

# High innate attractiveness to black targets in the blue blowfly, *Calliphora vomitoria* (L.) (Diptera: Calliphoridae)

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## ABSTRACT

*Calliphora vomitoria* is a myiasis-causing fly in many animal species including humans. The control of blowflies is still anchored on the use of chemicals. However, mass trapping and lure-and-kill techniques represent a promising alternative to pesticides. Visual and olfactory cues are the main stimuli routing the fly's landing behavior. Notably, color attractiveness has been barely explored in flies of medical and veterinary importance, with special reference to blowflies. In this study, we investigated the innate color preferences in *C. vomitoria* adults, testing binary combinations of painted targets under laboratory conditions. The identity of tested species *C. vomitoria* was confirmed by DNA sequencing (18S and *cox1* genes). *C. vomitoria* flies showed a significant preference for black colored targets in all tested binary color combinations, after 5, 15, 30 and 60 min of exposure. Black targets were significantly preferred over blue, red, yellow and white ones. Spectral characteristics of all tested color combinations were quantified and the innate attraction of blowflies towards black targets was discussed in relation to their behavioral ecology. To the best of our knowledge, this is the first report on innate color preferences in the *Calliphora* genus. Our findings can be useful to develop new, cheap and reliable monitoring traps as well as "lure and kill" tools to control blowfly pests.

## 1. Introduction

*Calliphora vomitoria* L. (Diptera: Calliphoridae) is a pest fly characterized by the capacity of its larvae to cause myiasis (Stevens, 2003). The *Calliphora* genus includes primarily carrion feeders (Aak et al., 2011), although several species can be classified as secondary or tertiary agents of myiasis, as in the case of *C. vomitoria* (Zumpt, 1965). Indeed, since the first description of *C. vomitoria* as an agent of myiasis (Hope, 1840), several cases have been reported both in animals and in humans (Edwards et al., 1984; Erzinlioglu, 1987; Ferrar, 1987; Rawlins, 1988; Zeltser and Lustmann, 1988; Gursel et al., 2002), mostly in the case of low hygienic conditions or of compromised host's health (Wood and Slight, 1970; Gursel et al., 2002). Fly-borne myiasis also occurs frequently in animals, particularly in sheep and goats (Sotiraki and Hall, 2012) and *C. vomitoria* may arouse ovine cutaneous myiasis, also called sheep strike, worldwide (Hall, 1997; Morris and Titchener, 1997). In addition, *C. vomitoria* may transmit *Clostridium botulinum* type C toxin in birds (Hubálek and Halouzka, 1991) or *Xanthomonas*

*campestris* pv. *campestris*, the etiological agent of crucifer black rot (Van der Wolf and Van der Zouwen, 2010).

Historically, the control of Calliphoridae has been based on the use of chemicals, such as organophosphates and pyrethroids (Hall and Wall, 1995), with insect growth regulators (IGRs) showing to be a good remedy (Graf, 1993). Ivermectin at a maximum dose of three µg has been commonly used for topical application for curing wounds caused by *C. vomitoria* larvae (Strong, 1989). However, the massive use of pesticides can lead to resistance problems and detrimental effects on non-target species and alternatives to chemical insecticides are urgently advocated (Aak, 2010). Therefore, novel insecticides have been developed. In particular, selected herbal preparations as well as macrocyclic lactones have been proposed to control myiasis-producing flies (Khater and Khater, 2009; Waliwitiya et al., 2010; Khater et al., 2011, 2013, 2018; Banumathi et al., 2018).

Mass trapping and lure-and-kill techniques have a relevant potential in blowfly control strategies (Hall and Wall, 1995), although they maximize their efficiency only if sheep congregate and are not

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appropriate over large land areas (Tellam and Bowles, 1997). Host location by blowfly females represent a critical phase in the life cycle of these species causing myiasis. Visual and olfactory cues are the main stimuli routing the fly's landing behavior by playing a role in boosting the performance of trapping systems (Hall, 1995). However, color attractiveness has been barely explored in flies of medical and veterinary importance (Bracken et al., 1962; Brady and Shereni, 1988; Wall and Fisher, 2001). Yellow sticky traps have been used for monitoring and control *Calliphora vicina* Robineau-Desvoidy in stock fish production (Aak et al., 2011), and *Lucilia cuprina* Wiedemann used brightness to discriminate color stimuli (Fukushi, 1989). Notably, limited knowledge on these topics has been exploited to develop effective and reliable control tools under field conditions.

In this study, we investigated, the first time in the *Calliphora* genus, the innate color preferences in *C. vomitoria* adults, testing binary combinations of painted targets. The identity of tested species, *C. vomitoria*, was confirmed by DNA sequencing (18S and *cox1* genes). Lastly, the spectral characteristics of all tested color combinations were quantified.

## 2. Materials and methods

### 2.1. Species identification, rearing and general observations

Third instar larvae of *C. vomitoria* were purchased from a commercial supplier (Fish Company Arco Sport, Pisa, Italy). They were placed in glass pots (diameter 150 mm, length 200 mm) to pupate. The pots were singly laid in cylindrical PVC cages (diameter 350 mm, length 600 mm), and aeration in the cages was allowed by a transparent chiffon fabric (mesh size 0.05 mm). The adults were transferred in cages, each containing 70 flies, with a sex ratio of 1:1, and maintained in laboratory [24 ± 1 °C, 45 ± 5% R.H., 16:8 (L:D) photoperiod]. The adults were fed with a dry mixture of yeast extract and sucrose (1:10, w:w), water was provided separately on a cotton wick (Romano et al., 2015).

For molecular identification, DNA was extracted from the flight muscles of *C. vomitoria* adult flies using the QIAamp<sup>®</sup>DNA Micro Kit (Qiagen, Milan, Italy). PCR amplifications were performed following Benelli et al. (2015). For the small subunit (SSU) rRNA nuclear gene (18S gene) amplification, we used the universal primers and the cycling conditions reported by Benelli et al. (2015), while the partial cytochrome oxidase I (*cox1*) mitochondrial gene was amplified using primers C1-J-1718 (5'-GGAGGATTTGAAAATTGATTAGTTC-3') and TL2-N-3014 (5'-TCCAATGCACTAATCTGCCATATA-3') (Simon et al., 1994) and the cycling conditions reported by Harvey et al. (2008).

The 18S gene was sequenced using the internal primers R536 (5'-CTGGAATTACCGCCGGCT-3'), R1052 (5'-AACCTTAAGGAACCCCGCCATGGCA-3') and F783 (5'-GACGAAATCAAAGAAATACCGTC-3') (Fokin et al., 2008). The *cox1* gene was sequenced using the external primers C1-J-1718 and TL2-N-3014, and the internal primers C1-J-2183 (5'-CAACATTTATTTGATTTTTTGG-3') and C1-N-2329 (5'-ACTGTA AATATATGATGAGCTCA-3') (Simon et al., 1994). A comparative analysis of the sequences obtained to those homologous recorded in GenBank/EMBL databases was carried out using a BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).






All experiments were conducted in a room illuminated with fluorescent daylight tubes [16:8 (L:D), lights on at 6:00]. The light intensity close to the testing cage was about 1000 lx (300–1100 nm waveband, LI-1800 spectroradiometer LI-COR Inc., Lincoln, NE, USA), with a remote cosine receptor. Temperature was 24 ± 1 °C, and R.H. was 45 ± 5%. Tested flies were 6–12 days old. Only insects with intact legs and wings were tested.

### 2.2. Color spectral measurements

The innate preference of *C. vomitoria* adults for colors was

Table 1

Spectral measurements of the tested colors using standard CIELab color space coordinates determined with the spectrometer Ocean Optic HR2000-UV-NIR (Ocean Optics, USA).

Color	L*	a*	b*	RGB-transformed CIELab color patch
Yellow	91.5	2.9	81.6	
Red	46.4	61.8	25.6	
Blue	46.3	-38.8	-31.8	
White	98.8	-0.7	0.6	
Black	9.5	-1.2	-2.5	

Parameters used to quantify the color characteristics of the cardboard models:  $L^*$  represent the lightness component and  $a^*$  (from green to red) and  $b^*$  (from blue to yellow) are the two chromatic components.

investigated using a two-choice assay. We tested binary combinations of colors for their ability to elicit innate attractiveness in blowflies. Cardboard models with different colors were used. Tested colors included black, white, yellow, blue and red. Standard acrylic pigments were used to prepare the cardboards (Polycolor<sup>®</sup>, Maimeri, Italy).

Parameters used to quantify the color characteristics of the targets were  $L^*$ ,  $a^*$  and  $b^*$ .  $L^*$  represents the lightness component and  $a^*$  (from green to red) and  $b^*$  (from blue to yellow) are the two chromatic components. Measurements were recorded using standard CIELab, color space coordinates determined using a spectrometer Ocean Optic HR2000-UV-VIS-NIR (Ocean Optics, USA). The color spectral features are detailed in Table 1.

### 2.3. Innate color attractiveness

The testing arena had four sections (A–D) (Fig. 1), each of which having an equal volume. At the end of the A and D sections the colored cardboards were presented. Following our preliminary observations, where blowflies were attracted by black targets we tested the following combinations of colors: (i) black vs. white, (ii) black vs. yellow, (iii) black vs. red, and (iv) black vs. blue. The B and C sections were considered as neutral areas. Food and the water sources were not provided in the testing arena. For each binary combinations of colors tested, flies were observed for 60 min.

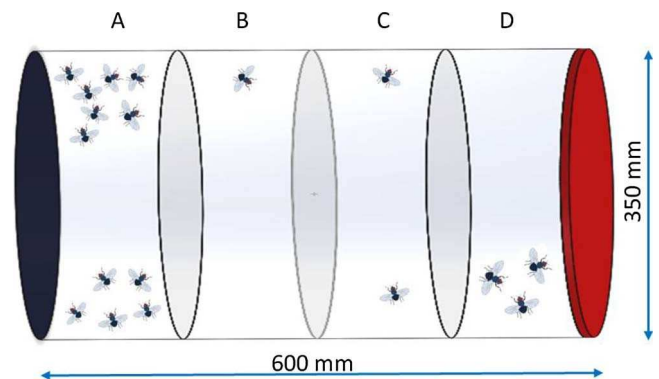


Fig. 1. Design of the testing arena where innate color preferences were tested. The arena was virtually divided in A, B, C and D sections, containing the blue blowflies, *Calliphora vomitoria*. Each section had an equal volume. The colored cardboards were positioned at the end of the A and D sections. B and C sections were considered as neutral areas (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

During the observations, we recorded the number of the blowflies that showed a preference for a particular section of the arena over different time intervals (i.e., 5, 15, 30 and 60 min from the beginning of the experiment). The final number of flies landed on each target was noted after 60 min. Flies not showing any color preference (e.g., flies stationing in the B or C sections) were not considered by statistical analysis. After each replicate, the arena was rotated clockwise 90° to avoid orientation bias. Twenty-four replicates were performed for each binary combinations of colors, in each replicate, 70 flies (sex ratio 1:1) were tested.

#### 2.4. Statistical analysis

Data on innate color preferences in *C. vomitoria* flies were transformed into arcsine√proportion, and analyzed with a mixed linear model (JMP 9® SAS) with two fixed factors (i.e., tested colors and time interval) and one random factor (i.e., replicate):

$$y_{ijw} = \mu + C_i + T_j + C_i T_j + R_{ij} + e_{ijw}$$

ijw

i

-

j

j

w

ijw

### 3. Results

#### 3.1. Innate color attractiveness

The spectral characteristics of the tested color targets are provided in Table 1. *Calliphora vomitoria* flies showed a significant preference for black colored targets in all tested binary color combinations, after 5, 15, 30 and 60 min of exposure (Fig. 2). Black targets were significantly preferred over blue ( $F_{1,22} = 757.64$ ;  $P < 0.001$ ), red ( $F_{1,22} = 135.51$ ;  $P < 0.001$ ), yellow ( $F_{1,22} = 219.01$ ;  $P < 0.001$ ) and white ones ( $F_{1,22} = 525.75$ ;  $P < 0.001$ ) (Fig. 2). Notably, no significant differences in color attractiveness over the tested time intervals were found for all tested binary color combinations ( $P > 0.05$ ), showing a stable higher attractiveness of the black targets over time (Fig. 2). The interaction color\*time interval ( $C*T_i$ ) was not significant in all tested binary color combinations ( $P > 0.05$ ).

#### 3.2. Molecular identification

The *cox1* gene nucleotide sequence displayed a 100% sequence identity with those of *C. vomitoria* (accession number: KF919009) recorded in the GenBank/EMBL databases. Furthermore, the 18S gene sequence displayed a 99% sequence identity with those of *C. nigribarbis* (accession number: AB466039), which is, in turn, the only *Calliphora* species of which is available the homologous sequence in the GenBank/EMBL databases.

### 4. Discussion

*Calliphora vomitoria* flies tested in our experiments showed a significant preference for black targets, while colored cardboards attracted only a limited number of flies. Although color traps have been used to control other blowflies (Aak et al., 2011), innate preferences for colors have never been investigated in the *Calliphora* genus, despite the importance of these flies as livestock pests and vectors. However, in agreement with our findings, data concerning several dipteran pests, including the tabanid *Tabanus illotus* Osten Sacken, showed the attractiveness of black color (Bracken et al., 1962). *Glossina morsitans morsitans* Wiedemann, commonly known as tsetse fly, is attracted to black (Brady and Shereni, 1988; Steverding and Troscianko, 2004) and blue cues (Green, 1986, 1988; Green and Flint, 1986), as well *Lucilia sericata* (Meigen) females, when selecting final landing site (Wall and Fisher, 2001).

In insect eyes, the variations in the number of color receptor types, their differential expression across the retina, as well as their tuning concerning the wavelength scale, can be related to the visual ecology of

a given species (Briscoe and Chittka, 2001), thus strong differences in the insect lifestyle can shape their color preferences. As a general trend, the newly emerged flower-visiting insects show innate preferences for flower-related colors prior to individual experience with flowers (Lunau and Maier, 1995). Based on the frequency of visits to differently colored flowers under natural conditions, it has been reported that bumblebees (*Bombus terrestris* L.) exhibit preferences for blue (Müller, 1881). In addition, bumblebees exhibit innate preferences for selected colors even after intense learning, when choosing among different new colors (Gumbert, 2000). More generally, many other examples of innate and learned color preferences are available in the insects' world. For instance, naive *Manduca sexta* L. moths exhibit innate preference for blue and after training experience they can switch color preferences (Goyret et al., 2008). Food-deprived parasitoids (*Cotesia rubecula* Marshall) showed a preference for yellow targets when foraging for food (Wäckers, 1994), while *Psytalia concolor* (Szépligeti) females showed no innate color preferences, but they can develop clear preferences for selected colors (i.e., red, cyan and yellow), after repeated oviposition experiences on *Ceratitis capitata* (Wiedemann) larvae associated with a given color (Benelli and Canale, 2012). Lastly, *Frankliniella occidentalis* Pergande thrips exhibited a significant preference for white traps during an experiment in lettuce farms (Yudin et al., 1987).

Concerning flies of medical and veterinary importance, despite several promising findings – including the present study – about the attractiveness of black colored targets, it should be noted that practical applications in the “real world” are sadly lacking (see also Aak et al., 2011), since most of the currently marketed traps for fly monitoring and mass-killing are still based on the employ of relatively expensive chemicals (Urech et al., 2009; Aak, 2010; Aak et al., 2010). In this framework, our findings concerning the innate color preferences for black of blowflies can be useful to develop new, cheap and reliable monitoring traps, as well as “lure and kill” tools to control these flies of economic importance. Indeed, the employ of pheromones and other VOCs useful for manipulating the chemical ecology of *C. vomitoria* is not necessary in these traps. In other words, black-colored sticky traps can be proposed as a not expensive strategy – combined to adjuvants treatments and good IPM practices – aiming at the reduction of blowfly young instars populations. Moreover, in areas highly infested by blowflies, it is advisable to avoid the employ of black colors for clothes and animal husbandry. Further trials in the field to test the efficacy of these traps in livestock farming areas are ongoing.

#### Conflict of interests

The authors declare no competing interests.

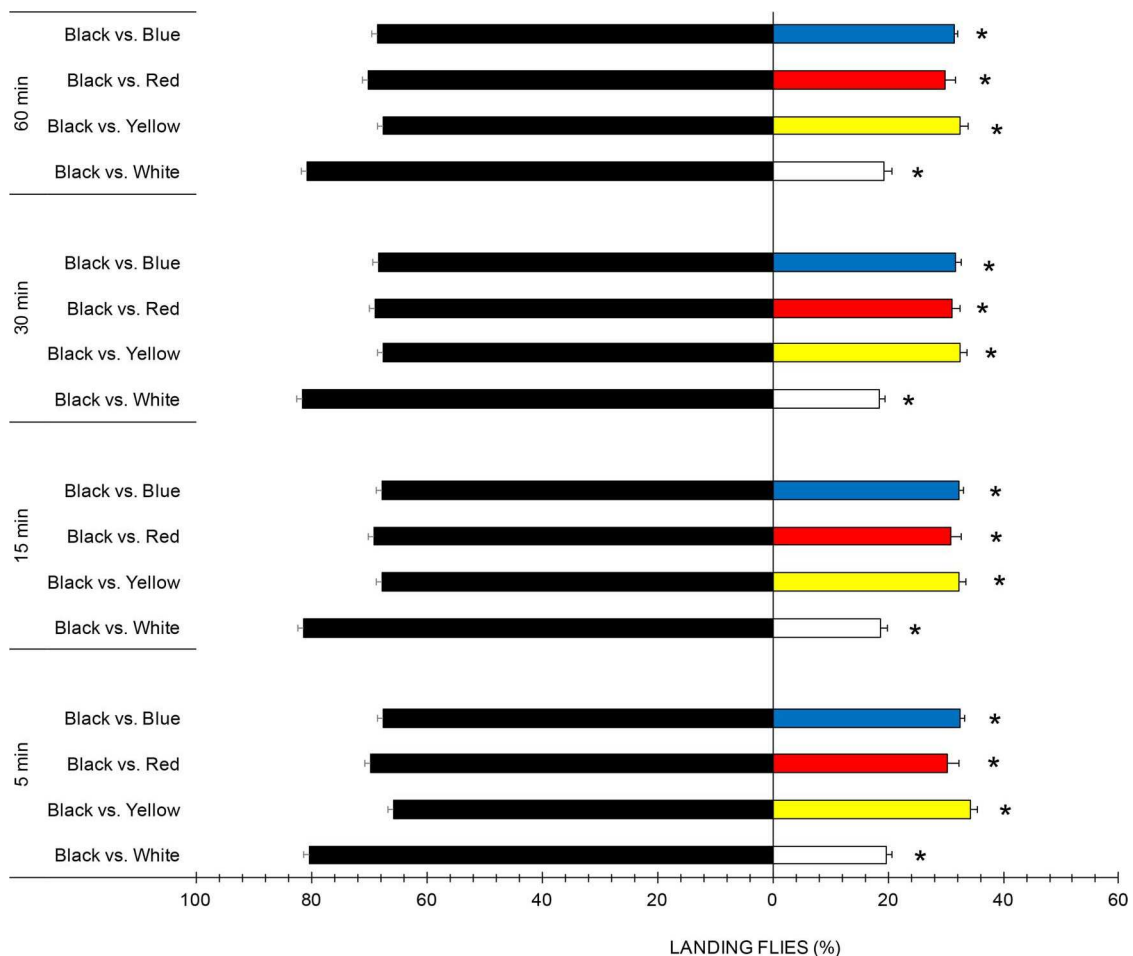


Fig. 2. Innate color preferences in adults of the blue blowfly *Calliphora vomitoria* after 5, 15, 30 and 60 min of exposure to colored targets. Each column indicates the percentage of flies landing on each colored target. T-bars represent standard errors. Asterisks highlight significant differences among means (general linear model,  $P < 0.05$ ) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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