Are males of the chafer Protaetia ishigakia (Coleoptera: Scarabaeidae) sexually deceived by flowers of the orchid Luisia teres (Aspargale: Orchidaceae)?

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Abstract

Males of the chafer *Protaetia ishigakia* (Fairmaire) (Coleoptera: Scarabaeidae) were observed to fly toward, hover around and land on flowers of the epiphytic orchid *Luisia teres* (Thumberg) Blume (Orchidaceae). On a flower, a male chafer inserted his head deep into the base of the flower column, where pollinia transferred between stigma of the flower and frons of the male. When solvent extract of the flower was applied on a cotton ball, male chafers landed on it and some of them showed pseudocopulatory behavior involving extrusion of the proboscis and/or copulatory organ. No such behaviors were observed in females. A chemical releasing these behaviors in males was identified as (*R*)-2,3-dihydroxypropyl isovalerate ((*R*)-2,3-DHPiV). Male chafers were observed to land frequently on the cotton balls treated with synthetic (*R*)-2,3-DHPiV, whereas a 95:5 blend of (*R*)- and (*S*)-enantiomers was more attractive than pure (*R*)-enantiomer. 2,3-DHPiV was strongly suggested to be a sex-pheromone component of *P. ishigakia*. These results that the flower of *L. teres* chemically deceived males of *P. ishigakia* while the flower secrete honey as an award in response of stimulation by chafer's proboscis were quite like to the interaction between the orchid *L. teres* and the congeneric chafer *P. pryeri pryeri* (Janson).

Introduction

Approximately 400 species of orchids have sexually deceptive flowers, which usually attract male bees and make them carry pollen from flower to flower (Barth 1991; Cozzolino and Wildmer 2005; Nilsson 1992). They attract the pollinator by fraudulent female signals but provide any reward such as nectar or pollen (Bergstöm 1978; Schiestl et al. 1999). Almost all known sexually deceptive orchids were from Australia and Europe (Gasket 2011) before the discovery of the case of beetle pollinated sexually deceptive *Luisia teres* (Thunberg) Blume in Okinawa, Japan (Arakaki et al. 2016; Bohman et al. 2016).

The epiphytic orchid *Luisia teres* is distributed in subtropical and temperate regions of Asia: the Ryukyu and Ogasawara Islands, Kyushu, Shikoku, and Honshu, Japan, and Sichuan, China (Seidenfaden 1971). This orchid releases floral scent that attracts males of the cupreous polished chafer *Protaetia pryeri pryeri* (Janson) (Coleoptera: Scarabaeidae) for pollination (Arakaki et al. 2016). Male chafers fly toward, hover around, and land on flowers of the orchid. On a flower, a male chafer holds the lip with his legs, and then inserts his head deep into the base of the flower column, where pollinia are often stuck onto his frons. Subsequently some males show pseudocopulation on the lip involving downward elongation of the abdomen and extrusion of the copulatory organ. When the chafer carrying pollinia visits another flower, he delivers the pollinia to the flower stigma.

The attractant chemical has been identified as (*R*)-2,3-dihydoroxypropyl isovalerate ((*R*)-2,3-DHPiV) from the solvent extract of petals of *L. teres* flower (Wakamura et al. 2020). This compound causes males of *P. pryeri pryeri* to display copulatory behavior toward a cotton ball treated with the extract of orchid flower petals or synthetic (*R*)-2,3-DHPiV. Because this compound was detected in a volatile sample collected from the virgin females of *P. pryeri pryeri*, it is considered to be a sex-attractant pheromone in *P. pryeri*

pryeri. This led us to conclude that the flower of this orchid chemically mimics the female sex pheromone of the pollinator.

On Okinawa Island, males of *Protaetia ishigakia okinawana* Y. Kurosawa were also found to be attracted to *L. teres* flower (Arakaki et al. 2016), but it was rather rare for further detailed analyses. *P. ishigakia* (Fairmaire) is divided into several subspecies that occur on different islands in the Ryukyu Islands (Hirashima 1989; Sakai and Fujioka 2007). Adults of this species are observed to visit ripe fruits, sap on stems or flowers (Sakai and Fujioka 2007) and to feed on leaves of mango (Nakasone et al., 1996; Yamaguchi 2012), but whether they visit flowers of native orchids was unclear. Therefore, we suspected that the orchid also attracted males of *P. ishigakia* by sexual deception as it did to *P. pryeri pryeri* (Arakaki et al 2016). The purpose of the present study was to confirm the chemical compound(s) released from the flower of *L. teres* that attracted males of *P. ishigakia*.

Materials and Methods

Plant

Stalks of *Luisia teres* were collected from epiphytic colonies on dead pine trees in the northern part of Okinawa Island in May 2005 and May 2012, planted on tree-fern board, and grown in a greenhouse or on tree branches until use. The stalks were covered with a mesh bag made of polyester (38 cm diameter, mesh size 3.5 mm) in order to prevent insect pollination.

Attraction of male of P. ishigakia by flower of L. teres

Orientation and subsequent pollination of *L. teres* flower by feral chafers of *P. ishigakia* were observed at the secondary forest, Manabi-no-mori (Manabi forest) located at the central area of Miyako Island. Intact stalks of *L. teres* with flowers were placed ca. 1 m above the ground on shrubs along pavement in the forest. Exposing intact flowers did not risk introduction of exotic gene since this orchid did not occur on this island.

Extraction of flower

Four orchid flowers were soaked in 1 cm^3 diethyl ether in a glass vial (1.6 cm diameter × 6.0 cm height) for 4 h and stored in a refrigerator (0–5°C) until use. Sepals, petals and lips separated from another four flowers were separately soaked in 1 cm^3 diethyl ether in the same manner as above. Each ether extract was impregnated onto a cotton ball, which was a wad of absorbent cotton wrapped with black polyester cloth and tied with plastic-coated wire to form a ball of 1.5 cm diameter.

For chemical analyses of the active compound, petals removed from 80 flowers were soaked in ether (13–35 flowers/5 cm^3 ether) at 0–5°C in a refrigerator for ca. 24 h. The extract was then removed to

other vials and the same volume of fresh ether was added. This procedure was repeated three times. The ether extracts were pooled, dried over anhydrous Na_2SO_4, and stored at − 28°C until use.

Silica gel column chromatography

Silica gel column chromatography
The solvent was removed from the crude extract of orchid petals at below 20°C and the residue was dissolved with 0.5 cm^3 hexane in a small vial. The hexane layer was poured onto 5.0 g of silica gel column and successively eluted with each 25 cm^3 of hexane, 5%, 15% and 50% ether in hexane, ether and methanol. When exchanging the solvent, the vial was rinsed with 1 ml of the new solvent, the rinse was adsorbed on the column top, and then a new solvent was poured.

Flight tunnel bioassay

A flight tunnel of the same type as described by Yasuda (1996) was used to evaluate the effect on male behavior of extracts of *L. teres* flower and fractions eluted from silica gel column chromatography. Fresh air was supplied at a flow rate of 10 cm/s to the flight tunnel (60 cm long, 18.5 cm wide and 18.5 cm height) made of transparent acrylic plates and exhausted to outside via draft chamber. The tunnel floor was covered with a sheet of white filter paper. Five males of *P. ishigakia miyakona* were placed on a filter paper disk (9 cm diameter) on the floor 5 cm from the leeward end. They were covered with a transparent plastic cup (9 cm diameter × 5 cm height) until the observation started. A cotton ball lure impregnated with test solution and another cotton ball with pure solvent (control) were placed on the floor in parallel with 10 cm interval and 5 cm from the windward end. Observations were started by removing the plastic cup to expose the males to the air in the tunnel. The males that arrived at and stayed on the cotton balls for more than 10 s were counted for 10 min.

Gas chromatography/mass spectrometry (GC/MS)

GC/MS analyses were conducted with Agilent 6890N Network GC System combined with an Agilent 5975 inert XL mass selective detector at EI mode (70 eV). The GC was equipped with split/splitless injector, an HP-5MS fused silica column (30 m × 0.25 mm inner diameter (ID) × 0.25 um film thickness), Agilent Technologies, Santa Clara, CA, USA). Samples were injected at splitless mode at 220°C for 1 min. Helium was used as a carrier gas at a constant flow mode of 1.0 ml/min. The column oven temperature was kept at 50°C for 1 min, programmed to increase at 10°C/min to 240°C, and held at the final temperature for 6 min.

Chemicals

(*R*)- and (*S*)-2,3-dihydroxypropyl isovalerate (2,3-DHPiV) and racemic mixture were the same lots as described in the previous paper (Wakamura et al. 2020). Opposite enantiomer was not detected in the

purified samples of either (*R*)- or (*S*)-enantiomer by HPLC analyses using a chiral column, CHIRALPAK® IC (4.6 mm ID × 25 cm length, Daicel Corporation, Osaka, Japan). (*R*)- and (*S*)-enantiomers and the racemic mixture showed single GC peaks in GC/MS analyses.

Field tests

Field tests were performed at coast forest along sea at Nanjo, Okinawa Island and at Manabi forest or Nishi-henna-zaki on Miyako Island in may-July 2014 – 2018. Each sample containing four flower-equivalents of crude extracts or fractions obtained from column chromatography was impregnated as an ether solution onto a cotton ball described above. The cotton balls with the samples were immediately attached to plastic bars at 20–30 cm above the ground and the bars were placed 1–2 m apart. Feral chafers of *P. ishigakia* that hovered around and landed on the cotton balls were recorded as hovering and landing, respectively. When authentic compounds were tested for their activity to attract male chafers, 2 μg (= 4 flower-equivalents) dissolved in ether were applied on a cotton ball and assayed as above. To minimize possible positional effects on the results, the positions of cotton balls were rearranged every 0.5 h or 1 h depending on the male density. Assay was repeated 4 to 6 times at different sites and times. All individuals attracted were examined to confirm that they were *P. ishigakia*.

For statistical analyses, data (X) were transformed to $(X + 0.5)^{1/2}$ and submitted to analysis of variance. The means were subsequently ranked by paired *t*-test and significant differences were determined using Bonferroni's correction (Sokal and Rohlf 2012). In the figures, the means accompanied with the same letter are not significantly different at the 5% level unless otherwise noted.

Results

Behavior of males of P. ishigakia miyakona around and on L. teres flowers

When intact *L. teres* flowers (Fig. 1A) were placed in the forest on 27 June 2014, males were observed to fly around them, hover and then land nearby. The males typically landed on a leaf or stem of the plant and walked toward one of the flowers, held the lip of the flower with their legs and then stuck their head toward the base of the column for few seconds (Fig. 1B). Some of the males occasionally extruded their proboscis on the flower (Fig. 1C). When males were leaving the flower, they had a pair of pollinia on their frons (Fig. 1D). When the males visited another flower, their pollinia were left on the flower's stigma.

Attraction of males with floral extracts

Male chafers of *P. ishigakia miyakona* were observed to land on the cotton balls treated with extracts of petals or whole flowers significantly more frequently than on those with extract of sepals or lips ($p < 0.01$, Fig. 2). Some of the males were occasionally observed to extrude the proboscis and/or copulatory organ on the cotton ball (Fig. 1E, F). Males landed on the cotton balls treated with extract of sepals or lips significantly less frequently than on those treated with petals or whole flowers Therefore, the results

suggested that the petals of the orchid flower were the main organ containing the attractive volatile cue(s) for the chafers.

Isolation and chemical analysis

Among the six fractions from the silica gel column chromatography of the extract of *L. teres* flower petals, males of *P. ishigakia okinawana* were attracted to the ether and methanol fractions at coast forest along sea at Nanjo, Okinawa (Fig. 3A). On Miyako Is., *P. ishigakia miyakona* were attracted to the same fractions at the Manabi forest; 13 males hovered around and three of them landed on the cotton ball treated with methanol fraction, and three males hovered around the ether fraction during 8-h observation period in total (Fig. 3B). To confirm the above results, the six fractions were also tested using a wind tunnel at KUAS laboratory. Males were observed to move toward, arrive at and mount on the cotton balls treated with the fraction eluted with methanol most frequently (Fig. 3C). The methanol fraction was as attractive as the crude extract of petals of *L. teres* flowers. We considered, therefore, that the major component of attractant was contained in the methanol fraction.

GC/MS analysis of this bioactive fraction showed a single GC peak at t_R = 11.23 min. The mass spectrum showed significant ions at *m/z* 145 (16), 134 (2.3), 128 (2.2), 103 (23), 101 (22), 93 (2.0), 85 (100, base), 69 (11), 57 (89), 43 (27) and 41 (33), while molecular ion was not observed at *m/z* 176. The mass spectrum and the retention time were identical to those for synthetic 2,3-DHPiV, and the enantiomeric isomerism had been determined to be (*R*) in our previous investigation (Wakamura et al., 2020). The same compound was detected in the ether fraction. Total amount of 2,3-DHPiV was corresponded to ca. 0.5 µg/flower in comparison to the value for the authentic compound.

Field attraction with 2,3-DHPiV enantiomers and racemic mixture

When synthetic enantiomers and racemic mixture of 2,3-DHPiV were assayed, feral males of *P. ishigakia miyakona* were observed to land most frequently on balls impregnated with (*R*)-enantiomer (Fig. 4). Small numbers of chafers landed on those with (*S*)-enantiomer and racemic mixture. When (*R*)-enantiomer was blended with (*S*)-enantiomer, more males were attracted to a 95:5 blend than to the other blends or pure (*R*)-enantiomer (Fig. 5).

Attractiveness was confirmed at Nanjo, Okinawa Is. on 3 July 2017; Significant numbers of males (6.0 ± 0.81 males/30 min, N = 12) were observed to land on cotton balls treated with 20 µg of 95:5 blend of (*R*)- and (*S*)-enantiomers while no males landed on negative controls ($p < 0.01$).

Discussion

The present study demonstrated that males of the chafer *Protaetia ishigakia okinawana* on Okinawa and *P. ishigakia miyakona* on Miyako Islands were attracted to flowers of the orchid *Luisia teres*, where no critical difference was found in their behavior between these two subspecies. Interestingly, the males

extruded the proboscis when they put the head into the flower and sometimes displayed copulatory behavior by extruding the copulatory organ. This type of behavioral responses to this orchid flower is similar to what has been observed in the other congeneric chafer, *P. pryeri pryeri* to the same orchid flower on Okinawa Island. (Arakaki et al. 2016). The males of both chafers were attracted to ether extract of petals (Fig. 1E, F) but no female was attracted to either such extract or flowers. When males of either species put their head into a flower, pollinia were often found on their frons. When they visited another flower, the pollens on their frons were transferred to the latter's stigma (Fig. 1D).

In the present study, a chemical produced by *L. teres* flower to attract *P. ishigakia* males was isolated and identified as (*R*)-2,3-DHPiV. When this compound or flower extract was presented, males were attracted and occasionally released precopulatory behavior including extruding copulatory organ (Fig. 1E). These facts strongly suggest (*R*)-2,3-DHPiV is a sex-pheromone component of *P. ishigakia*, and the orchid chemically deceive males by releasing this compound. Males of *P. ishigakia* were significantly more attracted with 95:5 mixture of (*R*)- and (*S*)-2,3-DHPiV than pure (*R*)-enantiomer or the other blends (Fig. 5). Small but substantial numbers of males were also attracted to different blends, even with pure (*S*)-enantiomer (Figs. 4 and 5). These redundant responses to 2,3-DHPiV by males of *P. ishigakia* were not observed in males of *P. pryeri pryeri* that were attracted neither to a racemic mixture nor to pure (*S*)-enantiomer (Wakamura et al. 2020). This suggests enantiomeric purity may involve species discrimination in mate orientation between these two congeneric species. This hypothesis remains to be chemically examined in future.

On the other hand, males of *P. ishigakia* were also observed to extrude their proboscis on the treated cotton ball (Fig. 1E). *L. teres* flowers secreted honey immediately after soft touch on the base of flower column (Arakaki et al., 2016); Males of *P. ishigakia* and *P. pryeri* may take the honey there. Thus, the orchid *L. teres* does not only deceive males of *P. ishigakia* and *P. pryeri* by mimics of sex-pheromone but also provide honey as an award for pollination. The orchid *L. teres* appeared to be at evolutionally transitional step, as considered by Arakaki et al. (2016) and Jersáková et al. (2006).

The fact that the subspecies *P. ishigakia miyakona* occurs on Miyako Island where no *L. teres* is present may suggest interesting possibilities about the origin of this chafer and orchid. According to the hypothesized paleogeography of the Ryukyu Islands (Kimura 2002), Miyako and Okinawa Islands joined the Taiwan and Asian Continent by land bridge in early Pleistocene (1.7–1.3 million years ago). Since the old Ryukyu Islands were formed by a rise in sea level in middle Pleistocene (1.2–0.2 million years ago), land bridge has never been formed between Miyako and Okinawa Islands (Kimura 2002; Muraji et al. 2012). The orchid *L. teres* has survived on Okinawa Island but not on the Miyako Island for an unknown reason, whereas the chafer *P. ishigakia* has been differentiated into five subspecies on Miyako, Okinawa, and the other Ryukyu Islands (Hirashima 1989; Sakai and Fujioka 2007). Present study would provide an interesting information to verify those geological hypotheses.

The chafer *P. ishigakia* is a minor pest species feeding on leaves of mango (Nakasone et al. 1996; Yamaguchi 2012). Probable sex pheromone 2,3-DHPiV would provide a useful tool for population

monitoring and/or direct control by communication disruption when *P. ishigakia* become a serious pest in future.

Declarations

Author Contribution

S.K., N.A. and S.Wak, conceived of the study, D.M., S.Wak. and N.A. Designed the study. N.A. Collected insects, D.M., S.Wak, M.O., A.K., S.Waj and N.A. performed the experiments, D.M. and S.Wak. analyzed the data, DM prepared the 2,3-DHPiV, and D.M. and S.Wak wrote manuscript. D.M., S.Wak. and N.A. wrote the main manuscript text and D.M., S.Wak. prepared figures 1-6. All authors gave final approval for publication.

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Figures

Fig. 1

Figure 1

Visit to *Luisia teres* flowers by males of *Protaetia ishigakia miyakona*. Flowers of *L. teres* (A), male inserting his head into space at the base of stigma (B), male extruding his proboscis (C) and carrying pollinia (arrow) on his frons (D, Inset is enlarged view), and males landing on a cotton ball impregnated with ether extract of *L. teres* flowers, occasionally extruding his proboscis (E, Inset is enlarged view) and/or copulatory organ (arrow) (F).

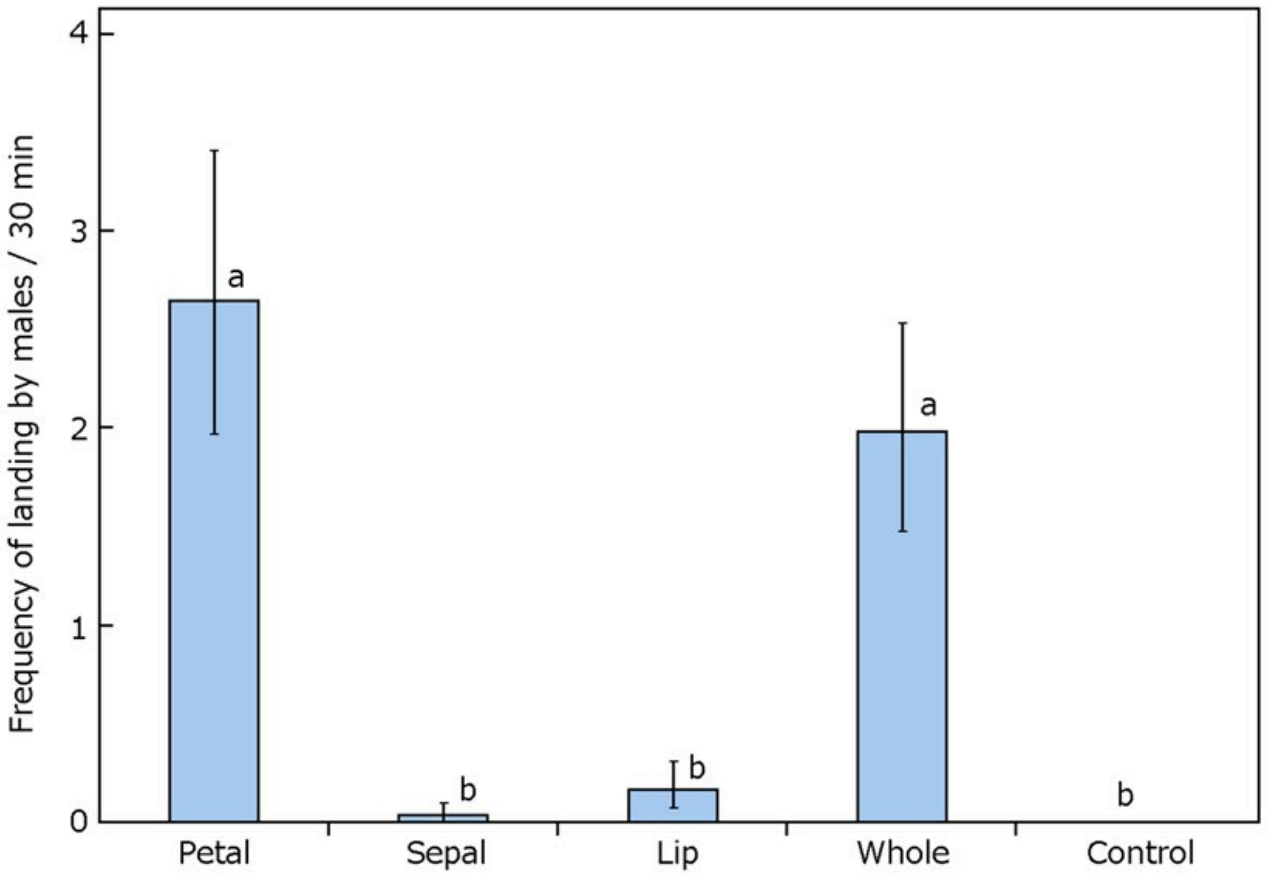

Fig. 2

Figure 2

Landing on cotton balls impregnated with solvent extracts of *Luisia teres* flowers by males of *Protaetia ishigakia miyakona* (24 June 2014, *N* = 16). Means accompanied by the same letter are not significantly different ($p < 0.05$).

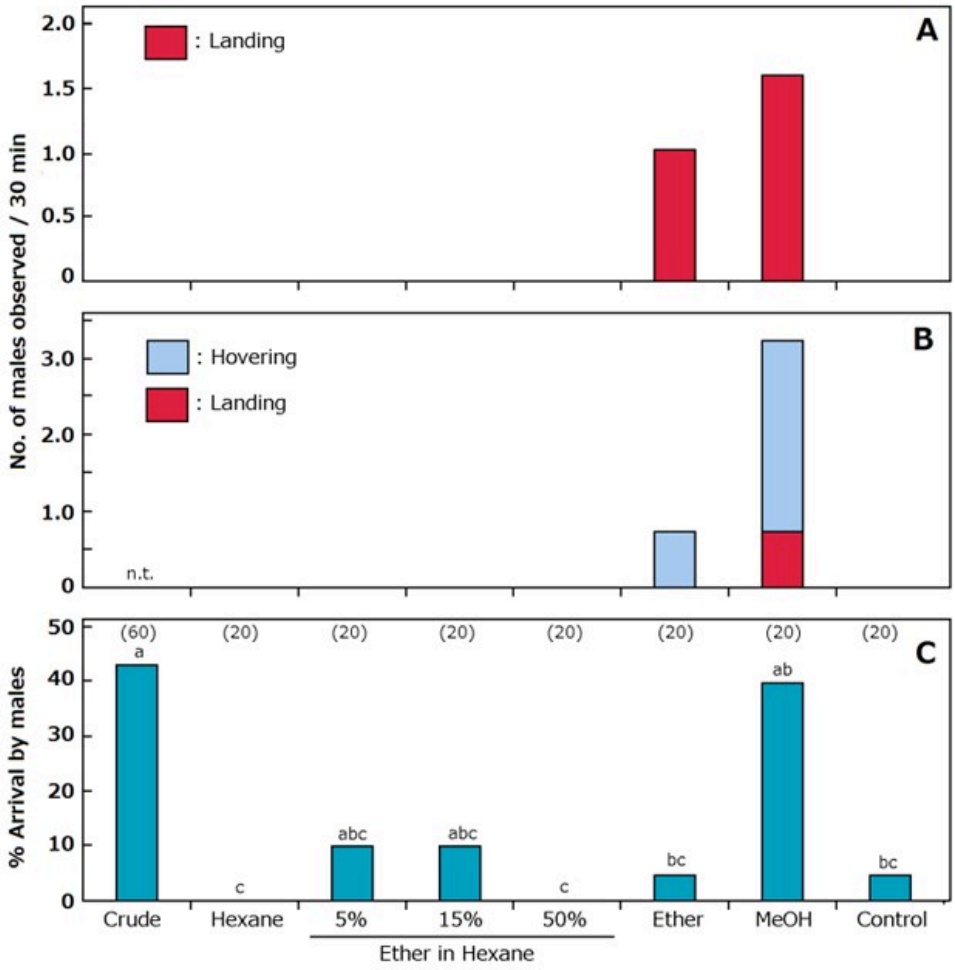

Fig. 3

Figure 3

Behavioral response to fractions of silica gel column chromatography by males of *Protaetia ishigakia okinawana* (A), and *P. ishigakia miyaikona*(B) in the field and in the wind tunnel (C). A: Landing by males during 2-h observation (1 July 2017, *N*= 2). B: Hovering and landing during 2-h observation (27 June 2016, *N* = 4). C: Arrival by males at treated cotton balls in wind tunnel assay. Numbers in parentheses are numbers of males tested (See text for details). Values accompanied by the same letter are not significantly different ($p < 0.05$). n.t.: not tested.

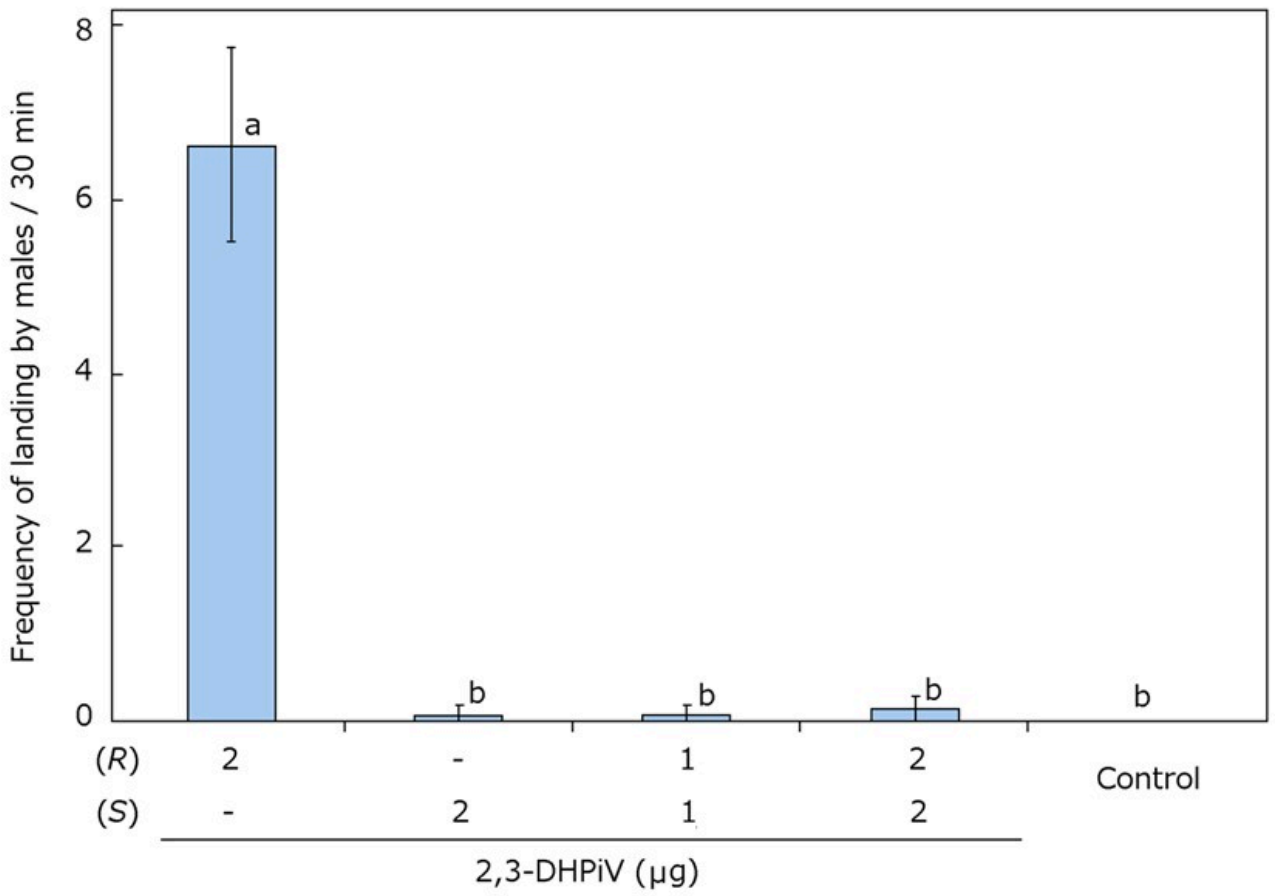

Fig. 4

Figure 4

Attraction of males of *Protaetia ishigakia miyakona* with synthetic (*R*)- and (*S*)-2,3-DHPiV and racemic mixture (17 June 2017, *N* = 10). Dose: 2 µg/cotton ball. Means accompanied by the same letter are not significantly different ($p < 0.05$).

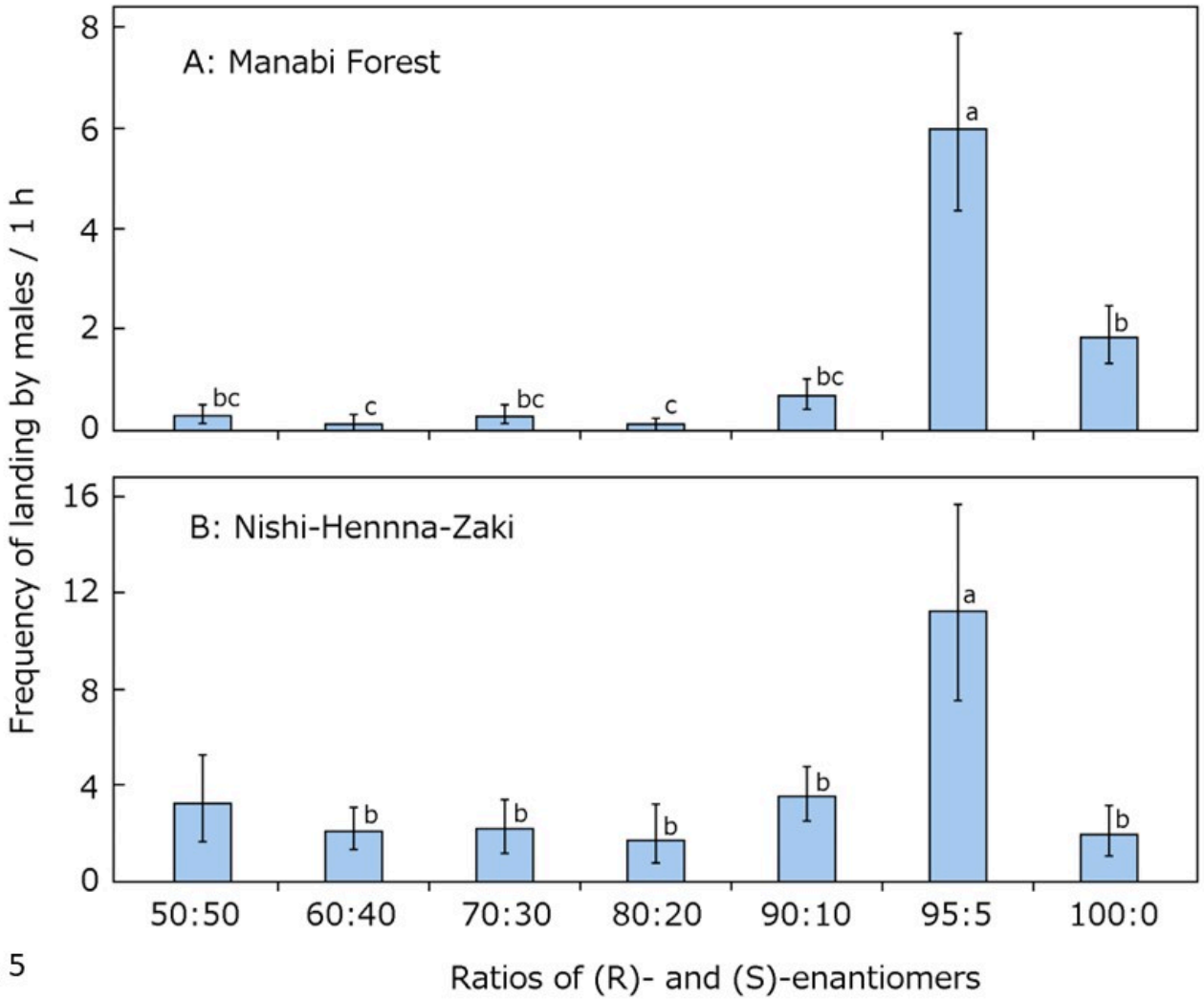

Fig. 5

Figure 5

Attraction of males of *Protaetia ishigakia miyakona* with synthetic 2,3-DHPiV at Manabi forest (A) and Nishi-Henna-Zaki (B) on Miyako Island (A: 17 June 2018, *N* = 12, B: 19 June 2018, *N* = 6). Means accompanied by the same letter are not significantly different ($p < 0.05$).