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Research

The Expression of Chemosensory Genes in Male Maxillary Palps of *Anopheles coluzzii* (Diptera: Culicidae) and *An. quadriannulatus*

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Abstract

Because of its importance as a malaria vector, *Anopheles coluzzii*'s Coetzee & Wilkerson olfactory system has been studied extensively. Among this work is a series of studies comparing the expression of chemosensory genes in olfactory organs in females and/or males of these species. These have identified species- and female-biased chemosensory gene expression patterns. However, many questions remain about the role of chemosensation in male anopheline biology. To pave the way for future work we used RNAseq to compare chemosensory gene expression in the male maxillary palps of *An. coluzzii* and its sibling species *An. quadriannulatus* Theobald. As expected, the chemosensory gene repertoire is small in the male maxillary palps. Both species express the tuning receptors *Or8* and *Or28* at relatively high levels. The CO_2 receptor genes *Gr22-Gr24* are present in both species as well, although at much lower level than in females. Additionally, several chemoreceptors are species-specific. *Gr37* and *Gr52* are exclusive to *An. coluzzii*, whereas *Or9* and *Gr60* were detected only in *An. quadriannulatus*. Furthermore, several chemosensory genes show differential expression between the two species. Finally, several *Irs*, *Grs*, and *Obps* that show strong differential expression in the female palps, are absent or lowly expressed in the male palps. While many questions remain about the role of chemosensation in anopheline male biology, these results suggest that the male maxillary palps could have both a sex- and species-specific role in the perception of chemical stimuli. This work may guide future studies on the role of the male maxillary palp in these species.

Key words: chemosensory gene, male behavior, maxillary palp, malaria vector

Like other mosquitoes, anophelines use olfaction to guide a range of behaviors. Females use odor cues to detect vertebrate hosts, sugar sources, and oviposition sites (Clements 1999). The role of olfaction in male anophelines is less well understood, although recent work by Mozüraitis et al. (2020) demonstrated that male *An. gambiae* Say and *An. arabiensis* Patton produce aggregating pheromones that attract males and virgin females from several of their sibling species, as well as the more distantly related *An. funestus* Giles. Furthermore, a contact pheromone, or one with low volatility has been postulated in this species (Pitts et al. 2014). Mating pheromones had been reported from several mosquito species previously. For example, *Culiseta inornata* (Williston, Diptera: Culicidae) uses a contact pheromone to identify conspecific females (Kliewer et al. 1966, Lang and Foster 1976, Lang 1977). This has also been demonstrated for various *Aedes* species (Nijhout and Craig 1971) and one study suggests that *Ae. aegypti* (L., Diptera: Culicidae) males produce volatiles that attract other males and females (Cabrera and Jaffe 2007).

Olfaction plays a role in male biology besides mating behavior as well. Like females, males use olfactory cues to locate sugar sources. These include volatiles produced by flowers and fermented fruit (reviewed in Pitts et al. 2014), with different plant species eliciting a variable response (Nyasembe et al. 2012). Based on a variety of studies looking at behavioral and electroantennographic responses, male and female culicines appear to have similar responses and preferences for plant volatiles (e.g., Jepson and Healy 1988, Jhumur et al. 2007, Otienoburu et al. 2012), and this has been found for *An. arabiensis* as well (Healy and Jepson 1988).

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Finally, males of some mosquito species are attracted to vertebrate hosts to locate conspecific mates. For example, males of *Aedes aegypti* and *Ae. albopictus* Skuse congregate around humans (Lumsden 1957, McClelland 1959, Hartberg 1971, Gubler and Bhattacharya 1972). While this behavior has not been reported for anophelines, some data suggests that male *An. coluzzii* respond to human odor (Foster and Takken 2004). Although males were significantly less attracted to human odors than females during a dual olfactometer assay, a small proportion of males (10%) preferred human odor to clean air (Foster and Takken 2004). However, this result is not conclusive and needs confirmation. Certainly, male *An. coluzzii* are much less attracted to human hosts than are females, as human odor baited traps collect far fewer males than females, (e.g., Qui et al. 2007). Intriguingly however, an extensive effort to map *An. coluzzii* mating swarms showed that they are located around human habitation (Diabaté et al. 2011). Therefore, it is conceivable that human odor is used by males to position mating swarms in locations where females are more abundant.

Males of both *An. coluzzii* and *An. quadriannulatus* express the gustatory receptor *Gr33* in their antennae at high levels, whereas this receptor is all but absent in female antennae (Pitts et al. 2011, Athrey et al. 2020). It is tempting to speculate that it plays a role in mating behavior, either during swarm formation or recognition of conspecific females.

The mosquito olfactory system is housed in three appendages: the antennae, the maxillary palps, and the proboscis (Pitts et al. 2004, Kwon et al. 2006). The neurons housed in the sensilla on these appendages express three types of olfactory receptors: the odorant receptors (*Ors*), variant ionotropic receptors (*Irs*), and gustatory receptors (*Grs*) (Clyne et al. 1999, Lu et al. 2007, Benton et al. 2009). Odorant binding proteins (*Obps*) in olfactory tissues are thought to carry hydrophobic odorants through the sensillum lymph to the receptors (Wojtasek and Leal 1999, Horst et al. 2001, Leal et al. 2005).

In contrast to the antennae, mosquitoes carry only a single type of olfactory sensillum on the maxillary palps: the capitate sensilla basiconica, also known as the capitate peg (McIver and Siemicki 1975). *Anopheles gambiae* sensu lato male palps have 28 capitate pegs, whereas female palps have 134 (McIver 1980). This relative scarcity of chemosensory sensilla suggests that the expression of chemosensory-related genes is lower in male maxillary palps. This hypothesis is supported by transcriptome analyses showing a lower abundance of reads associated with chemosensory-related genes in male maxillary palp than in those of females (Pitts et al. 2011).

Female *An. coluzzii* capitate pegs contain three chemosensory neurons. The cpA neuron co-expresses *Gr22, Gr23,* and *Gr24,* and responds to CO_2 and human skin odor (Lu et al. 2007, Tauxe et al. 2013). While it is well-known that female host-seeking is activated by CO_2 detection of the maxillary palps (Omer and Gillies 1971, Healy and Copland 1995, Lu et al. 2007), we know little about the ecological function of the male mosquito palp. We know that males of at least some mosquito species detect CO_2 with their palps because palp ablation largely removes their aversion for high CO_2 concentrations (Bässler 1958).

The cpB neuron co-expresses *Or8* and *Orco*, and is highly sensitive to 1-octen-3-ol. Finally, the cpC neuron co-expresses *Or28* and *Orco* and responds to a wider range of odorants (Lu et al. 2007). Besides the highly expressed *Or8, Or28* and the *Grs* that encode the CO_2 receptor, other chemosensory receptors are expressed in female palps at lower level, such as *IR100a, IR7w, IR75k,* and *Gr52* (Pitts et al. 2011, Athrey et al. 2017). Several of these receptors are differentially expressed in female palps between the anthropophilic *An. coluzzii* and its zoophilic sibling species *An. quadriannulatus*. Several odorant binding proteins (*Obps*) are also expressed at a higher level in the female palps of *An. coluzzii* than in those of *An. quadriannulatus*: *Obp25, Obp26,* and *Obp57* (Athrey et al. 2017). Whether these differences in chemosensory gene expression show that the palps have functionally diverged to some extent between these species remains an open question.

Understandably female mosquitoes are studied extensively compared to males, and much remains unknown about male biology. However, as sterile-insect techniques and transgenic insect releases are being explored as vector control tools, interest in male behavior has increased, as mating competitiveness could be crucial to the success of such programs. Furthermore, malaria vector control might be improved by targeting anopheline males using a lure-and-kill strategy based on sound, visual or chemical cues (Diabaté and Tripet 2015). Currently, the role of the maxillary palps in anopheline males remains largely understudied. To facilitate work in this area, we compared chemosensory gene expression in the maxillary palps of males from the anthropophilic *An. coluzzii* and the zoophilic *An. quadriannulatus*. These results may provide an impetus for future work that may be able to put the observed differences in an ecological and behavioral context.

Methods

Mosquito Rearing

We maintained mosquito colonies in the insectary at Texas A&M University, College Station, Texas, USA. The *An. coluzzii* M form (GASUA) strain was collected in Suakoko, Liberia in 1986 and *An. quadriannulatus* (SANQA) was established from female mosquitoes collected in South Africa in 1999. We kept mosquitoes at 25°C, relative humidity of 75–85% and a 12:12 (L:D) h photoperiod. Females were fed defibrinated sheep blood using a membrane feeding system. Approximately 150 larvae were reared in each tray containing approximately 4.0 liters of water, and fed with ground Tetramin (Tetra, Blacksburg, VA) fish food. Pupae were collected each day and placed in adult rearing cages composed of 3.8 liters size ice cream cartons (height: 16.5 cm, diameter: 17.4 cm) at a density of 200–300 per cage. Adults used in this study were given access to a 5–10% sucrose solution for at least 6 d prior to dissection.

Dissections and RNA Isolation

Male mosquitoes were kept with females and could mate. Six to eight days post-emergence, males were briefly placed at –20°C shortly after the start of the dark cycle to immobilize them. These mosquitoes were then placed on ice and their maxillary palps were dissected and stored in RNAlater (Life Technologies, Grand Island, NY) at 4°C for 24 h. Next, samples were stored at –20°C until RNA extraction. Maxillary palps were dissected from 200 to 300 males for each of two replicates sample per species. The variation in the number of tissues included per sample was normalized during analysis by using a TMM method (see below).

We used the RNeasy Mini (Qiagen, Hilden, Germany) column-based extraction kit, following standard protocol. A Qubit fluorometer (Life Technologies) was used to estimate RNA quantity, and RNA Pico LabChip analyses on an Agilent BioAnalyzer 2100 was used to assess RNA quality by the Texas A&M AgriLife Genomics and Bionformatics Services (College Station, TX). Next, mRNA was enriched from approximately 1 µg of total RNA and single-end cDNA libraries were constructed with an Illumina TruSeq RNA Library kit. Libraries were sequenced on two lanes of an Illumina

HiSeq 2000 in single-end mode. Texas A&M AgriLife Genomics and Bionformatics Services performed preparation and sequencing of the libraries. Library sequencing generated between 50 and 60 million reads with an average read length of 49 base pairs.

RNAseq Analysis

Illumina read quality was first assessed using FASTQC (ver 0.10.0). Trim_galore (Babraham software) was used to trim the reads and filter out reads with quality score < Q30. We mapped sequencing reads to the reference *An. gambiae* genome (AgamP4; downloaded January 2020) using STAR (version 2.7). Despite being mapped to a reference genome belonging to a different species, no reads were discarded due to mismatches. After processing using the SplitNCigarReads tool from the Genome Analyses Toolkit (McKenna et al. 2010), the featureCount tool from the Subread package (Liao et al. 2014) was used to estimate counts mapping to exon features. The R package EdgeR was used to test for differential expression of genes between samples (Robinson et al. 2010, McCarthy et al. 2012). Following normalization of each library, we excluded genes with low abundance (CPM < 1). Next, common and tagwise dispersion was estimated, followed by tests for significance using the 'exactTest' function, which implements the exact test proposed by Robinson and Smyth (2008). We considered genes differentially expressed at a false discovery rate (FDR) of $q < 0.05$ and fold-change (FC) > 2. It was recently shown that applying such a FC threshold results in a very low false-positive rate when using a variety of analysis tool (including EdgeR), even when low numbers of replicates are available (Schurch et al. 2016).

To facilitate interpretation and discussion of the results, we present transcripts per million (TPM) values calculated following the method described in Wagner et al. (2012). We converted Negative Log_2FC values to negative FC values using the equation $FC = -(2^{-Log_2FC})$. Genes expressed at < 1 TPM were considered not-detected. Furthermore, we focus our discussion on chemoreceptors expressed at >5 TPM and *Obps* expressed at >50 TPM. This is because chemoreceptors expressed at higher levels convey most of the functional significance of the female maxillary palps (Lu et al. 2007), and genes expressed at relatively low level are more likely to be noisy (Sha et al. 2015). We analyzed the gene ontology (GO) terms for molecular function using the database PantherDB (https://www.pantherdb.org). Unless otherwise indicated, all *Ors*, *Irs*, *Grs*, and *Obps* refer to *An. gambiae* s.l. genes.

Results

The sequencing generated between 50.6 and 59.3 million reads for each of the four libraries. Between 88.9 and 89.2% of reads mapped uniquely for each library, resulting in 45.1 to 52.7 million mapped reads for each library (Supp Table S1 [online only]). Of the 13,796 annotated genes, 9,843 were detected at >1 TPM (Fig. 1 and Supp Fig. S1 [online only]) in at least one species. Of these, 3,371 were differentially expressed between species, with 1,817 and 1,554 genes expressed at higher levels in *An. coluzzii* and *An. quadriannulatus*, respectively. Of the differentially expressed genes, 839 were unique to *An. coluzzii*, and 167 were unique to *An. quadriannulatus* (Supp Data S1 [online only]).

Overall *Or* expression was low compared to female palps (Pitts et al. 2011, Athrey et al. 2017), but showed a high correlation between the two species (R^2 = 0.718, Fig. 2A). Overall gene family expression levels were not subjected to a statistical test, but total expression of tuning *Ors* was 57.5 ± 6.5 TPM in *An. coluzzii* versus

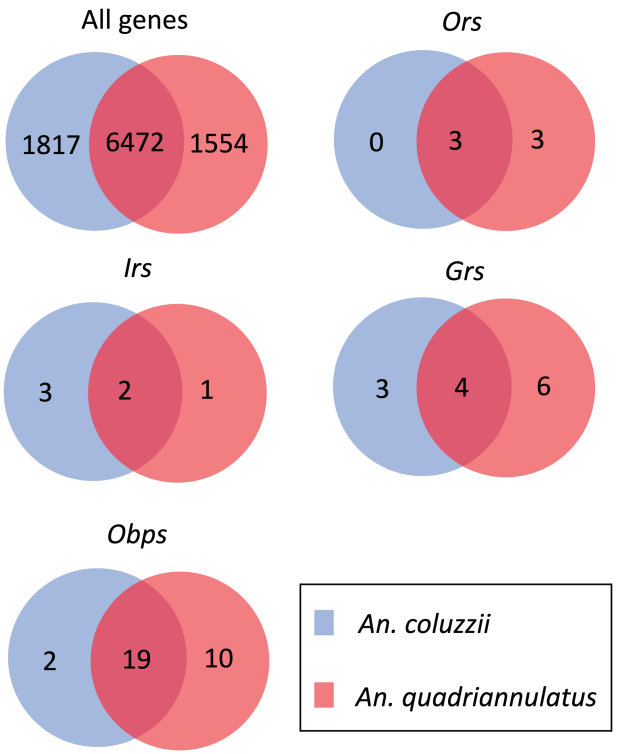

Fig. 1. Venn diagram indicating differential gene expression between the maxillary palps of male *Anopheles coluzzii* and *Anopheles quadriannulatus*. Genes are considered differentially expressed if $q > 0.05$ and fold change > 2.0.

79.1 ± 1.2 TPM in *An. quadriannulatus*. The expression of the co-receptor *Orco* was considerably lower than that of the tuning *Ors* combined (14.7 ± 6.6 vs. 42.8 ± 0.1 in *An. coluzzii* and 9.4 ± 4.4 vs.69.7 ± 5.5 in *An. quadriannulatus*). We detected only three *Ors* in *An. coluzzii*: *Orco, Or8,* and *Or28*. These genes are also the only *Ors* expressed in female palps of this species (Lu et al. 2007), but their expression in males is at a much lower level (Pitts et al. 2011). Interestingly, *Or9* is expressed at 14.2 ± 0.0 TPM in *An. quadriannulatus,* while absent in *An. coluzzii* ($q < 0.0001$). In addition, we detected *Or5* and *Or29* just above our threshold in *An. quadriannulatus* (Fig. 2A, Supp Data S1 [online only]).

Overall *Ir* expression is low in both species (24.8 ± 5.8 vs. 16.9 ± 6.2 TPM in *An. coluzzii* vs. *An. quadriannulatus,* respectively, Supp Data S1 [online only]). There is a moderate correlation between the two species in *Ir* expression (R^2 = 0.53, Fig. 2B). Only the co-receptor *Ir25a* is differently expressed between species, with a 3.5-fold higher expression in *An. quadriannulatus*. In addition, five *Irs* (*Ir64a, 41t.1, 41t.2, Ir76b, Ir75k*) were detected at very low levels in *An. coluzzii* and/or *An. quadriannulatus* (Fig. 2B, Supp Data S1 [online only]). Recently, an additional set of *Irs* was identified in the *An. gambiae* genome (Matthews et al. 2018); however, none were expressed in the male palps of either species.

The total level of *Gr* expression was 74.7 ± 5.0 in *An. coluzzii* versus 83.3 ± 8.0 TPM in *An. quadriannulatus* (Supp Data S1 [online only]), but there is little correlation between the two species (R^2 = 0.20, Fig. 2C). Males of both species express the CO_2 receptor genes (Gr22-24), but with much higher expression of *Gr22* than *Gr23* and *Gr24* in both species. When considering only those *Grs* that are expressed >5 TPM, *Gr52* and *Gr37* were expressed only in the male palps of *An. coluzzii* (25.8 ± 3.9 and 7.3 ± 3.9 TPM,

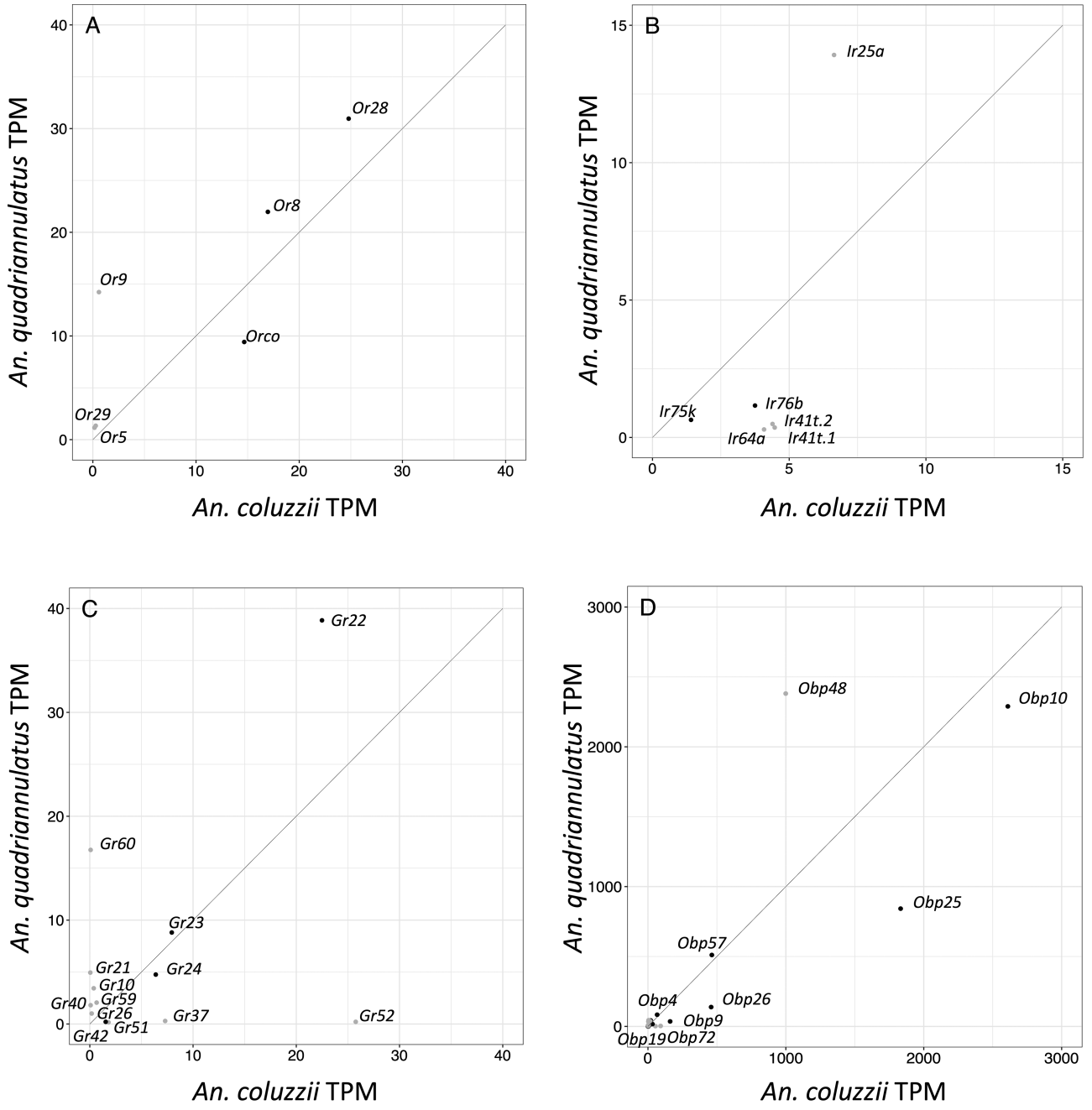

Fig. 2. A comparison of the expression in transcripts per million (TPM) of four chemosensory gene families in male maxillary palps of *Anopheles coluzzii* vs. *Anopheles quadriannulatus*. A: Odorant receptors (*Ors*), (B): Ionotropic receptors (*Irs*), C: Gustatory receptors (*Grs*), D: Odorant binding proteins (*Obps*). The line indicates equal expression between the two species. Genes with differential expression ($q < 0.05$, fold-change >2) are indicated by grey dots. Only genes expressed at >1 TPM are shown.

respectively, Table 1). *Gr60* was expressed only in the male palps of *An. quadriannulatus* (16.8 ± 3.5 TPM, $q < 0.0001$, Table 1). In addition, seven *Grs* (*Gr10*, *Gr21*, *Gr26*, *Gr40*, *Gr42*, *Gr51*, *Gr59*) were detected at low level in one or the other species (Fig. 2C, Supp Data S1 [online only]).

Overall *Obp* expression was 6,895 ± 1,127.1 TPM in *An. coluzzii* versus 6,585 ± 306.7 TPM in *An. quadriannulatus* (Supp Data S1 [online only]). The correlation of *Obp* expression between the species is relatively high ($R^2 = 0.72$, Fig. 2D). When considering *Obps* expressed above >50 TPM, only three *Obps* showed differential expression between the two species: *Obp72* and *Obp19* are expressed at higher level in *An. coluzzii*, although still at low levels relative to overall *Obp* expression (91.7 ± 2.2 and 54.0 ± 10.3 TPM, respectively, Table 1). The expression of *Obp48* is significantly higher in *An. quadriannulatus*. This gene is the most highly expressed gene among the four chemosensory gene families considered here and is the 76th most highly expressed gene overall in the male palps of *An. quadriannulatus*. Finally, an additional 23 detected *Obps* are expressed at low levels in one or both of the two species (Fig. 2B, Supp Data S1 [online only]).

Table 1. Chemosensory genes expressed at >5 TPM (chemoreceptors) and > 50 TPM (odorant binding proteins) in the male maxillary palps of *Anopheles coluzzii* versus *Anopheles quadriannulatus*

Gene ID	Name	*Anopheles coluzzii* TPM	*Anopheles quadriannulatus* TPM	Fold change	*q*-value
AGAP002560	*Orco*	14.67 (±6.57)	9.42 (±4.35)	−1.14	0.80154
AGAP002722	*Or28*	24.79 (±3.51)	30.96 (±4.99)	−2.27	0.24576
AGAP001912	*Or8*	16.96 (±3.67)	21.97 (±2.83)	−2.51	0.40123
AGAP008333	*Or9*	0.59 (±0.21)	14.23 (±0.02)	−40.84	**0.00000**
AGAP010272	*Ir25a*	6.64 (±0.22)	13.92 (±4.47)	−3.48	**0.00028**
AGAP001173	*Gr52*	25.76 (±3.88)	0.21 (±0.19)	77.91	**0.00000**
AGAP001117	*Gr37*	7.30 (±3.92)	0.29 (±0.16)	17.62	**0.00010**
AGAP001915	*Gr24*	6.39 (±2.48)	4.76 (±2.08)	−1.31	0.60764
AGAP003098	*Gr23*	7.94 (±1.70)	8.81 (±0.03)	−2.09	0.22193
AGAP009999	*Gr22*	22.49 (±1.66)	38.86 (±8.43)	−3.01	0.36954
AGAP001121	*Gr60*	0.08 (±0.08)	16.76 (±3.51)	−339.74	**0.00000**
AGAP012714	*Obp72*	91.66 (±15.37)	3.21 (±2.21)	19.04	**0.00000**
AGAP000278	*Obp9*	160.80 (±8.30)	35.71 (±15.55)	2.74	0.19918
AGAP012321	*Obp26*	458.26 (±329.17)	138.31 (±34.43)	2.23	0.38237
AGAP012320	*Obp25*	1832.21 (±503.99)	842.46 (±13.64)	1.28	0.64018
AGAP001189	*Obp10*	2609.74 (±316.58)	2289.37 (±336.60)	−1.59	0.32645
AGAP010489	*Obp4*	66.63 (±22.15)	83.61 (±54.48)	−1.81	0.34936
AGAP011368	*Obp57*	463.14 (±1.77)	510.89 (±114.58)	−1.89	0.28079
AGAP007286	*Obp48*	999.04 (±110.63)	2381.02 (±393.28)	−4.56	**0.00030**

Fold change values are indicated as a positive value if expression is higher in *Anopheles coluzzii* and vice versa.

Gene ontology analyses of molecular function showed that among the 1,423 genes that were differentially expressed between species and that showed functional hits, 525 were classified as having 'binding' (GO:0005488) as a molecular function, while 573 were classified as having 'catalytic activity' (GO0003824). Genes classified under molecular function categories of 'structural constituent ribosome' and 'protein binding' were significantly over-represented among differentially expressed genes (Fisher exact test; q = 0.019 and 0.015, respectively).

Discussion

Previously, we and others have shown species-, sex- and organ-specific patterns of chemosensory gene expression in the anthropophilic *An. coluzzii* and zoophilic *An. quadriannulatus* (Pitts et al. 2011, Rinker et al. 2013, Athrey et al. 2017, Saveer et al. 2018, Athrey et al. 2020). Here, we extended that work by comparing chemosensory gene expression in the male maxillary palps of these two species. As expected, the repertoire of chemosensory genes expressed in male palps is small. While much of it is shared between the two species, several chemosensory genes are highly species-biased or specific; specifically, *Gr37* and *Gr52* in *An. coluzzii*, and *Or9* and *Gr60* in *An. quadriannulatus*.

The function of the maxillary palps in male anopheline biology has remained largely unstudied. Nonetheless, a comparison of the species-specific or species-biased gene expression in the male maxillary palps provides a starting point for further inquiry. For example, male-specific chemosensory genes could play a role in mating biology (e.g., mate recognition), whereas female-specific chemosensory genes are more likely to play roles in behaviors such as oviposition and host-seeking.

The primary role of the maxillary palps in females is the detection of CO_2 (Lu et al. 2007), which is an important host kairomone for mosquitoes (Gillies 1980). The expression of CO_2 receptor genes in males suggests they detect this chemical, although the relatively low expression level suggests they do so with less sensitivity than females. This was not found in *Ae. aegypti*, in which CO_2 sensitivity appears to be similar in females and males (Grant et al. 1995). However, to our knowledge, the expression level of CO_2 receptors has not been compared between the two sexes in this species.

One possibility is that CO_2 detection in males may be useful during nectar seeking. Flowers emit more CO_2 when producing nectar, and hence this could signal an abundant food source (Thom et al. 2004). However, at higher concentrations, CO_2 likely repels male anophelines, as CO_2-baited traps rarely catch any male mosquitoes (e.g., Kweka et al. 2013, McPhatter and Gerry 2017). Carbon dioxide avoidance has been demonstrated in males of *Ae. aegypti,* who avoid airstreams with enhanced with CO_2 in an olfactometer (Bässler 1958).

The capitate pegs on the maxillary palps of *An. coluzzii* females are innervated by three olfactory neurons, designated the cpA, cpB, and cpC neurons (Lu et al. 2007). The cpA neuron expresses the CO_2 receptors, the cpB neuron co-expresses *Orco* and *Or8*, and the cpC neuron co-expresses *Orco* and *Or28* (Lu et al. 2007). These genes are among the most highly expressed chemoreceptors in the male palps as well, and presumably the capitate pegs on the male palps contain the same three neurons. In *An. coluzzii, Or8* is narrowly tuned to detect 1-octen-3-ol (Lu et al. 2007), an odorant found in both the breath and sweat of large herbivores and humans (Hall et al. 1984, Cork and Park 1996) and that serves as an attractant to *Anopheles* and *Aedes* mosquitoes (Takken and Kline 1989). Our results indicate that male *Anopheles* are capable of detecting 1-octen-3-ol as well. Nonetheless, the importance of octenol to host seeking is not entirely clear. For example, *Ae. aegypti* and *An. quadrimaculatus* Say females with removed palps readily locate a human arm (Roth 1951). Additionally, some plants also produce octenol and attract mosquitoes (Syed and Guerin 2004, Impoinvil et al. 2004). Work by Dekel et al. (2016) suggests that 1-octen-3-ol fulfills additional ecological roles beside host detection, as *Or8* in the female palps of non-blood feeding *Toxorhynchites amboinensis* (Doleschall, Diptera: Culicidae) also responds to 1-octen-3-ol.

Or28 is more broadly tuned (Lu et al. 2007). Out of a panel of 82 odorants tested, it shows its strongest response to 2,4,5-trimethylthiazole and acetophenone, two odorants not associated with humans (Lu et al. 2007). Acetophenone is a plant volatile which also elicits responses in other insect species (Wright

et al. 2005). This could suggest a role for *Or28* in nectar seeking (Lu et al. 2007). However, *Or28* also responds to 6-methyl-5-hepten-2-one (sulcatone) (Lu et al. 2007). Sulcatone was recently identified as one of five aggregation pheromones produced by male *An. gambiae s.s.* and *An. arabiensis,* although it is also a constituent of human sweat (Meijerink et al. 2000). A blend of these pheromones attracts males and females from a range of *Anopheles* species, including *An. coluzzii* (Mozūraitis et al. 2020). *An. quadriannulatus* was not included in this study, but given that the effect of the pheromones extends beyond the *An. gambiae* species complex to *An. funestus*, it is reasonable to assume that *An. quadriannulatus* is attracted to these as well. Certainly, the high expression of *Or28* in the male and female palps of both species indicates that its role is conserved both between sexes and species. This is consistent with a role either in nectar seeking or in mating behavior. Interestingly, the expression of *Or8*, *Or28*, and *Gr22* in females increases several-fold between 1 vs. 4 d post-emergence, which correlates with the onset of both host-seeking and mating (Omondi et al. 2015).

In female palps, several neurons express *Orco* without either *Or8* or *Or28* (Lu et al. 2007). It is possible that these neurons co-express *Orco* with the other chemoreceptors that are expressed at low levels (Pitts et al. 2011, Athrey et al. 2017). In *Drosophila*, there is some precedence for the co-expression of *Orco* and *Irs,* as *DmOr35a, DmOrco,* and *DmIr76b* are co-expressed in a subpopulation of coeloconic olfactory sensory neurons. However, this is exceptional, as *Irs* do not generally co-express with *Orco* (Benton et al. 2009). Additional chemoreceptors are expressed in males as well, and a notable difference between males and females is the relatively high expression level of these. At the moment, no information is available on which neurons express these genes in the male palps.

In contrast to the male antennae (Athrey et al. 2020), no chemosensory genes in the palps were male-specific in these species, i.e., all the genes expressed in males are expressed in females as well (Pitts et al. 2011, Athrey et al. 2017). However, several chemosensory genes were species-specific when comparing *An. coluzzii* and *An. quadriannulatus* male palps, which could indicate divergent odor responses between the males of these species. The gustatory receptors *Gr37* and *Gr52* are expressed only in *An. coluzzii*. Intriguingly, the expression of *Gr52* in *An. coluzzii* exceeds that of all other chemoreceptors and the expression of this gene is also exclusive or highly biased towards *An. coluzzii* in male and female antennae, as well as female palps (Athrey et al. 2020). Furthermore, the labella of both sexes of *An. coluzzii* express *Gr52* as well (Saveer et al. 2018). The expression pattern of *Gr37* is less consistent in other olfactory organs, as it is more highly expressed in male *An. quadriannulatus* versus *An. coluzzii* antennae, although at low levels (Athrey et al. 2020). It was not detected in the female antennae or palps of either species (Athrey et al. 2017).

The species-specific expression of particularly *Gr52,* and to lesser extend *Gr37,* suggests they could play a role in divergent behaviors between these two closely related species. On the other hand, both *Gr52* and *Gr37* were also detected in the whole male body by Pitts et al. (2011), suggesting a role beyond olfaction or gustation, such as internal nutrient sensing (Miyamoto et al. 2012). Given these expression patterns, *Gr52* and *Gr37* may perform multiple functions in *An. coluzzii*. Neither gene has an identified ortholog in *Drosophila*, but whereas *Or37* has orthologues throughout the Culicidae, *Gr52* appears to be specific to *Anopheles*.

Both *Or9* and *Gr60* are exclusive to *An. quadriannulatus* in male palps. However, *Or9* is more highly expressed in the female antennae of *An. coluzzii* versus *An. quadriannulatus* (Pitts et al. 2011, Athrey et al. 2017) but at similar levels in male antennae of the two species (Athrey et al. 2020). This gene is not expressed in the female palps of either species (Athrey et al. 2017, Pitts et al. 2017). A de-orphanization study showed that *Or9* has an intermediate tuning breadth and that it is highly sensitive to 3-methylphenol, 4-methylphenol (major component of pig odor and cattle urine), 2-ethylphenol, 4-ethylphenol (yeast), and 2,4,5-trimethylthiazole (fox urine, wheat flour, cooked beef) (Carey et al. 2010). The significance of this receptor in male biology is unclear, but one possibility might be that male *An. quadriannulatus* use host odors to position swarming sites near locations where females may be more abundant.

We know nothing about the function of *Gr60*. It does not have a known ortholog in *Drosophila*, and like *Gr52,* appears to be present only in *Anopheles*. This gene is specific to the maxillary palps of *An. quadriannulatus* in both sexes (Athrey et al. 2017, Athrey et al. 2020), but is absent from their antennae (Athrey et al. 2017, Athrey et al. 2020) and the *An. coluzzii* labellum (Saveer et al. 2018).

Besides the chemosensory genes expressed only in males of one of the two species, several showed differential expression. However, none of these genes stand out as particularly noteworthy. The *An. coluzzii*-biased *Obp19* and *Obp72* are expressed at very low level relative to overall *Obp* expression. Two chemosensory genes, *Ir25a* and *Obp48* were expressed at higher level in *An. quadriannulatus* male palps. *Ir25a* is a co-receptor, whose function has been linked to the detection of amines (Ai et al. 2010, Abuin et al. 2011, Silbering et al. 2011, Pitts et al. 2017), and cool sensing (Ni et al. 2016). However, no other *Irs* were expressed in the male palps for *Ir25a* to form complexes with other than one of the other co-receptors *Ir76b*. *Obp48* is among the most highly expressed *Obp* in the antennae of both sexes in both species (Pitts et al. 2011, Athrey et al. 2017), and is highly expressed in the labella of both sexes of *An. coluzzii* as well. (Saveer et al. 2018).

Our comparison between the male palps of *An. coluzzii* and *An. quadriannulatus* also provides some context for previous work on females. In particular, the expression of *Ir7w, Ir41n,* and *Ir100a* is highly biased towards *An. coluzzii* in female palps, whereas *Ir41a, Gr21,* and *Gr26* are exclusive to *An. quadriannulatus* in female palps (Athrey et al. 2017). Except for *Gr21,* these genes are absent from the male palps of both species, suggesting they may play a role in divergent female odor responses between species. *Gr21* is exclusive to *An. quadriannulatus* in male palps as well, although at a much lower level. Interestingly, *Gr21* is an ortholog of sugar receptors in *Drosophila* and is also expressed in *An. coluzzii* labella in both sexes (Saveer et al. 2018). Its exclusive expression in the palps of *An. quadriannulatus* is somewhat puzzling. In *Drosophila*, sugar receptors are multimeric heteromers (Fujii et al. 2015). No other sugar receptor genes were detected in the palps, so it is not clear that *Gr21* encodes a sugar receptor in *An. quadriannulatus* male palps. In *Drosophila* some sugar receptors are also expressed in olfactory neurons, and it has been speculated that these may dimerize with *Orco* to form a receptor tuned to new ligands (Fujii et al. 2015). This could also be the case in *Anopheles*.

Despite the recent exciting work on aggregation pheromones by Mozūraitis et al. (2020), important questions surrounding the role of chemosensation in anopheline male biology remain outstanding. For example, it is not known if anophelines use a sex pheromone for species recognition, although a close contact pheromone has been postulated (Pitts et al. 2014). The fact that the aggregation pheromones attract various species of the *An. gambiae* complex equally, but that very low levels of hybridization are observed is consistent with the presence of a contact or low volatility pheromone. Chemoreceptors with a species-specific expression profile could play

a role in these. That being said, species recognition within swarms can also be explained at least partially by acoustic cues (Cator et al. 2010, Pennetier et al. 2010). Furthermore, we suspect that males use host odor in positioning mating swarms, although the data supporting this is circumstantial at present (Foster and Takken 2004, Diabaté et al. 2011). Therefore, many of the odor cues that could play an important role in male anopheline biology are expected to be species-specific, and the chemosensory genes that were shown to have species- and/or sex-specific expression in the maxillary palps of these species may be promising candidate genes underlying various components of male mating behavior.

In conclusion, we characterized differences in chemosensory gene expression between the male maxillary palps of *An. coluzzii* and *An. quadriannulatus*. The expression of several chemoreceptors is specific to either *An. coluzzii* or *An. quadriannulatus*. We postulate that these genes may underly species-specific behavior, such as close contact mate recognition. Regardless, the results present here may be useful for future work on the functional divergence of the maxillary palps between the malaria vector *An. coluzzii* and one of its sibling species.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Figure S1. Plot of Log_2FC (Fold Change) against -log^{10}(q-values) between the maxillary palps of *Anopheles. coluzzii* vs. *Anopheles quadriannulatus* males.

Table S1: Mapping statistics for male maxillary palps samples.

Supplemental Data S1: Gene expression (TPM) and differential gene expression data for all genes in the male maxillary palps of *Anopheles coluzzii* vs. *Anopheles quadriannulatus*.

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Data Availability

The sequence data used in this study is deposited on the NCBI Sequence Read Archive, under BioSample accessions SAMN15539281–SAMN15539284. The full gene expression data are available in Supp data S1 [online only].

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