

1 A decision underlies phototaxis in an insect

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18

19 **Abstract**

20 Like a moth into the flame - Phototaxis is an iconic example for innate preferences. Such
21 preferences likely reflect evolutionary adaptations to predictable situations and have traditionally
22 been conceptualized as hard-wired stimulus-response links. Perhaps therefore, the century-old
23 discovery of flexibility in *Drosophila* phototaxis has received little attention. Here we report that
24 across several different behavioral tests, light/dark preference tested in walking is dependent on
25 various aspects of flight. If we temporarily compromise flying ability, walking photopreference
26 reverses concomitantly. Neuronal activity in circuits expressing dopamine and octopamine,
27 respectively, plays a differential role in photopreference, suggesting a potential involvement of
28 these biogenic amines in this case of behavioral flexibility. We conclude that flies monitor their
29 ability to fly, and that flying ability exerts a fundamental effect on action selection in *Drosophila*.
30 This work suggests that even behaviors which appear simple and hard-wired comprise a value-
31 driven decision-making stage, negotiating the external situation with the animal's internal state,
32 before an action is selected.

33

34 **Keywords**

35 Behavioral Flexibility; Octopamine; Dopamine; Decision-making; *Drosophila*; invertebrates

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39 **Introduction**

40 In their struggle for survival, animals need not just the capability to trigger behaviors at the
41 appropriate time, but these behaviors need to be flexible in response to or anticipation of
42 changes in environmental and internal conditions. What may be an appropriate response to a
43 given stimulus when the animal is hungry may be maladaptive when the animal is seeking a
44 mating partner, and *vice versa*. The relative values of extrinsic and intrinsic factors must be
45 analyzed and weighed in order to shape the behavior to be adaptive in a particular situation.
46 Across animal phyla, biogenic amines have been found to be part of a complex network
47 involved in such value-driven processes. In invertebrates, Dopamine (DA) and Octopamine
48 (OA) are two important modulators of behavior. OA, the invertebrate counterpart of the
49 adrenergic vertebrate system, has been implicated in state-dependent changes in visual-
50 processing [1,2], experience-dependent modulation of aggression [3], social decision-making
51 [4], and reward [5]. DA is also known for its countless roles in physiological and behavioral
52 processes across animal phyla such as reward [5–7], motivation [8–10] and value-based or
53 goal-directed decision-making [8,11–15]. Complementing such flexible behaviors are simple,
54 innate responses such as escape responses, taxis/kinesis behaviors, or fixed action patterns.
55 They are commonly thought to be less flexible and more automatic, but with the advantage of
56 either being especially efficient, fast, or with only a low cognitive demand. However, recent
57 research has shown that many of these behaviors are either more complex than initially
58 imagined [16–19] or liable to exploitation [20]. Moreover, several studies have shown that the
59 state of the animal modulates how sensory structures process identical stimuli [21–26] and
60 many of these modulations are caused by aminergic actions [1,2,21,27–29]. Due to
61 observations like these, the general concept of behaviors as responses to external stimuli
62 ('sensorimotor hypothesis') has come under ever more critical scrutiny in the last decade.
63 Studying what can arguably be perceived as the most iconic of stereotypic insect responses, the
64 approach of a bright light (phototaxis), we provide further evidence that the simple input-output

65 relationships long assumed to underlie most if not all behaviors, may only exist at the
66 observational level, dissipating at the neuronal level.

67 *Drosophila melanogaster* phototactic behavior has been studied for at least one hundred years.
68 As most flying insects, flies move towards a light source after being startled, showing positive
69 phototaxis. This innate preference for light appears to be species- and strain-specific and has
70 been described as part of a fly's personality [30]. Recently, it has been shown that mated female
71 flies transiently avoid UV light during egg-laying [31]. Interestingly, experiments described by
72 McEwen in 1918 and Benzer in 1967 demonstrated that wing defects affect phototaxis also in
73 walking flies. These early works showed that flies with clipped wings did not display the
74 phototactic response to light, whereas cutting the wings from mutants with deformed wings did
75 not decrease their already low response to light any further [32,33]. The fact that manipulating
76 an unrelated organ, such as wings, affects phototaxis contradicts the assumed hard-wired
77 organization of this behavior, suggesting that it may not be a simple matter of stimulus and rigid,
78 innate response, but that it contains at least a certain element of flexibility. In this work, we
79 systematically address the factors involved in this behavioral flexibility and begin to explore the
80 neurobiological mechanisms behind it.

81 **Methods**

82 **Strains and fly rearing.**

83 Flies were reared and maintained at 25°C in vials containing standard cornmeal agar medium
84 [34] under 12h light/dark cycles with 60% humidity, except for experiments involving *UAS-trpA1*
85 or *UAS-shibire^{TS}*, in which parental crosses and their offspring were maintained at 18°C under
86 12h light/dark cycles with 60% humidity.

87 Stocks obtained from the Bloomington *Drosophila* Stock Center (RRID:SCR_006457; NIH
88 P40OD018537) were used in this study: *UAS-TrpA1* (26263), *th-GAL4* (8848), *tdc2-GAL4*
89 (9313), and *PKC^δ* (18258). The *PKC^δ* mutant flies were intended for a different project when we

90 discovered that the flies do not even attempt to fly. To our knowledge, the molecular mechanism
91 behind the flightlessness is unknown.

92 The sources of other stocks are detailed here:

93 w^{1118} , w^{1118} ; *hs-Gal4* (heat shock inducible GAL4), and *UAS-PKCi* (inhibitory pseudosubstrate of
94 protein kinase C) were provided by Henrike Scholz (University of Cologne, Germany).

95 *WTB* is a Wild-type Berlin strain from our stock in Regensburg.

96 *CS^{RE}* is a *Canton S* strain bred in our lab in Regensburg.

97 *CS^{TZ}* and *FoxP³⁹⁵⁵* were provided by Troy Zars (University of Missouri, USA).

98 *rsh¹* was provided by B. van Swinderen (The University of Queensland, Australia).

99 *rut²⁰⁸⁰*, *mb247-GAL4* and *UAS-CNT-E* were provided by Martin Heisenberg (Rudolf Virchow
100 Center, Germany).

101 *act88F-Gal4* was provided by Juan A. Navarro (University of Regensburg, Germany).

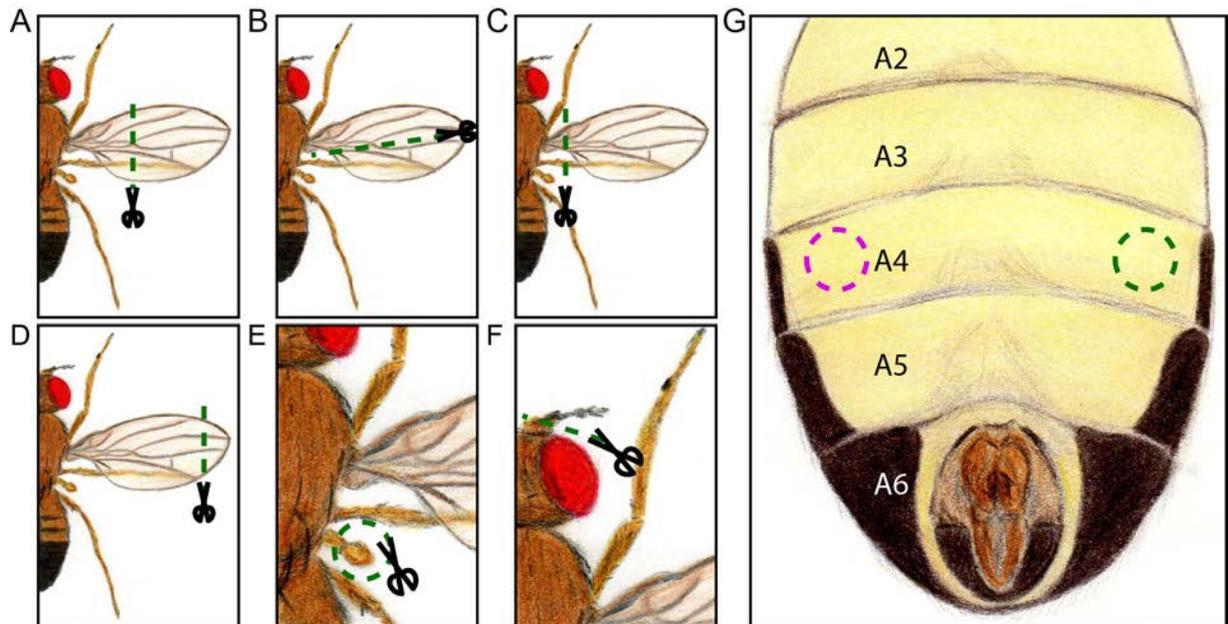
102 *A9-GAL4* and *UAS-baboon^{QD}* were provided by Florian Bayersdorfer (University of Regensburg,
103 Germany).

104 **Mechanical manipulations**

105 Unless described otherwise, 24h before the experiment 2-5 d old flies were briefly anesthetized
106 under CO₂. In the standard wing-clipping procedure, the distal two thirds from both wings were
107 clipped from half of the individuals (Fig. 1A). At least 30 flies with clipped wings and 30 flies with
108 intact wings were placed in the same vial until the experiment was performed, in which they
109 were tested together. For other manipulations, one of the different treatments (see Fig. 1) was
110 applied to half of the flies of a given group. At least sixty flies (half of them with injury) were
111 placed in vials for a 24h recovery period and tested together. Flies with abdominal injury were
112 not mixed with intact flies to avoid mistakes during the evaluation of the experiment due to the
113 inconspicuous nature of the injury.

114 Haltere removal was performed by pulling each haltere with forceps, while the antennal damage
115 was produced by clipping the third segment of the antenna (funiculus). The abdominal injury

116 was performed with a sharpened needle, and was always made ventrally in one side of the
117 fourth abdominal segment.



118
119 **Figure 1. Schematic representation of the different injuries made to the flies.** **A**, This was the
120 standard procedure, where the distal two thirds from both wings were removed. **B**, Longitudinal cut. Half
121 of the wing was removed. It was applied to both wings in experiments of Fig. 4A,B. **C**, Whole wing cut. It
122 was used in Fig. 4C,D to remove only one wing (the side was randomly selected), and in Fig. 4E,F to
123 remove both wings. **D**, End of the wing cut. Around 20% of each wing was removed. It was used in Fig.
124 4E,F. **E**, Haltere removal. Both halteres were removed and the effect on photopreference is presented in
125 Fig. 4G,H. **F**, Antennal damage. The third segment of both antennae was cut. This treatment was used
126 for experiments in Fig. 4I,J. **G**, Abdominal injury. Flies were stabbed on one side of the ventral fourth
127 abdominal segment (the side was randomly selected). The results of the effect of this injury in phototaxis
128 are depicted in Fig. 4K,L.

129 **Wing gluing**

130 Flies were cold anesthetized using a custom made cold air station and their wings were glued
131 together in their natural relaxed posture using a 3M sucrose solution. To unglue the wings flies
132 were cold anesthetized and their abdomen gently submerged in water to dissolve the sucrose.

133 After each process flies were left to recover overnight. Flies were discarded from the analysis if
134 their wings were damaged because of the treatments or unglued by chance.

135 **Countercurrent Apparatus**

136 Phototactic preference was evaluated using Benzer's classic countercurrent apparatus [32]
137 (<http://dx.doi.org/10.17504/protocols.io.c8gztv>). The apparatus was completely transparent and
138 consisted of two acrylic parts, a lower one with 6 parallel tubes (an initial tube + 5), and a
139 movable upper part with 5 parallel test tubes. Each plastic tube had a length of 6.8 cm, an inner
140 diameter of 1.5 cm, and an outer diameter of 1.7 cm. The test group was placed in the initial
141 tube and was left in darkness to acclimate for 10 min, with the apparatus placed horizontally.
142 Thereafter, flies were startled by tapping the apparatus, making all of them end up at the bottom
143 of the tube. The apparatus was placed horizontally and the upper part shifted, making the initial
144 tube face the first test tube for 15 seconds, allowing the flies to move towards the light if the test
145 tube was facing it (positive phototaxis test), or away from it if the initial tube was facing the light
146 (negative phototaxis test). Then, the upper part was shifted again and flies that moved to the
147 test tube were transferred to the next tube of the lower part by tapping the apparatus, and the
148 same test was repeated 4 more times. The light source was always placed at 30 cm from the
149 apparatus and consisted of a fluorescent warm white tube (OSRAM 18W/827), which delivers
150 1340 lux at that distance.

151 The Performance Index was calculated using the formula:

$$PI = \frac{(\#F_5 \times 5) + (\#F_4 \times 4) + (\#F_3 \times 3) + (\#F_2 \times 2) + (\#F_1 \times 1) + (\#F_0 \times 0)}{\#F_T}$$

152 where $\#F_n$ was the number of flies in the tube n (being 0 the initial tube and 5 the last test tube),
153 and $\#F_T$ was the total number of flies. If the test tubes were on the bright side a higher index
154 meant a more positive phototaxis. In each experiment a PI was calculated for the wingless flies
155 and other for the intact flies. The tubes were cleaned thoroughly after each test.

156 In order to facilitate comparisons in figures 3A and 6A, the effect size was calculated using the
157 Glass Δ estimator.

$$Glass \Delta = \frac{x_1 - x_2}{s_2}$$

158 where x_1 was the mean of treated group, x_2 the mean of the control group, and s_2 the standard
159 deviation of the control group. When positive phototaxis was tested, a negative *Glass Δ* value
160 reflected a reduction in positive phototaxis after wing-clipping; and when negative phototaxis
161 was tested, a positive value represented an increase in negative phototaxis after wing-clipping.

162

163 **T-Maze**

164 Light/Darkness choice was measured in a custom built, opaque PVC T-Maze with only one
165 transparent (acrylic) choice tube (<http://dx.doi.org/10.17504/protocols.io.c8azsd>). Flies were
166 placed in an initial dark tube (10 cm long, 1.5 cm inner diameter, and 2.5 cm outer diameter)
167 and were left to dark adapt for 10 min. Then, they were transferred to the cylindrical elevator
168 chamber (1.5 cm diameter, 1.5 cm height) by gently tapping the apparatus, where they
169 remained for 30s. Next, the elevator was placed between the dark and the bright tube (both 20
170 cm long, 1.5 cm inner diameter, and 2.5 cm outer diameter), and flies were allowed to choose
171 for 30s. As the source of light, the same fluorescent tube as for Benzer's Countercurrent
172 Apparatus was used, and placed 31.5 cm above the base of the T-Maze.

173 The Choice Index was calculated using the formula:

$$CI = \frac{(\#F_L \times 1) + (\#F_D \times -1) + (\#F_E \times 0)}{\#F_T}$$

174 where $\#F_L$ meant the number of flies in the transparent tube, $\#F_D$ was the number of flies in the
175 opaque tube, and $\#F_E$ was the number of flies that remained in the elevator. A *CI* of 1 meant all
176 the flies chose the light, while an index of -1 meant a dark photopreference. The tubes were
177 cleaned thoroughly after each round.

178 **Buridan**

179 Locomotion towards dark objects was evaluated using Buridan's paradigm as explained in
180 Colomb *et al.* [35]. Briefly, 3-6d old flies were selected and half of them had their wings clipped
181 under CO₂ anesthesia (<http://dx.doi.org/10.17504/protocols.io.c7vzn5>). They were left to recover
182 overnight within individual containers, with access to water and sugar (local store) before being
183 transferred to the experimental setup. The setup consists of a round platform (117 mm in
184 diameter) surrounded by a water-filled moat placed at the bottom of a uniformly illuminated
185 white cylinder (313 mm in height) with 2 stripes of black cardboard (30mm wide, 313 mm high
186 and 1 mm thick) placed 148.5 cm from the platform center one in front of the other. Flies were
187 prevented from escaping by a transparent lid over the platform. The experiment duration was
188 set to 900 seconds. Data were analyzed using BuriTrack and CeTrAn [35]
189 (RRID:SCR_006331), both available at <http://buridan.sourceforge.net>.

190 **Genetic manipulation of wing utility and neuronal activity**

191 For the experiments involving *TrpA1* and the *act88f-GAL4* driver, experimental flies and their
192 respective controls were raised at 18°C. 3-5d old flies were tested at room temperature (RT)
193 and recovered for 5-6h at 18°C. Then, they were transferred to a 37°C climate room where they
194 were placed in an acclimation vial for 15min. Next they were transferred to the first tube of the
195 T-maze placed in the 37°C climate room, and the experiment proceeded as explained above.
196 The choice step was reduced to 15s to compensate for the increased activity that flies showed
197 in pilot experiments. After counting the flies, they were transferred to fresh vials and placed at
198 18°C for 24h. After this recovery phase, they were tested again at RT. We noticed that the CI
199 obtained for wild types could differ between chambers at 37°C .

200 In the case of manipulation of dopaminergic and octopaminergic neural activity with *shi*^{TS} or
201 *TrpA1* the same protocol was applied but instead of 37°C, 32°C were used and the choice step
202 was 30s long.

203

204 **Statistical Analysis**

205 Statistical analyses were performed with InfoStat, version 2013 (Grupo InfoStat, Facultad de
206 Ciencias Agropecuarias, Universidad Nacional de Córdoba, Córdoba, Argentina) and R
207 (<http://www.r-project.org/>). Number of replicates in each experiment was adjusted to provide a
208 statistical power of at least 80% using pilot experiments. As dictated by the experimental design
209 and data composition, a paired T-test, a Randomized Block Design ANOVA or an ANOVA were
210 performed. Normality was tested using Shapiro–Wilks test, and the homogeneity of variance
211 was assessed with Levene’s test. A value of $p < 0.05$ was considered statistically significant.
212 After ANOVA, a Tukey least-significant difference or an orthogonal contrasts test was
213 performed. If an interaction between factors was significant in two-way ANOVAs, simple effects
214 were performed, and p values were informed. In figures 1A, 3B-E and H, and 7C and D,
215 homogeneity of variance was violated. In figures 1A, and 3B-E and H a Wilcoxon test was used,
216 while in figures 7C and D Kruskal-Wallis test was employed for multiple comparisons. The alpha
217 value was corrected using Bonferroni’s correction.

218 **Availability of data and materials**

219 The datasets supporting the conclusions of this article are available in the FigShare repository,
220 <http://dx.doi.org/10.6084/m9.figshare.1502427>.

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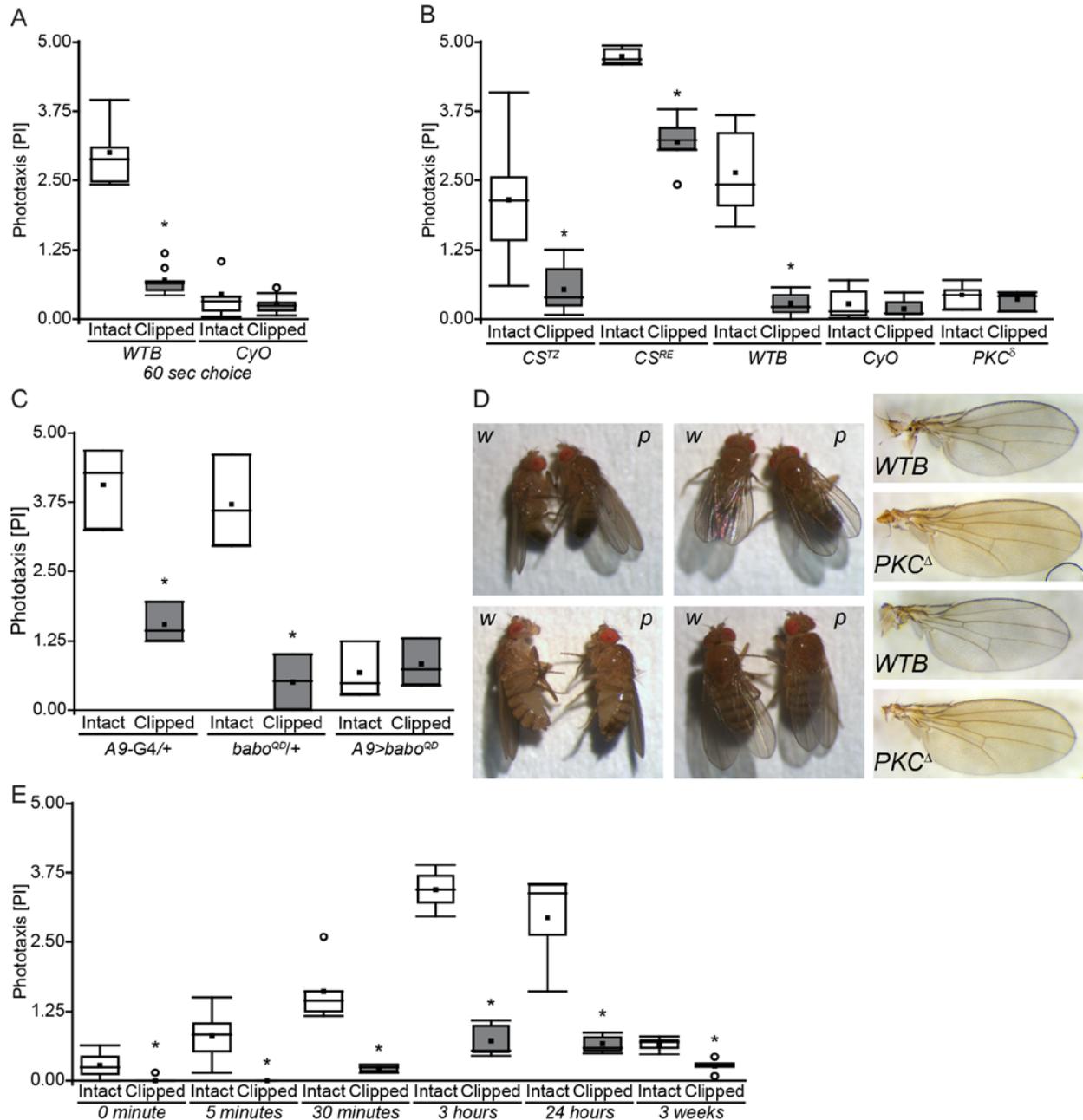
222 Results

223 Wing-clipping effect is absent in flightless flies.

224 Motivated by the findings of McEwen and Benzer, we decided to explore the nature of the
225 phototactic change observed in wingless flies. After replicating Seymour Benzer's original
226 results on wild type flies and mutant flies with deformed wings (Fig. 2A), we wondered if the
227 wing-clipping effect on phototaxis could be also observed in other genetic backgrounds.
228 Therefore, flies with and without wings from two Canton-S strains inbred in different laboratories
229 (CS^{TZ} and CS^{RE}) and from the Wild Type Berlin (WTB) line were tested in Benzer's
230 Countercurrent Paradigm (BCP). All three lines showed a significant reduction in BCP
231 performance index (PI) when the wings were cut (Fig. 2B). This reduction was apparent despite
232 large variations between the three lines in the PI levels from intact flies, showing that the
233 reduction in phototaxis due to wing-clipping can be observed across laboratory strains, with its
234 magnitude dependent on genetic background and/or associated differences in baseline levels of
235 phototactic performance.

236 Original experiments from McEwen, and then Benzer, showed that mutant flies with deformed
237 wings displayed a lower positive phototaxis than wild types [32,33] and a diminished wing-
238 clipping effect [33] (replicated in Fig. 2A). We wondered whether this simultaneous low
239 phototaxis and absence of wing-clipping effect was due to a specific effect of these mutations or
240 a general consequence of both manipulations altering the flies' wing utility. In order to tackle this
241 question, we tested three lines with flight impairments, the flightless PKC^{δ} mutant, the wings of
242 which are indistinguishable from wild type wings (Fig. 2D), the CyO balancer line with curly
243 wings, and a transgenic line in which the wings were deformed due to an overexpression of a
244 constitutively active form of the *baboon* receptor in wing imaginal discs ($A9>babo^{OD}$, [36]). Again
245 replicating previous experiments, CyO flies showed a reduced PI that remained unchanged in
246 wing-clipped animals (Fig. 2B). Similarly, $A9>babo^{OD}$ showed less attraction to light and no
247 significant wing-clipping effect (Fig. 2C), while all genetic controls behaved similar to wild type

248 flies. Remarkably, *PKC^Δ* mutants exhibited the same behavioral characteristics as *CyO* flies
 249 (Fig. 2B). Hence, we conclude that the reduction in phototaxis is not dependent on the origin of
 250 wing damage or the damage itself, but probably on wing utility.



251
 252 **Figure 2. The wing-clipping effect is observable across genetic backgrounds and throughout**
 253 **lifespan, but is absent in flightless flies. A**, Replication of the original BCP experiments using 60s of
 254 time in which the animals were allowed to walk towards the light. Wilcoxon test; *WTB*: N=8, $p < 0.001$;

255 CyO: N=8, $p=0.505$ **B**, BCP Performance Index (15s choice time) from three wild type strains and two
256 flightless mutants with intact and clipped wings. Paired T-test; CS^{TZ} : N=6, $p=0.003$; CS^{RE} : N=5, $p<0.001$;
257 WTB : N=12, $p<0.001$; CyO: N=14, $p=0.066$; PKC^{δ} : N=4, $p=0.413$. **C**, BCP Performance Index from flies
258 with a genetic manipulation of wing development ($A9>babo^{QD}$) and their genetic control groups ($A9-G4/+$,
259 $babo^{QD}/+$). Randomized Block Design ANOVA; N=3; Block $p<0.001$, Interaction Genotype vs Wings
260 Integrity: $p<0.001$, simple effect Genotype: $A9-G4/+$: $p<0.001$, $babo^{QD}/+$: $p<0.001$, $A9>babo^{QD}$: $p=0.401$.
261 **D**, Lateral and dorsal view of wing posture of WTB (w) and PKC^{δ} (p) males (upper panels) and females
262 (lower panels). Right panels: Examples of wing anatomy from WTB flies and PKC^{δ} mutant flies. **E**, BCP
263 Performance Index of WTB flies after different recovery time lengths. Paired T-Test, 0 minutes: N=6,
264 $p=0.023$; 5 minutes: N=6, $p=0.008$; 30 minutes: N=5, $p=0.007$; 3 hours: N=5, $p<0.001$; 24 hours: N=5,
265 $p=0.005$; 3 weeks: N=5, $p=0.004$. * indicates significant differences. Box plot show quantiles 0.05, 0.25,
266 0.75 and 0.95, median, mean (black square), and outliers (circle).

267

268 **The behavioral change is immediate**

269 If flies were able to assess wing utility, wing-clipping might have an almost instantaneous effect
270 on the behavior. Thus, to find out when the behavioral change takes place, we assessed wing-
271 clipped WTB flies at different time points after the injury was made. Flies from different groups
272 were tested either 3 weeks, 24h, 3h, 30min, 5min or immediately after the surgery. To diminish
273 the effects of anesthesia on phototactic behavior [37], we only used CO_2 anesthesia for
274 recovery times longer than 30min, and cold anesthesia for 0 and 5min recoveries. We found
275 that the reduction in phototaxis could be observed in all tested groups (Fig. 2E). Moreover, the
276 difference between intact and clipped flies increased with longer recovery phases, probably due
277 to the vanishing of the anesthesia effect, only to decrease again in aged flies, perhaps due to a
278 combination of a deteriorated locomotor activity and a decreased response to light in old flies
279 [38,39]. Even if flies were placed in BCP right after surgery and let to recover from anesthesia
280 only during the acclimation phase (0min group), it was possible to see a significant decrease in

281 phototaxis. These results are consistent with the hypothesis that flies continually (or at relatively
282 short intervals) monitor their ability to fly.

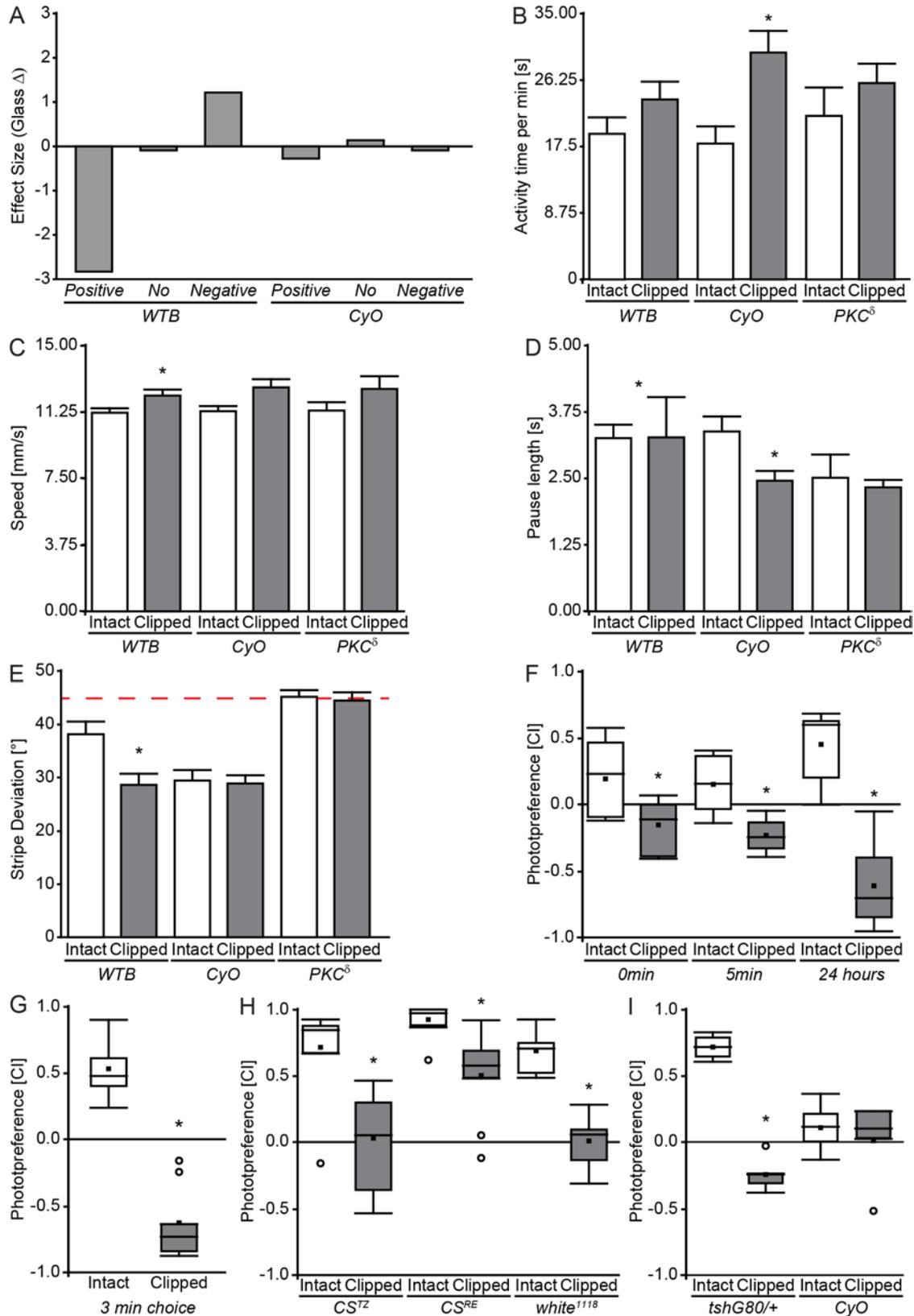
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284 **Wingless and untreated flies do not differ in their locomotor activity**

285 A potential explanation for the reduction in phototaxis is a possible reduction in locomotor
286 activity in treated flies. We tested this hypothesis by placing the light source not only in front of
287 the horizontal tubes of the BCP, but also above them, with the light shining perpendicular to the
288 trajectory of the flies. In addition, we tested for negative phototaxis by placing the light source on
289 the same side of the starting tube, such that we were able to count the flies with negative
290 phototaxis. This tripartite experimental design allowed us to directly compare all three situations:
291 light source on the opposite side of the starting tube (positive phototaxis), light source on top of
292 the BCP (no taxis; locomotor activity control), and light source on the same side as the starting
293 tube (negative phototaxis). In order to facilitate direct comparison of the behavioral
294 consequences of wing-clipping in the three situations, we assessed the proportion of behavioral
295 change with the *Glass Δ Effect Size* (ES). A negative ES in positive phototaxis indicates a
296 reduction in positive phototaxis after wing-clipping. A negative ES in the no-taxis situation
297 indicates a decrease in locomotor activity after wing-clipping, a positive ES an increase. A
298 positive ES in the negative phototaxis situation indicates an increase in negative phototaxis after
299 wing-clipping. We could not find any evidence for a reduced locomotor activity in these
300 experiments. If anything, there was a small tendency of wing-clipped flies, instead of reducing
301 their locomotor activity to actively avoid the light source (Fig. 3A).

302 We tested the generality of these results in two additional experiments, Buridan's paradigm and
303 a T-maze. Buridan's Paradigm, where the flies walk on a water-surrounded circular platform
304 with two opposing vertical black stripes on the walls of a round panorama illuminated in bright
305 white light from behind, has been used as a standard test for walking speed and locomotor
306 activity for several decades [35,40]. We compared total activity time, walking speed, and pause

307 duration in intact and wingless flies from three lines (*WTB*, *CyO*, *PKC^δ*) in a modified version of
308 Buridan's Paradigm, where a roof prevents the flies from escaping. The results show only
309 occasional small differences with the overall tendency of wingless flies exhibiting, if anything,
310 slightly higher general activity than intact flies (Fig. 3B, C, D).



312 **Figure 3. Flies without wings are not less active and prefer darker stimuli.** **A**, Effect Size of wing
313 clipping on BCP with the light source on the opposite side of the starting tube (positive phototaxis -
314 *Positive*-), light source on top of the BCP (no taxis *-No-*; locomotor activity control), and light source on
315 the same side as the starting tube (negative phototaxis *-Negative-*). **B-E**, Buridan's paradigm. *WTB*:
316 Intact, N=20; Clipped, N=21. *CyO*, N=17. *PKC^δ*, N=13. Wilcoxon test. **B**, Activity time. *WTB*: p=0.151.
317 *CyO*, p=0.002. *PKC^δ*, p=0.526. **C**, Speed. *WTB*: p=0.033. *CyO*, p=0.056. *PKC^δ*, p=0.159. **D**, Pause
318 Length. *WTB*: p=0.022. *CyO*, p=0.002. *PKC^δ*, p=0.426. **E**, Stripe deviation. *WTB*: p=0.004. *CyO*,
319 p=0.959. *PKC^δ*, p=0.98. Dotted line indicates 45°, the mean value for computer-generated data. **F**, T-
320 Maze Choice Index after different recovery time lengths. Paired T-Test; *WTB*: 0 minutes: N=7, p=0.003; 5
321 minutes: N=6, p=0.026; 24 hours: N=6, p<0.001. **G**, T-Maze Choice Index with 3 min choice step. Paired
322 T-test; *WTB*: N=8, p<0.001. **H**, Choice Index of *CS^{TZ}*, *CS^{RE}* and *w¹¹¹⁸* flies with intact and clipped wings.
323 Wilcoxon test. *CS^{TZ}*, N=8, p=0.003. *CS^{RE}*, N=11, p<0.001. *w¹¹¹⁸*, N=8, p<0.001. **I**, Choice Index of *CyO*
324 flies and their wild-type siblings. Two way ANOVA, N=5, Interaction Wings Integrity (intact or clipped) vs
325 Genotype p<0.001, simple effects: clipped vs intact: *tshG80/+* p<0.001, *CyO* p=0.487. See figure 2 for
326 detailed graph information.

327

328 **Black stripe fixation in Buridan's Paradigm is influenced by wing utility**

329 Interestingly, the wing-clipped wild type flies also showed a stronger fixation of the black stripes
330 in Buridan's Paradigm, compared to the intact flies, while the flightless flies did not show such a
331 difference (Fig.3E). This result is consistent with the tendency of the wild type flies to show
332 some negative phototaxis after wing clipping (Fig. 3A). One possible explanation for these two
333 congruent observations in such disparate experiments is that the darker stimuli become more
334 attractive after wing clipping in situations where the animals are faced with a choice of darker
335 and brighter stimuli. One prediction of this hypothesis is that other experiments where the
336 animals face a choice of bright and dark stimuli should also be affected by wing-clipping. To test
337 the generality of the wing-clipping effect and to obtain a third independent test of general

338 activity, we set out to develop a T-maze experiment, where the animals are forced to choose
339 between a dark and a bright arm.

340 **Wing-clipped flies can show negative photopreference in a T-maze**

341 After several pilot experiments with a variety of different T-maze designs, we arrived at an
342 experimental design where wing-clipped WTB flies would robustly avoid the transparent tube
343 and approach the dark tube (see Material and Methods). As for the BCP, we selected different
344 recovery times (0min, 5min or 24h). Congruent with the BCP results, intact flies showed a
345 positive photopreference, while wing-clipped flies switched to light avoidance and a negative
346 photopreference immediately after their wings were cut (Fig. 3F). These results hold even if the
347 flies are allowed three minutes to choose between the two arms of the T-Maze (Fig. 3G). Also
348 similar to the results in the BCP, we found that the magnitude of the baseline photopreference in
349 intact flies and the wing-clipping effect varied with the genetic background. In the case of the T-
350 Maze, the size of the effect determined whether or not the wing-clipped flies would show
351 positive or negative photopreference (Fig. 3H). Moreover, *CyO* balancer flies also displayed a
352 diminished photopreference, almost an indifference to light, which remained unchanged in wing-
353 clipped animals, in contrast to their siblings (carrying the *tshG80* construct) which showed a
354 clear shift after wing-clipping (Fig. 3I).

355 **Only manipulations affecting flight-related abilities cause a change in photopreference**

356 While the mutant or transgenic flies used so far may shift their photopreference due to unknown
357 side effects, the shift in wing-clipped flies could in principle be brought about either directly by
358 the injury or indirectly via a detection of flying ability. To distinguish between these two
359 hypotheses, we tested the effects of a series of manipulations (see Materials and Methods, Fig.
360 1), only some of which affecting some aspect of flight, in BCP and in the T-Maze. First, we
361 evaluated flies with a longitudinal cut through their wings and flies with only one of the two wings
362 completely removed (the side was randomly selected). Both manipulations cause flightlessness.
363 Again, we observed the same shift in photopreference as with standard wing-clipping (Fig. 4A-

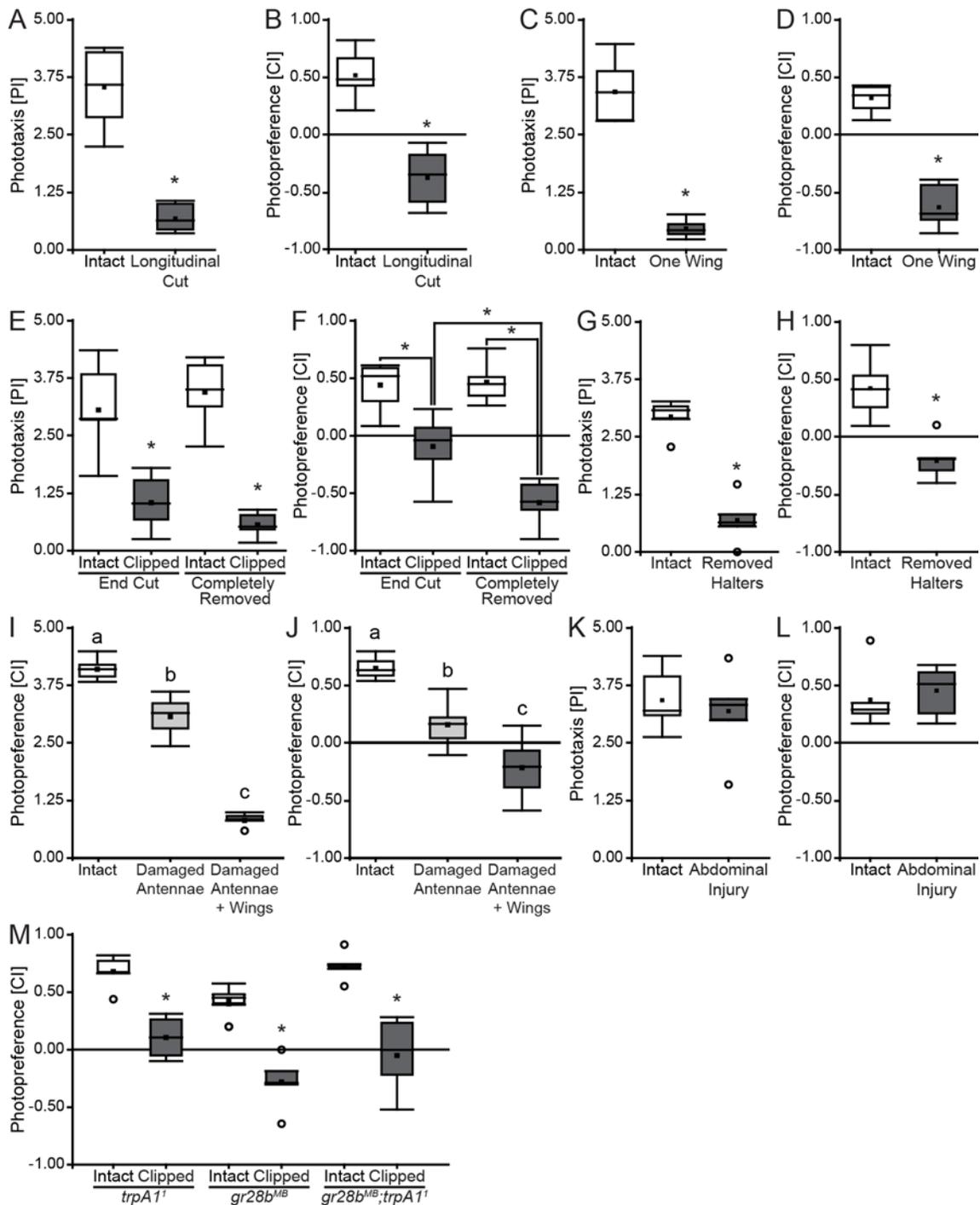
364 D). Both flies with longitudinally cut wings (Fig. 4A,B) and one wing removed (Fig. 4C,D)
365 exhibited diminished phototaxis in BCP and a negative photopreference in the T-Maze. During
366 our pilot experiments, we observed that flies with different degrees of injuries on their wings
367 behaved differently. Therefore, we hypothesized that manipulations affecting only some aspects
368 of flight behavior, rather than abolishing flight completely, might lead to less pronounced
369 behavioral changes. Thus, we next compared the behavior of flies whose wings were
370 completely removed, with those where only the tip of the wings had been removed. Flies with
371 partially removed wings are still able to fly, but with reduced torque during turns and reduced
372 lift/thrust [41]. It is worth mentioning that McEwen also attempted to test if the decrease in
373 positive phototaxis was directly proportional to the amount of wing removed, but his low number
374 of replicates, the use of ether as an anesthetic, and his different setup, prompted us to obtain
375 our own data (the same for antenna experiments – see below).

376 In both cases (complete and partial removal), injured flies showed a statistically significant
377 reduction in BCP phototaxis and T-Maze photopreference, but both indices were higher in flies
378 with only the end of the wing cut (Fig. 4E,F). In fact, the behavior from both types of injured flies
379 was significantly different from one another in the T-Maze paradigm (Fig. 4F). Therefore, we
380 conclude that behavioral change depends to some extent on the degree of the injury, and on
381 which aspects of flight behavior it affects. To test yet other aspects of flight behavior, we
382 administered injuries that did not affect the wings, in two organs related to flight (halteres and
383 antennae) and one unrelated to flight (the abdomen). In one group of flies, we removed the
384 gyroscopic halteres, mechanosensors involved in sensing body rotation and necessary for free
385 flight [42–45]. In another, we removed the distal segments of the antennae (funiculus and
386 arista), depriving the flies of their most important mechanosensor for airspeed and wind
387 direction [46–48]. The two different treatments both significantly decreased photopreference
388 values (Fig. 4G-J). However, only the manipulation abolishing free flight completely, haltere
389 removal, also led to negative photopreference in the T-Maze (Fig. 4H). Affecting flight

390 stabilization and speed by removing parts of the antennae renders the flies almost indifferent to
391 the light, on average (Fig. 4J). Fully abolishing flight ability in these antenna-damaged flies,
392 yielded negative choice indices (Fig. 4J). Thus, when flies are still able to fly, but individual
393 aspects of flight behavior are disrupted such as stabilization, torque, speed or lift/thrust, their
394 photopreference is less severely affected than when flight is abolished completely. These
395 findings extend the concept of flying ability beyond mere wing utility. To test whether any injury,
396 even one that does not affect any aspect of flight at all, can affect photopreference, we used a
397 small needle to carefully puncture the abdomen of the flies. Consistent with the results so far, a
398 wound in the abdomen did not produce any detectable shift in photopreference (Fig. 4K,L).

399 **Photopreference shift is not caused by sensory deprivation**

400 A byproduct of manipulations such as cutting the wings or damaging the antennae is the loss of
401 sensory inputs coming from those organs. Therefore, we wondered if any sensory deprivation
402 by itself could cause a dark photopreference in flies which are able to fly. We tested two
403 different thermosensation mutants in the T-Maze paradigm; *trpa1*¹, a long-term thermal
404 preference mutant [49,50], and *gr28b*^{MB} which is defective in rapid negative thermotaxis [50].
405 We also combined *trpa1*¹ and *gr28b*^{MB}, abolishing thermosensation completely. It is worth to
406 mention that the TrpA1 channel also mediates chemical avoidance via gustatory neurons
407 [51,52], and Gr28b is expressed in HC-neurons located in the same portion of the antennae
408 damaged with our manipulation [50]. The wings-intact mutants all showed a positive
409 photopreference (Fig. 4M), indicating that photopreference is not automatically affected when
410 any sensory modality is knocked out. Corroborating this observation was a sharp drop in
411 photopreference when the wings were clipped in these mutants (Fig. 4M).



412

413 **Figure 4. Only flight-affecting manipulations affect photopreference. A, C, E, G, I, K, BCP**
 414 **Performance Index from WT BCP flies with and without different injuries. B, D, F, H, J, L, T-Maze Choice**
 415 **Index from WT BCP flies with and without different injuries. A, B, Longitudinal cut of the wings. N=7, A:**
 416 **p<0.001, B: p<0.001. C, D, Only one wing cut. N=7, C: p<0.001, D: p<0.001. E, F, Wing clipped at**
 417 **different lengths. Randomized Block Design ANOVA; N=6; E: Block p= 0.094, Interaction Wings Integrity**

418 (intact or clipped) vs Degree of Injury (completely removed wings or end of the wings cut): $p=0.087$,
419 Wings Integrity: $p<0.001$, Degree of Injury: $p=0.797$; F : Block $p=0.238$, Interaction Wings Integrity vs
420 Degree of Injury: $p=0.007$, simple effects: end cut vs intact: $p<0.001$, completely removed vs intact:
421 $p<0.001$, end cut vs completely removed: $p<0.001$, intact (control from end cut) vs intact (control from
422 completely removed wings): $p=0.865$. **G, H**, Both halteres removed. **G**: $N=5$, $p<0.001$, **H**: $N=7$, $p<0.001$. **I**,
423 **J**, Both antennae damaged, and both antenna damaged and wings clipped (Damaged Antennae +
424 Wings) **I**: $N=5$, ANOVA $p<0.001$, Tukey's *post hoc* test ($p<0.05$; least-significant difference=0.54), **J**: $N=6$,
425 ANOVA $p<0.001$, Tukey's *post hoc* test ($p<0.05$; least-significant difference=0.29). Same letter indicates
426 no significant differences. **K, L**, abdominal wound. **K**: $N=6$, $p=0.377$, **L**: $N=6$, $p=0.552$. **A, B, C, D, G, H, K**,
427 **L**, Paired T-Test. **M**, Thermal sensory deprivation. $N=5$, T-Test, $trpA1^1$ $p<0.001$, $gr28b^{MB}$ $p<0.001$,
428 $gr28b^{MB};trpA1^1$ $p=0.001$. See figure 1 for detailed graph information.

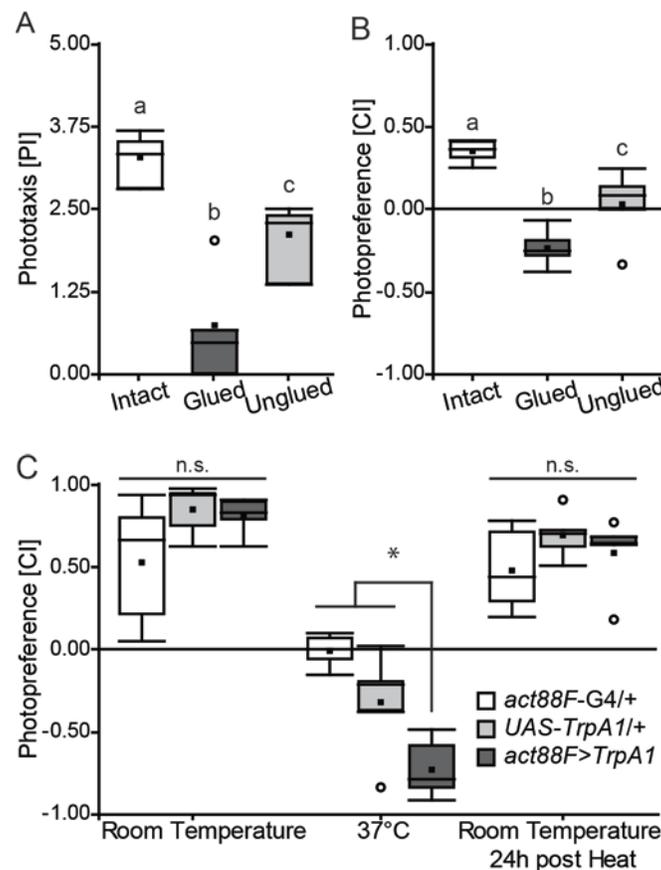
429

430 **The shift in photopreference is reversible and traces wing utility.**

431 If flies were monitoring the different aspects of their flying abilities and changing their
432 photopreference accordingly, one would expect that transient impairments in wing utility would
433 cause transient changes in photopreference. To examine the reversibility of the behavioral shift,
434 we designed two complementary experiments. In the first, we tested *WTB* flies in BCP and T-
435 Maze before and after gluing, as well as after ungluing their wings. Wing gluing perfectly
436 reproduced the wing-clipping effect, evidenced by a clear reduction of the PI and CI (Fig. 5A,B),
437 showing again that the shift in photopreference is independent from the cause of the
438 flightlessness. Remarkably, normal photopreference was restored after cleaning the wings of
439 the tested flies (Fig. 5A,B).

440 In our complementary approach, we manipulated wing utility by reversibly altering Indirect Flight
441 Muscle (IFM) contraction, expressing the temperature-sensitive *TrpA1* channel under the
442 promoter of the IFM-specific gene *actin 88F* (*act88F*), using the *act88F-GAL4* [53] driver. At
443 room temperature, experimental flies tested in our T-Maze were indistinguishable from their
444 genetic controls. However, at 37°C, when *TrpA1* caused a sustained IFM contraction disrupting

445 wing movements, the same flies showed a marked preference for the dark arm of the maze that
 446 fully recovered when they were tested back at room temperature on the following day (Fig. 5C).
 447 The genetic controls also showed a CI decrease at 37°C, but it was less pronounced and
 448 significantly different from the experimental group. In sum, these results show that flies adjust
 449 their photopreference in accordance with their wing utility. Moreover, these changes are
 450 immediate and reversible.



451
 452 **Figure 5. Photopreference changes together with wing utility in a reversible manner.** **A**, BCP tests
 453 in flies before, during and after their wings had been rendered useless by applying (and then removing)
 454 sucrose solution. Randomized Block Design ANOVA, N=4, Block p=0.091, ANOVA p<0.001, Tukey's *post*
 455 *hoc* test (p<0.05; least-significant difference=1.0257). **B**, T-Maze. Randomized Block Design ANOVA,
 456 N=5, Block p=0.173, ANOVA p<0.001, Tukey's *post hoc* test (p<0.05; least-significant difference=0.232).
 457 Same letter indicates no significant differences. **C**, Genetic manipulation of IFM contraction and wing
 458 utility. T-Maze Choice Index before, during and after 37°C exposure of experimental and control flies.

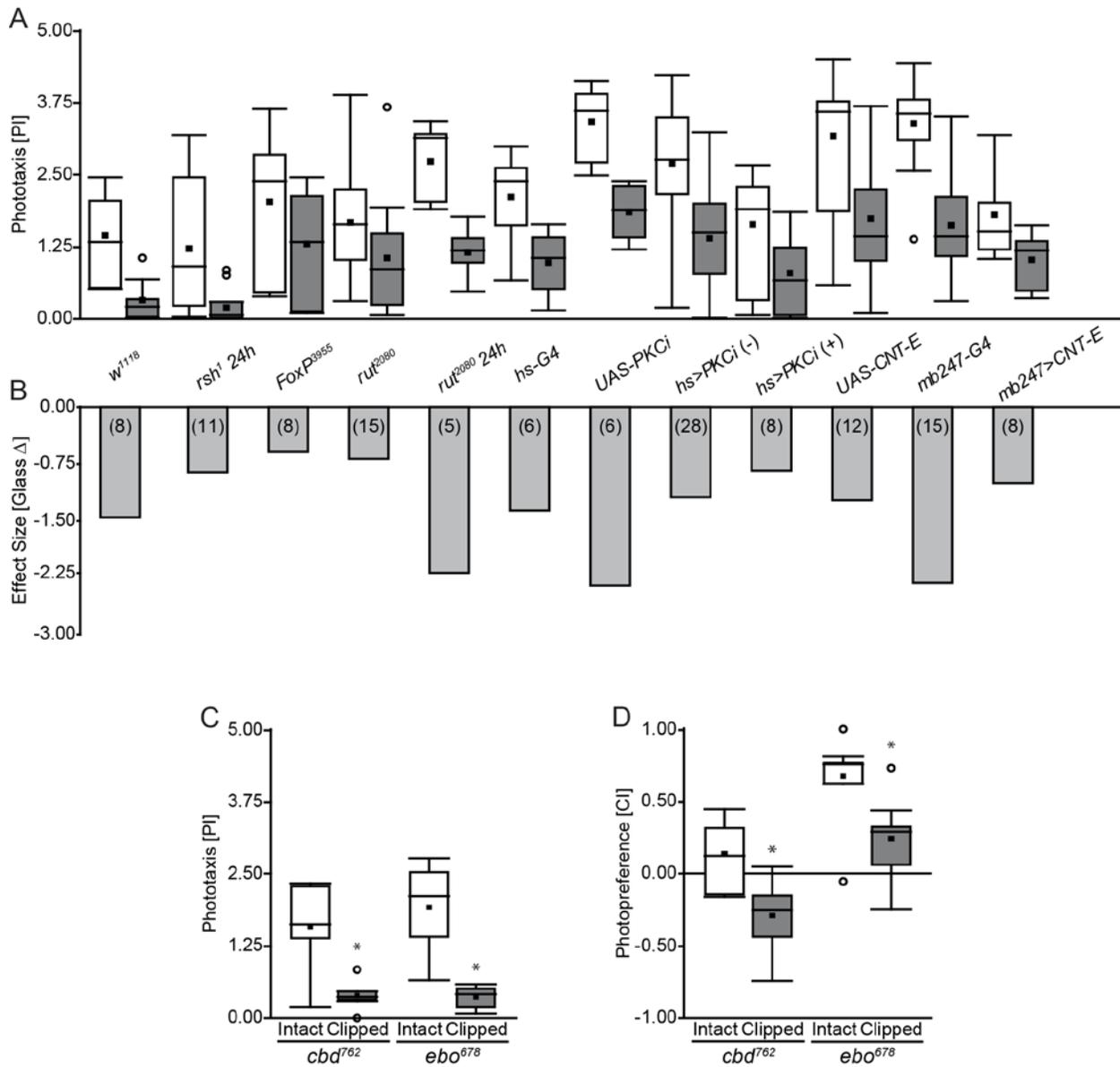
459 Randomized Block Design ANOVA, N=5, Block $p=0.152$, Interaction Genotype vs Temperature: $p<0.001$,
460 simple effects with Tukey's *post hoc* test ($p<0.05$): least-significant difference=0.349, Room Temperature:
461 $p=0.073$, 37°C: $p<0.001$, Room Temperature 24h post heat: $p=0.344$. * indicates significant differences,
462 n.s. means not significant. See figure 1 for detailed graph information.

463

464 **Wing-clipping effect is not dependent on known learning and memory processes**

465 The reversibility of the shift in photopreference is reminiscent of a learning process where the
466 animal may evaluate its flight capabilities at one point and then remember this outcome until the
467 next evaluation. For instance, the animals may attempt flight and immediately learn about the
468 futility of their attempt. Until the next attempt, the flies remember this state and shift their
469 photopreference accordingly. To test this hypothesis, we screened a selection of
470 mutant/transgenic fly lines with a variety of known learning and memory impairments using
471 BCP. We selected lines known to affect classical olfactory conditioning/operant world-learning,
472 operant self-learning, or any Mushroom Body-dependent learning processes. In order to avoid
473 differences related to specific locomotor characteristics from the different lines, here again the
474 wing-clipping effect was assessed with the Effect Size. None of the lines tested showed any
475 wing-clipping effect at all. All lines showed a clear behavioral change after wing-clipping,
476 evidenced by a decrease in their PI with an *Effect Size* around -0.6 or more, irrespective of the
477 baseline value (Fig. 6A, B).

478



479

480 **Figure 6. The wing-clipping effect is independent from known learning/memory processes or**

481 **neuropil areas associated with learning. A, Performance Index of mutants and transgenic flies with**

482 **learning and memory impairments, before and after clipping their wings. B, Effect Size of wing clipping on**

483 **BCP for several lines with learning and memory impairments and their controls. N=Numbers in brackets.**

484 **C, D, Behavioral performance from two structural Central Complex mutants with intact and clipped wings**

485 **on BCP (c) and T-Maze (d). Paired T-Test. c, *cbd⁷⁶²*, N= 6, p=0.005; *ebo⁶⁷⁸*, N= 6, p=0.004. d, *cbd⁷⁶²*, N=**

486 **8, p=0.002, *ebo⁶⁷⁸*, N= 7, p<0.001. See figure 1 for detailed graph information.**

487

488 **The behavioral switch is not central complex-dependent**

489 The central complex is a higher-order neuropil related to locomotion [54,55], visual information
490 processing [56], orientation [57], visual pattern recognition [58,59] and spatial working memory
491 [60]. As many of these functions may be important for either phototaxis or its flexibility, we
492 tested two structural mutants of this neuropil, Central Body Defect (*cbd*⁷⁶²) and Ellipsoid Body
493 Open (*ebo*⁶⁷⁸). However, wing-clipped *cbd*⁷⁶² as well as *ebo*⁶⁷⁸ flies both showed a clear
494 significant change in their photopreference measured either in BCP or T-Maze (Fig. 6C,D). We
495 note that, although *ebo*⁶⁷⁸ wingless flies still showed a preference for the bright tube in the T-
496 Maze, their PI was significantly decreased in comparison with intact *ebo*⁶⁷⁸ flies. While more
497 sophisticated manipulations of central complex function are clearly warranted, we tentatively
498 conclude that if the central complex plays a role in this process, it is likely not a crucial one, or
499 one that does not require an anatomically intact central complex.

500

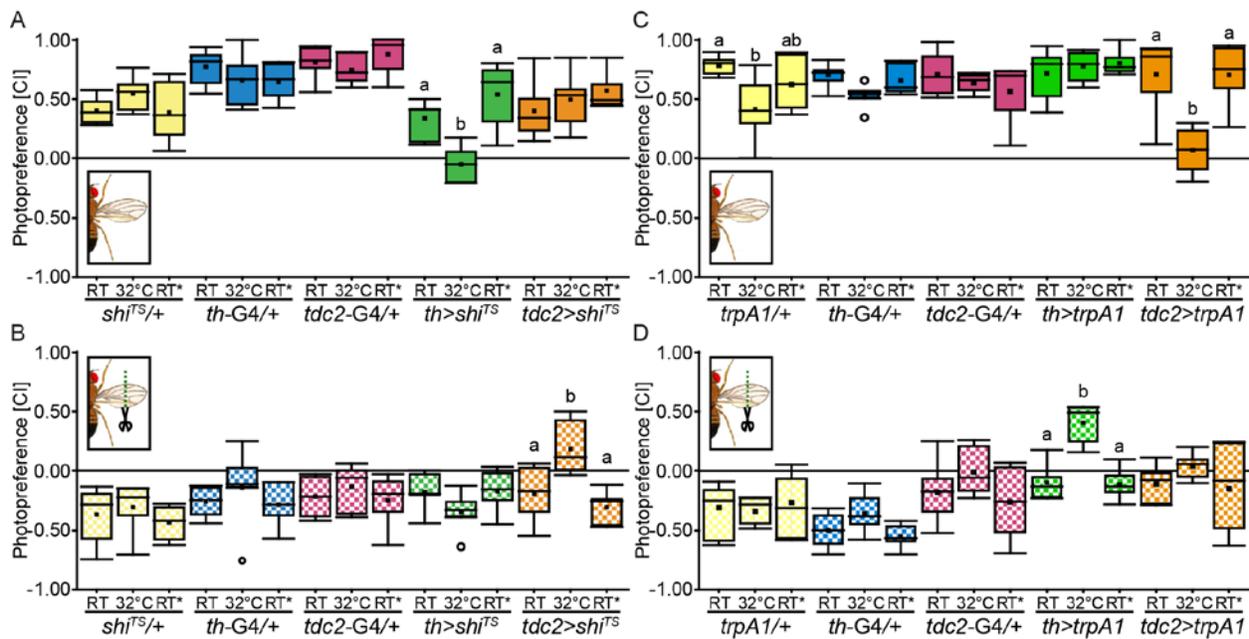
501 **DA and OA differently modulate intact and wingless fly behavior**

502 In the absence of any evidence that any of the known learning processes or neuropils known to
503 be relevant for learning or other aspects of orientation/choice behaviors are crucial for the shift
504 in photopreference, we explored the hypothesis that any unknown learning mechanism as well
505 as an unknown constant monitoring of flying ability may rely on a re-valuation of sensory input
506 after wing manipulation. That is, whether or not any memory is involved, the consequence of
507 being rendered flightless may be identical: a re-valuation of sensory input, such that previously
508 attractive stimuli become more aversive and previously aversive stimuli become more attractive.
509 Biogenic amines have long been known for their role in mediating the processing and
510 assignment of value [4,9,11–13,15,21,61–67]. If indeed it is the photopreference that is shifted
511 when a fly's flying ability is altered, it is straightforward to hypothesize that the two biogenic
512 amines most known for being involved in valuation in *Drosophila*, octopamine (OA) and
513 dopamine (DA), may be involved in this instance of value-based decision-making as well.

514 Moreover, mutant flies that lack tyrosine hydroxylase (*th*) only in the nervous system, i.e.
515 neuronal specific DA-deficients, show reduced phototaxis in BCP [66] further motivating the
516 manipulation of this amine pathway. Finally, flies without OA show a pronounced impairment in
517 flight performance and maintenance [68], making OA an interesting candidate for testing
518 photopreference as well.

519 To evaluate the involvement of DA and OA neurons for photopreference, we acutely disrupted
520 synaptic output from two separate groups of neurons by expressing the temperature-sensitive
521 form of dynamin (*Shibire*; *shi^{TS}*, [69]) either under control of the *th*-GAL4 driver (driving in
522 dopaminergic neurons) or under control of the *tdc2*-GAL4 driver (driving in octopaminergic, as
523 well as tyraminerbic, neurons). We tested the resulting transgenic flies with and without wings in
524 BCP and T-Maze. Although BCP and T-Maze results tended to agree, we only obtained clear
525 results in our T-Maze experiments. The reason for the less clear results in the BCP was a
526 genotype-independent and long-lasting effect of the temperature switch on the flies' PI in the
527 BCP. Hence, we show the results from the T-Maze experiments here and the BCP results are
528 available for download with the rest of the raw data. In the T-Maze at permissive room
529 temperature, when dynamin is in its wild type conformation, in all tested groups, flies with intact
530 wings showed positive CIs, while wing-clipped flies showed negative CIs (Fig. 7A,B). In
531 contrast, when the same experiment was performed at the restrictive 32°C (i.e., blocking
532 synaptic activity), we found opposite effects in flies with dopaminergic, and
533 octopaminergic/tyraminerbic neurons blocked, respectively. While disrupting synaptic output
534 from dopaminergic neurons appeared to have little if any effect on clipped animals, flies with
535 intact wings shifted their preference to the dark tube (Fig. 7A), rendering their CI
536 indistinguishable from that of their wingless siblings with which they were tested (Fig. 7B).
537 Conversely, blocking synaptic output from octopaminergic neurons only affected wingless flies,
538 which now preferred the bright arm of the maze (Fig. 7B), similar to their siblings capable of
539 flight with which they were tested (Fig. 7A). Replicating the reversibility described above, after a

540 24h recovery phase, flies tested at room temperature showed wild type behavior, meaning
 541 positive photopreference for intact flies and negative photopreference for wing-clipped flies (Fig.
 542 7A,B). The conventional interpretation of these results is that synaptic transmission from
 543 octopaminergic/tyraminergetic (OA/TA) neurons is necessary for shifting the photopreference
 544 towards darkness in flightless flies, while synaptic transmission from DA neurons is necessary
 545 for setting the preference of intact flies towards the bright arm.



546
 547 **Figure 7. Dopamine and Octopamine are necessary and sufficient to modulate phototactic**
 548 **behavior, but with opposite effects. A, B,** Photopreference from flies with (A) and without (B) wings
 549 before, during and after DA or OA/TA neuron silencing. **A,** Randomized Block Design ANOVA, Block
 550 $p=0.026$, Interaction Genotype vs Temperature $p<0.001$, simple effects with Tukey's *post hoc* test
 551 ($p<0.05$, least-significant difference=0.24, $tdc2>shi^{ts}$ least-significant difference= 0.263): $shi^{ts}/+$ $p=0.208$,
 552 $th-GAL4/+$ $p=0.417$, $tdc2-GAL4/+$ $p=0.428$, $th>shi^{ts}$ $p<0.001$, $tdc2>shi^{ts}$ $p=0.242$. $N=6$ except for $tdc2>shi^{ts}$
 553 RT^* ($N=5$). **B,** Randomized Block Design ANOVA, Block $p=0.006$, Interaction Genotype vs Temperature
 554 $p=0.02$, simple effects with Tukey's *post hoc* test ($p<0.05$, least-significant difference=0.278, $tdc2>shi^{ts}$
 555 least-significant difference= 0.288): $shi^{ts}/+$ $p=0.533$, $th-GAL4/+$ $p=0.394$, $tdc2-GAL4/+$ $p=0.6$, $th>shi^{ts}$
 556 $p=0.262$, $tdc2>shi^{ts}$ $p<0.001$. $N=6$ except for $tdc2>shi^{ts}$ RT^* ($N=5$). **C, D,** Photopreference from flies with
 557 (C) and without (D) wings before, during and after DA or OA neuron activation. **C,** Kruskal-Wallis for

558 temperature factor comparison within genotypes (alpha after correction=0.013): *trpA1/+* $p=0.012$, *th-*
559 *GAL4/+* $p=0.069$, *tdc2-GAL4/+* $p=0.667$, *th>trpA1* $p=0.97$, *tdc2>trpA1* $p=0.004$. *th-GAL4/+* and *th>trpA1*,
560 $N=6$; *trpA1/+*, *tdc2-GAL4/+* *tdc2>trpA1*, $N=7$. **D**, Kruskal-Wallis for temperature factor comparison within
561 genotypes (alpha after correction=0.013): *trpA1/+* $p=0.834$, *th-GAL4/+* $p=0.15$, *tdc2-GAL4/+* $p=0.126$,
562 *th>trpA1* $p=0.005$, *tdc2>trpA1* $p=0.415$. *th-GAL4/+* and *th>trpA1*, $N=6$; *trpA1/+*, *tdc2-GAL4/+* *tdc2>trpA1*,
563 $N=7$. Different letters indicate significant differences between temperatures for each genotype (only
564 shown for genotypes where the factor temperature had a statistically significant effect). See figure 2 for
565 detailed graph information.

566

567 We also transiently activated O/TA and DA neurons, respectively, using the temperature
568 sensitive *TrpA1* channel [49], while testing the flies for their photopreference. Again, at room
569 temperature, when the channel is closed, flies with and without wings behaved similar to wild
570 type animals (Fig. 7C,D). However, when tested in the same experiment at 32°C, where the
571 *TrpA1* channel is open and depolarizes the neurons in which it is expressed, the flies showed a
572 change in their behavior. Flies with clipped wings and activated DA neurons now preferred the
573 bright arm of the maze, with no effect on intact flies (Fig. 7D). Conversely, activating O/TA
574 neurons only had an effect on flies with intact wings, abolishing their previous preference for the
575 bright arm of the maze (Fig. 7C), rendering them indistinguishable from their wingless siblings
576 with which they were tested, but which did not show any significant effect (Fig. 7D). Again,
577 when tested back at room temperature 24h later, wild type behavior was restored. The
578 conventional interpretation of these results is that active O/TA neurons are sufficient for
579 shifting photopreference towards the dark arm of the maze, while the activation of DA neurons
580 is sufficient to set the flies' preference towards brightness.

581

582

583

584 Discussion

585 McEwen's discovery captured our attention because of its implications for the supposed rigidity
586 of simple behaviors. We first reproduced the findings of McEwen [33] and Benzer [32] that wing
587 manipulation leads to a decrease in *Drosophila* phototaxis (Fig. 2). Slightly altering the
588 conditions of the BCP and comparing performance between two additional experiments, we
589 found that the decrease in phototaxis is not due to hypoactivity of wing-manipulated flies, but to
590 a more general change in the flies' assessment of their environment (Fig. 3). We discovered
591 evidence that the BCP is just one of several experiments that can measure a fly's general
592 photopreference. Manipulating the wings modulated this preference in all of the selected
593 experiments such that compromised wing utility yielded a decreased preference for brightness
594 (bright stimuli) and an increased preference for darkness (dark stimuli) across the experiments
595 chosen (Fig. 3). However, of these experiments, only the BCP can be argued to test phototaxis
596 proper. In Buridan's Paradigm the flies walk between two unreachable black stripes; and in the
597 T-Maze, the flies choose between a dark tube and a bright one where the light is coming from
598 an angle perpendicular to their trajectory. Neither of the two paradigms is testing taxis to or
599 away from a light source. Interestingly, in our pilot experiments, we have tested phototaxis in
600 different variations of the T-maze with various LEDs placed at the end of one of two opaque
601 tubes and only found a reduction of phototaxis and never negative phototaxis (unpublished
602 observation). In fact, in these pilot experiments we have observed every possible difference
603 between flying and manipulated flies. In the end, we chose the experimental design that yielded
604 positive and negative scores, respectively, in WTB flies purely for practical reasons. Other wild
605 type strains, such as some Canton S substrains, do not show a negative photopreference in the
606 T-Maze after wing clipping (Fig. 3H). Taken together, these lines of evidence strongly suggest
607 that photopreference in *Drosophila* is a strain-specific continuum where experimental design
608 assigns more or less arbitrary values along the spectrum. In some special cases, this

609 photopreference manifests itself as phototaxis. If that were the case, phototaxis would constitute
610 an example of a class of experiments not entailing a class of behaviors.

611 This insight entails that manipulations of different aspects of flight ought to affect this continuum
612 in different ways. Complete loss of flight ought to have more severe effects than manipulations
613 affecting merely individual aspects of flight behavior, such as wing beat amplitude/frequency
614 (i.e., lift/thrust), torque, flight initiation, flight maintenance, proprioception or motion/wind-speed
615 sensation. We have found some evidence to support this expectation. For instance, clipping
616 only the tips of the wings does not eliminate flight, but affects torque as well as lift/thrust [41,70].
617 Flies with the tips of their wings cut behaved indifferently in the T-Maze and do not avoid the
618 bright tube (Fig. 4F). Flies without antennae are reluctant to fly and have lost their main sense of
619 air speed detection [46–48], but they are still able to fly. Also these flies do not become light
620 averse in the T-Maze after the manipulation, but indifferent. Only clipping the wings in these flies
621 abolishes their flight capabilities completely and yields negative scores (Fig. 4I). Flies with
622 removed gyroscopic halteres, on the other hand, are severely affected in their detection of
623 rotations and usually do not fly [42–44], despite being able to still beat their wings and control
624 flight direction using vision alone in stationary flight [42,43]. These flies avoid the bright arm of
625 the T-Maze. Finally, injuries to flight-unrelated parts of the fly's body did not affect
626 photopreference (Fig. 4K, L), ruling out the preference of darkness being a direct escape
627 response due to bodily harm. Further research is required to establish a quantitative link
628 between the many different aspects of flight behavior and their relation to photopreference.

629 Taken together, our experiments so far demonstrate that 1) the physical state of the wings with
630 regard to their shape, form or degree of intactness influences photopreference (Figs. 2-4). 2)
631 The capability to not just move the wings, but specifically to move them in a way that would
632 support flight (Figs. 2, 3, 5) also influences the flies' photopreference. 3) The state of sensory
633 organs related to flight such as antennae or halteres also exerts such an influence, while non-
634 flight-related sensory deprivation shows no such consequences (Fig. 4). This multitude of flight-

635 related aspects extends the concept of flying ability beyond mere wing utility: manipulating
636 seemingly any aspect of the entire sensorimotor complex of flight will affect photopreference,
637 and do so reversibly (Fig. 5). As it appears that any aspect of flight, sensory or motor, is acutely
638 linked to photopreference, it is straightforward to subsume all of these aspects under the term
639 'flying ability', emphasizing that flying ability encompasses several more factors in addition to
640 wing utility. The observation that each fly, when it is freshly eclosed from the pupal case and the
641 wings are not yet expanded, goes through a phase of reduced phototaxis that extends beyond
642 wing expansion until the stage when its wings render it capable of flying [71], lends immediate
643 ethological value to a neuronal mechanism linking flying ability with photopreference.

644 One possibility how the link between flying ability and photopreference may be established
645 mechanistically is via a process reminiscent of learning: at one time point, the flies register a
646 sensory or motor deficit in their flight system and at a later time point, they use this experience
647 when making a decision that does not involve flying. Once flying ability is restored, the same
648 choice situation is solved with a different decision again in the absence of flight behavior. How
649 the flies accomplish this learning task, if indeed learning is involved, is yet unknown, but we
650 tentatively conclude that it is unlikely that any of the known learning pathways or areas involved
651 in different forms of learning play more than a contributing role (Fig. 6). While the molecular
652 learning mechanism remains unidentified, the process appears to be (near) instantaneous (Figs.
653 2, 3). Even though we cannot rule out that an unknown learning mechanism exists which is
654 unaccounted for in our screen, we conclude that at least none of the known learning
655 mechanisms suffices to explain the complete effect size of the shift in photopreference. These
656 results corroborate the findings above, that the switch is instantaneous and does not require
657 thorough training or learning from repeated attempts to fly, let alone flight bouts. They do not
658 rule out smaller contributions due to these known learning processes or an unknown, fast,
659 episodic-like learning process. It is also possible, that the flies constantly monitor their flying
660 ability and hence do not have to remember their flight status. Despite these ambiguities, we

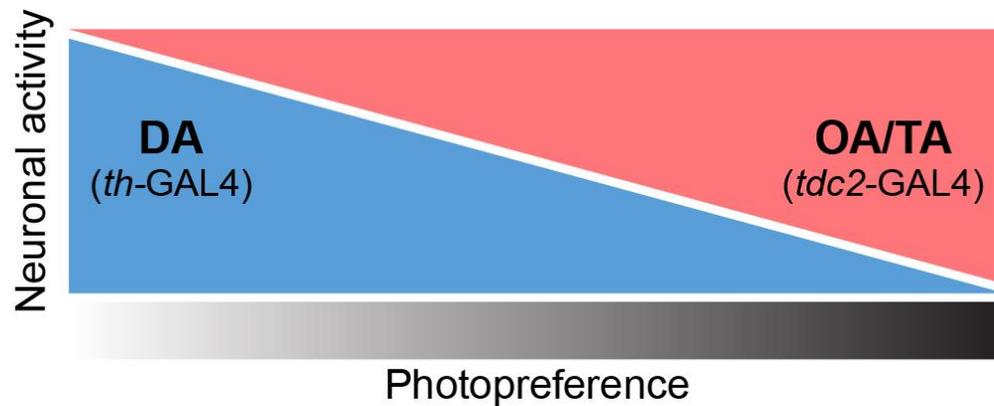
661 have been able to elucidate some of the underlying neurobiological mechanisms. Much as in
662 other forms of insect learning and valuation [72–76], neurons expressing the biogenic amine
663 neuromodulators OA and DA appear to have opposite functions in the modulation of
664 photopreference (Fig. 7).

665 Although both DA and OA play some role in different aspects of flight behavior [68,77–79],
666 these cannot explain our results. In general, our biogenic amine neuron manipulated flies
667 escape their vial via flight if granted the opportunity. Thus, flight is not abolished in any of our
668 transgenic lines affecting OA, TA or DA neurons. However, there may be more subtle deficits in
669 less readily perceived aspects of flight. Experiments performed with mutant flies lacking OA
670 demonstrated that OA is necessary for initiation and maintenance of flight [68]. However, in our
671 paradigm, silencing OA/TA neurons promoted approaching light, the opposite effect of what
672 would be expected for a flightless fly (Fig. 7 B). Activating these OA/TA neurons, however,
673 rendered the flies indifferent in the T-Maze. OA/TA appear to be involved in flight initiation and
674 maintenance via opponent processes [68]. Transient activation of OA/TA neurons may lead to a
675 subtle alteration of flight performance and reduce photopreference in these flies. Similarly, it has
676 been shown that altering the development of specific DA neurons results in flight deficits
677 (reduction of flight time or loss of flight, depending on the treatment [78,79]). Our manipulations
678 lasted for approximately 30 min during adulthood, ruling out such developmental defects. Work
679 in the laboratory of Gaiti Hasan has also found that silencing of three identified TH-positive
680 interneurons for several days in the adult animal compromises flight to some extent (wing
681 coordination defects during flight initiation and cessation [77]). Our much shorter manipulation
682 does not lead to any readily observable flight defect. However, one needs not discuss whether
683 or not our aminergic manipulations may have had subtle effects on some aspects of flight
684 behavior, as we can compare these flies to the wing-clipped siblings with which they were
685 tested simultaneously (i.e., the flies with the maximum shift in photopreference due to
686 completely abolished flight). Comparing the intact DA-inactivated flies and OA/TA-activated flies

687 (Fig. 7 A,C) with their respective wingless siblings (Fig. 7 B,D) reveals that the CIs of the pairs
688 of groups become essentially indistinguishable at the restrictive temperature. In other words,
689 intact flies where DA neurons have been inactivated or O/TA neurons have been activated
690 behave as if their wings had been clipped and their flight capabilities abolished completely,
691 despite them being capable of at least some aspects of flight. Hence, even if there were some
692 contribution of some aspect of flight behavior being subtly affected by manipulating these
693 aminergic neurons, there is a contribution of activity in these neurons that goes beyond these
694 hypothetical flight deficits. Therefore, we conclude that neither the O/TA, nor the DA effects
695 can be explained only by subtle defects in one or the other aspect of flight behavior in the
696 manipulated flies.

697 The precise neurobiological consequences of manipulating O/TA and DA neurons,
698 respectively, are less certain, however. Not only are the two driver lines (*th*-GAL4 and *tdc2*-
699 GAL4) only imperfectly mimicking the expression patterns of the genes from which they were
700 derived. Our effectors, moreover, only manipulated the activity of the labeled neurons. One
701 manipulation (*shi*^{TS}) prevents vesicle recycling and likely affects different vesicle pools
702 differentially, depending on their respective release probabilities and recycling rates. The other
703 effector (*TrpA1*) depolarizes neurons. It is commonly not known if the labelled neurons may not
704 be co-releasing several different transmitters and/or modulators in the case of supra-threshold
705 depolarization. Hence, without further research, we can only state the involvement of the
706 labelled neurons, which as populations are likely to be distinct mainly by containing either DA or
707 O/TA, respectively. If it is indeed the release of these biogenic amines or rather the (co-
708)release of yet unknown factors in these neuronal populations remains to be discovered. Further
709 research will also elucidate the exact relationship between the activities of these two neuronal
710 populations and whether/how it shifts after manipulations of flying ability (Fig. 8).

711



712

713 **Figure 8. Schematic illustration of the potential dependence of photopreference on the activity of**
714 **aminergic neurons.**

715 Depending on several factors (e.g., the status of its flight apparatus), individual flies may fall anywhere on
716 the photopreference spectrum (grayscale): approaching light, avoiding it or behaving indifferently.
717 Increasing neuronal activity in *tdc2*-GAL4 positive neurons (red) or decreasing neuronal activity in *th*-
718 GAL4 positive neurons (blue), each alone promoted a preference of darkness (shift to the right of the
719 spectrum) in flies able to fly, which normally prefer brightness over darkness. In contrast, increasing
720 neuronal activity in *th*-GAL4 neurons (blue) or decreasing neuronal activity in *tdc2*-GAL4 neurons (red),
721 each alone promoted preference of brightness (shift to the left of the spectrum) in wing-clipped flies,
722 which normally tend to avoid brightness. It is straightforward to hypothesize that the quantitative
723 relationship between two opponent processes (potentially based on OATA and DA action) constitutes
724 one mechanism mediating photopreference in *Drosophila*. In this figure, we depicted this relationship as
725 linear for illustrational purposes only.

726

727 In conclusion, our findings provide further evidence that even innate preferences, such as those
728 expressed in classic phototaxis experiments, are not completely hard-wired, but depend on the
729 animal's state and presumably other factors, much like in the more complex behaviors
730 previously studied [21–26]. This endows the animal with the possibility to decide, for example,
731 when it is better to move towards the light or hide in the shadows. Moreover, the fact that flies
732 adapt their photopreference in accordance with their flying ability raises the tantalizing possibility

733 that flies may have the cognitive tools required to evaluate the capability to perform an action
734 and to let that evaluation impact other actions - an observation reminiscent of meta-cognition.

735

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743

744 **Author's contribution**

745 EAG, JC and BB designed the experiments, EAG and JC performed the experiments, EAG and
746 BB wrote the manuscript.

747

748 **References**

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