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ORIGINAL ARTICLE

Innate floral object identification in a solitary pollinator employs a combination of both visual and olfactory cues

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Abstract

Object identification is fundamental to animal behaviour and identifying nutritive objects is key for survival. Solitary insects emerging far from food sources and without access to social learning must therefore employ innate identification of food cues to locate relevant nutritive objects from a distance. Such innate preferences for food cues should be both specific enough to allow discrimination between food and non-food objects and general enough to allow for the variety of food objects relevant to the insect species. Here, we examined innate floral object identification behaviour in solitary generalist insect pollinator *Eristalinus aeneus* using an artificial floral object with both visual and olfactory cues previously found to be attractive to several hoverfly species across multiple environments. We used a subtractive two choice assay to present flower-naïve *E. aeneus* with a choice between this object and a similar object that differed in either a single visual or olfactory cue. We found that innate floral choices of the hoverfly *E. aeneus* are a product of broad, plant-based olfactory cues and visual cues, where a combination of radial symmetry and reflectance in the 300–400 nm and 500–700 nm wavelength range was particularly important for innate floral object preference. Our study, therefore, shows how solitary animals without prior experience can efficiently employ multimodal cues to identify multiple relevant nutritive objects.

Keywords Floral signature · Olfaction · Innate object identification · Behavioural ecology · Neuroethology

Introduction

Identifying objects is a fundamental aspect of animal behaviour. All organisms must be able to distinguish objects that provide nutritional value from those that do not. Identifying food sources in the complex natural world can be particularly challenging and many organisms can learn to identify the features of objects individually or through social interactions over their lifetime. However, in some cases, the innate ability to identify certain objects is crucial for survival. Several vertebrates are able to innately identify cues associated with predators (Hawkins et al. 2004) or kin (Rosa-Salva et al. 2018). Insects like *Anopheles gambiae* also have an innate olfactory bias towards their prey (Dekker et al. 2001); generalist insect predators like *Orius* spp. are innately attracted to herbivore-induced volatiles released by host plants damaged by their herbivore prey (Ardanuy et al. 2016); Dipterans like blowflies are innately attracted to black targets (Benelli et al. 2018), and some Arachnids like the East African jumping spider *Evarcha culicivora* are also innately attracted to their prey (Dolev and Nelson 2014).

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Most insects are solitary, and therefore, the majority of insect species must identify initial food objects without any social learning or larval experience with food as found in social insects (Farina et al. 2005; Arenas et al. 2009). Essentially, solitary insects can employ three sensory strategies to discriminate between food and non-food objects innately. First, the insect can accept a large set of objects as food with little discrimination. Second, the insect can employ a minimal set of cues common to all nutritive objects. Third, the insect can use a specific combination of sensory cues signifying a particular object or set of objects.

Attraction to many objects with little discrimination could be opportunistic and computationally simple, but metabolically and energetically challenging, as it requires exploring several objects and a potentially low-quality diet. Exploration of low-quality resources can also result in the accumulation of metabolites that could be detrimental to fitness (Olazcuaga et al. 2023). Being attracted to a single or small and specific set of sensory cues, such as rare volatiles, has been observed in many specialist insects such as bees (Milet-Pinheiro et al. 2013), flies (Cha et al. 2011), aphids (Chapman et al. 1981), and weevils (Bartlet et al. 1997). However, a narrow preference could be inefficient when those particular resources are scarce, necessitating a generalist diet that can utilize a variety of resources as food. For generalists, a third strategy that employs a combination of broad ranges of cues from different sensory modalities to discriminate food from non-food objects could be more viable. Such a strategy can be seen in the case of attraction in mosquitoes towards multimodal integration of carbon dioxide, heat, and odour cues as a proxy for a variety of warm-blooded species (Mc Meniman et al. 2014).

Generalist solitary insects face a two-fold challenge. First, they must be able to discriminate nutritive objects from non-nutritive objects in the clutter of the natural world. Second, these insects should ideally be able to recognise multiple food sources as potential resources. As a result, the innate preferences of generalist solitary insects must be flexible to accommodate varied resources yet maintain high degree of accuracy in object identification. Thus, as a first principle, one would hypothesize that innate food identification in solitary generalist insects would be guided via attraction to a small set of cues indicative of several relevant food objects. In this study, we test this hypothesis using a solitary insect generalist pollinator, the hoverfly *Eristalinus aeneus* (Scopoli, 1763), and examine the set of floral cues significant for innately identifying floral objects.

Long-distance floral choice in generalist insect pollinators is mediated by several factors including olfactory cues (Andersson and Dobson 2003; Primante and Dötterl 2010) and visual cues such as hue (An et al. 2018; Giurfa et. al 1995; Trunschke et al. 2021), saturation (Lunau 1990; Koethe et al. 2018; Trunschke et al. 2021), brightness, which is intensity or amplitude of reflectance spectra (Free 1970; Trunschke et al. 2021), and shape (Free 1970; Howard et al. 2019). Some generalist pollinators like honeybees have been reported to use both hue and achromatic contrast (brightness) for flower choice depending on the angle subtended by the object, with achromatic parameters used for small angles and chromatic contrast used for targets subtending large visual angles (Giurfa et al. 1995). Within hoverflies, studies report hoverflies like *Episyrphus balteatus* can use olfactory cues alone for identifying flowers (Primante and Dötterl 2010), while other hoverflies, like *Eristalis tenax*, are known to exhibit true colour vision (Ilse 1949) and exhibit innate preference for yellow hues (An et al. 2018; Lunau and Watch 1997; Lunau 2014).

Pollinators are also known to utilize combinatorial signatures for foraging (Riffell and Alarcon 2013; Howard et al. 2019; Balamurali et al. 2020; Matoušková et al. 2023). Further, cross-modal interactions can alter the valence of a stimulus. For example, colour modulates proboscis extension reflexes (PER) to learnt odours in bees (Mota et al. 2011), and ovipositional odours decrease preference to specific colours in female pierid butterflies (Balamurali et al. 2020). These findings indicate the need to study the multimodal floral object using experimental stimuli with both olfactory and visual cues to understand the innate floral search preferences of pollinators from an ecologically relevant perspective. While we have some understanding of the innate preferences of pollinators to specific floral cues, the behavioural responses of solitary generalist insects to combinations of floral cues have been relatively understudied. Further, most research on floral search preferences has focused on social hymenopterans, such as bees, where it is difficult to differentiate innate attraction from socially learned cues acquired within the hive (Farina et al. 2005; Arenas et al. 2009). Hoverflies are important solitary non-bee pollinators that visit at least 72% of global food crops (Doyle et al. 2020). They exhibit colour vision (Ilse 1949), with a preference for yellow hue in human colour vision in some hoverfly species, like *E. balteatus* (Sutherland et al. 1999) and *E. tenax* (An et al. 2018). However, hoverflies also visit flowers with other colours and use multimodal cues for foraging in the wild (Lunau 2014; Nordstrom et al. 2017). Further, we know that colour processing in *E. tenax* involves four photoreceptors with sensitivity peaks at 330 nm, 340 nm, 460 nm and 540 nm (Horridge et al. 1975). Because the sensory physiology (Lunau 1990, 2014; An et al. 2018), ecological niches (Rotheray and Gilbert 2015; Nordstrom et al. 2017), attractive floral signatures (Nordstrom et al. 2017) and sensitivity peaks of the photoreceptors involved in colour processing (Horridge et al. 1975) are known for hoverflies, they make excellent model organisms to investigate the innate cues used to identify objects in generalist insects. Further, the solitary nature of hoverflies (Rotheray and Gilbert 2015)

removes confounding effects of social learning from the hive (Farina et al. 2005) and sensitivity (Arena et al. 2009) or floral biases (Farina et al. 2005) arising therein.

In this study, we assessed the floral cues that significantly impact the innate floral object identification behaviour in flower-naïve *E. aeneus*. As stimuli, we employed a multimodal floral lure composed of visual and olfactory cues derived from several flowering species attractive to hoverflies across continents in the field, henceforth referred to as v + o + (Nordström et al. 2017). Because Nordström et al. (2017) found that hoverflies of multiple species and in multiple ecosystems were attracted to v + o +, it suggests that these floral cues could be innately attractive. We first tested for this innate attractiveness using flower-naïve individuals and then engaged a subtractive approach to determine which visual and olfactory cues were important for innate floral object identification in hoverflies. In this approach, hoverflies (*E. aeneus*) were given a choice between the v + o + model and a similar model where one visual or odour feature of v + o + was altered. Evaluating the innate preferences of hoverflies helps us understand how this important pollinator identifies flowers in the natural world, with implications for our understanding how solitary animals without prior experience can efficiently employ multimodal cues to identify multiple relevant objects in our natural world.

Materials and methods

Study design

The floral characteristics tested in the current study were first derived from a cross-continental field study using wild hoverfly species (Nordstrom et al. 2017). This previous study found that models with a specific combination of visual and olfactory cues (henceforth referred to as the positive control, or v + o +) were ubiquitously attractive to hoverflies across multiple ecosystems without any nutritive rewards (Nordstrom et al. 2017). The visual and olfactory cues of the positive control (v + o +) had been previously standardized as a radially symmetric, petaloid, non-nutritive object measuring 36 ± 0.1 mm in diameter, with a blend of five floral volatiles, and this model appears yellow hued in human colour vision (Nordstrom et al. 2017). This preference across environments implies that v + o + could be innately attractive to flower-naïve hoverflies. To test this hypothesis, the current study conducted two choice assays with freely flying, flower-naïve *E. aeneus* to examine (1) if the v + o + model was innately attractive and (2), if so, which visual or odour characteristics are important for innate attraction. In the previous field study, the negative control was a dark circular disk with no odour, and it received significantly fewer visits (Nordstrom et al. 2017). Thus, in our study we chose a similar negative control (v − o −) of equivalent diameter to the positive control, without any odour. To control for confounding environmental factors, all experiments were performed in lab conditions in a black cage with a black background, black mesh sides, and checker board top (Supplementary Fig. S1). Preliminary tests indicated that an arena with this configuration increased the response rates of tested hoverflies compared to green or white arenas of the same size.

In the previous field study by Nordström et al. (2017), wild, free-ranging hoverflies were observed to land sparingly, with the majority of responses being directed flights to within 15 cm of the model, after which the hoverflies flew away. In our study we similarly defined a "response" as either landing from above or directed flights within 5 cm towards the model in our arena which was a cube of side measuring 50 cm (see "Two-choice assays"). We used a smaller distance of 5 cm in our study because the hoverflies introduced in our arena encountered restricted distances as compared to the original field assays. To determine which visual and olfactory cues were important for pollinator attraction, we used a reductive approach. In this approach, unless otherwise stated, the hoverflies (*E. aeneus*) were given a choice between models where one feature of the visual (v +) or olfactory (o +) cues was altered, while all other features were kept constant. This experimental design allowed us to isolate the specific cues that were most influential in attracting the hoverflies, compared to the full model (v + o +) and the negative control (v − o −).

Artificial floral models

Visual cues of the artificial floral models

The artificial floral models were printed using WoL 3D printer silk white PLA filament (WOL3D, India) and Raised N2 3D printer (Raise3D, USA), ensuring that they were identical.

The floral models were painted with Camlin acrylic paints (KOKUYO CO., LTD.; Japan). A white base coat was provided to all floral models, followed by specific ratios of paints mixed to achieve the needed reflectance spectrum (Table S1). All visual models, and their reflectance spectrums are shown in Fig. 1 (see Table S1 for full details). The surface area of all models except negative control is the same, the longest diagonal of Hexagon + (~ 32 ± 0.1 mm) is smaller than the diameter of v +, while the major chord of Asymmetric + (~ 40 ± 0.1 mm) is longer than diameter of v +. The four petals of asymmetric model are one identical ellipse of minor chord of 6.8 mm, and major chord 40 mm rotated at 0°, 45°, 200°, and 285° making it asymmetric.

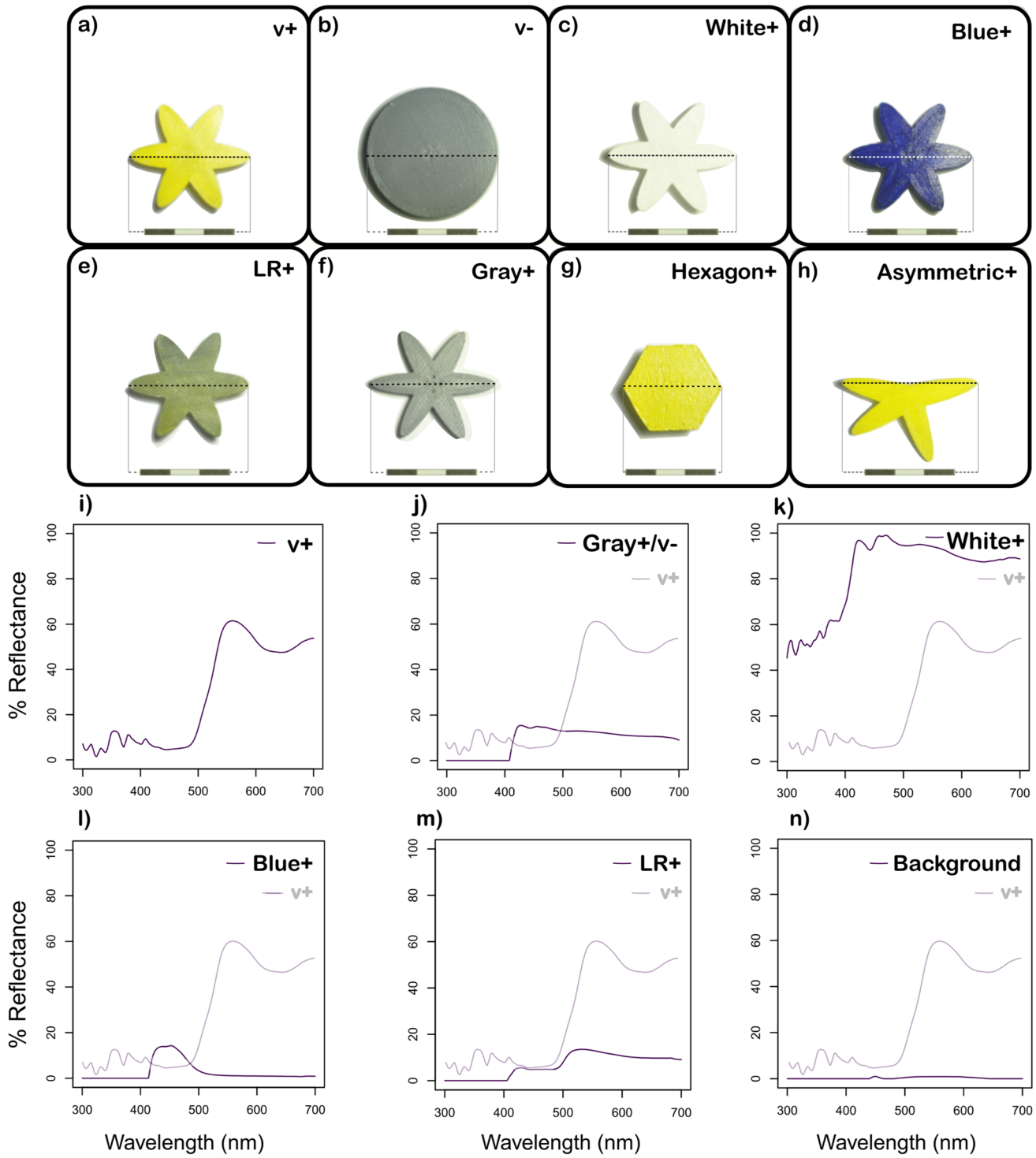

Fig. 1 Different visual models including different colour morphs used in the study and their respective reflectance spectra: **a, i** positive visual control (v+); **b, j** negative visual control (v−); **c, k** White+; **d, l** Blue+; **e, m** low reflectance model (LR+); **f, j** Gray+; **g, a** undisrupted model (Hexagon+); **h, a** asymmetric model (Asymmetric+); **n** spectra of the background of the experimental arena. Scale bar: 3 cm

Hoverflies, known to detect moving objects that subtend an angle smaller than 1° (Nordström and O'Carroll 2006), could be able to perceive this asymmetry.

The reflectance of all models was measured using a portable spectrophotometer (Ocean optics Jaz-200 spectrophotometer with the optic fibre tip of 25-cm, 600-µm

Premium Fiber, UV/Visible, FL, USA), after calibrating the spectrophotometer to a white balance (Labsphere certified reflectance standard USRS-99–010 AS-01158–060 S/N 99AA08-0615–3676) under the 5000 Lux LED lights used in the experimental setup (ORFLD-30W, Chromatic temperature 6500 K close to CIE standard illuminant D65 with a chromatic temperature of 6504 K). Output was the spectral power distribution curve of light reflected off an object (in percent reflectance) normalised to the white balance. For each visual stimuli used in the behavioural assays, we use empirically measured colourimetric variables to compare between the stimuli used in an assay. The colourimetric variables of the stimuli were obtained by analysing the reflectance spectra of the stimuli using *summary.rspec* function in *pavo* package (Maia et al. 2013) in the R programming environment and have been mentioned in Table 1.

For the scope of the study, we define the term "Colour" as the specific spectral reflectance of a stimuli. Thus, the term "colour morphs" indicates stimuli with different reflectance spectra. Further, all mentions to the term "total brightness" are for the colourimetric variable B1 obtained using the *summary.rspec* function from *pavo* package in R programming environment. B1 is called "total brightness" in the *pavo* documentation, and here, this terminology denotes the total area under the spectral reflectance curve. In other words, "total brightness" is the sum of the relative reflectance over the entire spectral range (Montgomerie 2006; Ornborg et al. 2002).

Odour cues of the artificial floral models

Models with o + in their name have the same odour blend of five floral volatiles derived from cues attractive in field conditions across multiple continents (2-Ethyltoluene, p-Cymene, Undecanal, r-Limonene, 6-Methyl-5-hepten-2-one [Fluka], in mineral oil; Nordstrom et al. 2017). Blends were provided by pipetting 2 µl of the blend on a grade 2 Whatman filter paper (1 cm × 5 cm) affixed to the artificial floral models. Refer to Table S2 for full details.

Emission rates for o + were calibrated empirically against measured release rates of component volatiles from real flowers in the field (Nordstrom et al. 2017; Table S3). The volatile emission from the models was assessed using a 100-µm PDMS solid-phase microextraction (SPME) fibre (model no: 548575-U, SUPELCO, USA). The fibre was placed within 10 cm of the models with volatile blends in a foil covered, cleaned and heated 500 ml beaker for 30 s. The fibre was injected into the inlet of an Agilent 7890B gas chromatograph in splitless mode, to desorb volatiles at 270 °C. The volatiles were then separated using an HP-5 MS column (30 m × 0.25 mm, i.e. 0.25 µm film thickness) coupled with a 5977A MSD mass spectrometer. The column oven was kept at 40 °C for 1 min, increased to 150 °C at 25 °C/min and then to 270 °C at 100 °C/min and held for

Table 1 Colourimetric variables of the stimuli used in the study obtained using *summary.rspec* function in *pavo* package (Maia et al. 2013)

Stimuli	B1[a]	B2[a]	B3[a]	S1U[a] (300–400 nm)	S1B[a] (400–510 nm)	S1G[a] (510–605 nm)	S1R[a] (605–700 nm)
Gray + /v −	3549.712	8.85	15.42	0	**0.3891**	0.3340	0.2837
White +	33,113.85	82.58	99.03	0.1693	**0.3132**	0.2692	0.2559
LR +	2654.877	6.62	13.51	0	0.2104	**0.4445**	0.3534
v +	11,246.51	28.05	61.48	0.0651	0.0739	**0.4433**	0.4249
Blue +	1120.568	2.79	14.26	0	**0.8187**	0.1079	0.0763
Background	116.4692	0.29	0.97	0	0.2012	0.6955	0.1145

The parameters (column headings) have been explained in footnotes. Bold values in S1U, S1B, S1G, and S1R are the part of the spectrum with highest contribution to B1 (total brightness)

[a]B1—Total Brightness: sum of the relative reflectance over the entire spectral range (area under the curve) (Montgomerie 2006; Ornborg et al. 2002)

[a]B2—Mean brightness: mean relative reflectance over the entire spectral range (Delhey et al. 2003)

[a]B3—Intensity: reflectance at wavelength of maximum reflectance (Montgomerie 2006)

[a]S1U—Relative contribution of spectral range (300–400 nm) to the total brightness (B1) (Ornborg et al. 2002)

[a]S1B—Relative contribution of spectral range (400–510 nm) to the total brightness (B1) (Ornborg et al. 2002)

[a]S1G—Relative contribution of spectral range (510–605 nm) to the total brightness (B1) (Ornborg et al. 2002)

[a]S1R—Relative contribution of spectral range (605–700 nm) to the total brightness (B1) (Ornborg et al. 2002)

5 min. Compounds were identified by mass spectroscopy using Masshunter Qualitative Analysis software (B.07.00) and the mass spectra library NIST (National Institute of Standards and Technology and libraries created from authentic standards). The models were sampled at 5-min intervals to check for contamination and determine release rates as a function of time. This enabled us to ensure similar release rates, retention times and absence of any contaminants during the behavioural assays (Fig. S2, Table S3).

Insect maintenance

The *E. aeneus* used in the study were obtained with required permissions from three sources: Kalimpong, West Bengal, India, from late May to early June 2018, Polyfly Almeria, Spain, from August 2018 to January 2020, and the rest of the hoverflies were reared in the lab according to protocols established for *E. tenax* (Nicholas et al. 2018).

For lab rearing, third instar larvae were collected from manure pits and incubated to pupation in fresh cow dung in plastic boxes (Nicholas et al. 2018). Nearing pupation, the larvae were provided dry sawdust around the boxes and the larvae crawled into the sawdust for pupation. The sawdust was then sifted, and pupae were hand collected and transferred into petri plates with autoclaved vermiculite (Nicholas et al. 2018). The hatched adults were then transferred into aphid-proof nylon mesh cages of side 30 cm (width × height × length: 30 cm × 30 cm × 30 cm). The adult *E. aeneus* were supplied with distilled water, 10% sucrose solution, and ground mustard pollen mixed with powdered sugar ad libitum without exposure to flowers or plants to keep them flower-naïve. The volatiles in the headspace of ground mustard pollen were analysed using thermal desorption on PDMS (Polydimethylsiloxane), to ensure that none of the volatiles used in the current study could be detected in the pollen headspace chromatogram (Fig. S2). All *E. aeneus* were sex-sorted by day 4 unless the experiment required mated *E. aeneus* and were housed in batches of 30 in chambers with temperature of 22 °C, humidity of 60% and 12-h light and 12-h dark cycle. Only flower-naïve hoverflies between the ages of 3 and 14 days were used for experiments, and they were starved for a period of 4–6 h prior to experimentation. The experiments were conducted in the day between 11 am and 6 pm.

Two choice assays by freely-flying insects

Insects were reared as stated above. All tests were performed in a black Nylon mesh cage of side 50 cm (width × height × length: 50 cm × 50 cm × 50 cm) with a solid black base as background for the models, and a checker board roof under two 100 W flicker-free white LED flood lights. The illumination of the experimental arena was 5000 ± 50 Lux (mean ± s.e.) as measured using a lux meter (LX-101A HTC instruments). Two models were provided in the centre of the arena—20 cm apart from each other, in the middle of the cage, 25 cm away from the front and the back of the cage. The model's stalks were 20 cm high. Directly on top of the floral models, an exhaust duct was installed to ensure minimal contamination of the odour from the other model. The flies were captured individually using 50-ml Falcon tubes (Tarsons, catalog number: 500041), in which they were carried to the experimental arena (1 fly per tube), within 5 min. Each fly was placed on the floor of the experimental arena at the mid-point of the line joining the base of the stalks of the two models, on the cap of the falcon tube in which it was transported to the arena (Fig. S1). The set-up and a behavioural assay are shown in Online resource 2 and Fig. S1.

All observations were conducted in real time, with the observers always sitting across from the transparent front panel of the experimental arena. The observer's line of sight was at the same plane as the models. A response was defined as landings or approaching at least 5 cm above the models, as per other studies (Nordstrom et al. 2017; Matoušková et al. 2023; Chapman et al. 2023). The time period for each observation was capped at 5 min. Only the first response by a hoverfly was counted, with $n_{\text{responder}}$ being the total number of hoverflies responding as described above. The testing arena was controlled for side bias by periodically switching the floral models. In the side bias assay, the positive control (v + o +) was placed at both sides. To investigate whether cues attractive to hoverflies in the field (Nordstrom et al. 2017) were innately attractive to naïve *E. aeneus*, hoverflies were given a choice between positive control (v + o +, Fig. 1a) and negative control models (v − o −, Fig. 1b) with similar cues as tested in the field (Nordstrom et al. 2017). To evaluate if either odour or visual cues alone were sufficient for *E. aeneus* to be attracted to our floral model, naïve *E. aeneus* were given a choice between the positive control (v + o +) and a negative visual model with floral odour (gray disk; v − o +) or the positive visual model without floral odour (v + o −). In all assays parsing visual cues, all visual morphs were paired with the positive control odour o + as indicated in their labels. All models used in assays parsing the impact of achromatic visual features had the same surface area as the positive control, and all our models were presented horizontally. In assays parsing odour cues, we kept the associated visual cues across the morphs same as the positive control visual cues (v +). To evaluate the impact of floral odour identity, *E. aeneus* were given a choice between the positive control (v + o +) and a positive visual model v + paired with a different odour blend in two-choice assays. The other odour blends used were green leaf volatile (GLV—a blend of cis-3-hexenyl acetate in mineral oil), IA (a blend of isoamyl acetate in mineral oil), coffee furanone (CF—a blend of 2-methyltetrahydrofuran-3-one in mineral oil), and meta

Cresol (MC—a blend of meta Cresol in mineral oil). Note that all assays were conducted using concentrations similar to empirically measured release rates of odours from flowers in nature and as described in "Odour Cues of the artificial floral models" (Nordström et al. 2017) (Fig. S2, Table S3, see Methods for details). To understand if the intensity of floral odour impacted innate choices, *E. aeneus* were given a choice between the positive visual control (v+) and an odour blend that had been diluted to 1% of the original loading concentration calibrated to concentrations measured from real flowers (Nordström et al. 2017).

Electroantennography (EAG)

E. aeneus between the ages of 4 and 10 days were used for EAG and prepared as previously described (Batra et al. 2019). Data was acquired using EAG2000 software, and insects were exposed to air, solvent blank, and volatile concentrations ranging from 10^{-1} to 10^{-8} g/ml presented in 10-µl aliquots on a filter paper inserted into Pasteur pipettes as in Batra et al. (2019). Data were acquired using Syntech intelligent data acquisition controller (IDAC-2, SYNTECH, Netherlands), and the stimuli was presented as pulses of 0.5 s for 10 s of recording using a custom-built odour delivery system, standardized as previously described (Batra et al. 2019). The electrophysiological response of flies was calculated as voltage deflections in millivolts (mV) as follows:

$$\text{Normalised deflections} = \frac{\text{voltage deflection for treatment (mV)}}{\text{voltage deflection for solvent blank (mV)}}$$

Statistical analysis

For each assay, a series of Binomial GLMMs (Generalized Linear Mixed Models) were performed to test for differences in responses to the two models. More details of the GLMMs used are given in Supplementary Material S1.

Phi coefficient (ϕ) was used to estimate the effect size of the difference, calculated as follows:

$$\phi = \sqrt[2]{\frac{\chi^2}{n}}$$

χ^2 is the chi square value. Phi-coefficient is a special case of Cramer's *V*, used to measure the effect size of associations among categorical variables, where a value of 0 signifies no effect, 0.1 a very small effect, and 0.5 a large effect (Thimmegowda et al. 2020). Preference index for a model in an assay was defined as the percentage of response received by a model in a two-choice assay, calculated as follows:

$$\text{Preference index (\%)} = \left[\frac{\text{response to model}}{n_{\text{responder}}}\right] * 100$$

We also conducted a power analysis to determine the number of flies needed in each assay to detect a statistically significant difference in the response of hoverflies to the two models if the difference is present. The "*biologically meaningful effect size*" (Johnson et al. 2014) that we were interested to detect was a difference between 70% of $n_{\text{responder}}$ flies visiting Model1 (and hence, 30% of $n_{\text{responder}}$ flies visiting Model2) as opposed to the null where both models are visited equally by the flies (50% to Model1 and 50% to Model2; see Supplementary Methods and supplementary material S2 for details).

Results

Two choice assays between visual and olfactory multimodal floral objects and unimodal floral objects

We found no significant side bias to either side of the arena ($n_{\text{responder}} = 105$; preference index to v+o+ on left = 48.54, $p = 0.768$, Table S4; Fig. 2a.1). The response of flower-naïve *E. aeneus* to the positive control v+o+ was significantly higher than that to the negative control ($n_{\text{responder}} = 96$, preference index to v+o+ = 73.95, $p = 7.18 \times 10^{-6}$, phi = 0.35, Table S4; Fig. 2a.2), indicating an innate preference for these cues.

We also found that the hoverflies responded to the positive control in significantly greater numbers over either unimodal model (v−o+: $n_{\text{responder}} = 90$, preference index to v+o+ = 73.33, $p = 2.2 \times 10^{-5}$, phi = 0.357, Table S4, Fig. 2a.3; v+o−, $n_{\text{responder}} = 108$, preference index to v+o+ = 70.37, $p = 4.05 \times 10^{-5}$, phi = 0.296, Table S4, Fig. 2a.4). This indicates that a combination of both odour and visual cues increases the innate attractiveness of the positive control v+o+.

Parsing visual cues

Two choice assays between floral objects of different colours

To assess if the colour of v+o+ had an impact on the innate floral object identification in *E. aeneus*, flower-naïve *E. aeneus* were given a choice between the positive control (v+o+) and Gray+o+ (Fig. 1f, j). These two models differ in several colourimetric parameters (Table 1) and have very different reflectance spectra (Fig. 1f, j). The hoverflies responded to the positive control v+o+ in significantly higher numbers than Gray+o+ ($n_{\text{responder}} = 56$, preference index for v+o+ = 73.21, $p = 0.000862$, phi = 0.3; Fig. 2b.5, Table S4). Hence, the colour of v+o+ had a significant impact on the innate object preference of flower-naïve hoverflies.

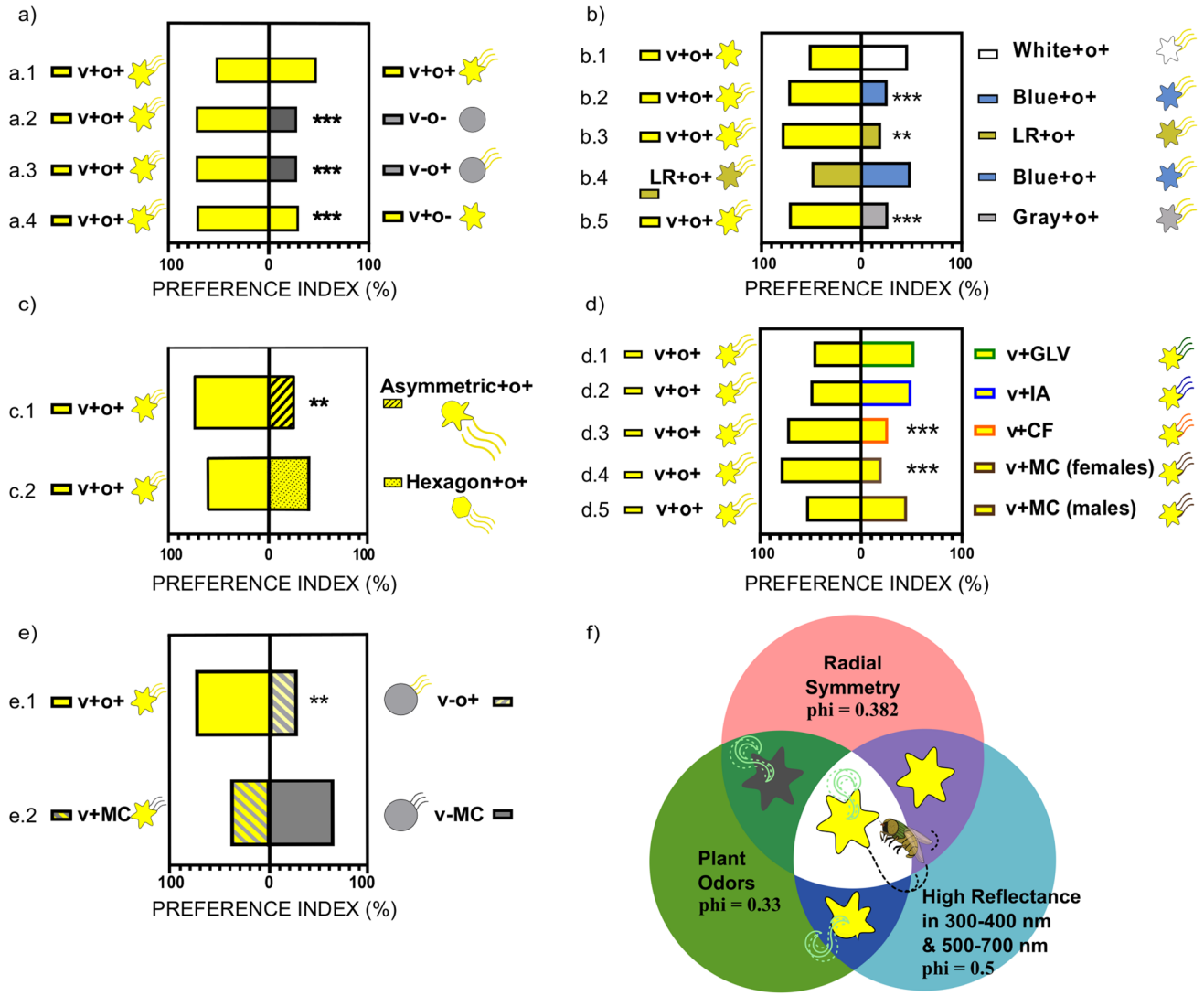

Fig. 2 Preference indices of naïve *E. aeneus* when given a choice between the positive control (v+o+) against various single cue perturbations as listed. Stars indicate significant differences based on Binomial GLMM (* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$; Table S4). **a** Assays for multimodality of positive control v+o+against (a.1) itself (side bias assay), (a.2) negative control, v−o−, (a.3) negative visual control with floral odour, v−o+, and (a.4) positive visual control with no floral odour, v+o−. **b** Assays for impact of colour of positive control v+o+against (b.1) White+o+, (b.2) Blue+o+, (b.3) low reflectance model LR+o+. (b.4) low reflectance model LR+o+against Blue+o+, and (b.5) positive control v+o+against Gray+o+. **c** Assays for impact of shape of positive control v+o+against (c.1) Asymmetric+o+and (c.2) undisrupted Hexagon+o+. **d** Assays for odour of positive control v+o+against (d.1) v+with green leaf volatile v+GLV, (d.2) v+with isoamyl acetate v+IA, (d.3) v+with coffee furanone v+CF, (d.4) v+with meta-cresol v+MC (female flies only), and (d.5) v+with meta-cresol v+MC (male flies only). **e** Female flies given a choice between (e.1) positive control v+o+and negative visual control with odour cues v−o+and (e.2) positive visual control with meta-cresol (v+MC) and negative visual control with meta-cresol (v-MC). Pictograms indicate visual cue presented and wavy lines indicate odour with different colours for different odour identities. **f** Set of floral cues with a significant impact (presented with their effect sizes) on innate floral object identification in flower-naïve hoverflies

Next, *E. aeneus* were given a choice between the positive control (v+o+) and White+o+(see Fig. 1c). The White+model has higher total brightness (B1: sum of relative reflectance over the entire spectral range; Table 1) and higher mean relative reflectance (B2 in Table 1) than v+. Further, White+reflects light across a broader range of wavelengths (Fig. 1k), with higher reflectance than v+across the entire range of measured wavelength spectrum from 300–700 nm (Fig. 1f) and relatively similar contribution of different spectral ranges (S1U, S1B, S1G, S1R in Table 1), as compared to v+. Hoverflies responded to both models equally ($n_{responder}=55$; preference index to v+o+ =52.73, $p=0.686$; Fig. 2b.1, Table S4). Thus, increasing the intensity of spectral reflectance across the entire

300–700 nm range did not have a significant impact on the response of flower-naïve hoverflies.

E. aeneus were then given a choice between the positive control v + o + and Blue + o + (Fig. 1d, Online resource 2). The Blue + o + model (Fig. 1l) has a peak at 440 nm, no reflectance in the 300–400 nm range (S1UV = 0, Table 1), and the highest relative contribution to total brightness in the 400–500 nm range (S1B = 0.8187, Table 1). The v + model exhibits some reflectance in 300–400 nm range (Fig. 1i, S1U = 0.0651, Table 1), has lower reflectance in the 400–500 nm (S1B = 0.0739, Table 1), and has high relative contribution to total brightness in the 505–605 nm and 605–700 nm ranges (S1G = 0.4433, S1R = 0.4249, Table 1). Thus, the Blue + o + model has lower total brightness (B1 in Table 1) and lower mean relative reflectance (B2 in Table 1), than v + . We found that hoverflies respond to v + o + significantly more than the Blue + o + model ($n_{responder}$ = 64, preference index to v + o + = 73.44, p = 0.000327, phi = 0.33, Table S4; Fig. 2b.2).

We also compared the response to v + o + against LR + o + (Fig. 1e). LR + has lower total brightness (B1 in Table 1), and lower mean relative reflectance (B2 in Table 1) than v + . The LR + model has no reflectance in the near UV 300–400 nm range (Fig. 1m, S1U = 0, Table 1) in contrast to v + (Fig. 1i, S1U = 0.0651, Table 1), and has near identical reflectance in the 400–500 nm range to v + . Beyond 500 nm, the reflectance spectra of v + and LR + have similar relative contribution to total brightness in the 510–605 nm (S1G) and 605–700 nm (S1R) ranges (Table 1). However, since v + has much higher total brightness than LR + , v + has much higher reflectance than LR + beyond 500 nm. We found that *E. aeneus* responded to the positive control v + o + significantly more than to the LR + o + model ($n_{responder}$ = 30, preference index for v + o + = 80, p = 0.00239, phi = 0.5; Fig. 2b.3, Online resource 2, Table S4). Together with the response of flower-naïve *E. aeneus* to Blue + o + , these results suggest that higher reflectance in the 300–400 nm and/or 500–700 nm ranges impacts innate floral object identification behaviour of flower-naïve *E. aeneus*.

In the final assay between different colour morphs, we presented flower-naive *E. aeneus* a choice between LR + o + against Blue + o + . Both Blue + and LR + have no reflectance in 300–400 nm region (Fig. 2.3 l, 2.3 m; S1U = 0, 0, Table 1) and have similar total brightness (B1 in Table 1), mean relative reflectance (B2 in Table 1) and intensity (reflectance at wavelength of maximum reflectance, B3 in Table 1). But while Blue + has most reflectance in the 400–510 nm range, as seen by the relative contribution of the 400–510 nm range to total brightness (S1B = 0.8187, Table 1), LR + has most reflectance beyond 510 nm (S1G = 0.4445, S1R = 0.3534, Table 1). The flies in this assay responded to both floral models in equal proportions ($n_{responder}$ = 64; preference index for LR + o + = 50, preference index for Blue + o + = 50, p = 1; Fig. 2b.4, Table S4), indicating that change in reflectance in either 300–400 nm range alone, or in 500–700 nm range alone, does not impact the innate floral object identification behaviour of flower naïve *E. aeneus*, especially when the total brightness and mean brightness of the objects are not high.

Two choice assays between floral objects of different shapes and symmetry

E. aeneus exhibited a preference for the positive control (v + o +) against an asymmetric model (Asymmetric + o + , Fig. 1h; Fig. 2c.1; $n_{responder}$ = 61, preference index to v + o + = 75.41, p = 0.00164, phi = 0.382, Table S4). However, *E. aeneus* responded to the v + o + and undisrupted model equally (Hexagon + o + , Fig. 1g; Fig. 2c.2; $n_{responder}$ = 65, preference to v + o + = 60, p = 0.181, Table S4). This suggests that symmetry, but not necessarily disruptive shape, impacts innate floral object preference in *E. aeneus*.

Parsing odour cues

Two choice assays between floral objects of different odour identities

We found that flies responded to v + o + and a positive visual model v + with the GLV odour blend (v + GLV) models equally ($n_{responder}$ = 61; preference index for v + o + = 47.54, p = 0.701, Table S4, Fig. 2d.1). Further, *E. aeneus* responded equally to v + o + and a positive visual model v + with the fruit odour isoamyl acetate (v + IA; $n_{responder}$ = 59; preference index for v + o + = 48.28, p = 0.696, Table S4, Fig. 2d.2). These results potentially suggest that odour identity does not impact floral object preference.

However, we found that *E. aeneus* responded to v + o + in significantly higher numbers than a positive visual model v + with the known repellent coffee furanone (v + CF; $n_{responder}$ = 60, preference index to v + o + = 73.33, p = 0.00053, phi = 0.33, Table S4; Fig. 2d.3). These results indicate that *E. aeneus* is attracted to a wide range of plant odours when coupled with visual cues in floral objects, but not all odours paired with visual cues are equally attractive to flower-naïve individuals.

Contextual pairing of odour and visual cues

We also examined preference of our model visual cues in the presence of an herbivore dung volatile, which is more likely to be an odour with neutral or even positive valence in a non-food (oviposition) context. In two choice assays between v + o + and v + MC we observed sexually-dimorphic response rates (ratio of $n_{responder}$ to total number of flies tested) and responses, with a statistically significant impact

of sex on both response rates (Table S5) and responses (Table S6). Female *E. aeneus* hoverflies responded significantly more to the positive control ($n_{responder} = 115$, preference index for v + o + = 80.87, $p = 1.2 \times 10^{-9}$, phi = 0.42; Table S4; Fig. 2d.4), and male *E. aeneus* responded equally to both models ($n_{responder} = 126$; preference index for v + o + = 51.59, $p = 0.469$; Table S4; Fig. 2d.5).

Mated female hoverflies tested in a two-choice assay between a negative visual model paired with meta Cresol (v-MC) against the positive visual model and meta-cresol (v + MC) visited both the models without any significant difference ($n_{responder} = 50$; preference index for v + MC = 40, preference index for v-MC = 60, $p = 0.16$; Table S4; Fig. 2e.2). This is in contrast to behaviour of females to v − o + presented against v + o + (Fig. 2e.1).

We note that there was no statistically significant difference in the sensitivity of male and female *E. aeneus* to meta Cresol and both males and females start detecting meta Cresol at the concentration of 10^{-2} g/ml ($n_{male} = 14$, $n_{female} = 18$, Fig. S3, Table S8, Table S9).

Two choice assays between floral objects of different odour concentrations

When presented with 100-fold differences in odour concentrations, no significant difference was observed between the 1% positive odour model (v + (1%o +)) and a model with GLV odour (v + GLV; $n_{responder} = 61$; preference index for v + (1%o +) = 40.98, preference index for v + GLV = 59.02, $p = 0.161$, Table S4; Fig. 3a.1), the v + 1%GLV vs. v + (1%o +) ($n_{responder} = 30$; preference index for v + (1%o +) = 43.33, preference index for v + (1%GLV) = 56.67, $p = 0.467$, Table S4; Fig. 3a.2), or the positive control v + o + vs. a 1% GLV model (v + (1% GLV); $n_{responder} = 57$; preference index for v + o + = 59.65, preference index for v + (1%GLV) = 39.35, $p = 0.148$, Table S4; Fig. 3a.3). However, a significant difference in preference index was observed between models containing the innately attractive odour blend at 100% or 1% concentration (v + o + against v + (1%o +); $n_{responder} = 62$, preference index for v + o + = 64.52, $p = 0.0243$; Table S4; Fig. 3a.4). In summary, odour intensity can impact innate floral object identification in flower-naïve hoverflies. Figure 2f shows the set of floral cues with a significant impact on response of flower-naïve hoverflies and their effect sizes.

EAGs indicated that the *E. aeneus* detected all the volatiles used in our study at the loading concentrations as used in the behavioural assays ($n \geq 20$, Fig. 3b–g). Further, we found that the concentration–response curves for the positive odour blend o + (Fig. 3d), and for green leaf volatile GLV (Fig. 3e) plateaued at a 10^{-5} g/ml range. Thus, the difference in behavioural response observed was not due to an inability to detect the odours at lower concentrations.

Discussion

Our results indicate that the generalist pollinator *E. aeneus* solves the challenge of innately identifying floral objects with a simple yet robust strategy of relying on a combination of visual and olfactory cues, rather than specializing on a single cue. First, we found that flower-naïve E. aeneus were innately attracted to the positive control (v + o +, Fig. 2a.2), as suggested by previous field experiments (Nordstrom et al. 2017). We then showed that flower-naïve hoverflies prefer multimodal visual and olfactory cues over unimodal cues (Fig. 2a.2, a.3). Odour alone can be sufficient for object identification in some hoverfly species (Primante and Dötterl 2010), but this was not our observation for the hoverfly species *E. aeneus*. Rather we found that the combination of olfactory and visual cues together was significant for innate floral object identification in *E. aeneus.*

Regarding visual cues, we found that the cues of high reflectance in 300–400 nm and/or 500–700 nm ranges (a yellow hue to human perception) significantly impacted innate floral object identification in flower-naïve hoverflies. Models with higher reflectance in 300–400 nm and 500–700 nm were preferred over objects reflecting in the 400–500 nm range. This suggests that floral objects with hues that appear yellow in human colour vision are preferred by flower-naïve hoverflies over objects with blue hues. This is similar to observations in other flower-naïve hoverflies like *E. tenax* (Matoušková et al. 2023; An et al. 2018; Lunau and Watch 1997; Lunau 2014). In addition, many insect pollinators, like bees, prefer symmetry and disruptive shape as a floral cue (Free 1970), and some hoverflies like *E. tenax* prefer both radially or bilaterally symmetric objects equally (Matoušková et al. 2023). In our study, symmetry, but not disruptive shape, was found to have a significant impact on the innate object identification behaviour of *E. aeneus* (Fig. 2c).

For olfactory cues, flower-naïve hoverflies responded to several types and concentrations of odours indicative of plant life in general, such as floral volatiles, green leaf volatiles (GLVs; cis-3-hexenyl acetate) and fruit volatiles (isoamyl acetate; Fig. 2d). The volatile cis-3-hexenyl acetate, also known as a green leaf volatile (odour used in v + GLV), is present in many floral scents (Effmert et al. 2005; Cozzolino et al. 2015; Nordström et al. 2017; Feng et al. 2017) and has been reported to increase pollinator attraction (Cozzolino et al. 2015). Since *E. aeneus* did not exhibit any preference between models with either floral, vegetative, or fruit odour, we also evaluated if the

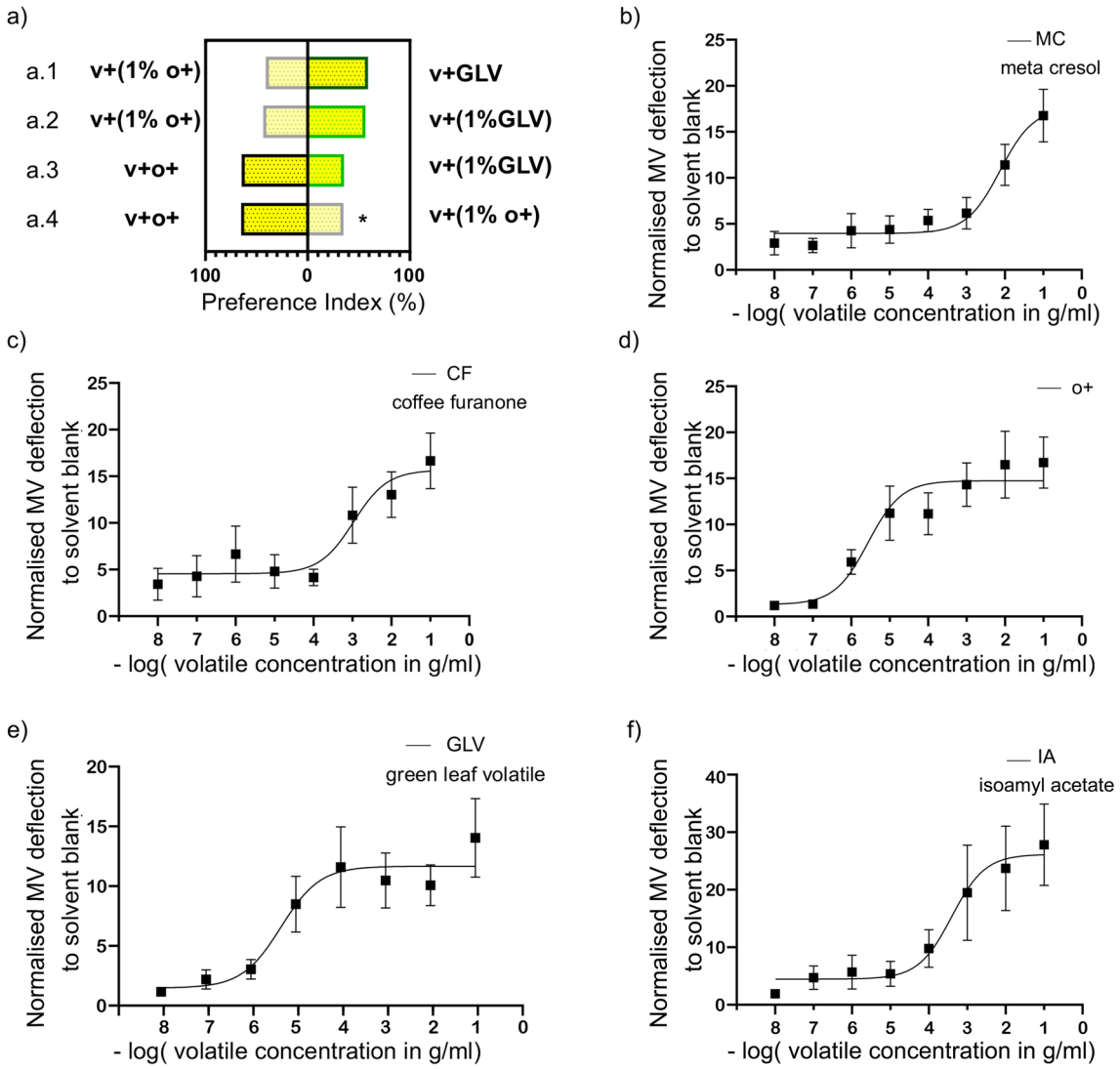

Fig. 3 Preference indices of naïve *E. aeneus* when given a choice between floral objects with positive visual control v + and different concentrations of odour cues, as well as electroantennogram responses to varying concentrations of these different odour cues ranging from 10^{-8} g/ml to 10^{-1} g/ml. Stars indicate significant differences based on Binomial GLMM (* for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$; Table S4). **a** Preference index of naïve *E. aeneus* when given a choice between (a.1) the positive visual control v + containing 1% positive odour blend (v + 1%o +) against the positive visual control v + containing GLV (v + GLV); (a.2) the positive visual control v + containing 1% positive odour blend (v + 1%o +) against the positive visual control v + containing 1% GLV (v + 1%GLV); (a.3) positive control (v + o +) against v + (1%GLV); (a.4) positive control (v + o +) against positive visual control with 1% positive odour blend (v + 1%o +). Concentration-dependent EAG response curves (least-squares fit with mean and standard error bars, $n \geq 20$) shown for **b** meta-cresol (MC), $n = 34$; **c** coffee furanone (CF), $n = 20$; **d** positive odour blend (o +), $n = 22$; **e** green leaf volatile (GLV), $n = 27$; and **f** isoamyl acetate (IA), $n = 20$, all normalised against the solvent control—hexane

presence of any odour was sufficient for attraction. The plant-derived compound 2-methyltetrahydrofuran-3-one, also known as coffee furanone, is known to repel many insects including dipterans like *Drosophila melanogaster* and *Aedes aegypti* at a 10^{-1} g/ml concentration (Batra et al. 2019). In our assay, flower-naïve hoverflies responded to the floral odour model v + o + significantly more than to a visual model with coffee furanone v + CF (Fig. 2d.3), even though electroantennography indicated that all tested volatiles including coffee furanone were detectable by hoverflies. In summary, *E. aeneus* can potentially accept a wide range of plant odours when coupled with visual cues in floral objects, but all odours are not equally attractive. This suggests that odour identity is flexible but important with broad ranges of plant odours attractive to flower-naïve *E. aeneus*. Further, odour intensity also significantly impacted innate floral object identification in flower-naïve *E. aeneus* (Fig. 3a.4). Similar to our observations, previous

studies have documented generalist pollinators utilizing the intensity of floral volatiles as a cue to identify flowers (Wright and Schiestl 2009).

We also found that some odour cues were context-specific with respect to the internal state, specifically sex. Flower-naïve female hoverflies preferred the positive control v + o + over a positive visual model paired with meta Cresol (v + MC), but males responded to both models equally (Fig. 2d.4, d.5). Herbivore dung piles are an important oviposition site for hoverflies (Rotheray and Gilbert 2015), and meta Cresol is an odour abundantly found in herbivore dung (Nair et al. 2018), a common oviposition site for female *E. aeneus*. Both male and female hoverflies have to forage for survival; hence, foraging is not expected to be a sexually dimorphic trait in hoverflies. However, we found that the response of hoverflies to the model v + MC was sexually dimorphic. Sexual dimorphism was not observed when v + was paired with the other volatiles (o +, IA, GLV or CF; Table S5, Table S7). Importantly, in our study, meta Cresol was not repulsive to females, because naïve, mated female *E. aeneus* responded equally to meta Cresol when paired with a negative visual model (gray disk) and meta Cresol paired with the positive visual model (Fig. 2e.2). Context matching and sexual dimorphism have been observed in other pollinators when foraging cues are juxtaposed with ovipositional cues (Roswell et. al, 2019; Balamurali et al. 2020). Thus, in specific contexts, the same olfactory cue could trigger discrimination in females but not in males. It should be noted that the sexually dimorphic response to meta Cresol was not due to differences in sensitivity to meta Cresol in male and female hoverflies (Fig. S3, Fig. S3, Table S8, Table S9). Further, while we did not detect the impact of sex on response in other assays, it should be noted that the sample size in these assays was not enough to detect the impact of sex on differences in response with sufficient power, and future studies aimed at detecting the impact of sex on the behaviour of hoverflies should use larger sample sizes.

Thus, we conclude that the innately preferred floral object of *E. aeneus* consists of radial symmetry, high reflectance in 300–400 nm and/or 500–700 nm wavelength ranges, and plant odours of high concentration (Fig. 2f). Since these features encompass a broad range of cues, it is probable that large regions of the peripheral olfactory and visual nervous systems detect these multiple cues rather than specialized neurons sensitive to specific floral features. As a consequence, innate floral object identification behaviour in *E. aeneus* most likely results from an innate search strategy similar to global image matching found in bees, ants and wasps (Dittmar 2011), or snapshot matching in rodents for odour object identification (Gottfried 2010). Thus, the default state could be a lack of attraction until the right combination of cues exhibiting radial symmetry, high reflectance in 300–400 nm and/or 500–700 nm ranges, and high concentrations of plant odours are present. Therefore, we hypothesize that the positive visual model with meta Cresol is not attractive to female *E. aeneus* (Fig. 2d) because the right combination of visual and olfactory cues is absent, rather than an aversion to the cues themselves.

A candidate region for the integration of these cues in the fly brain could be the ventrolateral protocerebrum (vlPr). The vlPr is known to receive input from both the antennal lobe and the optic lobe in insect pollinators like bees (medulla and lobula; Paulk et al. 2009; Liang et al. 2013). Inhibitory projection neurons necessary for attraction have also been seen to suppress activity in vlPr in response to food odour in Dipterans like *Drosophila melanogaster* (Strutz et al. 2014). Further, the lateral horn (LH) of the fly brain is implicated in innate behaviour (De Belle and Heisenberg 1994). Interestingly, activity in the anterior-lateral LH, the region of LH that is active in innate aversion in *D. melanogaster* (Strutz et al. 2014), is mediated by odour-induced activity in the vlPr. Finally, when activity is suppressed in the vlPr, it has been shown to suppress aversive behaviour (Strutz et al. 2014). Thus, the vlPR could be a good candidate region in the brain of Dipterans, like hoverflies, for processing the right combination of visual and olfactory cues and attenuating attraction towards objects that are identified as floral by a flower-naïve hoverfly.

The attraction to a combination of visual and odour cues could allow *E. aeneus* to innately identify objects with multiple perturbations in odours, colours, and shapes, while still maintaining a robust search strategy. Relying on a combination of cues could facilitate identification of multiple food objects in hoverflies, not only because these multimodal cues co-occur in specific objects in nature, but also because many of these cues indicate flower maturation and quality. In many flowering species, floral volatile emissions are maximized with flower maturation to increase pollinator visits (Schiestl et al. 1997; Muhlemann et al. 2006; Parachnowitsch et al. 2012). Pollination senescing flowers are fragrance-poor to avoid detection from seed predators (Dani et al. 2021). Conversely, intensification of petal colour is also observed in mature flowers with pollen (Schiestl et al. 1997). In addition, previous studies show that disruption of radial symmetry by artificial manipulation and natural florivory deters pollinator activity (McCall 2008; Tsuji and Ohgushi 2018), with even small perforations reducing seed set (Tsuji and Ohgushi 2018). Thus, the innate preferences of *E. aeneus* for radially symmetric objects with concentrated plant olfactory cues and high reflectance in 300–400 nm and/or 500–700 nm region could function together as a gestalt that is flexible yet effective in identifying floral objects, allowing solitary insects with tiny nervous systems to increase foraging efficiency without the need to increase neural processing.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00114-025-01965-5.

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Author contribution A.M.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, writing—original draft, and writing—review and editing; A.J.: methodology and investigation; P.S.I.: investigation; A.S.: investigation; K.N.: conceptualization, funding acquisition, and writing—review and editing. S.B.O.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing—original draft, and writing—review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein. No ethical approvals were required for our work, and all required permissions were taken for the collection of specimens.

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Data availability Supplementary data are provided in the electronic supplementary material.

Declarations

Competing interests The authors declare no competing interests.

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