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ORIGINAL ARTICLE

Olfactory responsiveness of *Culex quinquefasciatus* and *Aedes albopictus* (Diptera: Culicidae): Interactions between species, age and attractants

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Key words. Diptera, Culicidae, *Aedes albopictus*, *Culex quinquefasciatus*, olfactory response, age, attractants, CO_2 synergistic effect

Abstract. Invasive mosquitoes are vectors of important human and animal pathogens and a serious threat to public health. *Aedes albopictus* (Skuse) (Diptera: Culicidae) and *Culex quinquefasciatus* (Say) (Diptera: Culicidae) are good examples because of their wide occurrence, host range and vector competence. An understanding of the responsiveness of mosquitoes to olfactory stimuli is essential for implementing effective surveillance and developing repellents. The present study evaluated the behavioural responses of *A. albopictus* and *C. quinquefasciatus* to CO_2 and human skin odour in an olfactometer. In addition, CO_2 synergistic effect was assessed in association with human skin odour. Mosquitoes of different ages (3–5 and 10–15 day old) were included in the study in order to determine changes in responsiveness to attractants during an insects' lifetime. The highest numbers of mosquitoes captured associated with CO_2 were (*A. albopictus*, 48/77, 62.34%; *C. quinquefasciatus*, 117/126, 92.86%) and hand odour (*A. albopictus*, 211/232, 90.95%; *C. quinquefasciatus,* 320/374, 85.56%) in the "CO_2 vs blank" and "hand vs blank" treatments. Skin odour was the most attractive for both species (*A. albopictus*, 279/309, 90.29%; *C. quinquefasciatus*, 292/306, 95.42%) in "CO_2 vs hand" experiment. The highest mosquito responsiveness was recorded in the "CO_2 + hand vs hand" bioassay (*A. albopictus*, 174/265, 65.66%; *C. quinquefasciatus*, 231/425, 54.35%). Similar trends were recorded for 10–15 and 3–5 day old mosquitoes of both species in all the experiments. In addition, a linear mixed model was used to evaluate the interactions between species, age and attractants. Human skin odour and CO_2 were effective attractants for both *A. albopictus* and *C. quinquefasciatus* (attractant-species interaction, $p\text{-value} < 0.05$). CO_2 synergistic effect was recorded for both species (species-attractant interaction, $p\text{-value} < 0.05$) even when CO_2 was not directly combined with skin odour ($p\text{-value} < 0.05$). The interaction between attractant and age revealed ($p\text{-value} < 0.05$) that in both species, 10–15 day old mosquitoes were more responsive to CO_2 and human skin odour, than younger (3–5 days) adults. The species-age interaction ($p\text{-value} < 0.05$) showed that 3–5 and 10–15 day old *C. quinquefasciatus* were more receptive to CO_2 and skin odour, especially when used in combination, than *A. albopictus.*

INTRODUCTION

Invasive mosquitoes (IMSs) are important vectors of public health pathogens. Their incidence and geographical distribution in Europe have increased since the 1990s (ECDC, 2012), as a consequence of globalization (international trade and tourism), anthropogenic environmental and climatic changes (Medlock et al., 2012). IMSs have colonized new territories (Schaffner et al., 2013). Their spread is often associated with biotic homogenisation and reduction in biodiversity (Wilke et al., 2020) and putative vectorial competence for native viruses, bacteria or parasites (Juliano & Lounibos, 2005). In addition, IMSs may be vectors of important exotic pathogens (Schaffner et al., 2013), such as, the mosquito-borne arbovirus outbreaks that occurred in Europe over the last few decades (Delisle et al., 2015; Succo et al., 2016; Wiwanitkit & Wiwanitkit, 2016; Sieg et al., 2017; Wong et al., 2017; Vasquez et al., 2018).

Among the IMSs, *Aedes albopictus* (Skuse) (Diptera: Culicidae) and *Culex quinquefasciatus* (Say) (Diptera: Culicidae) stand out in terms of their vector status. *A. albopictus* is recognised as a vector of at least 22 arbovirosis (including West Nile disease, Dengue and Chikungunya) (Medlock et al., 2015) and transmission of dirofilariosis in urban environments (Paupy et al., 2009). *C. quinquefasciatus* is an important vector of bancroftian filariosis and a competent vector of dirofilariosis, several arbovirosis (including West Nile disease) and protozoa (Bhattacharya et al., 2016). *A. albopictus* now occurs widely in Europe after its first identification in Albania in 1979 (Medlock et

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al., 2015), while *C. quinquefasciatus* has an extra-European geographic distribution mostly south of latitude 39°N (Bartholomay et al., 2010).

Both species have a wide anthropophilic/zoophilic host range and thrive in rural, semi-urban and urban settings (Gratz, 2004; Eritja et al., 2005). These features of *A. albopictus* and *C. quinquefasciatus* indicate they could be a potential vector for transmitting pathogens between different hosts (human and animal) and locations.

Particular attention has been focused on the geographical distribution and spread of IMSs and mosquito-borne disease epidemiology in order to understand their role in the emergence and spread of novel diseases and the recurrence of old ones. Hence, guidelines for the implementation of the surveillance of IMSs in Europe were proposed in 2012 in order to detect the spread of IMSs, assess the sanitary risk to human health and implement effective control measures (repellents and biological control) (Abramides et al., 2011; ECDC, 2012).

Determining the response of mosquitoes to different olfactory stimuli is imprtant for successful surveillance and developing repellents. Host kairomones or their synthetic derivatives are used as attractants in pest monitoring and reprllent substances for individual protection (Kline et al., 2003). Kairomones are volatile substances emitted by hosts (Dekker et al., 2005) and involved in mosquito-host interaction, in particular, the identification of a blood source (Pitts et al., 2014). Under natural conditions, mosquito activation and host-seeking behaviour are stimulated by host secretions, including carbon dioxide (CO_2) and skin odour. Specifically, CO_2 is considered to be a universal attractant and host indicator (Gillies, 1980; Pappenberger et al., 1996). Fluctuations in CO_2 are associated with vertebrates breathing and is therefore associated with a living prey (Dekker et al., 2005). In addition, CO_2 may act synergistically with other compounds in eliciting host finding behaviour in different species (Gillies, 1980; Kline et al., 1991; Cork, 1996; Takken & Knols, 1999). Skin odour consists of a hundred compounds, with variable attractiveness for different species of mosquitoes (Bernier et al., 2002; Krockel et al., 2006). Among them, lactic acid, ammonia and several carboxylic acids are the most attractive skin-related olfactory stimuli for mosquitoes (Costantini et al., 1998; Geier et al., 1999a; Bosch et al., 2000). There are several different studies (Moboera et al., 2000; Roiz et al., 2005; Cilek et al., 2012; Lacey et al., 2014; Dekker et al., 2016) on the olfactory preferences for different natural and synthetic attractants for *A. albopictus* and C. *quinquefasciatus*. Results often differ and frequently do not consider potential synergistic effects of different combinations of stimuli or focus on similarities between species of mosquitoes in terms of age and sensitivity to different attractants.

The present study evaluated and compared the behavioural response of *A. albopictus* and *C. quinquefasciatus* to CO_2 and human skin odour. The synergistic effect of CO_2 associated with human skin odour was investigated for both species. Different age groups (3–5 days and 10–15 days) were considered in order to evaluate the olfactory reaction at different stages in the life cycles of these mosquitoes. The aim of this study was to identify relationships between species, age and attractants, which could be useful for improving methods of capturing mosquitoes and for developing repellents.

MATERIALS AND METHODS

Mosquitoes and testing groups

C. quinquefasciatus was originally obtained from a colony reared by Biogents (Germany) in 2014, while *A. albopictus* was collected in the field in several years up to 2015. Subsequently both species were bred in the laboratory of Entostudio S.r.L. as described below.

Eggs of *A. albopictus* and *C. quinquefasciatus*, were collected from filter paper placed in black plastic cups or directly from the surface of dechlorinated water, respectively. Larvae were reared in 1 l buckets (500 larvae/bucket) and fed 1328 Hybrid-pellet (Altromin, Germany) (0.3–1.3 grams according to larval age). On reaching the pupal stage, they were transferred into small containers to complete their development. Their lifespan was approximately 6–8 weeks. Adult rearing conditions were as follows: temperature, 25 ± 1°C for *A. albopictus*, 27 ± 1°C for *C. quinquefasciatus*; photoperiod, 12L : 12D for both species; light intensity, 300 lux at 6000°K for both species; humidity, 60 ± 5% for *A. albopictus*, 70 ± 5% for *C. quinquefasciatus*. Adult mosquitoes were fed a 10% glucose solution. In addition, bovine blood at 37 ± 0.5°C was administered via a Hemothek feeder (Discovery Workshops, Lancashire, UK) twice monthly.

For each species two different age-class were selected, hence the identification of 4 test groups: (i) 3–5 day old *A. albopictus*; (ii) 10–15 day old *A. albopictus*; (iii) 3–5 day old *C. quinquefasciatus*; (iv) 10–15 day old *C. quinquefasciatus*.

Each age group consisted of 30 female mosquitoes, fed only 10% glucose solution before testing. Test mosquitoes were allowed to acclimatize in the flight chamber for 60 min before starting the bioassays. In addition, *C. quinquefasciatus* had undergone an inverted photoperiod for at least 24 h before the acclimatization. The different mosquito test groups were used in each replicate of this experiment.

Olfactometer

A home-made dual-choice olfactometer (Fig. 1) was used to test the effectiveness of attractants in this study. The device was composed of a cubic plexiglas flight chamber (50 × 50 × 50 cm), connected at the front to two tubes (A and B) (inner diameter: 10 cm) and at the back to a flexible tube for extracting air (C).

Each tube (A and B) is divided into two parts by a 1 mm mesh net (D), to prevent mosquitoes leaving the device during a test. The first section of each tube (12 cm) was made of plexiglass and the second (35 cm) of PVC. There is a PVC sliding door (E) at each of the entrances to the tubes from the flight chamber, which were removed at the beginning of each test.

The air extraction tube (C) was connected to an extractor fan, which controlled the airflow through the device at 0.2 m/s. A mesh net (D) between the extraction tube and the flight chamber prevented mosquitoes leaving via the extraction tube.

Olfactometer walls were covered with white paper in order to reduce the level of optical stimulation during each test.

Attractants

The olfactory stimuli were carbon dioxide and human skin odour.

Carbon dioxide from a pressurized gas cylinder (100% CO_2, E290, 600 g, Watergas, Italy) was administered through a 4 mm

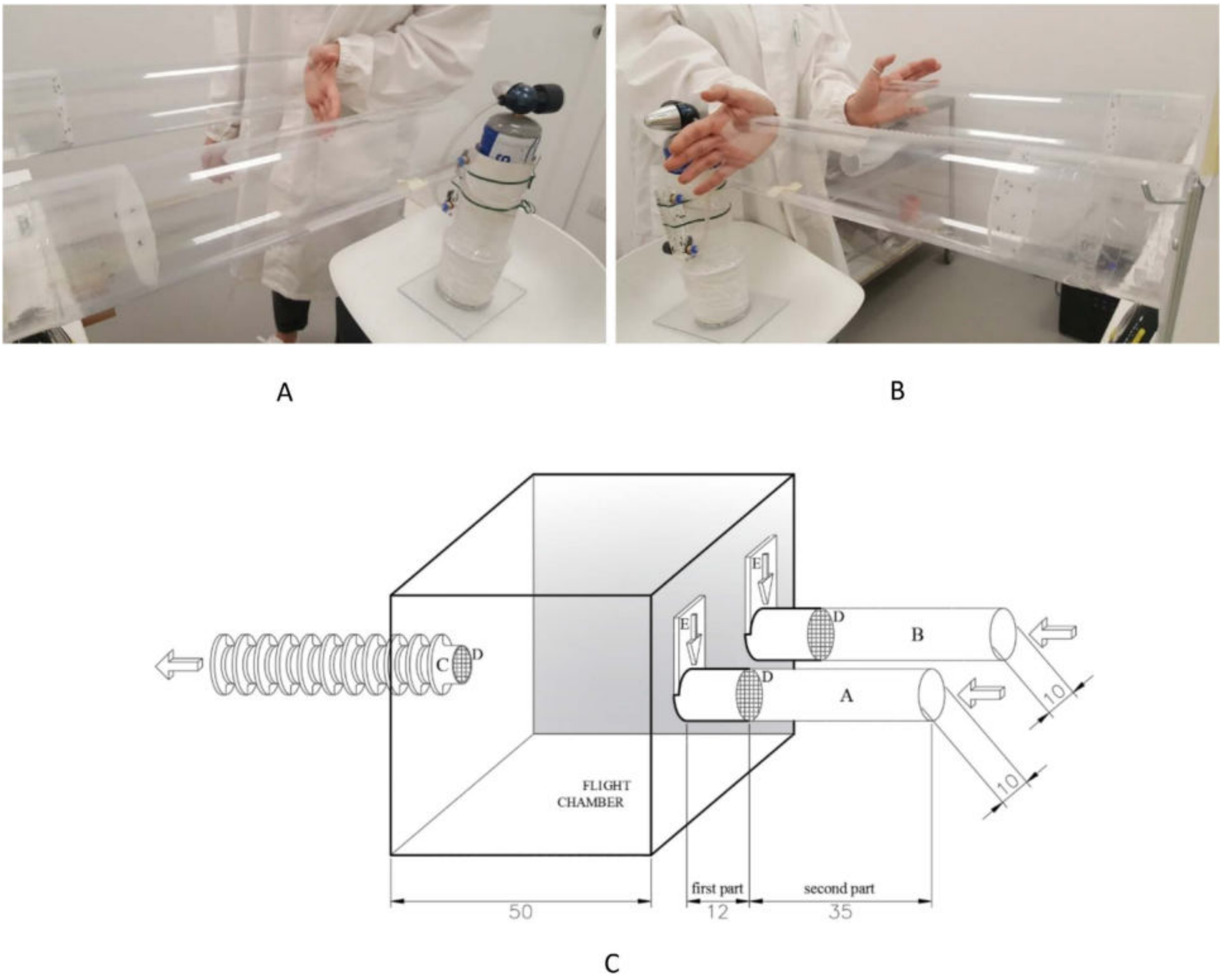

Fig. 1. Photographs of (A) the "hand vs CO_2" experiment and (B) the "hand + CO_2 vs hand" experiment. C – schematic diagram of the olfactometer used in this study. A, B – olfactometer tubes; C – air extractor tube; D – mesh net; E – sliding door. Dimensions are in cm.

diameter plastic tube inserted into the entrances of the tubes A and B. A flowmeter (Model LZM-6T, Cheng Xin, China) regulated the flow rate of carbon dioxide, which was set constant at 1L/min. In the olfactometer CO_2 was mixed with atmospheric air, producing air enriched to 1% CO_2. This concentration simulates the situation when close to a host and is within the CO_2 range mosquitoes usually encounter in the environment (4.5% CO_2, in human breath; 0.035% CO_2, in the atmosphere) (Gillies, 1980; Stange, 1996).

Human skin odour (hand) was tested by placing a volunteer's hand at a distance of 5 cm from the entrances of the olfactometer tubes. Six volunteers (3 females: A, B, C; 3 males: D, E, F) were used in this study and three of them were randomly selected for each experiment (Table 2A, 2B, 2C, 2D).

Atmospheric air ("blank") was used as a control (absence of olfactory stimuli) in "CO_2 vs blank" and "hand vs blank" experiments.

Experiments

Four experiments were carried out to evaluate: (i) the attractiveness of human skin odour compared to atmospheric air (blank); (ii) the attractiveness of carbon dioxide compared to atmospheric air (blank); (iii) the attractiveness of human skin odour compared with carbon dioxide; (iv) the attractiveness of carbon dioxide plus skin odour, compared to skin odour alone. Each experiment was repeated for both age-classes of *A. albopictus* and *C. quinquefasciatus*.

Experiments consisted of 4 assessments. During the assessments the position of the stimuli in the olfactometer arms was switched, from tube A to B and vice versa. Each experiment was repeated 3 times, changing the mosquito group tested for each repetition. A schematic representation of the experiments is presented in Table 1.

All the experiments were done in a room in which the temperature (25 ± 1°C) and relative humidity (60 ± 5%) were controlled. Lighting was provided by artificial lights with a solar spectrum LED at 6000°K of 300 lux of intensity. *C. quinquefasciatus* experiments were done in a dark room and results recorded using red light since this species is nocturnal.

Tests

The attractant combination was placed in front of the entrance of a tube once the the mosquito had completed their acclimatisation in the flight chamber. Air extractor was switched on and the sliding doors were removed to allow the mosquito to repond to the olfactory stimuli coming from the two tubes.

Each assessment lasted for 3 min for *A. albopictus*. A longer period (5 min) was used for *C. quinquefasciatus* as it is less active.

Table 1. Schematic representation of the experiments. AA – *Aedes albopictus*; CQ – *Culex quinquefasciatus*.

Attractant combination	Groups tested (n)	Experiment								Experiment repetition
		Assessment 1		Assessment 2		Assessment 3		Assessment 4		
		A	B	A	B	A	B	A	B	
CO$_2$ vs blank	3–5 day old AA (30) 3–5 day old CQ (30) 10–15 day old AA (30) 10–15 day old CQ (30)	CO$_2$	blank	blank	CO$_2$	CO$_2$	blank	blank	CO$_2$	3
hand vs blank	3–5 day old AA (30) 3–5 day old CQ (30) 10–15 day old AA (30) 10–15 day old CQ (30)	blank	hand	hand	blank	blank	hand	hand	blank	3
CO$_2$ vs hand	3–5 day old AA (30) 3–5 day old CQ (30) 10–15 day old AA (30) 10–15 day old CQ (30)	CO$_2$	hand	hand	CO$_2$	CO$_2$	hand	hand	CO$_2$	3
CO$_2$ + hand vs hand	3–5 day old AA (30) 3–5 day old CQ (30) 10–15 day old AA (30) 10–15 day old CQ (30)	CO$_2$ + hand	hand	hand	CO$_2$ + hand	CO$_2$ + hand	hand	hand	CO$_2$ + hand	3

Sliding doors were closed at the end of each experiment, trapping the mosquitoes in the first part of the olfactometer tubes. Mosquitoes were counted and then gently transferred to a cage. Airflow was kept constant for 10 min after the assessment to remove any residual attractant in the olfactometer. The next assessment was carried out 30 min after the beginning of the previous one.

Statistical analysis

Descriptive statistics (relative frequencies and percentages) are provided for the numbers of mosquitoes captured of both species and age groups in all the experiments (“CO$_2$ vs blank”, “hand vs blank”, “CO$_2$ vs hand”, “CO$_2$ + hand vs hand”). Relative frequencies were calculated as the number of mosquitoes captured in a specific tube divided by the total number of mosquitoes captured in both tubes.

A linear mixed model (LMM) was used to evaluate mosquito olfactory preference (in terms of absolute percentage response i.e., the number of mosquitoes captured in each tube divided by the total number of mosquitoes tested in a particular treatment) for the various combinations of olfactory stimuli (“hand vs blank”, “CO$_2$ vs blank”, “hand vs CO$_2$” , “hand + CO$_2$ vs CO$_2$”), for both age groups and species of moquito. The random-effect variable was “repetition”, whereas “species”, “age” and “attractant” were incorporated into the model as fixed-effect variables. Evaluation of the interactions between “species”, “age” and “attractant” was done and a p-value of < 0.05 was considered statistically significant.

Finally, one-way analysis of variance (ANOVA) was used to compare the results of the “hand vs blank” and “hand vs CO$_2$” experiments and if a CO$_2$ synergistic effect occurred in both species and whether it was effective also when not directly combined with skin odour. A p-value of < 0.05 was considered statistically significant.

Statistical analysis was performed in R version 3.6.1.

Table 2A. Responsiveness of three-five day old *A. albopictus* to different olfactory stimuli in the different experiments. A, B – olfactometer tubes.

Attractant combination	Volunteer	Number of mosquitoes/ assessment	Experiment								Experiment repetition
			Assessment 1		Assessment 2		Assessment 3		Assessment 4		
			A	B	A	B	A	B	A	B	
CO$_2$ vs blank			CO$_2$	blank	blank	CO$_2$	CO$_2$	blank	blank	CO$_2$	
	A	30	0	1	0	0	0	0	0	0	1
	B	30	4	2	0	1	0	2	0	0	2
	D	30	0	2	2	2	0	0	0	0	3
hand vs blank			hand	blank	blank	hand	hand	blank	blank	hand	
	C	30	1	2	3	0	0	3	3	1	1
	E	30	1	3	7	2	0	1	1	0	2
	F	30	1	6	7	0	0	8	3	0	3
CO$_2$ vs hand			CO$_2$	Hand	Hand	CO$_2$	CO$_2$	Hand	Hand	CO$_2$	
	A	30	0	4	7	2	1	3	7	0	1
	B	30	2	8	7	1	0	6	5	1	2
	F	30	4	3	4	1	1	3	6	0	3
CO$_2$ + hand vs hand			CO$_2$ + Hand	Hand	Hand	CO$_2$ + Hand	CO$_2$ + Hand	Hand	Hand	CO$_2$ + Hand	
	A	30	3	2	3	3	3	2	4	3	1
	B	30	7	5	3	5	4	3	3	5	2
	E	30	8	3	5	7	8	2	4	6	3

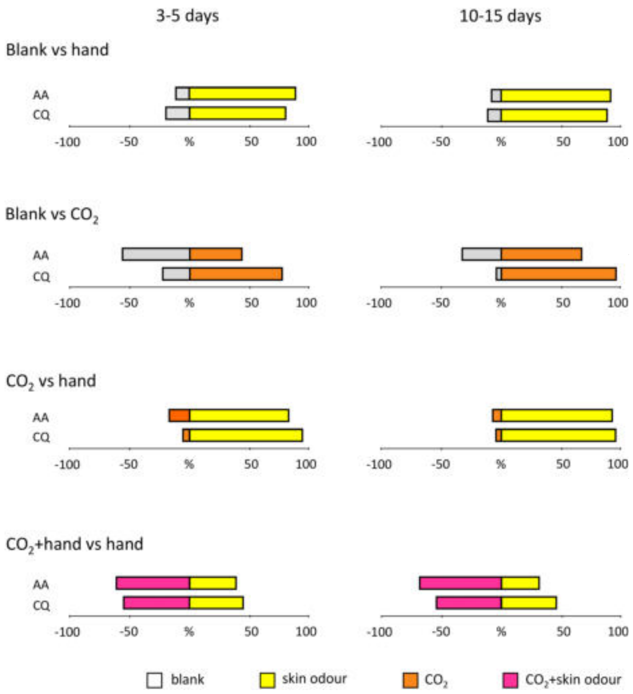

Fig. 2. The relative attractiveness of different combinations of olfactory stimuli, according to species and age. Data are the responses of the mosquitoes in all experiments (each one based on 3 replicates). AA – *Aedes albopictus*; CQ – *Culex quinquefasciatus*.

fasciatus, 17/21, 80.95%; 10–15 day old *C. quinquefasciatus,* 100/104, 96.15%) and hand odour (3–5 day old *A. albopictus*, 47/53, 88.68%; 10–15 day old *A. albopictus*, 164/179, 91.62%; 3–5 day old *C. quinquefasciatus*, 110/136, 80.88%; 10–15 day old *C. quinquefasciatus*, 210/237, 88.60%) in the "CO_2 vs blank" and "hand vs blank" treatments. An exception was the 3–5 day old *A. albopictus*, of which only 7/16 (43.75%) were attracted to CO_2 in the "CO_2 vs blank" treatment.

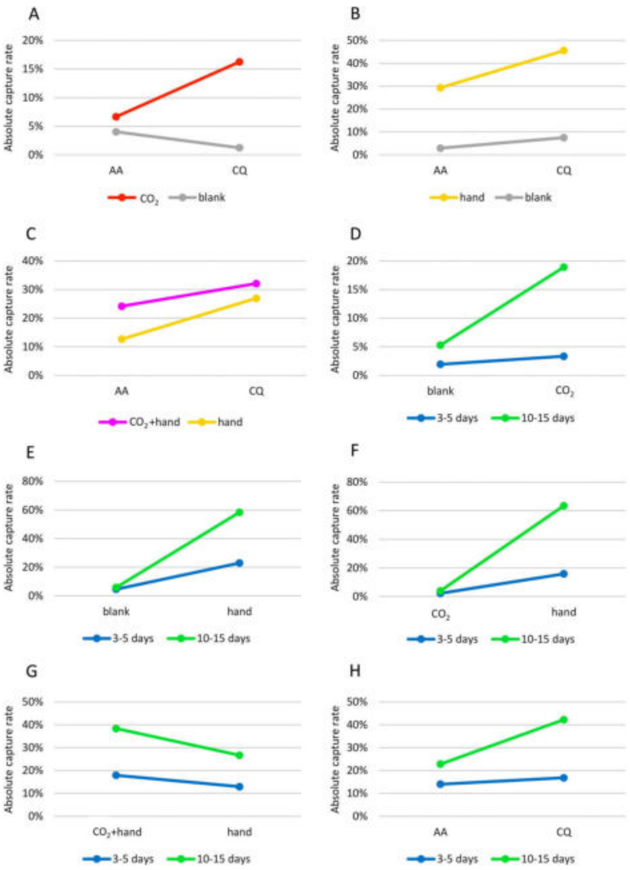

Fig. 3A–H. Significant species, age, attractant interactions recorded for the different combinations of attractants evaluated in this study. A – species-attractant interaction in "CO_2 vs blank" treatment; B – species-attractant interaction in "hand vs blank" treatment; C – species-attractant interaction in "hand + CO_2 vs hand" treatment; D – attractant-age interaction in "CO_2 vs blank" treatment; E – attractant-age interaction in "hand vs blank" treatment; F – attractant-age interaction in "CO_2 vs hand"; G – attractant-age interaction in "hand + CO_2 vs hand"; H – species-age interaction in "hand + CO_2 vs hand" treatment. The y-axis is the number caught. AA – *Aedes albopictus*; CQ – *Culex quinquefasciatus.*

Table 2B. Responsiveness of ten-fifteen day old *A. albopictus* to different olfactory stimuli in the different experiments. A, B – olfactometer tubes.

Attractant combination	Volunteer	Number of mosquitoes/ assessment	Experiment								Experiment repetition
			Assessment 1		Assessment 2		Assessment 3		Assessment 4		
			A	B	A	B	A	B	A	B	
CO_2 vs blank			CO_2	blank	blank	CO_2	CO_2	blank	blank	CO_2	
	A	30	3	3	3	1	2	4	2	1	1
	B	30	3	0	0	4	4	2	1	4	2
	E	30	4	0	1	5	5	2	2	5	3
hand vs blank			hand	blank	blank	hand	hand	blank	blank	hand	
	C	30	1	15	17	0	1	9	11	2	1
	D	30	1	11	10	3	1	9	16	0	2
	E	30	2	19	14	1	0	18	15	3	3
CO_2 vs hand			CO_2	Hand	Hand	CO_2	CO_2	Hand	Hand	CO_2	
	A	30	1	20	21	0	2	17	19	1	1
	C	30	0	25	23	2	3	16	19	3	2
	F	30	2	18	10	2	1	18	10	0	3
CO_2+hand vs hand			CO_2+ Hand	Hand	Hand	CO_2+ Hand	CO_2+ Hand	Hand	Hand	CO_2+ Hand	
	A	30	12	4	7	12	11	3	4	10	1
	C	30	8	4	7	6	8	2	1	7	2
	E	30	9	5	6	10	7	4	5	12	3

Table 2C. Responsiveness of three-five day old *C. quinquefasciatus* to different olfactory stimuli in the different experiments. A, B – olfactometer tubes.

Attractant combination	Volunteer	Number of mosquitoes/ assessment	Experiment								Experiment repetition
			Assessment 1		Assessment 2		Assessment 3		Assessment 4		
			A	B	A	B	A	B	A	B	
CO$_2$ vs blank			CO$_2$	blank	blank	CO$_2$	CO$_2$	blank	blank	CO$_2$	
	A	30	0	2	0	0	1	0	1	1	1
	C	30	0	0	0	2	3	0	1	2	2
	E	30	0	0	0	4	2	1	0	2	3
hand vs blank			hand	blank	blank	hand	hand	blank	blank	hand	
	B	30	1	7	6	4	2	9	8	1	1
	D	30	0	7	9	5	0	10	8	3	2
	F	30	4	12	13	3	3	10	11	1	3
CO$_2$ vs hand			CO$_2$	Hand	Hand	CO$_2$	CO$_2$	Hand	Hand	CO$_2$	
	A	30	0	7	4	0	1	3	6	0	1
	C	30	0	2	4	1	0	3	3	0	2
	F	30	0	0	5	0	1	4	10	0	3
CO$_2$ + hand vs hand			CO$_2$ + Hand	Hand	Hand	CO$_2$ + Hand	CO$_2$ + Hand	Hand	Hand	CO$_2$ + Hand	
	A	30	3	2	0	4	3	2	5	4	1
	B	30	1	4	7	2	6	6	10	7	2
	D	30	7	5	6	6	11	6	1	13	3

The highest number of mosquitoes captured was recorded in response to skin odour in the "hand vs CO$_2$" experiment for both age groups (3–5 day old *A. albopictus*, 63/76, 82.89%; 10–15 day old *A. albopictus*, 216/233, 92.70; 3–5 day old *C. quinquefasciatus*, 51/54, 94.44%; 10–15 day old *C. quinquefasciatus,* 241/251, 96.02%). CO$_2$ synergistic effect was detected in 10–15 day old (*A. albopictus*, 112/164, 68.29%; *C. quinquefasciatus*, 164/304, 53.95%) and 3–5 day old mosquitoes of both species (*A. albopictus*, 62/101, 61.39%; *C. quinquefasciatus*, 67/121, 55.37%).

Comprehensive data on the responsiveness of the different age groups and species of mosquitoes to different olfactory stimuli are presented in Table 2A, 2B, 2C and 2D.

Results of the LMMs describing the effect of attractants on mosquitoes of different ages and species are summarised in Table 3. Interactions between the three parameters investigated ("age", "species", "attractant") were identified and are described below.

"Attractant" had a significant effect ($p < 0.01$) in all experiments ("CO$_2$ vs blank"; "hand vs blank"; "CO$_2$ vs blank"; "CO$_2$ + hand vs hand"), with specific interactions with the other parameters, such as species and age, considered in this study.

Specifically, "species-attractant" interactions were recorded in "CO$_2$ vs blank" (Fig. 3A), "hand vs blank" (Fig. 3B) and "hand + CO$_2$ vs hand" (Fig. 3C) treatments. *C.*

Table 2D. Responsiveness of ten-fifteen day old *C. quinquefasciatus* to different olfactory stimuli in the different experiments. A, B – olfactometer tubes.

Attractant combination	Volunteer	Number of mosquitoes/ assessment	Experiment								Experiment repetition
			Assessment 1		Assessment 2		Assessment 3		Assessment 4		
			A	B	A	B	A	B	A	B	
CO$_2$ vs blank			CO$_2$	blank	blank	CO$_2$	CO$_2$	blank	blank	CO$_2$	
	A	30	2	0	0	1	10	0	1	4	1
	B	30	9	0	0	6	4	0	1	8	2
	E	30	15	1	0	16	12	0	1	13	3
hand vs blank			hand	blank	blank	hand	hand	blank	blank	hand	
	B	30	0	8	13	4	1	20	12	4	1
	C	30	2	18	25	2	1	21	14	3	2
	E	30	1	22	24	5	3	14	19	1	3
CO$_2$ vs hand			CO$_2$	Hand	Hand	CO$_2$	CO$_2$	Hand	Hand	CO$_2$	
	A	30	1	20	19	1	0	23	19	0	1
	C	30	2	16	17	1	1	21	23	2	2
	F	30	2	16	22	0	0	23	22	1	3
CO$_2$ + hand vs hand			CO$_2$ + Hand	Hand	Hand	CO$_2$ + Hand	CO$_2$ + Hand	Hand	Hand	CO$_2$ + Hand	
	A	30	13	12	14	6	16	6	13	15	1
	B	30	15	14	13	13	14	12	10	18	2
	F	30	14	10	10	14	16	11	15	10	3

Table 3. LMM results for the different combinations of attractants. The baseline for the covariate species is AA, and for the covariate age 10–15 days and covariate attractant are: (a) blank in the "blank vs CO_2" model; (b) blank in the "blank vs hand" model; (c) CO_2 in the "CO_2 vs hand" model; (d) CO_2 + hand in the "CO_2 + hand vs hand" model. SE – standard error; DF – degree of freedom; * – significant at $p < 0.05$; ** – significant at $p < 0.01$; *** – significant at $p < 0.001$.

Attractant combination	Estimate	SE	DF	t-value	p-value
Blank vs CO_2					
species	–0.002	0.039	8	–0.058	0.955
attractant	0.101	0.023	81	4.460	0.000***
age	0.012	0.039	8	0.304	0.769
species:age	–0.051	0.052	8	–0.990	0.351
species:attractant	0.124	0.026	81	4,752	0.000***
attractant:age	–0.149	0.026	81	–5.706	0.000***
Blank vs hand					
species	0.022	0.040	8	0.557	0.593
attractant	0.403	0.031	81	1.314	0.000***
age	–0.036	0.040	8	–0.906	0.391
species:age	0.047	0.051	8	0.935	0.377
species:attractant	0.117	0.035	81	3.296	0.002**
attractant:age	–0.278	0.035	81	–7.850	0.000***
CO_2 vs hand					
species	0.006	0.0318	8	0.195	0.850
attractant	0.576	0.026	81	2.226	0.000***
age	0.012	0.032	8	0.368	0.722
species:age	–0.057	0.040	8	–1.433	0.190
species:attractant	0.040	0.030	81	1.351	0.181
attractant:age	–0.460	0.030	81	–1.539	0.000***
CO_2 + hand vs hand					
species	0.162	0.042	8	3.885	0.005**
attractant	–0.149	0.027	81	–5.519	0.000***
age	–0.121	0.042	8	–2.888	0.020*
species:age	–0.167	0.055	8	–3.038	0.016*
species:attractant	0.064	0.031	81	2.056	0.043*
attractant:age	0.067	0.031	81	2.147	0.035*

quinquefasciatus was more responsive to both CO_2 and skin odour than to clean air than *A. albopictus*. A similar result was recorded for CO_2 combined with skin odour than for skin odour alone, which was more attractive for *C. quinquefasciatus*.

The "attractant-age" interaction was recorded in all combinations ("CO_2 vs blank", Fig. 3D; "hand vs blank", Fig. 3E; "CO_2 vs hand", Fig. 3F; "hand + CO_2 vs hand", Fig. 3G) used in this study. The older group of mosquitoes were more sensitive than the younger group (3–5 day old) for all the olfactory stimuli studied.

Interestingly, "age" and "species" were only significant in the "hand + CO_2 vs hand" model. The detected "species-age" interaction (Fig. 3H) revealed that both 3–5 and 10–15 day old *C. quinquefasciatus* were more attracted to skin odour and carbon dioxide than both age groups of *A. albopictus*.

A significant increase in mosquito attractiveness ($p < 0.05$) was recorded for skin odour in "hand vs blank", "CO_2 vs hand" experiments (Table 4) for mosquitoes of both age groups (Table 4).

DISCUSSION

Airborne olfactory stimuli are important for mosquito host-seeking behaviour (Takken & Knols, 1999; Lupi et al., 2013; Takken & Verhulst, 2013). Carbon dioxide and human skin odour are both involved in this process (Wooding et al., 2020). In particular, CO_2 is a long-distance universal mosquito activator, indicating a vertebrate host is nearby (Gillies, 1980; Pappenberger et al., 1996). Skin odour is considered to be a short-distance stimulus, prompting landing and feeding (Lupi et al., 2013; Lacey et al., 2014).

The efficacy of CO_2 and skin odour in host location by *A. albopictus* and *C. quinquefasciatus* is well demonstrated (Mboera et al., 2000; Lacey & Cardé, 2011; Cilek et al., 2012; Hao et al., 2012). Similar results are reported in our study, which recorded a higher number mosquitoes responding to CO_2 (*A. albopictus*, 62.34%; *C. quinquefasciatus*, 92.86%) and skin odour (*A. albopictus*, 90.95%; *C. quinquefasciatus*, 85.56%) in the "CO_2 vs blank" and "hand vs blank" treatments (Figs 2, 3A, 3B).

In the "CO_2 vs hand" experiment, the skin odour effect (percentage captured: *A. albopictus*, 90.29%; *C. quinquefasciatus*, 95.42%) was higher than that for CO_2 (*A. albopictus*, 9.71%; *C. quinquefasciatus*, 4.58%) for both *A. albopictus* and *C. quinquefasciatus*.

It is well known that a turbulent flow of CO_2, with rapid fluctuations in carbon dioxide content, indicates a nearby presumptive host, regardless of the background level of CO_2 (Dekker et al., 2001; Dekker & Cardé, 2011). Skin odour is considered to be a more important stimulus for nocturnal mosquitoes, since hosts are then stationary and exhaling a reduced and constant concentration of CO_2 (Dekker et al., 2005). In these species, other attractants, such as skin odour, may be more important. This might account for the high response to hand odour recorded for the nocturnal *C. quinquefasciatus*.

On the other hand, diurnal mosquitoes usually feed on conscious and active hosts and high and fluctuating levels of CO_2 may indicare the presence of a host nearby (Gillies, 1980; Dekker et al., 2005). The low CO_2 effect, compared to skin odour, recorded for the diurnal *A. albopictus* could

Table 4. Mean (± SD) response of mature and young adults of *Aedes albopictus* and *Culex quinquefasciatus* to skin odour and atmospheric air (in "hand vs blank" experiment) and skin odour and CO_2 (in "hand vs CO_2" experiment). Means were analysed using ANOVA. * – significant at $p < 0.05$; ** – significant at $p < 0.01$; AA – *Aedes albopictus*; CQ – *Culex quinquefasciatus*; SD – standard deviation.

Species	Age	Hand vs blank	Mean ± SD	Hand vs CO_2	Mean ± SD	p-value
AA-CQ	3–5 days	hand	6.54 ± 3.49	hand	4.75 ± 2.23	0.0395*
AA-CQ	10–15 days	hand	15.58 ± 4.81	hand	19.04 ± 3.77	0.0080**
AA-CQ	3–5 days	blank	1.38 ± 1.53	CO_2	0.67 ± 0.96	0.0608
AA-CQ	10–15 days	blank	1.75 ± 1.39	CO_2	1.17 ± 0.96	0.09796

be due to the constant CO_2 flow in the olfactometer. Indeed, homogeneous CO_2 plumes could inhibit mosquitoes from flying upwind as the CO_2 receptor cells habituate to continuous stimulation (Geier et al., 1999b).

Various studies have investigated the olfactory preference of mosquitoes for both CO_2 and human skin odour or its synthetic derivatives (Mboera et al., 1998; Puri et al., 2006; Lacey & Cardé, 2011). However, the lack of standardized techniques for evaluating attractant effectiveness has made it difficult to compare our data with that of previous studies.

There are many experimental conditions to consider when studying mosquitoes. For example, number of specimens, age, origin, light exposure and humidity, to mention a few. It is also important to choose the right flow rate for a one or a multi-choice wind tunnel system, which allows the release of one or more stimuli simultaneously. Once a system is chosen, deciding the appropriate concentration of the attractant, exposure and natural vs synthetic, is also crucial. (Mboera et al., 1998, 2000; Cooperband et al., 2008; Lacey & Cardé, 2011; Cilek et al., 2012; Hao et al., 2012; Scott-Fiorenzano et al., 2017; Xie et al., 2019; Wilke et al., 2020). It is essential to establish standardized methods for evaluating attractants in order to reduce variability in the response of a mosquito due to the experimental set-up. This allows us to obtain realistic and comparable data on the attractants mosquitoes prefer. Finally, some attractants are incredibly hard to evaluate. For example, human skin odour is a mixture of more than 500 compounds (Meijerink & van Loon, 1999; Dormont et al., 2013; de Lacy Costello et al., 2014). Even for the same species of prey these compounds can be very variable (Bernier et al., 2000) as the profiles of skin odour depend on the microbial flora on skin (Takken & Knols, 1999; Zwiebel & Takken, 2004; Verhulst et al., 2011; Takken & Verhulst, 2017). Comparisons of data should consider stimuli complexity, which would prevent incorrect universal assumptions about mosquito host finding behaviour.

In both of the species studied, the combination CO_2 and hand odour was significantly more attractive (*A. albopictus*, 65.66%; *C. quinquefasciatus*, 54.35%) than skin odour alone (*A. albopictus*, 34.34%; *C. quinquefasciatus*, 45.65%) (Fig. 3C). Similar results are reported for other species of mosquitoes, including *A. albopictus* (Dekker et al., 2005; Dekker & Cardé, 2011; Lacey et al., 2014; Roiz et al., 2016) and *C. quinquefasciatus* (Mboera et al., 2000; Lacey & Cardé, 2011; Spanoudis et al., 2020).

Why CO_2 has a synergistic effect on the attractiveness host odour is unknown. The mosquito olfactory system is complex as it consists of olfactory (ORs), ionotropic (IRs) and gustatory receptors (GRs) (Guidobaldi et al., 2014; Ray, 2015), with the ORs primarily involved in olfactory host detection.

ORs include specific receptors (Gr1, Gr2, Gr3) located on the olfactory receptor neurons cpA in capitate peg sensilla on the maxillary palps. Gr1, Gr2, Gr3 are able to detect both CO_2 and skin odour (Tauxe et al., 2013; Ray, 2015). Their activation is probably associated with a reduction in the skin odour threshold response in the presence of CO_2.

Notably, a significant increase in the number of mosquitoes captured was recorded when the responsiveness to hand odour was compared in the "hand vs blank" and "CO_2 vs hand" experiments in both age groups, independently of the species (Table 4). These results indicate that the synergistic effect of CO_2 is also effective when CO_2 is not directly combined with skin odour, but mixes with skin odour in the flight chamber.

Interestingly, *C. quinquefasciatus* was significantly more attracted to CO_2 (in "CO_2 vs blank" treatment) (Fig. 3A) and skin odour (in "hand vs blank" experiment) (Fig. 3B) than *A. albopictus*. A significantly higher synergistic effect of CO_2 (in "CO_2 + hand vs hand") (Fig. 3H) was also detected in *C. quinquefasciatus* of both ages.

Our findings indicates that *C. quinquefasciatus* is more responsive than *A. albopictus* to all of the olfactory stimuli tested in this study. As previously described, the higher attraction to skin odour is probably associated with the circadian rhythm in *C. quinquefasciatus*. As a nocturnal species, it feeds on a stationary host exhaling a constant concentration of CO_2 (Dekker et al., 2005). Evolution may have resulted in *C. quinquefasciatus* specializing in the reception of skin odour. The high number captured recorded in the presence of CO_2 could be due to the experimental set-up and olfactory sensory system. The constant flow of CO_2 in the olfactometer system may have resulted in a reduction in the response of *A. albopictus*, possibly due to the adaptation of its CO_2 receptor. Consequently, the higher reponse to CO_2 recorded for *C. quinquefasciatus* could be a misleading result.

Compared to *Aedes* spp., *C. quinquefasciatus* has a greater number of antennal trichoid and grooved peg sensilla, which are known to house ORs involved in odour detection (Hill et al., 2009) The high attractiveness recorded for *C. quinquefasciatus* could be explained by it greater number of cpA neurons. Further studies are needed on the anatomy of the olfactory sysyem of *C. quinquefasciatus*, specifically the distribution of cpA neurons and Gr1, Gr2, Gr3 receptors.

Interestingly, in all the experiments, 10–15 day old *A. albopictus* and *C. quinquefasciatus* were significantly more responsive to olfactory stimuli than 3–5 day old individuals of the same species (Fig. 3D, E, F, G).

Age seemed to be an important factor influencing the response to an attractant. Mosquito reactiveness to different stimuli changes during its lifetime and depends on the plasticity of the olfactory system. Physiological states, such as, age, feeding state, circadian rhythm and mating, influence the response to attractants (Gadenne et al., 2016). In particular, sexual maturation in early adulthood plays an important role in neural modulation. Indeed, 24–72 h after adult emergence, mosquitoes show blood-feeding behaviour (Klowden, 1990) and an increase in their response to CO_2 (Grant & O'Connell, 2007; Bohbot et al., 2013). In addition, the expression of the odour receptor gene in olfactory receptor neurons (ORNs) on the antennae intensifies from day 1 to day 6 post emergence (Bohbot et al., 2013). Hence, early adulthood could be associated with incomplete maturation of the olfactory system, which could account for the reduced olfactory sensitivity.

To the best of our knowledge our study is the first to describe differences in the responsiveness of different age groups of *C. quinquefasciatus* to attractants. Similarly, Xue et al. (1996) and Xue & Bernard (1996) also report a higher responsiveness to stimuli in old (10–20 days) than in young *A. albopictus* (5–10 days).

Briefly, this study confirmed that CO_2 and human skin odour are important attractants for both *A. albopictus* and *C. quinquefasciatus*, with *C. quinquefasciatus* the most responsive. The response to skin odour was stronger in both species than their response to CO_2. A synergistic effect of CO_2 on the response to skin odour was detected in both species and age groups. CO_2 resulted in a significant increase in response even when not directly associated with skin odour. The higher responsiveness to attractants recorded for old (10–15 day old) than young (3–5 day old) adults is probably due to the time needed for the development of the olfactory system.

Further studies are needed on the structure of the olfactory system and its development in mosquitoes. In addition, standardised methods for testing attractant effectiveness are needed to obtain representative and comparable results.

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