

SHORT COMMUNICATION

Acetoin is a key odor for resource location in the giant robber crab *Birgus latro*

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ABSTRACT

The terrestrial and omnivorous robber crab *Birgus latro* inhabits islands of the Indian Ocean and the Pacific Ocean. The animals live solitarily but occasionally gather at freshly opened coconuts or fructiferous arenga palms. By analyzing volatiles of coconuts and arenga fruit, we identified five compounds, including acetoin, which are present in both food sources. In a behavioral screen performed in the crabs' habitat, a beach on Christmas Island, we found that of 15 tested fruit compounds, acetoin was the only volatile eliciting significant attraction. Hence, acetoin might play a key role in governing the crabs' aggregation behavior at both food sources.

KEY WORDS: Volatiles, Food preference, Ionotropic receptors, Olfaction, Behavior

INTRODUCTION

The robber crab *Birgus latro* (Linnaeus 1758) (Coenobitidae) is a terrestrial hermit crab, and the world's largest extant land-living arthropod. The species inhabits the islands of the Indian Ocean and the Pacific Ocean (Drew et al., 2010) with Christmas Island (Indian Ocean) holding one of the largest and most undisturbed populations in the world. Individuals of this species may live for over 100 years (Drew et al., 2013), and are usually lone feeders with an average density of only 29.5 animals per hectare at the coastal site of this island (Drew and Hansson, 2014). The food spectrum of these omnivorous animals is extremely diverse, ranging from plant material, to large insects, to carrion of vertebrates and invertebrates. On Christmas Island, the robber crab also actively predares on a terrestrial brachyuran crab species, the endemic *Gecarcoidea natalis* (Gecarcinidae; Krieger et al., 2016). Here, we found that robber crabs gather at freshly opened coconuts (*Cocos nucifera*). However, only large individuals are able to open a coconut with their claws (Fig. 1A), and even for them it takes about 2 days (Drew et al., 2010). Coconuts are very abundant on Christmas Island, but unopened fruit usually are not attractive for most of these crabs. However, as soon as a single robber crab has managed to open a coconut, other animals approach this fruit. In addition, we observed similar accumulations of robber crabs when fruit of an arenga palm

start to ripen. In this case, many of these animals assemble in the vicinity and feed on the fallen fruit (Drew and Hansson, 2014) (Fig. 1B). Finally, we found animals accumulating at and feeding from the palm core of an arenga rotting on the ground. Behavioral and neurobiological analyses provide evidence that robber crabs and their closest relatives, the terrestrial hermit crabs (Coenobitidae), like insects, have evolved an olfactory sense that allows the animals to detect airborne molecules (Krang et al., 2012; Krieger et al., 2010; Stensmyr et al., 2005), and this helps the crabs to find and evaluate food (Stensmyr et al., 2005; Rittschof and Sutherland, 1986; Thacker, 1998) and shelter (Small and Thacker, 1994). Because carrion odors that are behaviorally active for robber crabs have been described (Stensmyr et al., 2005), we hypothesized that the number of crabs building up around open coconuts and fructiferous arenga palms is also olfactory driven. Furthermore, because robber crabs share this food preference with other arthropods like the vinegar fly (fermented fruit; Knaden et al., 2012) and the desert ant (dead insects; Buehlmann et al., 2014), we asked whether this similarity is based on preference for the same key volatile compounds.

MATERIALS AND METHODS

Aggregation behavior

We opened coconuts and placed them at the crabs' habitat on Christmas Island, to observe whether robber crabs would become attracted to them. We furthermore counted the number of animals in an area (5 m diameter) surrounding arenga palm trees either without fruit or with fruit at different ripening stages. None of the animals was harmed during the behavioral observations.

Odor collections

We collected volatiles from both coconuts and arenga for chemical analyses. Coconut fruit volatiles were trapped by solid-phase micro-extraction (SPME), and volatiles were subsequently analyzed by coupled gas chromatography-mass spectrometry (GC-MS). We collected headspace from intact young (i.e. green) and old (i.e. brownish) fruit, and from the milk of both ripening stages. During collection of headspace from the fruit, the coconuts were wrapped in polyester bags (Toppits Bratschlauch, Germany). The coconut milk was transferred into a clean glass vial and sealed with a Teflon-coated lid.

SPME analyses were carried out using a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB; Sigma-Aldrich, Steinheim, Germany) coated fiber (Ho et al., 2006; Santos et al., 2011) that was conditioned by heating for 20 min in the GC injection port at 250°C. Static SPME headspace collections were performed by exposing the fiber for 15 min to the headspace of the nuts or milk. All volatile collections were performed within an incubator at 35°C in order to imitate the ambient temperature on Christmas Island. Empty polyester bags and blank SPME fibers served as controls.

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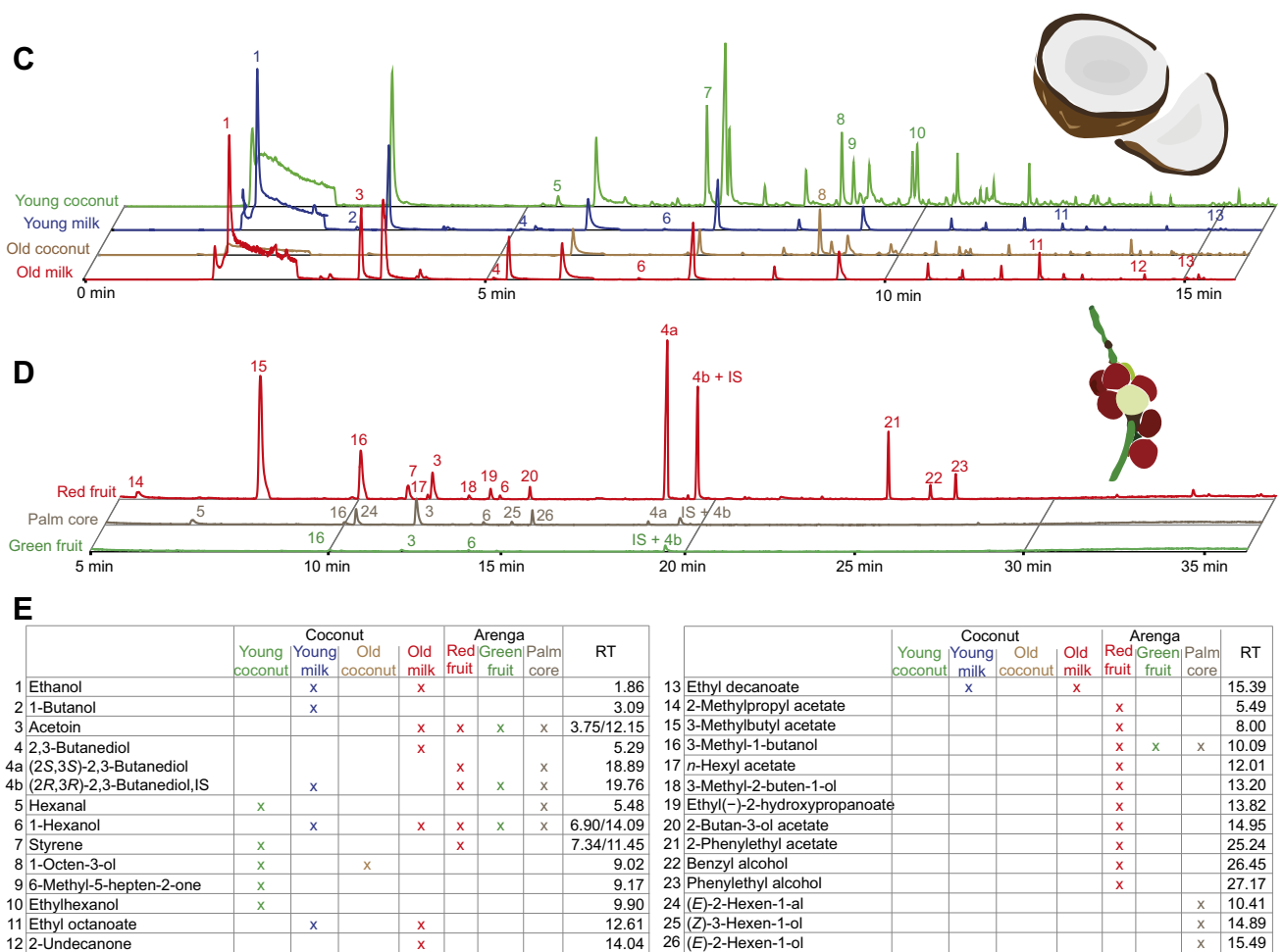
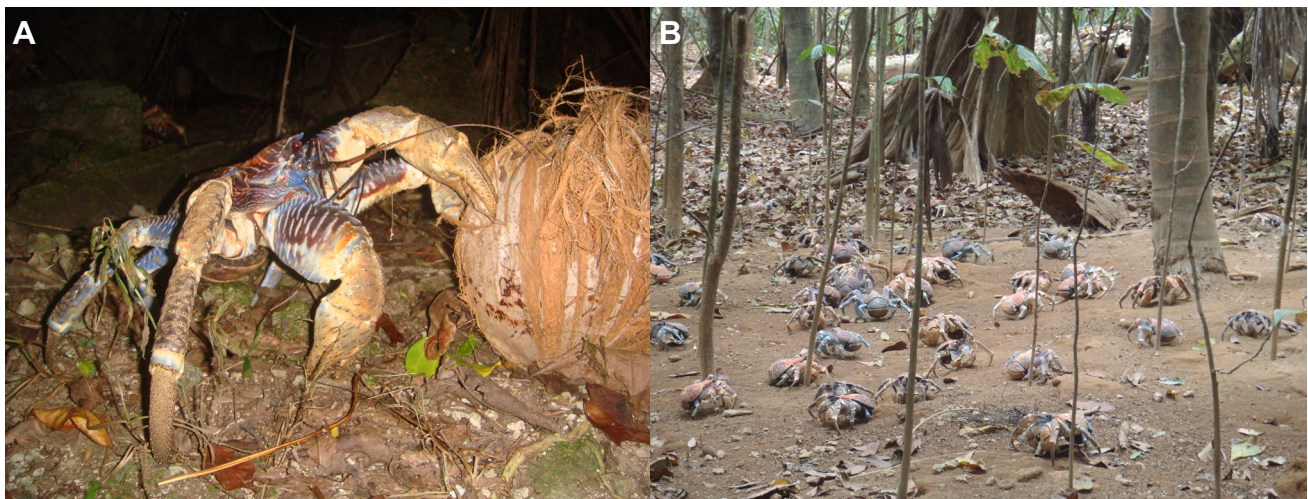


Fig. 1. Giant robber crabs are attracted to coconuts and fruit of the arenga palm. (A) A crab opening a coconut. (B) Crabs aggregating under a fructiferous arenga palm. (C) Gas chromatography (GC) profiles from coconut samples. (D) GC profiles from arenga samples. (E) List of 26 odorants that were identified by coupled gas chromatography-mass spectrometry (GC-MS) in coconut, arenga or both samples. IS, internal standard; RT, retention time (min).

Arenga volatiles were collected from unripe and ripe fruit as well as from palm core of this plant placed in clean and silanized 250 ml glass flasks. Activated charcoal-filtered air was pumped through the flask at 250 ml min⁻¹ for 10 h. Volatiles were trapped on filters charged with 25 mg each of Carbotrap C, B and X. After elution with 400 µl dichloromethane containing 400 ng bromodecane as internal standard, the solutions were

concentrated under a gentle stream of nitrogen to a final volume of 25 µl.

Chemical analysis

Coconut volatile compounds collected with the SPME fiber were thermally desorbed and analyzed using a GC-MS (GC 6890 and MS 5975, Agilent Technologies, Palo Alto, CA, USA). The GC

was equipped with a non-polar column (HP5; 30 m×0.25 mm i.d., 0.25 μm film thickness, Alltech Associates, Deerfield, IL, USA). The carrier gas was helium with a flow rate of 1.1 ml min⁻¹. After exposure, the SPME fiber was immediately injected into the GC injector port, followed by a 1 min desorption and column trapping period at 250°C. The GC temperature program was: initial temperature of 40°C for 3 min, rising to 240°C at 10°C min⁻¹, held for 3 min, followed by a post-run at 300°C, held for 10 min.

Aliquots of 1 μl per arenga volatile sample were analyzed on a GC-MS (as above), but equipped with a polar column (HP Innowax, 30 m×0.25 mm i.d., 0.25 μm film thickness) and operated under constant flow (1 ml min⁻¹), and the GC oven was programmed to hold at 40°C for 3 min, rise at 5°C min⁻¹ to 240°C, and hold for 15 min. The inlet and the MS transfer line were operated at 240 and 250°C, respectively. Compounds were tentatively identified by comparing their mass spectra with those of the NIST MS-library (National Institute of Standards and Technology, Gaithersburg, MD, USA, 2008), and confirmed by comparing MS and retention times with authentic standards.

Behavioral experiments

Experiments were conducted during dusk and dawn at Dolly Beach on Christmas Island (10°31'36S, 105°40'22E). The beach is inhabited by about 30 robber crabs of both sexes. In a former study, long-term experiments showed that robber crabs accumulate under hanging baits containing coconut flesh, ripe arenga fruit or the carrion odor dimethyl trisulfide (DMTS) (Stensmyr et al., 2005). However, to be able to screen the attraction of many odors to individual crabs, we established a novel experimental paradigm: we pipetted either pure coconut milk or DMTS (2 μl of odorant diluted in 200 μl of mineral oil) onto a foam plug (5 cm diameter, 2 cm height) and placed the plug 2 m upwind of a resting robber crab. We observed that 7 out of 12 animals approached the coconut milk (DMTS: 8 out of 12 animals) and contacted the foam plug in less than 2 min. In contrast, only 1 out of 24 crabs was attracted to a plug that contained mineral oil only (Fig. 2A), showing that neither the visual cue of a foam plug nor the solvent alone induced attraction. Therefore, we conclude that this assay was suitable to screen the behavioral activity of odors under field conditions. During the experimental period (14–30 October 2012), this beach was steadily exposed to east winds (i.e. the wind blew from the sea straight

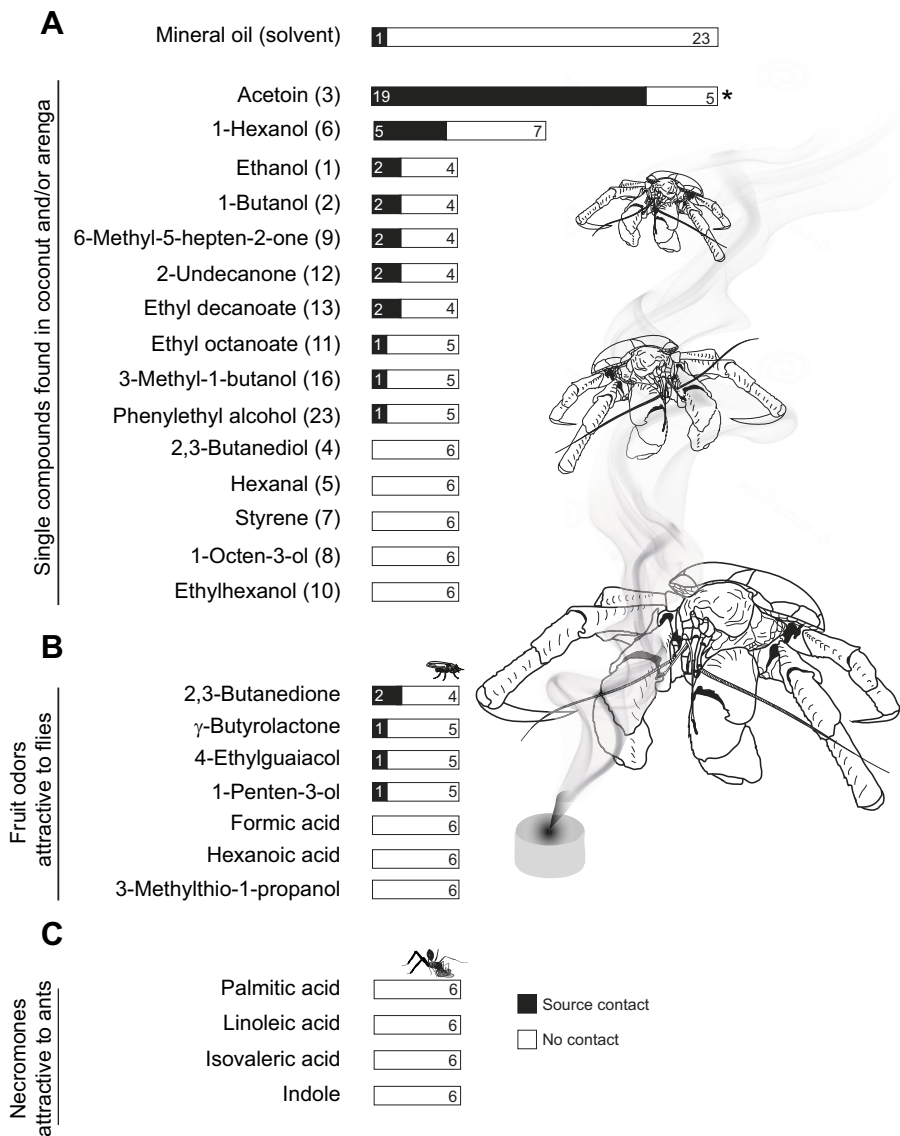


Fig. 2. Behavioral screen of odor attractiveness to robber crabs. (A) Coconut and arenga odorants tested for attraction to robber crabs; numbers in parentheses refer to corresponding numbers from the GC-MS analysis in Fig. 1E. (B, C) Published *Drosophila* (B) and *Cataglyphis* attractants (C) tested for attractiveness to robber crabs. Contact with source is indicated. Numbers in bars indicate the number of responding and non-responding animals. *Significantly different from the solvent control (Fisher's exact test with Bonferroni–Holmes correction for multiple comparisons). Schematic drawing shows robber crab approaching a foam plug containing an attractive odor.

landwards). By moving along the beach with our stimulus sources, we could therefore consecutively test individual crabs, while crabs that were 90 deg to the right or left were not exposed to any straight-downwind plume of the test odor. Crabs reacted to attractive odors with a sequence of behaviors: (1) flicking of the antennules, i.e. the peripheral olfactory organs of *Birgus* (Stensmyr et al., 2005), (2) turning towards the source and (3) approaching the source until source contact. When the crab exhibited this sequence and finally touched the foam plug within 2 min, the response was counted as positive. Any other outcome (e.g. no response or only flicking of antennules and/or locomotion that did not end up in plug contact) was counted as negative. Individual crabs were tested only once per odorant and a single crab was not tested more than 4 times per day.

RESULTS AND DISCUSSION

In initial field studies, we observed that robber crabs seemed to accumulate around arenga palms having ripe but still attached fruit approximately 5–10 m above the forest floor. Large groups of crabs would already be increasing in numbers before the fruit started dropping from the tree, and some crabs were even observed climbing up the tree to reach the ripening fruit. Although we counted 6–25 crabs for each tree exhibiting ripe fruit (average, 13.9 crabs; $n=7$ trees), almost no crabs at all accumulated around trees having unripe (average, 0 crabs; $n=4$ trees) or no fruit (average, 0.1 crabs, $n=16$ trees). In areas where coconut palms were present, we opened coconuts and observed immediate attraction of robber crabs from distances over 30 m. We hypothesized that the attraction towards arenga fruit and coconuts was governed by olfaction. To identify potential active compounds in the blends of both food sources, we collected the headspace from young and old coconuts, and young and old coconut milk (Fig. 1C), as well as from arenga fruit in different ripening stages, and from arenga palm core (Fig. 1D). We identified a total of 26 compounds in the different headspaces. Five odorants (acetoin, 2,3-butanediol, hexanal, 1-hexanol, styrene) occurred in both arenga and coconut samples, while 13 odorants were specific for arenga and 8 odorants for coconut, respectively (Fig. 1E).

We then tested the attraction of robber crabs to 15 of the identified individual compounds and complemented the behavioral screen with odorants we had identified from fermenting fruit or dead insects that had been shown to be highly attractive either to the vinegar fly *Drosophila melanogaster* (Knapen et al., 2012) or to the desert ant *Cataglyphis fortis* (Buehlmann et al., 2014).

Because of the limited number of accessible animals at the experimental site, we first conducted six replicates per odor and only increased the sample size if more than two animals became attracted. However, the only attractive single odorant turned out to be acetoin, which attracted 79% of the tested animals (Fig. 2A). This odorant is found in high amounts in preferred robber crab food sources like old coconut milk, ripe arenga fruit and arenga palm core, but is absent or only present at trace levels in young coconut milk, closed coconuts and green (unripe) arenga fruit (Fig. 1C,D), none of which are eaten by the animals. Thus, acetoin seems to be a key odorant governing the crabs' attraction to coconuts and arenga.

Interestingly, acetoin is a fermentation product that in a blend seems to be involved in governing the attraction of vinegar flies to yeast, while as a single compound it is not attractive to the flies (Becher et al., 2010). We also found that none of the tested single odorants that are known to attract vinegar flies to fermenting fruit (Knapen et al., 2012) or govern the desert ants' attraction to insect carcasses (Buehlmann et al., 2014) were attractive to the robber crabs (Fig. 2B,C). Hence, attraction to the same kind of food seems

to be governed by different key odors in robber crabs versus insects. This is in line with the finding that robber crabs do not express insect-like odorant receptors but possess only the more ancient group of olfactory ionotropic receptors (Groh et al., 2014). In vinegar flies, which express both kinds of receptors, these ionotropic receptors have a clearly different receptive profile from the odorant receptors (Silbering et al., 2011).

To summarize, we found that apart from acetoin, none of the additional 25 tested odorants elicited attraction in the robber crab. Interestingly, presenting a plug soaked with acetoin not only immediately attracted the tested robber crab but also very often resulted in the approach of robber crabs that were hidden in the coastal forest before the experiment started. Some of these animals approached over distances of more than 10 m, suggesting that robber crabs are extremely sensitive to this odor. These long-distance approaches never occurred when we tested any of the other odorants, but could be observed when robber crabs approached a conspecific that succeeded in opening a coconut. The gathering of these usually solitary animals at freshly opened coconuts and under trees with ripening arenga fruit presents a remarkable exception in the daily routine of these crabs. Our data suggest that these feeding assemblages are governed by olfaction, with acetoin being one of the key odorants involved.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.K., S.H., B.S.H.; Methodology: M.K., S.B.-K., J.L., A.R.; Validation: M.K., S.B.-K.; Formal analysis: M.K., J.L., A.R.; Investigation: M.K., S.B.-K., J.K., S.E., B.S.H.; Writing - original draft: M.K.; Writing - review & editing: S.B.-K., J.L., A.R., J.K., S.E., S.H., B.S.H.; Visualization: M.K., S.B.-K.; Project administration: M.K.; Funding acquisition: S.H., B.S.H.

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