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Functional development of carbon dioxide detection in the maxillary palp of *Anopheles gambiae*

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Summary statement

Onset of host seeking behaviour in malaria mosquitoes is correlated with an increased receptor gene expression, physiological and behavioural sensitivity to CO_2.

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Abstract

Olfactory information drives several behaviours critical for the survival and persistence of insect pests and vectors. Insect behaviour is variable, linked to their biological needs, and regulated by physiological dynamics. For mosquitoes, carbon dioxide (CO_2) is an important cue that signifies the presence of a host, and which elicits activation and attraction. To investigate the genetic basis of olfactory modulation in mosquitoes, we assayed changes in CO_2 detection from receptor gene expression through physiological function to behaviour, associated with the onset of host seeking in the malaria vector, *Anopheles gambiae*. The gene encoding for a subunit of the CO_2 receptor, *AgGr22*, was found significantly upregulated in host seeking females, consistent with a significant increase in sensitivity of CO_2-responsive neurons (cpA) housed in capitate peg sensilla of the maxillary palp. In addition, *AgOr28*, which is expressed in cpC neurons, was significantly upregulated. In contrast, *AgOr8*, which is expressed in cpB neurons, was not affected by this change in physiological state, concomitant with results for the obligate co-receptor *Orco*. Moreover, the sensitivity of the cpB neuron to (*R*)-1-octen-3-ol, a well-known mammalian kairomone, did not change in response to the onset of host seeking. The concentration of CO_2 flux influenced both the propensity of *An. gambiae* to take off into the wind and the speed with which this activation occurred. Female *An. gambiae* mosquitoes responded to CO_2 whether mature for host seeking or not, but onset of host seeking enhanced sensitivity and speed of activation at relevant doses of CO_2.

Introduction

Olfaction plays a vital role in the location and discrimination of resources in insects, and is a candidate target for sustainable pest control (Carey and Carlson, 2011; Pask et al, 2013; Tauxe et al., 2013). Blood feeding insects, such as the African malaria mosquito, *Anopheles gambiae*, respond to plant volatiles and emanations from their potential blood hosts, including metabolic by-products of animals and their cutaneous microbes (Bohbot et al., 2010; Foster and Takken, 2004; Takken and Knols, 1999; Verhulst et al., 2010). The behavioural response to these cues is not static but dependent on endogenous regulatory mechanisms related to the physiological state of the insect (Bohbot et al., 2013; Brown et al., 1994; Grant and O'Connel 2007; Nyasembe et al., 2014). For example, upon eclosion, female *An. gambiae* do not seek blood hosts for up to 24 – 48 hours, after which they will readily orient towards such hosts and take a blood meal (Foster and Takken, 2004). Mating is not a prerequisite for blood feeding but influences egg development in blood fed females (Lounibos 1994; Verhulst et al., 2010). Following a successful blood meal, these mosquitoes again ignore potential sources of a blood meal until after egg-laying (Anton et al., 2006; Klowden and Briegel, 1994; Klowden and Lea, 1979a; Klowden and Lea, 1979b; Klowden and Lea, 1998; Qui et al., 2013; Takken et al., 2001). Such physiological changes provide a practical model of studying olfactory modulation in insects, especially in mosquitoes (Anton et al., 2006; Rinker et al., 2013a; Rinker et al., 2013b; Saveer et al., 2012). Moreover, as blood-feeding preference is a key determinant of the epidemiological role of mosquitoes as disease vectors, an understanding of its modulation has important implications for human and animal health (Carey and Carlson, 2011; Cohuet et al., 2011; Potter 2014).

Carbon dioxide (CO_2), emitted by all potential blood hosts, is a key kairomone for mosquitoes, which signifies the presence of a blood source and sensitises them to other host sensory cues (Dekker et al., 2005; Gillies 1980; McMeniman et al., 2014; Webster et al., 2015). Activation to CO_2 is a component of source searching, which would make the mosquito

more liable to detection of the source given other odours (Dekker et al., 2005; Webster et al., 2015). CO_2 is an attractant in itself, but also synergises with host odours and primes take-off, sustained flight behaviour and landing in host-seeking mosquitoes (Costantini et al., 1996; Spitzen et al., 2008; Webster et al., 2015). Flowers also emit CO_2, however the role of this compound in floral quality evaluation in teneral stages of mosquitoes has not received particular attention, when compared to other non blood-feeding species of insect e.g. moths (Thom et al., 2004). Detection of CO_2 with the heteromeric gustatory receptor system is basal in several insect orders, and well conserved among insects (Robertson and Kent, 2009). In *An. gambiae*, three subunits (*AgGr22*, *AgGr23* and *AgGr24*) function together to mediate CO_2 detection (Lu et al., 2007). Functional analyses of these genes through heterologous expression (Lu et al., 2007), gene knock-out (McMeniman et al., 2014) and transient knockdown of orthologous *Grs* in the yellow fever mosquito *Aedes aegypti* (Erdelyan et al., 2011) have suggested a conserved role of these genes as CO_2 receptors. These receptors are expressed in one of the olfactory sensory neurons (OSNs), referred to as cpA, within the capitate peg sensilla on the maxillary palp of mosquitoes (Grant et al., 1995; Lu et al., 2007). In *Ae. aegypti*, these neurons exhibit an age-dependent increase in sensitivity, suggesting that changes in the sensory capability of the system is timed with the onset of host-seeking behaviour (Grant and O'Connel, 2007). Similar changes in OSN sensitivity have been observed in the cpB neuron of *Ae. aegypti*, which expresses the odorant receptor *Or8* along with the canonical receptor *Orco*. The latter receptor is tuned to (*R*)-1-octen-3-ol, a kairomone cue emitted by most mammals (Bohbot et al., 2013).

Transcription profiling has been used to infer the function and modulation of several insect receptors, where mRNA transcript abundance has been linked with protein (receptor) function (Abrieux et al., 2013; Bohbot et al., 2013; Iatrou and Biessmann, 2008; Poivet et al., 2013; Rinker et al., 2013a, Rinker et al., 2013b). Although the sensitivity of the OSNs detecting CO_2 and (*R*)-1-octen-3-ol has been shown to increase with age in *Ae. aegypti* (Bohbot et al., 2013; Grant and O'Connel, 2007), the relationship between transcript abundance and be-

haviour has yet to be investigated. We hypothesised that higher transcription may lead to an increased sensitivity to a ligand, a stronger response or wider dynamic range. This would enhance the insect's ability to detect and track fluctuations in the concentration of the ligand it perceives. In this study we assayed gene expression and odorant detection in the maxillary palp system of *An. gambiae* to evaluate the molecular, physiological and behavioural modulation of odorant reception.

Results

Gene regulation

Real time PCR assay showed that of the three CO_2 receptor subunits, only *AgGr22* transcripts were significantly enhanced in 4-day-old relative to 1-day-old mosquitoes (Fig. 1) ($t = 5.254$, $p \leq 0.003$, $df = 5$). Similarly, *AgOr28* transcripts were significantly enhanced in 4-day-old compared to 1-day-old mosquitoes (Fig. 1) ($t = 6.746$, $p \leq 0.001$, $df = 5$). Changes in the rest of the receptor transcripts were not statistically significant between age groups (Fig. 1) (*AgOr8*: $t = 2.304$, $p \leq 0.069$, $df = 5$; and *Orco*: $t = 2.294$, $p \leq 0.070$, $df = 5$).

Neural activity

Single sensillum recordings (Fig. 2A) showed a significantly enhanced response to CO_2 in 4-day-old relative to 1-day-old mosquitoes at all concentrations above 600 ppm, with a significant interaction between age and treatment ($F_{5,108} = 4.83$, $p \leq 0.0005$) (Fig. 2B). Moreover, 4-day-old mosquitoes had a lower CO_2 detection threshold than 1-day-old mosquitoes (Fig. 2B). Detection threshold and strength of response to (*R*)-1-octen-3-ol were not significantly different between 1-day-old and 4-day-old mosquitoes (Fig. 2C).

Activation by CO_2

In the bioassay, optimum CO_2 activation occurred between 600 ppm and 1200 ppm for both age classes, but 4-day-old mosquitoes were more responsive to CO_2 stimulation at these concentrations (Fig. 3A). At ambient CO_2 stimulation, the activation pattern was similar to random uniform flight with only < 5% of mosquitoes activated (Cox-Mantel test, I = 5.55 U = 0.71 $p \leq 0.76$; and I = 3.21 U = -0.70, $p \leq 0.69$ respectively, Fig. 3B). Enhanced levels of CO_2 above ambient concentration, however, resulted in both greater proportion activated and faster instantaneous activation of both age classes compared to random uniform activation, resulting in activation functions described by a convex-shaped line compared to the hypothetical diagonal line between the origin and maximum activation (Figs. 3C-D, Table 2), A significantly higher proportion of 4-day-old mosquitoes were activated by all CO_2 treatment levels but at 4800 ppm CO_2 a lower activation rate occurred, resulting in a diminished difference between the two age classes (Fig. 3).

Discussion

The olfactory receptors expressed in the maxillary palps of *An. gambiae* are active within 24 hours of emergence, but undergo transcriptional changes as the mosquito matures for blood host seeking. Increased transcription of a subunit of the CO_2 receptor (AgGR22) is mirrored in increased neural and behavioural sensitivity to CO_2. Behavioural activation by CO_2 was greatest at low concentrations (600 to 1200 ppm). Higher concentrations in fact reduced activation of both 1- and 4-day-old female *An. gambiae*, even though the highest concentration tested was just 10% of that in human breath. The cpC-expressed receptor (AgOR28) was also significantly upregulated. This receptor is less specific than other maxillary palp receptors with several potential ligands identified, and their behavioural function has not yet been ascribed (Lu et al., 2007; Smallegange et al., 2012). The transcript levels of the rest of the receptor genes *AgOrco*, *AgOr8*, *AgGr23* and *AgGr24* were not significantly changed between one and four days, consistent with the functional stability of AgOR8 expressing OSNs. Although we did not test

the receptor protein abundance in the neurons directly, these results show a correlation between transcript abundance and physiological activity in two different receptors, suggesting a direct relationship between transcription function and hence the role of the ligand decoded in behaviour at specific physiological conditions.

Activation of functionally required receptor proteins enables an efficient use of energy while amplifying a signal whose importance is relevant to a specific physiological state (Rinker et al., 2013a; Webster et al., 2015). The Ors and Grs are heteromers (Larsson et al., 2004; Sato et al., 2008), so one would expect that an equimolar presence of the receptor subunits would be necessary for optimal function (Bohbot et al., 2013). The regulation of a single subunit of the heteromeric receptors in both the OR and GR systems, suggests a very simplified modulation mechanism, which modifies a necessary and sufficient component to achieve down- or up-regulation of function. Lu et al. (Lu et al., 2007) showed that *Gr22* is necessary for CO_2 detection in *An. gambiae*, while Sengul and Tu (2008) and McMeniman et al. (2014) demonstrated that the knockdown of the orthologous gene (*Gr2*) in *Ae. aegypti* was sufficient to abolish CO_2 detection. These observations are consistent with our finding. Contrary to our results, Bohbot et al. (2013) reported up-regulation of all olfactory receptors expressed in the maxillary palps of *Ae. aegypti* throughout maturation (1 day, 6 days and 10 days post eclosion) and linked this to cellular and behavioural responses. Although we used two different quantification and expression normalisation protocols, this difference would nevertheless point at an interesting biological difference between Culicine and Anopheline mosquitoes. In *Anopheles*, changes associated with host seeking appear to involve regulation of only a subset of receptors implying that a small subset of the odour space may drive host seeking. It would be interesting to compare this among closely related species with divergent host seeking strategies (specialists and opportunists).

Modulatory mechanisms may shape the contextual meaning of a single olfactory signal. CO_2 has previously been reported to be associated with the host seeking behaviour of mosquitoes (Bohbot et al. 2013; Grant and O'Connel 2007), consistent with our observations.

CO_2 is a ubiquitous compound whose fluctuation in regular pulses above ambient is a reliable indicator of vertebrate blood hosts for mosquitoes. However, inconsistencies in the effectiveness of CO_2 based traps to catch mosquitoes suggest that anthropophilic vectors do not depend solely on this chemical cue to locate humans (Costantini et al., 1996; Takken an Knols 1999). Thus, an understanding of the stability of the CO_2 plume structure and the functional role of CO_2 will be important issues to resolve in the future. The observation that 4-day-old mosquitoes were more easily stimulated to fly with ambient CO_2 levels supports the earlier observation that host seeking input has a non-neuronal maturation component other than the receptor function (McMeniman et al., 2014). *Aedes aegypti*, however, do not get activated by CO_2 unless mature enough to blood feed at 6 and 10 days, respectively (Bohbot et al., 2013; Grant et al., 1995). Interestingly, we also observe dose-dependent behavioural response to CO_2 in 1-day-old mosquitoes suggesting that this compound is not restricted to blood host location. The functional and behavioural dynamic range of CO_2 observed is consistent with concentrations that would be expected in the medium to long-range following dilution of the 40,000 ppm CO_2 exhaled by humans for example.

The observed functional changes in receptor transcripts and neurons suggest an association of sensory signals with physiological needs. The cpB neuron expresses *AgOr8/AgOrco* that showed stable transcription and unchanged sensitivity to its key ligand (*R*)-1-octen-3-ol between 1 and 4 days post emergence, unlike in *Ae. aegypti* where it is both upregulated and the receptor sensitivity increased (Bohbot et al., 2013). As *AgOr8* is exclusively expressed in adults, this implies that transcriptional changes during pupation deliver a fully functional receptor at eclosion, and that (*R*)-1-octen-3-ol l may be used at both nectar feeding and host seeking stages, or that its importance at host seeking is dependent on co-detection with another compound. (*R*)-1-octen-3-ol is also a common compound emitted by fungi (Inamdar et al., 2013). Therefore, its role in the context of sugar source seeking would be interesting to investigate.

The significant upregulation of *AgOr28* transcripts suggests that the key ligand(s) of this receptor is important in the host-seeking behaviour of *An. gambiae*. Heterologously expressed *AgOR28* is more broadly tuned compared to *AgOr8*, responding to 2,4,5-trimethylthiazole, acetophenone, 2-acetylthiophene and fenchone, all associated with mammalian odour (Carey et al., 2010; Lu et al., 2007; Xia et al., 2008). Addition of either acetophenone or 2-acetylthiophene to a basic human odour blend decrease landing of *An. gambiae* (Smallegange et al., 2012), suggesting that this receptor may be involved in mosquito host selection or discrimination.

We show that peripheral modulation may explain behavioural changes towards host seeking and demonstrate a correlation between receptor gene expression, neuronal sensitivity and behaviour. Receptor sensitivity reliably modulates the olfactory signal and contextual relevance of components of an odour plume, and might also sharpen host selection. As the maxillary palp system is a secondary olfactory organ, an investigation of the functional structure of an odour plume, involving antennal-expressed receptors would be interesting. Such studies would also reveal suitable candidates for molecular manipulation of mosquito behaviour towards sustainable control of the diseases they transmit.

Materials and Methods

Mosquitoes

Anopheles gambiae sensu stricto (Suakoko strain, now renamed *An. colluzzi* (Coatzee et al., 2015), were reared according to standard protocols (http://www.mr4.org). Larvae were reared in plastic trays (30 cm x 15 cm x 5 cm), half-filled with distilled water, and fed every other day on Tetramin Baby fish food (Tetra GmbH, Germany). Rearing medium was refreshed with distilled water every other day. Pupae were collected into adult rearing cages (30 cm x 30 cm x 30 cm, Bugdorm, MegaView Science, Taiwan) and allowed 24 hours to eclose. Adult mosquitoes were fed on 10% sucrose solution *ad libitum*. When needed for colony maintenance, adults were fed on human blood by offering a human arm for 30 minutes. Non-blood fed fe-

male mosquitoes, either 1 day (12 to 24 hours) or 4 days after eclosion, were used for experiments.

RNA extraction and qPCR

The transcript abundance of receptor genes was compared between paired 1 day old and 4 day old female *An. gambiae* in six biological replicates. For each replicate, a single cohort of mosquito pupae was divided into two cages (*ca* 60 pupae each) and allowed to eclose: one cage was sacrificed the day after emergence, and the other cage at 4 days post emergence to constitute a single replicate of paired treatments. Maxillary palps and proboscis of female mosquitoes from each treatment group were dissected into 300 µl Trizol (Invitrogen Corporation, Life Technologies, Carlsbad, California, USA) and stored at -80°C until RNA extraction. All dissections occurred between 14:00 and 16:00 h to limit potential circadian changes in gene expression, The olfactory tissues of both treatment groups were processed side by side until the reverse transcription (RT) step to minimise variation arising from day-to-day differences, since the biological replicates and the RT step had been shown in a nested pilot study to be the greatest sources of variation. Total RNA was extracted in 500 µl Trizol reagent according to manufacturer's protocol. The RNA pellet was washed in 70% ethanol and then in 90% ethanol, dried briefly and re-suspended in 30 µl RNAse free water (BIORAD) on ice. RNA was quantified using absorbance measure (Nanodrop 2000c, Thermo Scientific, Wilmington, Delaware, USA) prior to DNAse treatment. Treatment with TURBO DNAse (Ambion, Life Technologies, Carlsbad, California, USA) was immediately carried out according to manufacturer's protocol and the reaction stopped using TURBO DNAse inactivator (Ambion, Life Technologies, Carlsbad, California). The supernatant was immediately used for the RT step using the iSCRIPT reaction mix (BIORAD; Bio-Rad Laboratories, Inc., Hercules California, USA) in three technical replicates. A 1:1 mix of oligo dT and random hexamer primers was used, in final volumes of 20 µl each, containing 8 µl of the RNA sample. The cDNA sample was diluted three times with PCR grade water to obtain the template for qPCR assays.

Primer design

All primers were designed using Primer 3 software (www.justbio.com) from available *An. gambiae* genome sequence information (www.vectorbase.org). All primers were designed to have a melting temperature (T_m) of 60°C, and a product size of 120 to 180 bp. Primer pairs were generally designed in adjacent exons or intron-straddling so as to exclude genomic DNA from the qPCR. Three sets of primers were designed for each target, usually in the first two exons to maximise product despite RT efficiency. The best primer combinations were selected by analysing the specificity, and compatibility of each primer set *in silico* using BLASTn and Oligoanalyser (Integrated DNA Technologies; http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer). The best two combinations were tested by qPCR and a selection made by comparing the consistency of amplification in three technical replicates.

Reference genes

RpS7 and *RpL18* genes are the most commonly used reference genes for the quantification of transcripts in mosquito olfactory tissue (Iatrou and Biessmann, 2008; Pelletier and Leal, 2011; Sengul and Tu, 2008; Thiberge et al., 2007). However, as we found no report of the systematic testing of these genes in treatments and tissues similar to this study, six other genes, commonly used in insect qPCR studies, were obtained and tested to produce the most stable combinations for this study (Bustin et al., 2009; Omondi et al., 2015) (Table 1). The expression was normalised to a reference factor comprising of the geometric means of the best combination reference genes in Genex version 5 (MultiD Systems, Göteborg, Sweden).

Quantitative Real-Time PCR

Quantitative PCR was done using the SYBR Green fluorescent dye for product detection. The reaction was carried out in a 20 µl reaction mix containing 10 µl iQ Supermix (Bio-Rad La-

boratories, Inc., Hercules, California, USA), 200 µM of each primer mix, 1.5 µl cDNA sample and PCR grade water. Amplification was done on a BIORAD CFX 96 (Bio-Rad Laboratories, Inc), using the following programme: a single 10 minute cycle at 94°C, followed by 40 cycles of 12 seconds each at 95°C, 59°C and 72°C. Data acquisition was done for each cycle just following each elongation step. A high resolution melting analysis (65°C to 94°C in 0.5°C steps) was done to test the fidelity of the PCR. For each plate and primer set, a no template and no RT control was set. The transcript levels of each of the chemoreceptor genes previously shown to be expressed in the maxillary palp of *An. gambiae*, *AgGr22*, *AgGr23*, *AgGr24*, *AgOrco*, *AgOr8* and *AgOr28*; (Lu et al., 2007) and of potential reference genes were assayed for each treatment.

Single sensillum recordings

Single unit electrophysiology was performed with sharpened tungsten electrodes from the capitate peg sensilla of the maxillary palps of female *An. gambiae*, as previously described (Bohbot et al., 2010). A single set of recordings from the cpA and cpB neurons across a dose spectrum of CO_2 and (*R*)-1-octen-3-ol, respectively, was taken from each preparation, with ten replications. A mounted 1-day or 4-day-old female mosquito was placed in front of a continuous humidified stream of synthetic air (80% Nitrogen, 20% oxygen, (Strandmöllen AB, Ljungby, Sweden)), which passed over the maxillary palp via a glass tube (7 mm i.d.) at 1.5 l min^{-1}. Delivery of CO_2 was regulated by two-way Teflon solenoid valves (Teddington, Skogås, Sweden) controlled via the digital output of an IDAC-4 (Syntech, Germany). The valves were connected to gas cylinders containing metered amounts of CO_2 (150, 300, 600, 1200, 2400, 4800 ppm) and oxygen (20%), balanced by nitrogen (Strandmöllen AB, Ljungby, Sweden). (*R*)-1-octen-3-ol (a gift from James Logan, Rothamsted Institute, UK; CAS: CAS: 3391-86-4), dissolved in GC-grade hexane (99.9% purity, Sigma Aldrich), was used to describe the dose response relationship of cpB. Pasteur pipettes (VWR International) containing a piece of filter paper (5x10

mm) (Whatman, GE Healthcare, UK) were loaded with 10 µl each of a (*R*)-1-octen-3-ol solution in a series of increasing concentrations (0.001- 1000 ng/µl). All pipettes were prepared in a fume hood and left for 30 min for the solvent to evaporate prior to use. In all experiments, insects were presented with a stimulus for 0.5 seconds, and pipettes replaced between replicates.

Behavioural assay

A glass non-choice bioassay tube, 80 x 9.5 cm i.d., (Majeed et al., 2014) with a laminar flow (20 cm s^{-1} wind speed) was used to assay the response of mosquitoes at each age to 380 (ambient), 600, 1200, 2400 and 4800 ppm CO_2. CO_2 stimulation was turned on or off manually by directing the inlet from the controller either into the bioassay tube or into the exhaust tube, to avoid pre-exposure of test animals to unintended doses of CO_2. The CO_2 pulses of 0.5 s ON/2 s OFF, embedded within the background of ambient CO_2, were generated by the stimulus controller (IDAC-4, Syntech, Kirchzarten, Germany) through two-way Teflon solenoid valves (Teddington, Lanna, Sweden) to simulate human host breath (Dekker et al., 2005). Air intake into the tube was charcoal filtered, and humidified (69 – 85% RH), with pulse originating from pure CO_2 (Strandmöllen AB, Ljungby, Sweden) to produce desired mix. Between each test, CO_2 levels were monitored at the downwind end of the bioassay using a CO_2 analyser (LI-COR Biosciences, LI-820, Nebraska, USA). Wind speed and stability of flow was tested using an anemometer (ThermoAir3, Svenska Schiltknecht Mestechnik AG, Switzerland). Mosquitoes to be tested were starved on water *ad libitum* for 12 hours prior the test. Females were then transferred into release cages and kept in the bioassay room for six hours prior to use, under the same conditions as during rearing and with *ad libitum* access to water through a moist cotton ball. Release cages consisted of a Perspex tube of the same diameter as the bioassay, sealed on one end with 1.0 mm gauge netting and a rotating mesh covered door on the other end. The release cages with test insects were set into the bioassay under red light (≈ 280 lux), left for ca.10 minutes to acclimatise, after which the butterfly door of the release cage was

carefully opened. Testing was done between 20:00 and 22:00h representing the first quarter of the scotophase. For each insect, the time taken to activation was recorded. Non-responders by 3 minutes were included in the analysis as censored individuals and contributed to the determination of activation levels per group. In total, 63 to 135 insects were tested per treatment (Table 2).

Data analysis

Gene expression levels were determined using the ΔΔCq method (Livak and Schmittgen, 2001) on Genex Version 5 (Multi D Systems, Sweden). Gene expression levels per sample were normalised a reference factor comprising of the geometric means of the three most stable reference genes, and expressed relative to the mean of the control group 1-day-old females. Transcription levels were compared between genes per group (1 day and 4 days) using a two tailed paired Students t-test implemented in Genex v5 after checking data for normality and homogeneity of residuals using Kolmogrov's test. Statistical significance values were adjusted for multiple comparisons.

Repeated measures 2-way ANOVA, followed by a Bonferroni *post hoc* test was performed to compare the physiological activity between 1-day and 4-day-old female mosquitoes to each ligand dosage using Statistica Version 8 (Statsoft, 2007). The interaction between independent variables (age and concentration) was assessed. A binomial function was used to test the proportion of mosquitoes taking flight and to calculate the confidence interval of the result for each treatment at $p \leq 0.05$ in R (R Development Core Team, 2011). The probability of activation function (Kaplan and Meier survivorship function) was compared among respondents using Cox-Mantel test to test the null hypothesis that activation probability functions do not differ between pairs of age and CO_2 activation regimes using Statistica. A censure variable was used enabling the non-responders to contribute to the observation.

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Author contributions:

ABO and RI designed the study, SM carried out SSR for experiment resulting in Figure 2. ABO carried out the rest of the experiments, analysed the data and wrote the paper. RI edited the manuscript, supervised and sourced funding for the study.

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Tables and figures

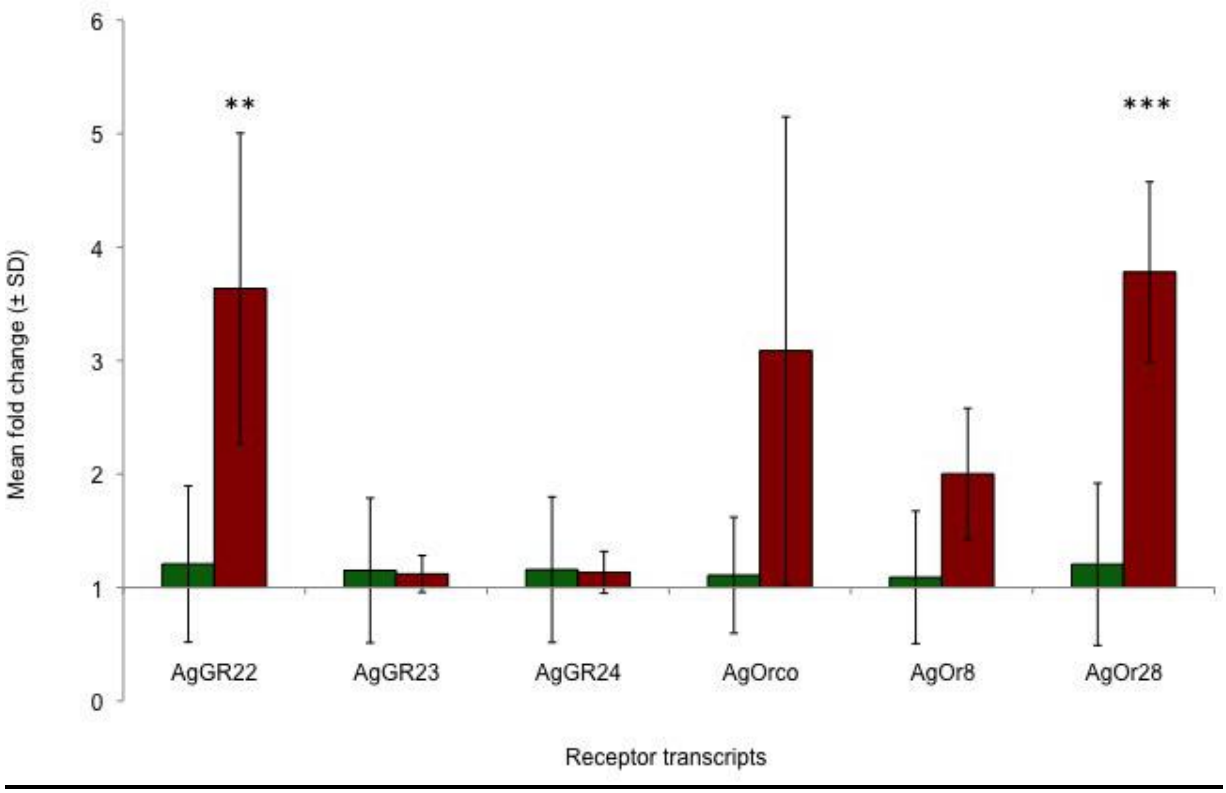

Fig. 1: Expression of CO_2 receptor repertoire significantly increases with age: The relative transcription levels of CO_2 receptor genes, *AgGr22*, *AgGr23* and *AgGr24*, in one day- and four day-old *An. gambiae*. Relative transcription level increases significantly for *AgGr22* but not for the other two sub-units: and for *AgOr28* but not other *AgOr* transcripts over the same period.

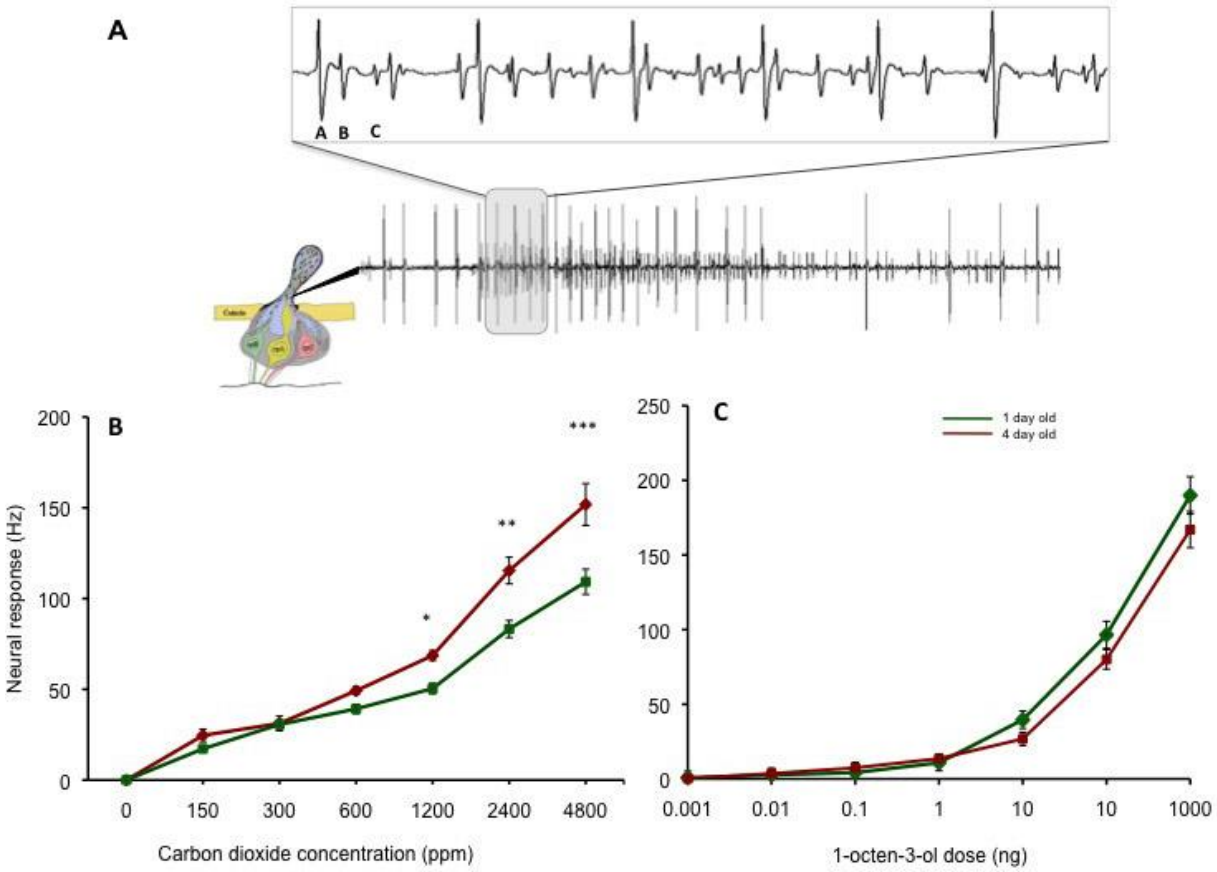

Fig. 2: The sensitivity of the CO_2 OSN is correlated with receptor transcription. A: The capitate peg sensillum houses three neurons, classified according to spike amplitude: cpA expresses the three subunits, *Gr22 – 24*, that mediate CO_2 detection, cpB expresses *Or8* and *Orco* and responds to (*R*)-1-octen-3-ol, while the cpC neuron expresses *Or28* and *Orco*. **B**: The sensitivity of cpA to CO_2 is increased 4 days post eclosion (4 dpe) relative to 1-dpe mosquitoes, **C:** cpB does not change its sensitivity to (*R*)-1-octen-3-ol over the same time period.

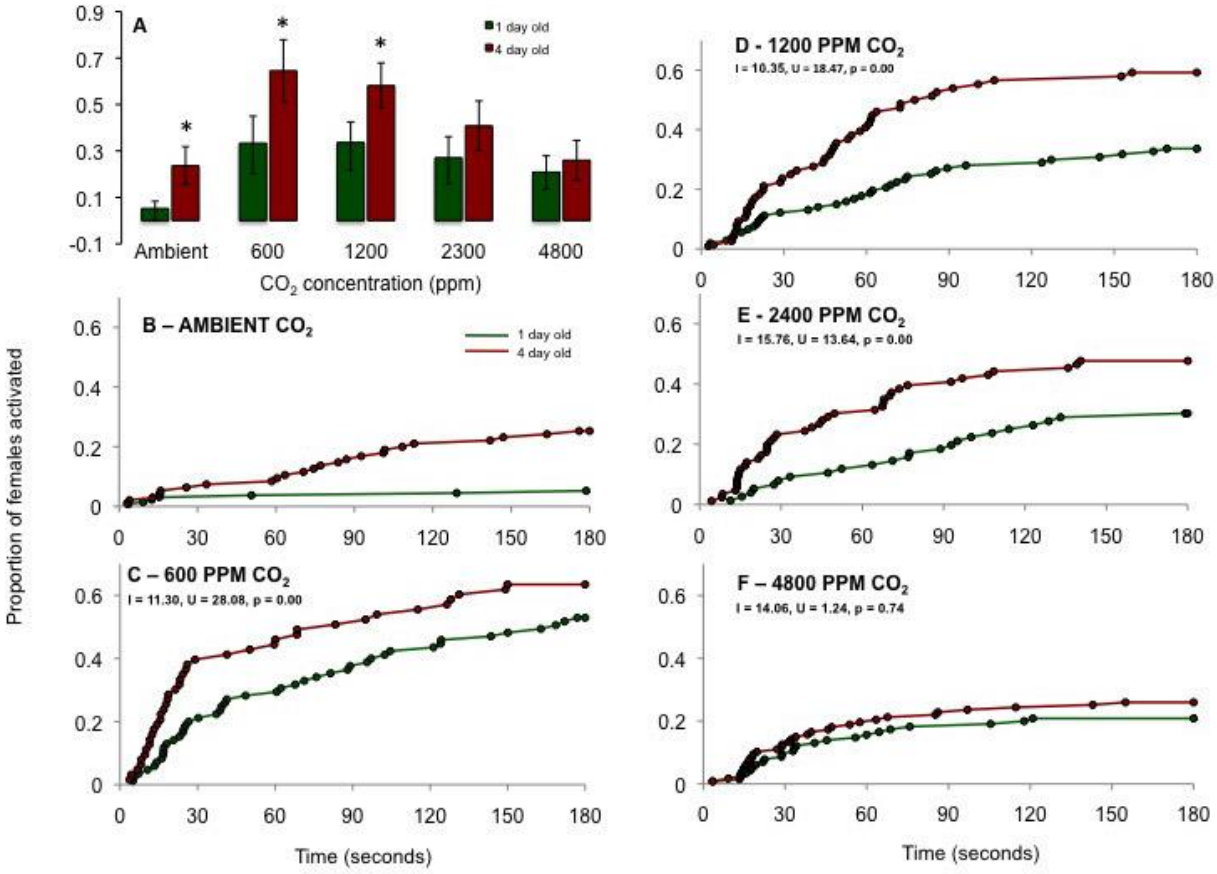

Fig. 3: Activation profiles of 1 day and 4-day-old *An. gambiae* females by CO_2 flux is dependent on the concentration of CO_2 stimulation. **A:** Both 1 day and four day old adult mosquitoes are activated by CO_2 but at different rates, both with an optimum range of 600 to 1200ppm. Error bars represent 95% confidence interval for binomial probability for each result (see also Table 2). **B to F**: Stimulation by additional CO_2 above ambient increased the propensity and speed of activation. Most mosquitoes take off as soon as CO_2 is detected giving a positive skew to the response-time function relative to the diagonal line (describing uniform interval activation).

Table 1: Details of the genes and primers sequences used for qPCR assay.

Gene name	Primer	Sequence 5' to 3'	Product		Citation
			cDNA	gDNA	
Ribosomal protein S7	AgRpS7F	CACCGCCGTGTACGATGCCA	132	132	Pelletier and Leal 2011
	AgRpS7R	ATGGTGGTCTGCTGGTTCTT			
Alpha tubulin elongation factor	EF1α-F	TGGAAAGCGTCCTTGTCAG	186	752 +186	new, Ponton et al., 2011
	EF1α-R	GCCATGCTCCAGACAGTACA			
	AgDLAPF	TTCTCGGATATGCCGATTTC	113	454 + 113	this study
	AgDLAPR	TGGGGCTCCATACTTCAGAC			
TATAA Box binding protein	AgTBPF	CTGTCCGAGATCAGAAAGCAC	151	-	this study
	AgTBPR	ATCCACCCATCCAGGTAGAGT			
Ubiquitin	UBQ-F	GCAAGCTAGTAGTGCCGTCTG	190	-	Robertson and Kent 2009
	UBQ-R:	TCGCGTGTGTGATTTATTCAG			
Ribosomal protein L13	RpL13F:	ATCCTGTCTGGTAACTCGGTG	169	83 + 169	Robertson and Kent 2009
	RpL13R	CCTTCCACAACATACGGCTC			
Ribosomal protein S4	RpS4F:	CGAGGTGACGAAGATTGTGA	124	124	this study
	RpS4R:	AAGTATTCGCCGGTCTTGTG			
CO_2 coreceptor	GR22F:	TTTGCAACGAAGCTCATCAC	166	419 + 166	this study
	GR22R:	GCGTACCCGTCAAGATTCAT			
CO_2 coreceptor	GR23F:	ATGAATCCGGCAATCGTAAG	106	70 +160	this study
	GR23R	ACTGCAGCAAAACGATCAGA			
CO_2 coreceptor	AgGR24F:	TGAAAGCTCCAAACCGATCT	101	-	this study
	AgGR24R	ATGTACACGGTGAGCAGCAG			
OR Coreceptor	AgOrcoF	GACTATTTCCGGAGCCAAGTTT	88	122 + 88	this study
	AgOrcoR	CAGCACCATGAAGTAGGTGACA			
Odorant receptor	AgOr8-F	TGCAGGTCTTTCTGCTGTGTTAC	177	199+92	Iatrou an Biessmann 2008
	AgOr8-R	TGGAGAATTTCAGCGCAGTC			
Odorant receptor	AgOr28F	CCATCCTGCAGCTCTTTCTG	200bp	-	Iatrou an Biessmann 2008
	AgOr28R	GGTTTGCAGCGTAACCATGT			

- intron straddling

Table 2. Median instantaneous activation time varies significantly between 1 day old and 4 day old *An. gambiae* by different concentrations of carbon dioxide.

CO_2 concentration ppm	Median activation time (seconds)[§]			
	Median activation time (s)		sample size (n/N)	
	1 day	4 days	1 day	4 days
380	12.64^{aA}	75.72^{aA}	8/135	24/95
600	41.31^{aA}	23.27^{aB}	45/85	41/63
1200	56.81^{aAB}	44.84^{aB}	36/107	45/76
2400	76.94^{aC}	38.77^{bB}	23/85	41/86
4800	33.15^{aB}	31.47^{aB}	24/114	33/128

[§]Comparisons are based on survival functions across groups defined by CO_2 dose and age. Medians with the same letters denote activation functions that are not significantly different between CO_2 concentrations within age class (Upper case) and between Age classes within CO_2 dose levels (lower case) (Cox-Mantel test, $p \leq 0.05$). For each treatment the sample sizes: N – number of mosquitoes tested and n - number of respondents - are given.