Formica* species emitting antacids (Ohkawara unpublished data). Birds may practice anting using other ant species to exclude the ectoparasites. To clarify the process of exclusion of mites, feather mites and lice, the effects of other maintenance behaviours should be considered and further examined.

The present study shows that anting behaviour using the two *Lasius* ant species has evolved as an exclusion mode specialised to the dipteran insect group. Anting seems to be one of the behaviours for self-treatment and preventive medication in birds. However, the ant species used in anting may differ across local sites[12, 14]. It is possible that the effects and advantages of anting are different in each interaction between birds and ants. To explore the interactions, they should be confirmed at the local

level, where bird and ant species mutually interact during anting. Furthermore, if anting is performed for self-treatment and preventive medication in birds, it must be tested whether the infection and transmission of any bird diseases are controlled by anting in bird populations and communities. In future, these aspects should be verified through field observations and experiments.

Methods

Birds and ants as materials

Japanese gray thrush *Turdus cardis* was used for field observations and experiments (Fig. 1a). This is a mid-sized thrush (body length: 21.0–22.0 cm)[57]. It visits the region from northern to western Japan for breeding from May to September and is found in deciduous or mixed forests. The two ant species, *Lasius spathepus* Wheeler and *L. nipponensis* Forel, belonging to the subgenus *Dendolasius*, were also used in the experiments. In Japan, *L. spathepus* is widely distributed from Hokkaido to Kyushu Island, whereas *L. nipponensis* is distributed in Hokkaido, Honshu, and the Shikoku Islands[58]. The colonies are composed of more than 250 thousand workers[59, 60]. All species in the subgenus *Dendolasius* have a distinctive smell owing to their species-specific volatile chemicals[61, 62].

Field collections of the ant workers were conducted at Mt. Iouzen (36.5295°N, 136.7705°E, 513 m a.s.l.) in the Ishikawa Prefecture and Mt. Ota (35.9775°N, 136.0189°E, 482 m a.s.l.) in the Fukui Prefecture, central Japan. During late August and early September in 2019, 2020, and 2023, approximately 400 workers were collected from the nests of *L. spathepus* and *L. nipponensis* using a mouth aspirator. During the experimental period, they were kept in plastic boxes supplied with honey water and used for the experiments. For the collection of the ants, no permission was required.

Comparison of chemical components on the body surface of birds before and after anting

In the study, the capturing and experiments of wild birds were performed in accordance with the relevant guideline and regulation of permission by Ministry of the Environment, Government of Japan (Permission No, 2019: 181311, 2020: 1911261, 2023: 2211251). In this time, the animal ethics approval in Kanazawa university and other institutes was not required, because there were no works with any serious damage to birds in the research process. Therefore, the works were performed solely under the permit of Ministry of the Environment, Government of Japan. Japanese gray thrush *T. cardis* were captured at the Otayama Bird Banding Station on Mt. Ota. Capture was conducted for approximately 10 d during September 2019, 2020, and 2023. Eleven mist nets were set up and kept open from 6:00 to 12:00. The nets were checked hourly and *T. cardis* captured were kept in cloth bags (35 cm L × 30 cm W). After recording sex and age, they were used for the observation and experiments.

To test whether the chemical components on the body surface of birds are altered by anting, an artificial anting experiment was conducted in 2019. Before the experimental treatment, a few feathers were

collected from the belly of each *T. cardis* individual. The collected feathers were stored in glass vials (length: 5 cm, diameter: 1.8 cm). Thereafter, we exposed *T. cardis* individuals to approximately 50 worker ants from the boxes by opening their wings similar to that done during anting under natural conditions (Fig. 1b). Handling time was approximately 5 min. Occasionally, multiple ants climbed on the bodies of *T. cardis*. Next, a few feathers from the belly were collected again and kept in glass vials. In the experimental treatments, the two ant species were used separately. After the treatments, *T. cardis* individuals were marked with number rings and released.

A monolithic hybrid absorptive material, MonoTrap (RSC18, GL Sciences) was used to absorb volatile chemical substances. One MonoTrap rod (φ2.9 mm × 5 mm) was placed in each glass vial and exposed to volatiles from the feathers for 10 min. A MonoTrap rod placed in an empty, clean glass vial served as the negative control. As a positive control, the rod was placed in a vial after removing 10 workers of either *L. spathepus* or *L. nipponensis* that had been kept and irritated in the vial. All the rods were collected and stored in clean glass vials at below 10°C in a freezer. Each MonoTrap rod was immersed in 200 µL dichloromethane in an extraction vial tightly capped with PTFE septum and then the vial was placed in an ultrasonic cleaner (UT-105S, Sharp Japan) for 5 min at 20°C.

The extracted chemicals were analysed using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC analysis was performed on a Shimadzu GC-14A equipped with a polar capillary column DB-WAX (30 m × 0.25 mm × 0.25 µm; Agilent J&W, USA) and flame ionisation detector. Helium was used as a carrier gas at a head pressure of 97.5 kPa. The injection port and detector temperatures were set at 220°C, and the column oven temperature was maintained at 40°C for 5 min, then programmed to 220°C at a rate of 5°C/min and maintained at the final temperature for 20 min. Peak areas were computed using a Shimadzu C-R6A integrator. GC-MS analysis was conducted using a Shimadzu GCMS-QP5000 system connected to a Shimadzu GC-17A chromatograph with a DB-WAX column of the same standard. Helium was used as a carrier gas at a head pressure of 63 kPa. The GC conditions were the same as those used in the GC analysis. Electron ionisation mass spectra were obtained at 70 eV and scanned from 40 to 500 m/z at two scans/s. Retention indices were determined in relation to a homologous series of n-alkanes (C_{10} – C_{24}) under the same GC conditions. Further identification was performed by comparison with the NIST MS library data [63].

For the comparison of components in chemical substances on the body surface before and after anting, the components in the plumage and ant samples were classified and compared using principal component analysis (PCA). For this analysis, we used the ratio of GC peak area for each component and all peak areas were integrated by GC analyses. Each ratio was normalised using the average and standard deviation values among the samples. It was performed by the software JMP ver. 14.2.0.

Effects of anting on the removal of ectoparasites

The effects of anting were tested through artificial treatment and collection of ectoparasites. In the capture of *T. cardis*, we carefully removed individuals from the mist nets and immediately placed them in

cloth bags. In the laboratory, as experimental treatments, 30 workers of *L. spathepus* or *L. nipponensis* were added to the bags (Fig. S2a, S2b and S2c). After 5 min, the *T. cardis* individuals were carefully taken out and released. The cloth bags were immediately put into large plastic bags (38 cm L × 32 cm W) and stored below 18°C in a freezer. After 1 h, all arthropods, including ectoparasites, in the cloth bags were collected under a binocular microscope. The samples were stored in 70% ethanol and the species were identified. As a control, cloth bags in which only *T. cardis* were kept for 10 min were also put in plastic bags and stored in the freezer. Similarly, arthropods, including insects, in the bags were collected after 1 h. The experiments were mostly conducted in 2020. Additional trials were conducted in 2023. Among the anting treatment and control groups, the rate of the instances when ectoparasites were collected relative to all cases compared using GLM with a logit function of a binomial. Also, the average collection number of ectoparasitic arthropods, including insects, was compared using GLM anova. Moreover, considering the effects of other factors, GLMM was performed. As random effects, the year of the experiments and the sex of individuals were used.

Furthermore, the insecticidal effect of ant's volatile substances on ectoparasites was tested through laboratory experiments. Particularly, the experiments were performed for dipteran insects as ectoparasites. For the tests, some louse flies, louse flies, blood-sucking mites, feather mites, and lice were collected from the bodies of captured *T. cardis*. For the collection of the ectoparasites, no permission was required. As the first step in the experiments, 20 workers of *L. spathepu*s or *L. nipponensis* were placed into a glass vial bottle (length: 5 cm, diameter: 1.8 cm). After 30 min, they were removed from the bottle (ant treatment). Continuously, a few individuals of ectoparasites were placed into the bottles in which ant's volatile substances were included. After 30 min, whether the ectoparasites were dead or survived was recorded. As a control, ectoparasites were only placed into non-treated bottles and survival was determined after 30 min. Since the collection number was small, the experiments for feather mites were conducted using only *L. nipponensis.* The insecticidal effects were compared using GLM with a logit function of a binomial. All statistical analyses for the experiments of removal and insecticidal effects were performed using the software JMP ver. 14. 2. 0 and the packages 'lme4' and 'MuMIn' in R 3.6.2[64].

Repellent effects of anting on mosquitoes

It has been suggested that the chemical substances emitted by ants of the subgenus *Dendrolasius* function as repellants to dipteran insects, including parasitic flies[65, 66]. Therefore, in 2019, laboratory experiments were performed to confirm whether the chemical substances of *L. spathepus* and *L. nipponensis* prevented dipteran insects from accessing bird feathers. In this experiment, the feather of *T. cardis* was so small that dipteran insects could access it and remain. Therefore, for large feathers, a few primary feathers of the black kite *Milvus migrans* were prepared and cut into square pieces (5 cm × 5 cm). The pieces were wiped with 99% ethanol and dried to exclude the original smell. Then, they were placed in plastic bags (20 cm L × 10 cm W) with a few *L. spathepus* or *L. nipponensis* workers for more than 30 min to attach the ant odour. The two feather pieces used as experimental samples were placed

in mesh cages (length: 35 cm, width: 20 cm, height: 20 cm, Fig. 4a), in which more than 100 individuals of mosquitoes, *Aedes albopictus* and *Ae. flavopictus*, were placed. The two mosquito species are dominant in the forests of central Japan. The eggs and larvae were collected at some ponds in Kanazawa City and they were kept in the cages until emergence. For the collection of them, no permission was required. The number of mosquitoes remaining on the pieces for more than 5 seconds was counted for 10 min (Fig. 4b). As a control, a similar observation was made using untreated feather pieces. The experiments were performed 20 times for both treated and untreated pieces. The number of mosquitoes remaining on the pieces was compared using GLM anova.

Additionally, to investigate what components have repellent effects, experiments on avoidance of the ant's chemical substances were conducted. Using the same methods in the experiments of chemical component comparison on the body surface of birds following anting treatments, the chemical substances were extracted from 20 *L. spathepus* and *L. nipponensis* workers. After the GC-MS analyses, each chemical fraction was divided and extracted. The substances as main components were concentrated and created as an approximately 90.0% hexane solution. To test the repellent effect of each component, 20 µl of each solution was added to the tips of a cotton swab. Thereafter, they were approached by individual *Ae. albopictus* and *Ae. flavopictus* mosquitoes in mesh cages (Fig. S3). After the approach, the number of mosquitoes that avoided and flew away within 5 seconds was counted. In the experiment, the trials were performed 20 times. For each substance, the experiments were conducted seven times. As controls, cotton swabs to which hexane was added and untreated swabs were used. The number of mosquitoes that stayed on pieces and avoided from tips was compared using GLM anova and the Tukey-Kramer HSD test. For the experiments of repellent effects on mosquitoes, all statistical analyses were performed using the software JMP ver. 14. 2. 0. and the packages 'lme4' in R 3.6.2[64].

Declarations

Ethics statement

All applicable institutional and/or national guidelines for the observing and use of animals were followed.

Competing interests

The authors declare they have no competing interests.

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Author Contribution

K. O. performed field observation, field experiments and data analyses. K. O. was also a major contributor in writing the manuscript. Y. K. performed field observation and field experiments. T. A. performed chemical analyses of the chemical substances. All authors read and approved the final manuscript.

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Data Availability

The datasets generated and/or analysed during the current study are available in the Kanazawa University Repository for Academic resources (KURA) repository, https://kanazawa-u.repo.nii.ac.jp/

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Figures

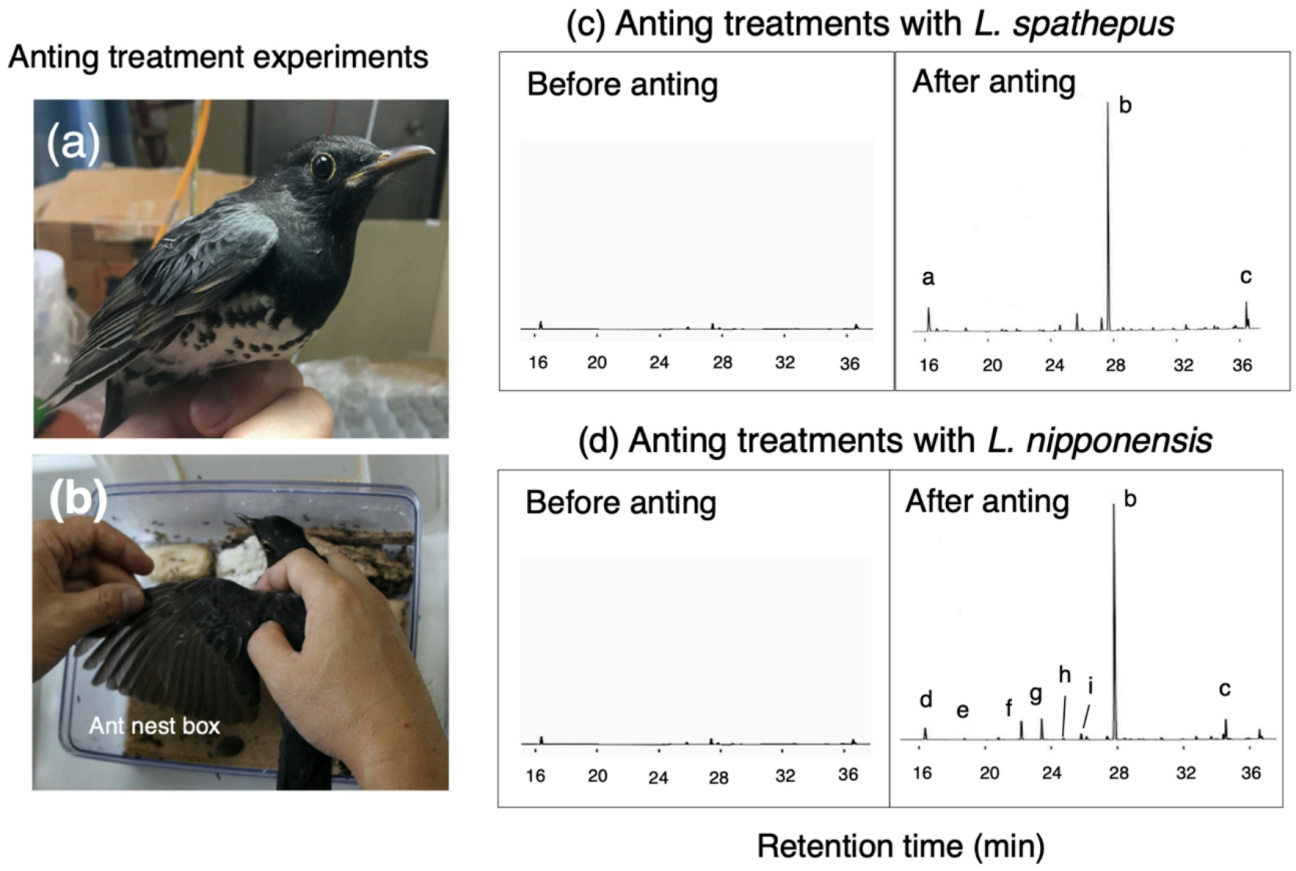


Figure 1

(a) Japanese gray thrush *Turdus cardis* as studied material. (b) Process of artificial anting experiment. GC peaks of volatile chemical substances in the plumage before and after the experimental treatments using (c) *L. spathepus* and (d) *L. nipponensis*. The chemical structures of each peak are as follows, a: beta-citronellal, b: Dendrolasin, c: Farnesal (3,7,11-trimethyl-2E,6E,10-dodecatrienal), d: unknown 1, e: Citronellal (3,7-dimethyl-6-octenal), f: Citral (3,7-dimethyl-2Z,6-octadecadienal, g: Citral (3,7-dimethyl-2E,6-octadienal), h: 3,7-dimethyl-octanol, i: unknown 2.

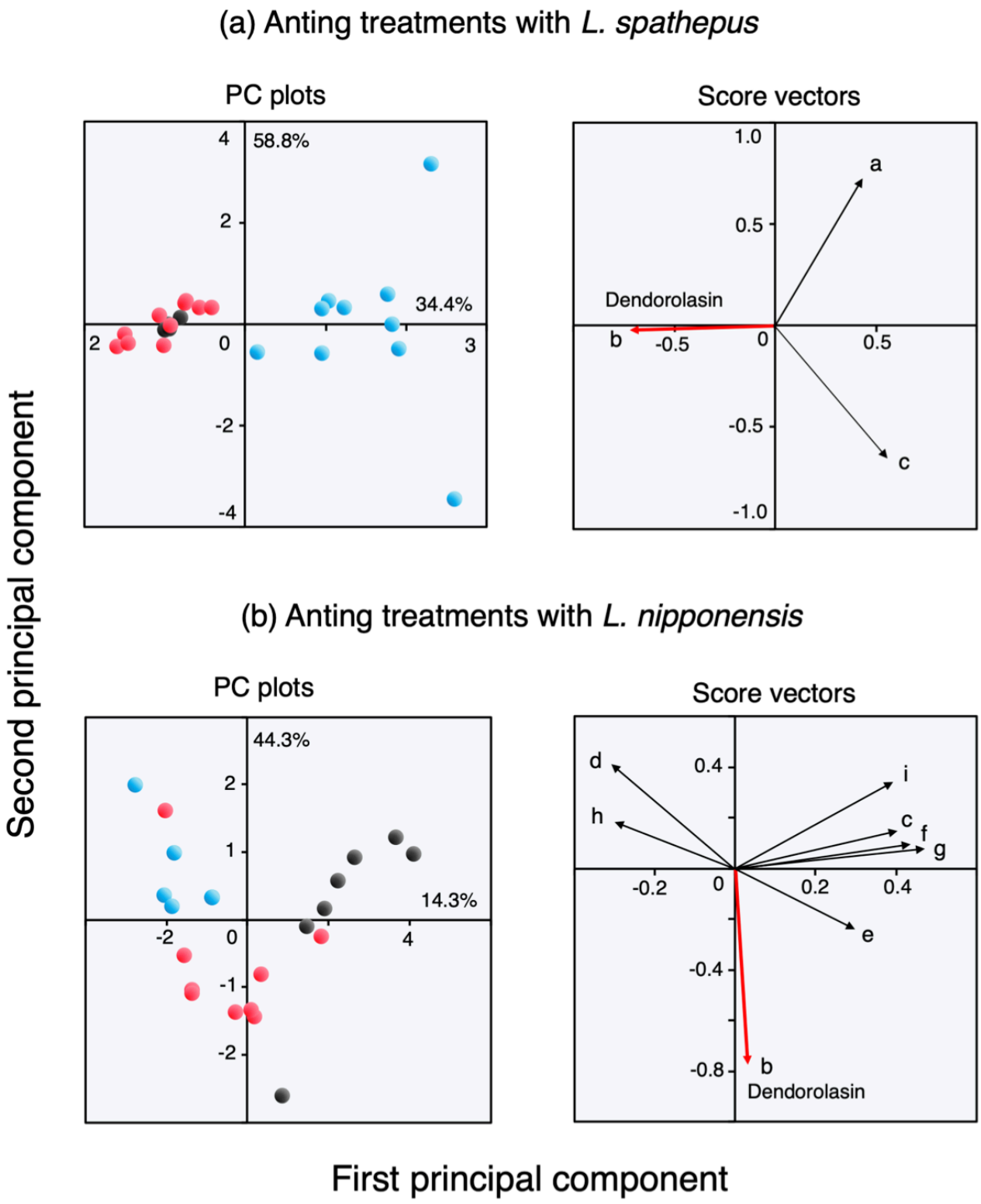


Figure 2

Classification of chemical substances in anting experiments with PCA based on the GC peaks in the analyses using (a) *L. spathepus* and (b) *L. nipponensis*. Biplots of the first and second principal components and the score vectors of GC peaks. Blue and red circle mean the chemical substances in the plumage before and after the experimental treatments. Black circle means the chemical substances of the two ant species. Letters show the chemical structures of each peak.

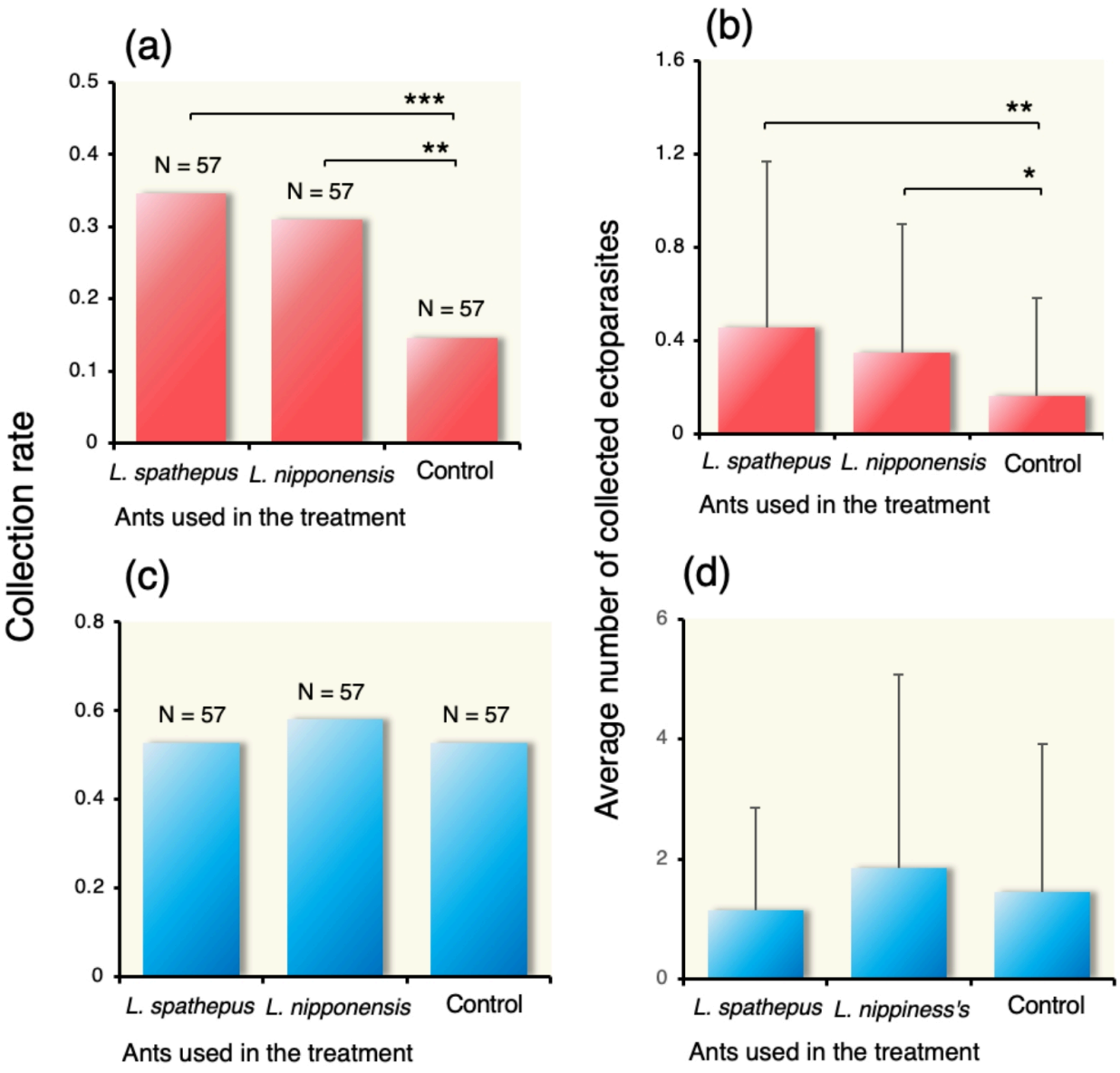


Figure 3

In the experiments of anting effects on ectoparasite removal, the rate of cases that (a) dipteran insects and (c) mites were collected to all cases and average collection number of (b) dipteran insects and (d) mites. The error bar shows the standard deviation value. The difference of collection rate and average number was tested using GLM and GLM anova, ***: $p < 0.02$, **: $p < 0.05$, *: $p = 0.05$.

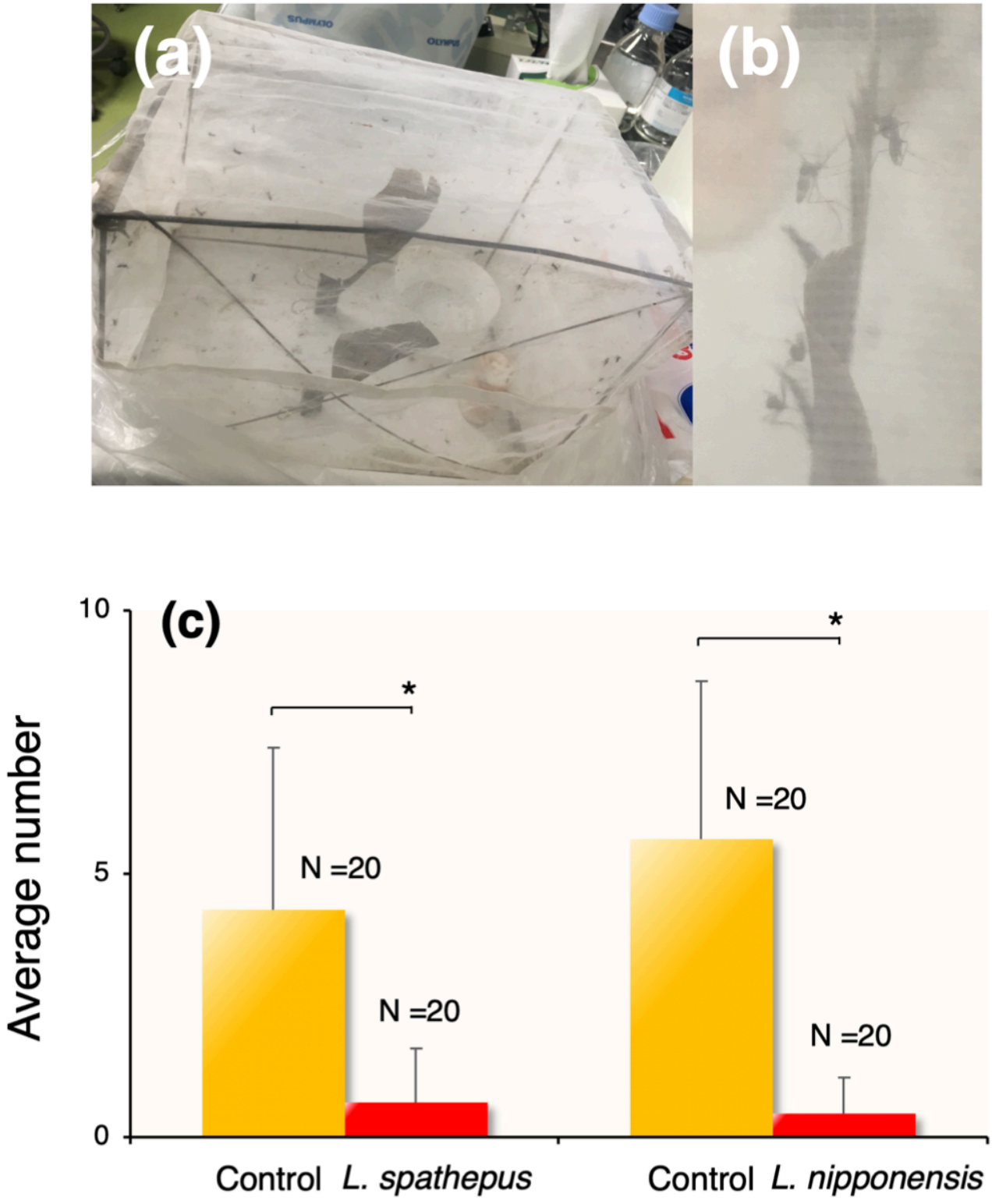


Figure 4

Experiments of repellent effects in anting on mosquitoes. (a) The mesh cages in which mosquitoes and feather pieces were placed for experiments. (b) The mosquitoes *Aedes albopictus* and *A. flavopictus* remaining on the feather pieces in the cage. (c) Average number of mosquitoes remaining for more than 5 seconds on feather pieces of anting treatments (red bar) and no treatment (yellow bar) were shown.

Ant name means species using the experimental treatment respectively. The error bar shows the standard deviation value. The difference of average number was tested using GLM anova, *: $p < 0.0001$.

Supplementary Files

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