1 ***Title:*** Indolergic receptors of the elephant mosquito *Toxorhynchites amboinensis*

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Abstract

The conservation of the mosquito indolergic receptors across the Culicinae and Anophelinae mosquito lineages, which spans 200 million years of evolution, is a testament to the central role of indolic compounds in the biology of these insects. Indole and skatole have been associated with the detection of oviposition sites and animal hosts. To evaluate the potential ecological role of these two compounds, we have used a pharmacological approach to characterize homologs of the indolergic receptors *Or2* and O*r10* in the non-hematophagous elephant mosquito *Toxorhynchites amboinensis*. We provide evidence that both receptors are narrowly tuned to indole and skatole like their counterparts from hematophagous mosquitoes. These findings indicate that indole and skatole are operating in a non-animal-host seeking context in *Toxorhynchites* and underscore the importance of understanding their roles in hematophagous mosquitoes.

Keywords

Odorant receptor, indole, skatole, mosquito, *Toxorhynchites amboinensis*, indolergic receptor

1. Introduction

It is well-established that resource-locating mosquito behaviors are mainly mediated by olfactory signals (Takken and Knols, 1999; Zwiebel and Takken, 2004). However, only a few relevant animal/plant hosts and oviposition odorants have been identified (Davis and Bowen, 1994). In this regard, the ecological roles of indole and its close analog skatole (3-methylindole), two nitrogen-containing aromatic compounds, are complex (Figure 1A). Indole and skatole alone or as a mixture, have been proposed to act as oviposition attractants in *Aedes aegypti* (Baak-Baak et al., 2013), *Culex* spp. (Beehler et al., 1994; Blackwell et al., 1993; Du and Millar, 1999; Mboera and Takken, 1999; Millar et al., 1994; 1992; Mordue et al., 1992) and *Anopheles gambiae* (Lindh et al., 2008). These two compounds, products of the metabolic activity of the microflora, are present in significant amount in mammalian waste products (Garner et al., 2007; Yokoyama and Carlson, 1979), human skin (Bernier et al., 2000; 2002) and human sweat (Meijerink et al., 2000), which indicate they may also act as animal-host attractants (Cork, 1996). Indole is a ubiquitous component of flower scents of many plant families (Knudsen et al., 2006) and it has been identified from host plants of *Ae. aegypti* and *An. gambiae* (Nyasembe et al., 2018).

Both indole and skatole are electrophysiologically active compounds detected by the antennae of *Ae. aegypti* (Siju et al., 2010), *C. quinquefasciatus* (Du and Millar, 1999) and *An. gambiae* (Blackwell and Johnson, 2000; Meijerink et al., 2000; 2001; Qiu et al., 2006). Two highly conserved odorant receptors (ORs), OR2 and OR10, are highly sensitive and selective towards indole and skatole, respectively in *Ae. aegypti* (Bohbot et al., 2011), *C. quinquefasciatus* (Hughes et al., 2010; Pelletier et al., 2010) and *An. gambiae* (Carey et al., 2010; Wang et al., 2010). The *Or2* and *Or10* genes are expressed in the antennae of adult male and female mosquitoes, while *Or2* is only expressed in the antennae of larvae along with a third paralog named *Or9*. The functional conservation of these receptors across the two mosquito subfamilies in both sexes and in larvae suggest that indole and skatole play important and multiple roles in the biology of these insects (Figure 1A) (Cork, 1996; Nyasembe et al., 2018).

Our objective was to explore the role of these two compounds in the context of animal-host selection by functionally characterizing candidate homologs of the indole and skatole receptor genes in the non-

hematophagous elephant mosquito *Toxorhynchites amboinensis*. Lack of functional conservation would argue the case for a role of the OR2-indole and OR10-skatole pairs in animal-host seeking in hematophagous insects. Our study shows that *T. amboinensis* OR2 (TaOR2) and OR10 (TaOR10) share high sequence identity with their *Aedes* counterparts in support of a highly conserved role in mosquitoes outside animal-host seeking. We provide pharmacological evidence that the elephant mosquito TaOR2 and TaOR10 are indole and skatole receptors operating in a non-animal host context, including oviposition site selection and/or plant-host-seeking.

2. Materials and Methods

2.1. Cloning *TaOr* genes and sequence analyses.

Cloning and sequencing of *TaORco* was described elsewhere (Dekel et al., 2016a). *TaOr2* and *TaOr10* were custom-synthesized (Bio Basic Inc., Markham Ontario, Canada), subcloned into into the pENTR™ vector using the Gateway® directional cloning system (Invitrogen Corp., Carlsbad, CA, USA) and subcloned into the *Xenopus laevis* expression destination vector pSP64t RFA. Plasmid purification was carried out using the The ZR Plasmid Miniprep™-Classic (Zymo Research, Irvine, CA, USA) and sequenced by Macrogen Europe (Amsterdam, the Netherland). DNA and amino-acid sequences for *TaOr2*, *TaOr10* and *TaORco* have previously been published (Zhou et al., 2014) and can be accessed at Figshare (https://figshare.com/articles/Transcriptome_assembly_of_T_ambionsis/2182684/2, last accessed on Dec 26, 2018).

Amino-acid sequence alignments (Supplementary Figure 1) were executed using MAFFT version 7 (Nakamura et al., n.d.). Phylogenetic analysis was performed using the neighbor-joining statistical function and 10,000 Bootstrap replications of the MEGA 7 software (Kumar et al., 2016).

2.2. Chemical reagents.

For establishing the tuning curve, we used 30 odorants, including 19 compounds from Sigma-Aldrich (Milwaukee, WI, USA), including 1-hepten- 3-ol (CAS 4938-52-7), 3-methylbutanol (CAS 123-51-3), *E*-2-

hexen-1-al (CAS 6728-26-3), heptaldehyde (CAS 111-71-7), octanal (CAS 124-13-0), propyl-acetate (CAS 109-60-4), 3-octanone (CAS 106-68-3), 6-methyl-5-hepten-2-one (CAS 110-93-0), 2,4,5-trimethylthiazole (CAS 13623-11-5), diallyl-sulfide (CAS 2179-57-9), benzaldehyde (CAS 100-52-7), indole (CAS 83-34-1), histamine (CAS 51-45-6), (+)-limonene oxide (CAS 203719-54-4), geranyl-acetate (CAS 105-87-3), (+)-fenchone (CAS 4695-62-9), 2-oxopentanoic acid (CAS 1821-02-9), (±)-1-octen-3-ol (CAS 3391-86-4) and 3-methylindole (CAS 83-34-1); 7 compounds from Merck (Darmstadt, Germany), including methyloctanoate (CAS 111-11-5), ethyl-hexanoate (CAS 123-66-0), 2-heptanone (CAS 110-43-0), dimethyl-sulfide (CAS 2179-57-9), tryptamine (CAS 61-54-1), octanoic-acid (CAS 124-07-2) and D-glucuronolactone (CAS 32449-92-6); 2 compounds from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA), including methyl-salicylate (CAS 119-36-8) and octopamine (CAS 770-05-8); and 2 compounds from Alfa-Aesar (Ward Hill, MA, USA), including L-lactic acid (CAS 79-33-4) and δ-Decalactone (CAS 705-86-2).

2.3. Two-electrode voltage clamp electrophysiological recording of *Xenopus* oocytes expressing TaOR2, TaOR10 and TaORco.

The methodologies and protocols used in this study have been described elsewhere (Bohbot and Dickens, 2009). Briefly, *TaOr2*, *TaOr10* and *TaORco* cRNA were synthesized using linearized pSP64tRFA expression vectors as template for in vitro transcription according to the instructions of the mMESSAGE mMACHINE® SP6 Transcription Kit (ThermoFisher Scientific). Stage V-VI oocytes were manually separated and enzymatically defolliculated using a 1 mg/mL collagenase (Sigma-Aldrich, Milwaukee, WI, USA) solution (calcium-free ND96 buffer, [pH 7.6]) for 40-50 min at 18 °C. Oocytes were then successively washed in calcium-free ND96 and gentamycin-supplemented (10 mg/mL, Sigma-Aldrich, Milwaukee, WI, USA) calcium-free ND96. Oocytes were then washed and incubated in ND96 buffer supplemented with cal- cium (0.1 M), 5% heat-inactivated horse serum (ThermoFisher Scientific), 50 mg/ml tetracycline (Carl Roth GmbH), 100 mg/ml streptomycin (Sigma-Aldrich, Milwaukee, WI, USA) and 550 mg/ml sodium pyruvate (Sigma-Aldrich, Milwaukee, WI, USA) for four to five days. Oocytes were injected with 27.6 nL (27.6 ng of each cRNA) of RNA using the Nanoliter 2010 injector (World Precision Instruments, Inc., Sarasota, FL,

105 USA). Odorant-induced currents of oocytes expressing *TaOr2/10* and *TaORco* were recorded using the two-
106 microelectrode voltage-clamp technique (TEVC). The OC-725C oocyte clamp (Warner Instruments, LLC,
107 Hamden, CT, USA) maintained a −80 mV holding potential.

108 For the establishment of concentration-response curves, oocytes were exposed to indole or skatole alone
109 (10^{-10} M to 10^{-4} M). Data acquisition and analysis were carried out with the Digidata 1550 A digitizer and
110 pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA).

111 The tuning curve was generated using a panel of 30 odorants including indole, skatole and other
112 compounds known to elicit physiological or behavioral responses in mosquitoes. All chemicals used were
113 administered at 90 nM, which approximates the EC_{50} of indole and skatole. All the data analyses were
114 performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

115

116 **3. Results**

117 3.1. TaOR2 and TaOR10 are highly conserved.

118 The antennae of *Toxorhynchites amboinensis* express two highly conserved indolergic receptor homologs
119 (TaOR2 & TaOR10). Multiple sequence alignments and phylogenic analyses show that they share about 80%
120 amino-acid identity with their *Aedes* counterparts (Figure 1b). TaOR2 and AaOR2 encode 376 amino-acid
121 proteins sharing 82.67% overall sequence identity. TaOR10 and AaOR10 encode 375 amino-acid proteins,
122 which share 78.34% amino-acid identity. The OR10 alignment requires only one gap to be introduced
123 (Supplementary Figure 1). Amino-acid divergence is evenly distributed throughout the peptide sequence.
124 Both TaOR2 and TaOR10 grouped with their *Aedes* counterparts supported by bootstrap support with values
125 above 95%.

126

127 3.2. TaOR2 and TaOR10 are highly sensitive to indolic compounds.

128 Our next question was to investigate whether the observed sequence conservation determined functional
129 orthology. To do so we expressed both receptors in the frog *Xenopus* oocyte system for pharmacological
130 characterization.

Because *Aedes* and *Anopheles* OR2 are highly sensitive to indole (Bohbot et al., 2011; Wang et al., 2010), we challenged TaOR2 with ten-fold increasing concentrations of this odorant and skatole, a methylated analog of the former. Like its *Aedes* counterparts, TaOR2 proved to be a challenging receptor to express in *Xenopus* oocytes as it consistently generates comparatively lower currents than other mosquito ORs such as OR10 or OR8 (Figure 2A). The resulting concentration-response relationships derived from the maximum amplitudes elicited by each tested concentrations provided EC_{50} values of 88 nM for indole and 1,380 nM for skatole (Figure 2B). Indole was about 15 times more potent than skatole and the indole dynamic concentration range occurred in the nanomolar range.

We applied the same approach to TaOR10 informed by the sensitivity of AaOR10 towards skatole (Bohbot and Dickens, 2012). Indeed, AaOR10 dynamically responds to skatole in the nM range while it is much less sensitive to indole (mM range). TaOR10 consistently generated robust and larger currents than the TaOR2 paralog (Figure 2A). The relative potency of indole and skatole was however reversed with TaOR10 being 105 times more sensitive to skatole (EC_{50} = 87 nM) than to indole (EC_{50} = 9,163 nM) (Figure 2B).

3.3. TaOR2 and TaOR10 are narrowly tuned to indole and skatole, respectively.

We further tested the odorant selectivity of these two receptors using a panel of 30 compounds belonging to diverse chemical classes, including alcohols, aldehydes, esthers, ketones, sulfur compounds, aromatics, amines, terpenes, carboxylic acids and lactones (Dekel et al., 2016b) (Figure 3B). In order to avoid receptor adaptation, antagonist effects and technical artefacts such as broad molecular receptive ranges (Bohbot and Pitts, 2015) associated with high chemical concentrations (Bohbot and Pitts, 2015), the screens were carried out at low 90 nM odorant concentration, which nearly corresponds to the EC_{50} values of the TaOR2-indole and TaOR10-skatole pairs. We controlled for possible position effects by administering odorant sets in reverse order (Supplementary Figure 2). At this concentration, indole and skatole elicited the strongest responses for their respective cognate receptors (Figure 3A). We did not observe any modulation of receptor activity in response to the cognate ligands at the end of the recording sessions.

Overall, TaOR2 exhibited a medium response profile with a kurtosis value of 19 (Figure 3B). TaOR10 was

157 narrowly tuned to skatole with a maximal kurtosis value of 30. These findings confirm the selectivity of these
158 two receptors for skatole and indole, respectively. The activation of TaOR2 elicited by indole was 2.7 times
159 greater than the next most potent odorant ((±)-1-octen-3-ol) and 3.8 times greater than the third most active
160 odorant (methyl-octanoate). The activation of TaOR10 by skatole was 23.1 times higher than the next most
161 activating odorant (3-octanone) and 29 times greater than indole.
162
163 **4. Discussion**
164 It is unclear whether indole and skatole act as oviposition or as animal-host (Cork, 1996; Millar et al.,
165 1992). To complicate the matter, these benzenoid compounds participate to the flower scent of plants (Jürgens
166 et al., 2010; Smith and Meeuse, 1966)1 and are preferred by Ae. aegypti in the context of plant-host attraction
167 (Nyasembe et al., 2018). To explore the ecological role of indole and skatole in the biology of blood-feeding
168 mosquitoes, i.e., to determine whether these receptors may operate in a non-animal host seeking context, we
169 have pharmacologically tested the responses of the OR2 and OR10 homologs from the strict-nectar feeding
170 mosquito *T. amboinensis*. Our criteria for determining functional orthology included both pharmacological
171 sensitivity and selectivity properties. On both accounts, we provide evidence that TaOR2 and TaOR10 are
172 functional orthologs of their counterparts from blood-feeding mosquito species, as they are highly sensitive
173 (nanomolar range EC_{50} values) and narrowly tuned (kurtosis values) to indole and skatole, respectively.
174 While, the biological significance between these two receptors in terms of tuning breadth remains
175 unresolved, the nanomolar level sensitivity of these receptors do suggest cognate relationships between these
176 pairs. TaOR2 and TaOR10 exhibit high-level of sensitivity (nM range) among *T. amboinensis* ORs. For
177 comparison, the EC_{50} value for (*R*)-1-octen-3-ol in relation to TaOR8 is 401 nM (Dekel et al., 2016a), about
178 4 times higher than TaOR2-indole and TaOR10-skatole interactions. The high sensitivity of TaOR2 and
179 TaOR10 towards indole and skatole underscores their fundamental ecological significance.
180 TaOR10 exhibited outstanding specificity towards skatole, second to OR8 towards (*R*)-1-octen-3-ol
181 (Bohbot and Dickens, 2009; Dekel et al., 2016a). Using a panel of 29 compounds identical to the ones tested
182 here but excluding skatole, we find that the kurtosis value (k = 29) for TaOR8 was maximal akin to TaOR10.

(*R*)-1-octen-3-ol was 30.3 times more potent than the next most activating odorant. Such ligand specificity is suggestive of the ecological significance of this odorant. Additionally, such a high degree of specificity may reflect an adaptation for high signal to noise ratio at the peripheral level (Lu et al., 2007).

We have provided evidence that TaOR2 and TaOR10 share the same function as their counterparts from the blood-feeding mosquitoes *Ae. aegypti*, *An. gambiae* (Bohbot et al., 2011; Bohbot and Dickens, 2012; Wang et al., 2010) and *Culex quinquefasciatus* (Hughes et al., 2010; Pelletier et al., 2010). In *Toxorhynchites* sp., these receptors may mediate oviposition site selection (Collins and Blackwell, 2002) and exhibit sensitive physiological responses (Collins and Blackwell, 1998). Our findings exclude the role of indole and skatole in animal-host seeking as far as *T. amboinensis* is concerned and underscore the need to decipher the role(s) of these compounds in blood-feeding mosquitoes using detailed behavioral studies. The unusual sequence and functional conservations of OR2 and OR10 during mosquito evolution reflect the importance of indole and skatole to mosquito ecology and behavior. Indeed, not only do adult detect indole but the larva antennae of *Ae. aegypti* also express *Or2*, suggesting a separate ecological role of this compound in aquatic environments. The prevalence and abundance of indole and skatole present us with a challenge that is to understand their potential role in foraging, mate searching, habitat finding and oviposition site seeking. In addition, odorants can elicit different activities from different mosquito species (Xu et al., 2015), which means that indole and skatole may operate in different contexts within and between species.

The conservation and central role of the mosquito-specific *Or2* and *Or10* genes may be leveraged for the development of future mosquito control agents, including receptor (agonists and antagonists) and behavioral modulators (repellents and attractants). However, comprehensive behavioral studies are wanting to develop such tools for vector population control and personal protection.

Figure legends

Figure 1. Indolergic receptors may operate in multiple ecological contexts. (A) Genes encoding the *Aedes aegypti* odorant receptors 2 (AaOR2) and 10 (AaOR10) proteins are expressed in the adult antennae and are respectively activated by indole and skatole. These two compounds have been linked to oviposition site

209 selection and animal-host seeking in *Aedes aegypti*. Indole is also a major component of flower scents and
210 may play a role in plant-host attraction. (B) Phylogenetic analysis of the candidate indolergic receptor from
211 *Toxorhynchites amboinensis*. Amino-acid identity between OR2 and OR10 are colored in blue and red,
212 respectively. Amino-acid differences are shown in black. AaOR9 was used as an outgroup.
213
214 **Figure 2. TaOR2 and TaOR10 are highly sensitive to indole and skatole, respectively.** (A) Representative
215 current traces elicited by increasing concentrations of indole and skatole recorded from *Xenopus* oocytes co-
216 expressing the *TaOR2 or TaOr10* and *TaORco* receptor complexes. (B) Concentration-response relationships
217 of TaOR2+TaORco and TaOR10+TaORco elicited by indole (blue curve) and skatole (red curve). Responses
218 were normalized to the maximum amplitude response. Extrapolated EC_{50} values are shown with yellow
219 circles. Lower and upper EC_{50} values (standard error) are in upper case. Asterisks represent statistically
220 significant differences of the OR responses (one-way ANOVA followed by Tukey's multiple comparisons
221 post test; ****$P < 0.0001$). Odorant concentrations were plotted on a logarithmic scale. Each point represents
222 the mean and error bars indicate s.e.m.
223
224 **Figure 3. TaOR2 and TaOR10 are narrowly tuned.** (A) Representative current trace elicited by indole,
225 skatole, (±)-1-octen-3-ol (1), heptaldehyde (2), propyl-acetate (3), 3-octanone (4), benzaldehyde (5) and
226 octopamine (6) recorded from *Xenopus* oocytes co-expressing the *TaOr2* or *TaOr10* and the *TaORco* receptor
227 complexes. (B) TaOR2 and TaOR10 are narrowly tuned (k, kurtosis value) to indole and skatole, respectively
228 (one-way ANOVA followed by Dunnet post-test; ****$P < 0.0001$). Mean responses (± s.e.m., $n = 6$) to 90
229 nM of 30 odorants were normalized to indole or skatole.
230
231 **Supplemental information**
232 **Supplementary Figure 1.** (A) Amino-acid sequence alignment of OR2 and OR10 between *Toxorhynchites*
233 *amboinensis* and *Aedes aegypti*. (B) Percentage of amino-acid sequence identity.
234

Supplemental Figure 2. The order of odorant administration does not affect the relative receptor activity. Representative current traces elicited by 90 nM of indole, skatole, (±)-1-octen-3-ol (1), heptaldehyde (2), propyl-acetate (3), 3-octanone (4), benzaldehyde (5) and octopamine (6) recorded from *Xenopus* oocytes co-expressing the *TaOr2* and the *TaORco* receptor complex.

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