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Orientation to polarized light in tethered flying honeybees

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Abbreviations:

clockwise; CW

counterclockwise; CCW

dorsal rim area; DRA

preferred e-vector orientation; PEO

25 **Summary statement**

26 Tethered flying bees exhibited polarotaxis under a zenithal rotating e-vector stimulus, in which
27 their right-and-left abdominal movements were coincident with the rotation of the stimulus.

28

29 **ABSTRACT**

30 Behavioral responses of honeybees to a zenithal polarized light stimulus were observed using a
31 tethered animal in a flight simulator. Flight direction of the bee was recorded by monitoring the
32 horizontal movement of its abdomen, which was strongly anti-correlated with its torque. When
33 the e-vector orientation of the polarized light was rotated clockwise or counterclockwise, the bee
34 responded with periodic right-and-left abdominal movements; however, the bee did not show any
35 clear periodic movement under the static e-vector or depolarized stimulus. The steering frequency
36 of the bee was well coordinated with the e-vector rotation frequency of the stimulus, indicating
37 that the flying bee oriented itself to a certain e-vector orientation, i.e., exhibited polarotaxis. The
38 percentage of bees exhibiting clear polarotaxis was much smaller under the fast stimulus (3.6°
39 s^{-1}) compared with that of the slow stimulus (0.9 or $1.8^\circ s^{-1}$). The bee did not demonstrate any
40 polarotactic behavior after the dorsal rim region of its eyes, which mediates insect polarization
41 vision in general, was bilaterally covered with black paint. The bees demonstrated a high
42 preference for e-vector orientations between 120 to 180° . Each bee exhibited similar e-vector
43 preferences under clockwise and counterclockwise stimuli, indicating that each bee has its own
44 e-vector preference, which probably depends on the bee's previous foraging experience. Our
45 results strongly suggest that the flying honeybees utilize the e-vector information from the
46 skylight to deduce their heading orientation for navigation.

47 INTRODUCTION

48 As a result of sunlight scattering in the atmosphere, the skylight is partially plane-polarized and
49 the celestial e-vectors are arranged in a concentric pattern around the sun (Strutt, 1871; Wehner,
50 1997). It is well known that many insects exploit this skylight polarization for visual compass
51 orientation and/or navigation (for review: Wehner, 1994; Wehner and Labhart, 2006; Heinze,
52 2014). There have been an enormous number of studies about insect polarization vision, not only
53 at the behavioral level (e.g., Dacke et al., 2003; Reppert et al., 2004; Henze and Labhart, 2007;
54 Weir and Dickinson, 2012), but also at the neural network level, such as sensory (e.g., Blum and
55 Labhart, 2000; Weir et al., 2016) and central brain mechanisms (e.g., Labhart 1988; Heinze and
56 Homberg, 2007; Sakura et al., 2008; Heinze and Reppert, 2011; Bech et al., 2014). The e-vector
57 detection in insects is mediated by a group of specialized ommatidia located in the most dorsal
58 part of the compound eye, the dorsal rim area (DRA), in which the photoreceptors are
59 monochromatic and highly polarization-sensitive (for review: Labhart and Meyer, 1999; Wehner
60 and Labhart, 2006). The neural pathway of polarization vision in the brain has been documented
61 in several species, and the central complex, one of the higher centers of the insect brain, is
62 considered to be the location of an internal compass (for review: Homberg et al., 2011; Heinze,
63 2017), although it is still unclear how the central complex controls the animal's steering during
64 navigation.

65 Foraging behavior in social insects, such as ants and bees, is a useful model system for
66 studying insect navigation because they repeatedly forage back and forth between the nest and a
67 feeding site. In particular, the path integration mechanisms in the desert ants *Cataglyphis* have
68 been extensively studied in regard to insect navigation (Wehner, 2003; Collett and Carde, 2014),
69 and *Cataglyphis* is well known to choose their heading direction using celestial polarization cues
70 during long distance navigation (Fent, 1986; Wehner, 1997; Wehner and Müller, 2006). In
71 addition to path integration based on the polarization compass, ants could learn visual landmarks
72 or panoramic views at familiar locations and use them for local navigation (Collett et al., 1992;
73 Wehner et al., 1996; Collett et al., 1998; Graham and Cheng, 2009; Narendra et al., 2013).
74 Honeybees also undertake long-distance foraging trips, that may reach over 40 km (Couvillon et
75 al., 2014). The foragers, after returning from a food source, transfer the food location to their
76 nestmates by the waggle dance, in which the vector information from their nest to the food
77 location is encoded. This clearly indicates that honeybees navigate based on path integration
78 (Frisch, 1967). The waggle dance has also been used to clarify the utilization of polarization
79 vision in their navigation. Under unpolarized light, the dancing honeybees failed to transfer the

80 correct directional information to the food source and their waggle dance orientations were
81 strongly affected by artificially-polarized light (Frisch, 1967; Rossel and Wehner, 1987; Sherman
82 and Visscher, 2002). More recently, it was reported that they changed their waggle dance
83 orientations depending on the e-vectors of polarized light experienced during their foraging trip
84 (Evangelista *et al.*, 2014). These results suggest that the honeybees use polarization vision for
85 path integration, i.e., deducing the direction to the food source. In contrast to investigations
86 regarding path integration into the waggle dance, to our knowledge, there has been only one
87 study that examined whether a flying bee can use celestial e-vector information to choose its
88 flight route. Kraft *et al.* (2011) showed that bees trained to fly in a four-armed maze to a feeder,
89 in which the bees received polarized light stimulus from above, chose their foraging routes as
90 they received e-vector information, similar to what they experienced during the training. This
91 suggests that the flying bees actually sense the e-vector orientation from the sky and use it for
92 navigation.

93 Similar to ants, honeybees display both path integration and visual landmark navigation.
94 Bees use familiar landmarks to find the correct location (Cartwright and Collett, 1983; Fry and
95 Wehner, 2005), and in some cases, visual landmarks dominate path integration (Chittka and
96 Kunze, 1995). Furthermore, a single bee can memorize several food locations simultaneously
97 based on the landmarks at each location and visit the best one among the destinations in a
98 context-dependent manner (Collett and Kelber, 1988; Zhang *et al.*, 2006). To accomplish those
99 complicated navigational tasks, foraging bees must use multiple navigational strategies, as the
100 situation demands, by using several visual cues such as polarized light and landmarks. However,
101 it still remains largely unknown how honeybees behaviorally select and use polarized light or
102 landmark cues during navigational flight. This is probably because of the technical difficulties of
103 presenting artificial visual stimuli to freely flying bees (Evangelista *et al.*, 2014).

104 Recently, some behavioral studies have overcome this difficulty by using tethered bees
105 (Luu *et al.*, 2011; Taylor *et al.*, 2013), with which we can observe responses in flying behavior of
106 the dorsally tethered bees to lateral optic flow and frontal air-flow stimuli. It was observed that
107 the tethered bees showed “streamlining” responses, whereby they raised their abdomen in a
108 correlated manner with the speed of the optic and air-flow stimuli. In the present study, we
109 investigated how the flying bees respond to polarized light stimuli using the tethered system. We
110 constructed a flight simulator, in which we could examine the tethered bee’s flight response to a
111 rotating polarized stimulus and found that they tended to orient themselves to a certain e-vector
112 direction, i.e., they exhibited clear “polarotaxis,” during the flight.

113

114 MATERIALS AND METHODS

115 Animals

116 The honeybees, *Apis mellifera L.*, used in this study were reared in normal ten-frame hives on the
117 campus of Kobe University. Forager honeybees with pollen loads were collected at the hive
118 entrance before the experiment and anesthetized on ice or in a refrigerator. An L-shaped metal
119 rod for tethering was attached to the pronotum of an anesthetized bee, as previously described
120 (Luu *et al.*, 2011). Briefly, the hair on the pronotum was gently shaved using a small piece of a
121 razor blade, and the metal rod was adhered using a small amount of light-curing adhesive
122 (Loctite; Henkel, Dusseldorf, Germany). The following image analyses of bee behavior (see
123 below) were conducted by marking the tip of the abdomen with a white light-curing dental
124 sealant (Conseal f; SDI Ltd., Bayswater, Australia). Next, the bees were placed in a warm room
125 to recover from anesthesia and fed several drops of 30 % sucrose solution before the experiment.

126 Setup

127 The experiments were performed using a custom-made black box (Fig. 1) in a dark room. A
128 tethered bee was mounted in the box by attaching the end of the metal rod to a three-dimensional
129 manipulator such that the bee's location could be adjusted manually. The flying behavior of the
130 tethered bee was enhanced by stimulating the bee with a headwind from an air circulator and
131 optic flow from a PC monitor. The circulator was located outside of the box and connected to a
132 tunnel that carried the wind stimulus into the box. The end of the tunnel (diameter, 8 cm),
133 consisting of many fine plastic straws to reduce the turbulent flow of wind, was fixed at 10 cm
134 from the bee's head. The wind speed was 1.7–2.0 m s⁻¹. The PC monitor (RDT1711LM;
135 Mitsubishi Electric, Tokyo, Japan), covered with a sheet of tracing paper to eliminate any
136 polarized components of the light, was located 5 cm beneath the tethered bee. The optic flow
137 stimulus of moving black-and-white stripes was displayed on the monitor using a self-made
138 program in Microsoft Visual C⁺⁺. The spacing of the stripes and the speed of the stimulus as seen
139 by the bee were approximately 40 ° s⁻¹ and 900 ° s⁻¹, respectively.

140 Light from a xenon lamp (LC8; Hamamatsu Photonics, Hamamatsu, Japan) was applied
141 above the bee using a quartz light guide. The light was filtered using a depolarizer (DPU-25;
142 ThorLabs, Newton, NJ) at the end of the light guide to eliminate any polarized components of the
143 light and a holographic diffuser (48-522; Edmund Optics, Barrington, NJ) was clamped under the
144 end of the light guide. The diffuser reduced illuminance irregularity and increased the size of the
145 light fit around a linear polarizer (HN42HE; diameter, 15 cm; Polaroid Company, Cambridge,

146 MA) beneath the diffuser. The polarizer was mounted on a circular holder that could be rotated
147 using a DC motor. The stimulus was centered at the bee's zenith (with respect to flying head
148 position) at a distance of 15 cm, providing a dorsal, polarized stimulus of 53 ° in diameter. In the
149 experiments, in which unpolarized light stimulus was used, the depolarizer was clamped just
150 above the bee's head instead of at the end of the light guide such that the size of the light stimulus
151 covered the entire receptive field of the bee's DRA. The intensity of the polarized and
152 unpolarized white light at the animal level was approximately 1000 lx.

153 **Behavioral experiments**

154 A bee with the metal rod was fixed in the experimental box after complete recovery from
155 anesthesia. First, we let the bee hold a small piece of paper so that it could not start flying. The
156 e-vector angle of the polarizer was set at 0° with respect to the bee's body axis, and static
157 black-and-white stripes were displayed on the PC monitor. After the bee had been familiarized
158 with the box, the paper was removed to allow the bee to start flying, and the wind and optic flow
159 stimuli were simultaneously presented. After the bee's flight became stable, the polarizer started
160 rotating slowly (0.9, 1.8, or 3.6 ° s⁻¹), and the behavior of the bee was monitored for 600 s. When
161 a bee stopped flying before 600 s, the data were not used in the analysis. In some cases, the bee
162 was tested 3 times under different stimulus conditions—clockwise (CW), static, and
163 counterclockwise (CCW). The order of these three stimuli was randomly changed for each
164 experiment. In other cases, a bee was tested only with the CW stimulus.

165 The flying behavior of the tethered bee was monitored using a USB camera
166 (IUC-300CK2; Trinity Inc., Gunma, Japan) placed behind the bee (see Fig. 1). Images of the bee
167 were recorded at a rate of 1 Hz, i.e., 600 images for 10 min data. For each image, the
168 x-coordinate of the bee's abdominal tip was determined manually to estimate flying orientation
169 (see Fig. S1). A series of x-coordinates was then calibrated into actual distances (in mm) from the
170 center, where the tethering wire was fixed and used for further analysis (see below).

171 Whether the DRA of the compound eye was involved in flying behavior under the
172 polarized light stimulus was determined using bees whose DRAs were painted (Fig. 7C, D). The
173 DRAs were painted as in our previous work (Sakura et al., 2012) with black acrylic emulsion
174 paint (Herbol; Cologne, Germany) under a dissecting microscope just before the tethering
175 procedure described above. The DRA of a compound eye is visually identifiable because the
176 cornea appears slightly grey and cloudy (Meyer and Labhart, 1981). Because it was technically
177 not possible to cover the DRA alone, which consists of only 4–5 horizontal rows of ommatidia
178 (see Meyer and Labhart, 1981; Wehner and Strasser, 1985), a small area of the unspecialized

179 dorsal region next to the DRA was also painted. After the experiments, the paint cover was
180 checked in all the experimental animals under a dissecting microscope. Data for cases in which
181 any of the paint was missing were excluded from further analysis. The three ocelli, which are not
182 involved in polarization vision (Rossel and Wehner, 1984), were not painted in the experiments.

183 **Analysis and statistics**

184 All data analyses were performed using self-made programs in MATLAB (MathWorks Inc., MA,
185 USA). Periodicity of the time course of the abdominal tip location was analyzed using the fast
186 Fourier transform (FFT). For FFT, data for only the last 400 s of each trajectory (600 s in total)
187 were used because the periodicity of a bee's flight was occasionally unstable at the beginning of
188 the stimulus (e.g., see Fig. 2Ac). The relative power spectrum was calculated, and peak
189 frequencies were determined. We defined a bee to be aligned with a certain e-vector orientation
190 or showing "polarotaxis", when the power spectrum of the bee showed a peak at the stimulus
191 frequency, i.e., 0.5, 0.01, and 0.02 Hz for 0.9, 1.8, and 3.6 ° s⁻¹ stimuli, respectively. Distributions
192 of bees showing polarotaxis were statistically analyzed using Fisher's exact test or Cochran's
193 *Q*-test with post-hoc McNemar test for among- or within-group comparisons, respectively. In
194 addition, the largest peak in the power spectrum of each bee was determined to compare the
195 distribution of the peaks by a bee.

196 In the case of experiments where 1.8 ° s⁻¹ CW stimulus was used, a preferred e-vector
197 orientation (PEO) for each bee that demonstrated polarotaxis was obtained from a phase of the
198 stimulus frequency component (0.01 Hz) in the division signal after FFT. The uniformity of the
199 distribution of PEOs was statistically analyzed using the Rayleigh test (Batschelet, 1981). For the
200 bees showing polarotaxis to both CW and CCW stimuli, differences in the PEOs between these
201 two stimuli were also calculated for each bee by subtracting the value of the CW stimulus from
202 that of CCW stimulus, and the distribution of the differences was analyzed using the *V*-test with
203 0 ° as an expected mean angle (Batschelet, 1981). All circular statistics were performed using
204 Oriana software (ver. 3.12; Kovach Computing Services, UK).

205

206 **RESULTS**

207 **Polarotactic behavior of tethered bees**

208 Under our experimental condition, approximately two-thirds of the experimental tethered bees
209 could stably fly for over 10 min. A representative horizontal trajectory of a bee's abdominal tip

210 under the three different polarized light conditions is shown in Fig. 2A. When the e-vector of the
211 polarized light stimulus was gradually (1.8° s^{-1}) rotated clockwise or counterclockwise, the bee
212 showed a periodic right-and-left abdominal movement, regardless of the rotational direction (Fig.
213 2Aa, c). The FFT analysis of the last 400 s of the trajectory data clearly showed that these
214 abdominal movements were synchronized with an e-vector rotating frequency of 0.01 Hz (Fig.
215 2Ba, c). Conversely, a bee did not show such periodic movement under the static e-vector
216 stimulus (0° with respect to the body axis; Fig. 2Ab), and the peak of the power spectrum (PS)
217 was detected at 0.0025 Hz instead of at 0.01 Hz, which is coincident with the entire data length
218 (Fig. 2Bb). In total, over half of the experimental bees showed a clear peak at 0.01 Hz in the PS
219 under the rotating e-vector stimulus (12 and 14 of 21 bees for CW and CCW, respectively);
220 however, under the static 0° e-vector stimulus, only 2 of the 21 bees showed a 0.01 Hz peak in
221 the PS, which was significantly smaller than the number of bees showing a peak at 0.01 Hz under
222 the rotating stimulus (data not shown; CW: $p = 0.008$, CCW: $p = 0.001$, Cockran's Q -test with
223 post-hoc McNemar test). In addition, a significantly higher number of bees (7 and 6 of 21 bees
224 for CW and CCW, respectively) displayed the highest peaks at 0.01 Hz in the PS compared with
225 that (none of the 21 bees) under the static 0° e-vector stimulus (Fig. 3; CW: $p = 0.008$, CCW: $p =$
226 0.014, Cockran's Q -test with post-hoc McNemar test). In the averaged PS, a clear peak was noted
227 at 0.01 Hz under the CW or CCW stimulus, although another strong peak was detected at 0.0025
228 Hz (Fig. 3A, C), and the peak was only detected at 0.0025 Hz under the static stimulus (Fig. 3B).

229 To determine whether the periodic movements were not elicited by the rotation of the
230 e-vector, but rather by a slight fluctuation in light intensity caused by the polarizer rotation, we
231 projected an unpolarized light stimulus through the depolarizer beneath the rotating polarizer (see
232 Materials and Methods). Under the unpolarized light stimulus, the bees did not show any clear
233 movements coincident with the polarizer rotation (Fig. 4A). Furthermore, no detectable peak at
234 0.01 Hz was noted in the averaged PS, and none of the five experimental bees demonstrated the
235 highest peak at 0.01 Hz (Fig. 4B). Only one bee showed a small PS peak at 0.01 Hz, which was
236 not significantly different from that under the static e-vector stimulus ($p = 0.4885$, Fisher's exact
237 test). These results indicate that the abdominal periodic movements were elicited by the rotation
238 of the polarized e-vector orientation.

239 We also determined the relationship between a tethered bee's abdominal location and its
240 flying behavior (see Fig. S1). Simultaneous recordings of the abdominal images and the yaw
241 torque of a flying tethered bee showed a strong negative correlation, i.e., the bee's abdominal tip
242 moved right when the bee turned left and *vice versa*. Therefore, a bee's periodic abdominal
243 movement under the rotating e-vector stimulus could be considered a kind of polarotaxis to adjust

244 the flying direction to a certain e-vector orientation.

245 **Polarotaxis under the different speeds of the stimulus**

246 Next, we observed polarotaxis of the tethered bees under CW rotating e-vector stimulus at twice
247 the speed (3.6° s^{-1}) or 2-times slower the speed (0.9° s^{-1}) to confirm that the periodicity in the
248 abdominal movement (Fig. 2, 3) was not elicited by internal rhythm but by external polarized
249 light stimuli. Under the faster stimulus, some bees still showed right-and-left abdominal
250 movements synchronized to the stimulus rotation (Fig. 5A). However, in contrast to the 1.8° s^{-1}
251 stimulus, the PS of the abdominal trajectory showed only a small peak at a stimulus frequency of
252 0.02 Hz (Fig. 5B). Moreover, in the averaged PS of all 14 experimental bees, a small, but
253 detectable, peak at 0.02 Hz and the highest peak at 0.0025 Hz were noted (Fig. 5C). The number
254 of bees showing the peak at 0.02 Hz in the PS was significantly different from experiencing the
255 static or 1.8° s^{-1} stimulus (7 of 14 bees for 3.6° s^{-1} and none of the 21 bees for static and 1.8° s^{-1}
256 stimulus; $p = 0.0005$, Fisher's exact test), although only one of the 14 experimental bees showed
257 the highest peak at 0.02 Hz (Fig. 5C). These results indicated that the bees exhibited weak
258 polarotactic behavior to the fast rotating e-vector stimulus.

259 Under the slower rotating stimulus, the tethered bees showed clear right-and-left
260 abdominal movements, the PS of which had the highest peak at the stimulus frequency of 0.005
261 Hz (Fig. 6A, B). Four of the 10 experimental bees exhibited the highest peak at 0.005 Hz in each
262 PS of the abdominal trajectory (Fig. 6C), whereas only one of the 21 bees did so under the 1.8° s^{-1}
263 stimulus, which was significantly lower ($p = 0.0274$, Fisher's exact test). This result indicated
264 that the bees also responded to a slow stimulus. However, we could not detect a 0.005 Hz peak in
265 the averaged PS, although the power at 0.005 Hz was relatively high compared with that under
266 other stimulus conditions (Fig. 6C); this could have occurred because the peak could not be
267 clearly separated from the peak at 0.0025 Hz owing to data interference from unresponsive bees
268 (see Figs 3B, 4C). Probably for similar reasons, the number of bees demonstrated the highest
269 peak at 0.005 Hz was not significantly different from that of the static e-vector stimulus (4 of the
270 21 bees; $p = 0.3809$, Fisher's exact test).

271 **Selective stimulation of eye regions**

272 Polarization vision in insects is known to be mediated by the DRA of the compound eye. To
273 confirm the sensory input area for polarotaxis in the eye, we covered a part of each compound
274 eye and restricted the area receiving light stimulation to the DRA (Fig. 7D, E). The bees whose
275 DRA was covered did not show polarotactic abdominal movement even under the 1.8° s^{-1}

276 rotating polarized light stimulus to which intact bees responded (Fig. 7A), and no clear peak was
277 noted at the stimulus frequency of 0.01 Hz in the power spectrum (Fig. 7B). The averaged power
278 spectrum of all eight experimental bees did not exhibit a peak at 0.01 Hz (Fig. 7C), indicating
279 that the bees with covered DRA lost the ability to orient to certain e-vectors. Similar to the
280 response of intact bees to a static stimulus, none of the eight bees displayed the highest peak at
281 0.01 Hz (Fig. 7C, see also Fig. 3B), and their response was not significantly different ($p = 1$,
282 Fisher's exact test). Conversely, the number of bees showing the highest peak at 0.01 Hz was also
283 not significantly different than that of the intact bees under the CW stimulus (see Figs 3A, 7C; p
284 = 0.1421, Fisher's exact test), probably owing to the small number of experimental bees used.

285 **Preferred e-vector orientation**

286 We assessed the PEOs of the 21 bees that showed polarotaxis under the 1.8° s^{-1} CW stimulus.
287 The PEO of each bee varied from 0 to 180° (Fig. 8A) However, more than half of the bees (14 of
288 21) showed PEOs between 120° to 180° and the distribution was not significantly random ($p =$
289 0.009 , Rayleigh test). To confirm whether each bee had a specific PEO, we compared the PEO to
290 the CW and CCW stimuli in the same bee. The angular differences in PEOs between CW and
291 CCW stimuli of 7 bees, which showed clear polarotaxis under both conditions, are shown in Fig.
292 8B. The distribution of the angular differences was not significantly random ($p = 0.01$, Rayleigh
293 test), but was concentrated around 0° ($p = 0.026$, V-test), suggesting that each bee had a certain
294 PEO and adjusted the flight direction by aligning to a particular e-vector angle.

295

296 **DISCUSSION**

297 **Behavioral response to the polarized light stimulus in the honeybee**

298 It is well known that honeybees use skylight polarization to detect their intended travel direction.
299 It was first described by von Frisch (1967) through a series of sophisticated behavioral studies on
300 the waggle dance. Thereafter, the waggle dance orientations of the nest-returning bees from a
301 certain feeder have been intensively studied. These studies were conducted under a patch of
302 polarized light stimulus or part of the sky and an internal representation of the celestial e-vector
303 map has been proposed (Rossel and Wehner, 1982, 1986, 1987; Wehner, 1997). These systematic
304 studies have focused on modification of the waggle dance orientation and not on how the bees
305 perceive polarized light from the sky en route to/from the nest. More recently, direct observation
306 of polarized light detection in flying bees has been performed using a four-armed tunnel maze
307 with a polarizer on top (Kraft et al., 2011). In this experiment, it was revealed that bees choose

308 their flying direction based on zenithal polarized light information. Moreover, it has also been
309 demonstrated that bees memorize the e-vector orientations experienced during their foraging
310 flight and use that memory for the subsequent waggle dances (Evangelista *et al.*, 2014). In the
311 present study, we directly showed that bees tended to orient to the certain e-vector angles during
312 their flight under the tethered condition, i.e., they referred polarized light information to control
313 their flight direction. The fact that fewer bees responded to the fast stimulus (3.6° s^{-1} , Fig. 5) than
314 the slow stimuli (0.9° s^{-1} and 1.8° s^{-1} , Fig. 2, 3, 6) is also indicative of the use of e-vector
315 orientation as a global cue for orientation. Probably, they did not refer to the e-vector when it
316 quickly changed because they did not expect such a situation, except when they quickly changed
317 their flight direction.

318 The polarotactic behaviors were not observed when the bee's DRA was blinded (Fig. 7).
319 It is well known that detection of skylight polarization in insects is mediated by ommatidia in the
320 DRA (for review: Labhart and Meyer, 1999; Wehner and Labhart, 2006). In honeybees,
321 UV-sensitive photoreceptors of the ommatidia in DRA are highly polarization-sensitive, and their
322 receptive field covers a large part of the celestial hemisphere, which is suitable for observing the
323 sky (Labhart, 1980, Wehner and Strasser, 1985). Behaviorally, it has also been demonstrated that
324 covering the DRA impaired correct coding of food orientation by the waggle dance orientation
325 (Wehner and Strasser, 1985). These results clearly show that bees utilize polarized light detected
326 by the ommatidia in the DRA for orientation.

327 **Polarotaxis in insects**

328 Polarotactic behavior in insects has been demonstrated in several species. Obviously, orientation
329 to a certain e-vector direction is a common occurrence among insect species that utilize skylight
330 polarization for navigation. Classically, it has been tested using a treadmill device in the cricket
331 *Gryllus campestris* (Brunner and Labhart, 1987) and the fly *Musca domestica* (von Philipsborn
332 and Labhart, 1990). Using such a device, the insect was tethered on an air-suspended ball and its
333 walking trajectory could be monitored through the rotation of the ball. In these species, the insect
334 on the ball showed clear polarotactic right-and-left turns when the e-vector of the zenithal
335 polarized light stimulus was slowly rotated, as we showed in this study in flying honeybees. This
336 kind of behavior does not merely demonstrate they have polarization vision but also allowed us to
337 clarify fundamental properties of insect polarization vision, e.g., perception through the DRA in
338 the compound eye (Brunner and Labhart, 1987), monochromatic spectral sensitivity (Herzmann
339 and Labhart, 1989; von Philipsborn and Labhart, 1990), and sensitivity to the degree of
340 polarization (Henze and Labhart, 2007).

341 Orientation to the polarized light has been investigated in tethered flying insects as well.
342 In the locust *Schistocerca gregaria*, direct monitoring of yaw-torque responses showed clear
343 polarotactic right-and-left turns to rotating polarized light (Mappes and Homberg, 2004). In
344 tethered monarch butterflies *Danaus plexippus*, measuring flight orientations using an optical
345 encoder revealed that their flight orientation under natural skylight was clearly affected by a
346 dorsally presented polarization filter (Reppert et al., 2004; but see also Stalleicken et al., 2005).
347 Similar orientation to polarized skylight has also been demonstrated in *Drosophila* (Weir and
348 Dickinson, 2012). In this study, a fly was magnetically tethered in the arena and its flight heading
349 was recorded from above by an infrared camera. A potential problem in investigating polarization
350 vision in tethered flying insects is that sometimes the tethering apparatus, including the torque
351 meter or other recording devices, interrupt a part of the visual field of the tested animal. In the
352 present experiments, we succeeded in evaluating the bee's polarotactic flight steering by simply
353 monitoring the horizontal position of the abdominal tip that was strongly anti-correlated with the
354 torque generated by the bee (see Fig. S1). Using these methods, the entire visual field of the
355 animal remained open; therefore, it had an advantage for investigating the animal's responses
356 under various stimulus conditions.

357 **Preferred e-vector orientation**

358 The PEO distribution has been reported in several species. In crickets and flies, a weak
359 preference to an e-vector orientation perpendicular to their body axis was demonstrated, although
360 the reason of this behavior was not clear (Brunner and Labhart, 1987; von Philipsborn and
361 Labhart, 1990). On the other hand, in laboratory-reared locusts, the PEOs were randomly
362 distributed and they did not show any directional preferences (Mappes and Homberg, 2004). In
363 the present study, the bees showed a significantly higher preference between 120 and 180 ° (Fig.
364 8A). In each bee, the PEOs under CW and CCW stimulus were quite similar (Fig. 8B), and this
365 suggested that each bee has its own PEO and used it not only as a reference for maintaining
366 straight flight but also to deduce its heading orientation.

367 Considering that central place foragers, such as honeybees, have to change their
368 navigational directions depending on the currently available food locations, their PEOs would
369 reflect their previous foraging experiences. In the present study, we collected the bees with a
370 pollen load at the hive entrance; therefore, all experimental forager bees were returners.
371 Consequently, we could no longer assess their feeding locations when we measured their flight
372 responses in the laboratory. Moreover, their path-integration vector should be reset to a zero-state
373 in such a situation (Sommar et al., 2008), and they might not have had a strong motivation to use

374 polarized light cues for navigation. To further clarify the role of polarization vision in flying
375 foragers, testing the PEOs in the bees in different navigational states will be crucial.

376

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381 **Competing interests**

382 No competing interests declared.

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387

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504 **Figure legends**

505 **Fig. 1. Experimental setup.** Light from a xenon lamp was equally depolarized and then linearly
506 polarized using a UV-transmitted polarizer. A bee was tethered under the polarizer and its flight
507 was monitored by a USB camera. For the stable flight of a tethered bee, rectified wind from a
508 circulator and moving black-and-white stripes on a PC monitor were presented.

509

510 **Fig. 2. A bee's abdominal movement under the polarized light stimulus.** Trajectories of the
511 abdominal tip (**A**) and the power spectrum (**B**) under the clockwise (1.8° s^{-1} ; **a**), static (**b**), and
512 counterclockwise (1.8° s^{-1} ; **c**) stimulus. The lower trace in each trajectory (Pol.) indicates the
513 e-vector orientation of the polarizer with respect to the bee's body axis. Under rotating e-vector
514 (**a** and **c**), the abdomen showed periodical movements from side to side. Dashed lines indicate the
515 peaks at the stimulus rotation frequency (0.01 Hz).

516

517 **Fig. 3. Averaged power spectrum of the abdominal movements under the polarized light**
518 **stimulus.** Power spectrum curves (black line) and histograms of the maximum peak in each
519 power spectrum (gray bars) under the clockwise (1.8° s^{-1} ; **A**), static (**B**), and counterclockwise
520 (1.8° s^{-1} ; **C**) stimulus are shown ($N = 21$). Dashed lines indicate the peaks at the stimulus rotation
521 frequency (0.01 Hz).

522

523 **Fig. 4. Abdominal movements under the depolarized light stimulus. A.** An example of the
524 bee's abdominal trajectory. A UV-transmitted depolarizer was put just below the rotating
525 polarizer (1.8° s^{-1}). The lower trace (Pol.) indicates the e-vector orientation of the polarizer with
526 respect to the bee's body axis. **B.** The power spectrum of the abdominal trajectory shown in A. **C.**
527 Averaged power spectrum (black line) and the histogram of the maximum peak in each power
528 spectrum (gray bars) are shown ($N = 5$).

529

530 **Fig. 5. Abdominal movements under the rotating polarized light stimulus (3.6° s^{-1}).** **A.** An
531 example of a bee's abdominal trajectory. The lower trace (Pol.) indicates the e-vector orientation
532 of the polarizer with respect to the bee's body axis. **B.** The power spectrum of the abdominal
533 trajectory shown in A. **C.** Averaged power spectrum (black line) and the histogram of the
534 maximum peak in each power spectrum (gray bars) are shown ($N = 14$). Dashed lines indicate the
535 peaks at the stimulus rotation frequency (0.02 Hz).

536

537 **Fig. 6. Abdominal movements under the rotating polarized light stimulus (0.9° s^{-1}).** **A.** An
538 example of a bee's abdominal trajectory. The lower trace (Pol.) indicates the e-vector orientation
539 of the polarizer with respect to the bee's body axis. **B.** The power spectrum of the abdominal
540 trajectory shown in **A.** **C.** Averaged power spectrum (black line) and the histogram of the
541 maximum peak in each power spectrum (gray bars) are shown ($N = 10$). Dashed lines indicate the
542 peaks at the stimulus rotation frequency (0.005 Hz).

543

544 **Fig. 7. Abdominal movements of the DRA-covered bees under the rotating polarized light**
545 **stimulus (1.8° s^{-1}).** **A.** An example of a bee's abdominal trajectory. The lower trace (Pol.)
546 indicates the e-vector orientation of the polarizer with respect to the bee's body axis. **B.** The
547 power spectrum of the abdominal trajectory shown in **A.** **C.** Averaged power spectrum (black
548 line) and the histogram of the maximum peak in each power spectrum (gray bars) are shown ($N =$
549 8). **D.** Head of the bee after its DRAs were painted. The area surrounded by the dashed line was
550 painted. Arrow heads indicate the ocelli. CE: compound eye. **E.** Lateral view of the compound
551 eye of the bee shown in **D.**

552

553 **Fig. 8. Preferred e-vector orientations (PEOs) of the bees caught at the hive entrance.** **A.**
554 PEOs of the bees that showed polarotaxis under clockwise rotating stimulus (1.8° s^{-1}) with
555 respect to the bee's body axis. The distribution was significantly random ($p > 0.1$, Rao's spacing
556 test). **B.** Angular differences in the PEOs of each bee between clockwise and counterclockwise
557 stimulus. The distribution was not significantly random ($p < 0.05$, Rao's spacing test), but
558 concentrated around 0° ($p = 0.026$, V-test).















