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EDITED BY
Kounbobr Roch Dabiré,
Research Institute for Health Science-Direction Régionale de l'Ouest (IRSS-DRO), Burkina Faso

REVIEWED BY
Merid Negash Getahun,
International Centre of Insect Physiology and Ecology (ICIPE), Kenya
Rajnikant Dixit,
National Institute of Malaria Research (ICMR), India

*CORRESPONDENCE
Constância Flávia Junqueira Ayres
constancia.ayres@fiocruz.br
Tatiany Patricia Romão
tatiany.romao@fiocruz.br

†These authors have contributed equally to this work

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Elevated expression of odorant receptors and odorant-binding proteins genes detected in antennae of *Culex quinquefasciatus* field females

Suzane Alves Santos[1†], Tatiany Patricia Romão[1*†], Elisama Helvecio[1], Rosangela Maria Rodrigues Barbosa[1], Walter Soares Leal[2] and Constância Flávia Junqueira Ayres[1*]

[1]Entomology Department, Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, Brazil, [2]Department of Molecular and Cellular Biology, University of California, Davis, Davis, CA, United States

Culex quinquefasciatus is responsible for the transmission of filarial worms and several arboviruses. Olfaction plays a crucial role in disease transmission as it influences behaviors that are essential for the survival and reproduction of the mosquito, such as the host-seeking behavior, courtship, and oviposition. Understanding the molecular events that coordinate how mosquitoes find their host may lead to alternative methods to reduce diseases transmission. Our aim was to investigate the differential expression profile of *odorant receptor* (*ORs*) and *odorant-binding proteins* (*OBPs*) genes in *Cx. quinquefasciatus* field females compared with CqSLab laboratory mosquito colony. Seventeen genes of interest were evaluated for their qualitative and specific expression by RT-PCR on RNAs extracted from female antennae, female legs, complete male bodies, incomplete female bodies (no head and no legs), and L4 larvae. The general expression mapping of olfactory genes revealed that all analyzed genes were expressed in antennae. Some genes showed different qualitative expression profiles, such as *CquiOR2*, *CquiOR64*, *CquiOR93*, *CquiOBP11*, and *CquiOBP16*, which were expressed exclusively in female antennae. On the other hand, *CquiOR37*, *CquiOBP2*, and *CquiOBP43* are expressed in all sample types, and *CquiOBP10* was expressed in female antennae and legs and in the complete male bodies. The expression of *CquiOBP5* was detected in the female's antennae and body, but it was absent in the legs. The quantitative differential expression analysis of six of the 17 genes by RT-qPCR was performed from RNA samples from antenna pools collected in three physiological states, post-emergence, post-mating, and post-blood feeding of the field females and CqSLab. A total of 3,600 antennae were analyzed, in pools containing 100 pairs. Most genes screened showed a higher expression level in field mosquitoes when compared with the laboratory strain CqSLab. The expression of *CquiOBP5* and *CquiOBP10* genes was significantly different

between the post-mating and post blood-meal samples of laboratory females ($p < 0.05$). Our results suggest specialization of the function of the genes studied and divergence in the expression pattern of field mosquitoes compared with laboratory mosquitoes, and therefore, caution should be exercised in the interpretation of data from laboratory mosquito studies.

KEYWORDS

differential gene expression, RT-qPCR, olfactory genes, mosquito vector, host seeking behavior

Introduction

The southern house mosquito *Culex quinquefasciatus* mosquito is one of the most prevalent vectors of lymphatic filariasis, and females of the species have a hematophagic feeding behavior to complete their gonadotropic cycle, the main characteristic that involves them in the pathogens transmission (1, 2). For insects, the perception of different contexts in the environment depends on their sense of smell, which is considered essential for their survival and reproduction (3). Behavioral aspects such as the search for food, search for a mate, search for oviposition sites, and group communication, whether intraspecific or interspecific, are linked to the olfactory perception of insects (4–6). In mosquitoes, blood supply is a physiological requirement for egg maturation, and it is directly related to olfaction. The main organs directly linked to olfactory perception are the antennae and adaptive evolution resulted not only in morphological variations of this organ but also in functional specialization (3, 7).

In contact with biologically active chemical stimuli, insects respond variably in accordance with biotic or abiotic factors, experience from previous situations, and physiological state (5, 8). The olfactory nervous system is highly conserved among insects and is composed of olfactory receptor neurons (ORNs) and chemosensory proteins responsible for detecting peripheral odor molecules, including odorant receptors (ORs) and odorant-binding proteins (OBPs), which are located inside the sensilla in the antennae (5, 8, 9). The plasticity of the insect's sensory system response occurs through changes in the expression of olfactory genes, activity, or neural structures (5). The response mechanism to odor stimuli occurs when odor molecules penetrate through the sensilla cuticle pores and reach the lumen filled by sensilla lymph, carried by OBPs to the ORN membrane, where these molecules interact with ORs, generating action potentials. From there, the stimulus travels throughout the insect's nervous system to finally culminate in a behavioral response (8, 10).

OR and OBP proteins are responsible for modulating the peripheral olfactory sensory system (3, 8, 10). The ORs are integral membrane proteins that form heterodimers, giving rise to a ligand-dependent ion channel, activated by one or more types of odor molecules (4, 6, 11, 12). When odor molecules bind to ORs, ionic pore opens, providing the depolarization of the ORN and consequent propagation of an action potential (4). In *Cx. quinquefasciatus*, there is a large repertoire of ORs (13). The OBPs are small, soluble, low–molecular weight proteins with excellent stability to temperature variations, proteolysis, and against denaturing agents (14–16). They are found in large quantities in the lymph of insect sensilla and are classified on the basis of the number of amino acid residues found in their primary structure. They are further classified into subfamilies in terms of amino acid sequence and tissue expression. There are currently 109 OBPs annotated in the *Cx. quinquefasciatus* genome (13). These proteins play the important role of carrying hydrophobic odorant molecules through the lymph of the sensilla to the membrane of the ORNs. Previous studies indicate that insect OBPs have a modulating effect on olfactory physiology and on behavior linked to specific odorants (10). To characterize the functions of individual olfactory genes, recent studies have been used several techniques to silence these genes and identify their roles in insect behavior (17, 18).

A mosquito's behavioral responses are directly related to gene expression within its cells. The fluctuation in gene expression through different physiological states is responsible for commanding what behavior will be performed. The example of behavior permutation can be seen after the blood feeding of a female mosquito, and the satiety state will make the female no longer look for hosts to feed on blood but will start the detection of odorants related to the search for good places for oviposition (19). There is a functional correlation between the behavioral and sensory response and the increased expression of chemosensory genes, which suggests a close relationship between the transcription of OR and OBP genes, and the

function of their gene products, which translates into behaviors regulated by the condition physiological effect of each individual within a population of mosquitoes (19).

Considering the large diversification of the two gene families, in the present study, we aimed to describe the expression patterns of OR and OBP genes in a tropical *Cx. quinquefasciatus* population, compared with a laboratory colony, to identify putative subfuncionalization among these gene members. We focused on genes among the most abundantly expressed in the *Cx. quinquefasciatus* antenna described in Leal et al. (13). Our hypothesis here is that these genes may play different roles in crucial biological process for mosquitos' adaptation and survival, and gene expression differentiation could be the first step to observe this. Here, the expression of *OR* and *OBP* genes in different tissues and in three physiological states of *Cx. quinquefasciatus* females was investigated using reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (RT-qPCR) assays. The differential expression of *OR* and *OBP* genes were screened in field mosquito antennae compared with laboratory mosquitoes antenna. Our results showed that four olfactory genes (*CquiOBP2*, *CquiOBP10*, *CquiOR2*, and *CquiOR93*) are significantly more expressed in field populations when compared with the laboratory colony at the post-mating stage. This allow us to speculate that these genes may play a role related to the detection of odorant stimuli in female field mosquitoes after mating, and they could be a target for the development of new methodologies and biotechnological products, which may help reduce disease transmission by interfering with the vector's host-seeking behavior.

Material and methods

Mosquito colonies

Samples of *Cx. quinquefasciatus* were obtained from two colonies: CqSLab, a reference colony from the city of Recife, maintained for more than 10 years under laboratory conditions; and a field population, established from eggs rafts of the mosquito *Cx. quinquefasciatus* collected from two municipalities of Recife (S 8°03′36.6″, W 34°53′21.1″), Mustardinha and Cidade Universitária, both belonging to the Metropolitan region of Recife (MRR) and with high rates of infestation and proliferation by mosquitoes of the species *Cx. quinquefasciatus*. Only the F_1 generation from the field population was used in the present study. All mosquitoes were kept in the insectary of the entomology department under controlled conditions of temperature (26 ± 2°C), humidity (70% ± 20%), and a photoperiod of 12-h light/12-h darkness (20).

OR and *OBP* genes selected

Seventeen genes were selected from two families of chemosensory genes, among those reported in Leal et al. (13) as the most abundant in antenna samples: OR (seven *ORs* genes) and OBP (10 *OBPs* genes). Specific primers for each gene were designed using PrimerSelect and EditSeq softwares, DNASTAR 7.0.0 package (Supplementary Table 1) according to the VectorBase code, published by 13. The *18S ribosomal* gene was used as the endogenous gene (21).

Qualitative tissue-specific gene expression assays of ORs and OBPs by RT-PCR

For the RT-PCR assays, five types of tissue samples were produced in pools: 1) 100 pairs of female antennae; 2) 60 female legs; 3) five complete bodies of male mosquitoes; 4) five incomplete bodies of females (headless and legless); and 5) five fourth instar larvae from the CqSLab colony, to map the tissue-specific expression of target genes through the presence of transcripts. RNA extractions were conducted using TRIzol™ (Invitrogen) following the manufacturer's protocol. Samples were treated with DNase™ (Ambion-Invitrogen), quantified in NanoDrop 2000c spectrophotometer (Thermo Scientific), and then were converted to cDNA with SuperScript™ II RT (Invitrogen) following the manufacturer's protocol and quantified. Finally, reactions were performed with the Taq PCR Master Mix Kit (Qiagen) in Mastercycler® nexus X2 gradient thermal cycler (Eppendorf). The amplified products were analyzed by electrophoresis in 2% agarose gel in 0.5× TBE (Tris-Borate-EDTA running buffer pH 8.0) and stained with ethidium bromide (10 mg/ml).

Tissue collection for RT-qPCR

Experiments to obtain female mosquitoes in three specific physiological states (post-emergence, post-mating, and post-blood feeding) were optimized.

Post-emergence: The experiment consisted of six small cages (20 cm × 15.2 cm × 16 cm), three with field mosquitoes and three with CqSLab mosquitoes, containing 100 female pupae each cage, to obtain newly emerged adult females. The aspiration of the cages was performed with an automatic manual aspirator; the females collected were frozen at −80°C, after 20 min, and transferred to petri dishes to avoid losing the antennae. The antennae were dissected using entomological tweezers and placed in 1.5-ml microtubes, to produce homogenates

containing 100 antennae pairs. The samples were macerated with a plastic pestle, and finally, the homogenates were stored at −80°C.

Post-mating: The experiment consisted of six small cages, three cages with field mosquitoes and three cages with CqSLab mosquitoes, containing 100 females and 200 males each, in the adult phase. After 7 days of contact, mosquitoes were captured with an automatic manual aspirator, and only females were selected and frozen at −80°C. The insemination status was verified through the dissection and analysis of the spermatheca, fixed and stained with Giemsa in a ZEISS West Germany Axiophot optical microscope, present at the Nucleus of Technological Platforms (NPT-IAM) under 20× to 40× objectives. Only the antennae of inseminated females (positive sperm) were dissected to produce homogenates, composed of 100 antennae pairs of inseminated females and 100 µl of ultra-pure water. Lastly, the homogenates were stored at −80°C.

Post blood feeding: The experiment consisted of six small cages, three cages containing field mosquitoes and three cages containing CqSLab mosquitoes, each one with 100 females and 200 males. After 7 days of contact, they received blood meal and defibrinated rabbit blood (*Oryctolagus cuniculus*) in an artificial apparatus. The engorged females were captured 2 h after the blood meal with an automatic manual aspirator and frozen at −80°C. The antennae were dissected with entomological forceps, and the homogenates were prepared with 100 pairs of antennae for each cage used and 100 µl of ultra-pure water.

Evaluation of the *OR* and *OBP* genes differentially expressed in three physiological states through RT-qPCR

After RT-PCR assays, nine genes (*OR2*, *OR93*, *OBP16*, *OR64*, *OBP11*, *OBP2*, *OBP5*, *OBP10*, and *OR151*) were tested by real-time quantitative reverse transcription PCR (RT-qPCR) using absolute quantification. These genes were selected among the 17 genes analyzed by RT-PCR based on tissue specificity; *OR2*, *OR64*, *OR93*, *OBP11*, and *OBP16* were exclusively expressed in antenna; and the remaining displayed a diverse qualitative expression profile (*OBP2*, *OBP5*, *OBP10*, and *OR151*). From these nine genes, only a subset of six genes (*OBP2*, *OBP5*, *OBP10*, *OBP11*, *OR2*, and *OR93*) resulted in good PCR efficiency to be used in real-time qPCR assays according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (22) and was selected for one-step quantitative RT-PCR, using relative quantification. These genes have been shown to play roles in the physiological states progression in insects such as reproduction, pheromone perception, oviposition, and others (23–28). The evaluation of the differential expression of the target genes was conducted between three physiological states of *Cx. quinquefasciatus* females. Total RNAs were extracted from pools of 100 pairs of antennae of female mosquitoes from the CqSLab colony and from the field colony, with TRIzol™ (Invitrogen), according to the manufacturer's instructions. RNA samples were treated with DNase™ (Ambion-Invitrogen) followed by quantification in Qubit™ 3.0 Fluorometer (Thermo Scientific). Samples were normalized at concentration of 10 ng. The *18S* ribosomal gene was used as the endogenous control of reactions. The RT-qPCR experiments were carried out in a ABI 7500 real-time PCR system (Applied Biosystems), samples were tested in biological triplicate with technical replicates, and relative quantification analyses were performed by the $2^{-\Delta\Delta CT}$ method (29), with post-emergence CqSLab used as a calibrator sample in analyses, through the 7500 software version 2.0.4 (Applied Biosystems). Statistically significant differences in gene expression by RT-qPCR were evaluated with unpaired t-test, using the GraphPad Prism 8 software.

Results

RT-PCR

Transcripts of all the 17 genes evaluated were detected by RT-PCR, producing PCR products with the expected size. All tested genes were expressed in antenna, but five of them showed a tissue-specific transcription being exclusive to antennae, and they were *CquiOR2* (CPIJ002479), *CquiOR64* (CPIJ006216), *CquiOR93* (CPIJ039866), *CquiOBP11* (CPIJ006551), and *CquiOBP16* (CPIJ012715). Three genes—*CquiOR37* (CPIJ004163), *CquiOBP2* (CPIJ007617), and *CquiOBP43* (CPIJ017326)—were ubiquitously expressed and displayed expression in all samples analyzed (female antennae, female legs, incomplete female bodies, whole males, and L4 larvae), and the remaining genes—*CquiOBP3* (CPIJ007611), *CquiOBP5* (CPIJ007608), *CquiOBP7* (CPIJ001365), *CquiOBP10* (CPIJ013976), *CquiOBP23* (CPIJ001876), *CquiOBP46* (CPIJ010782), *CquiOR121* (CPIJ014392), *CquiOR125* (CPIJ015178), and *CquiOR151* (CPIJ017911)—produced transcripts in at least two tissues. Seven genes (CquiOR37, *CquiOR151*, *CquiOBP2*, *CquiOBP3*, *CquiOBP7*, *CquiOBP10*, and *CquiOBP43*) were expressed in both males and females (Figure 1).

Differential gene expression by RT-qPCR at female adult life stages

In general, the RT-qPCR results showed that most genes, apart from *OR93* at post-blood feeding, were predominantly more expressed in the field samples when compared with the laboratory colony CqSLab (Figure 2A–C). This result was statistically significant ($p < 0.05$) for four genes (*OBP2*, *OBP10*, *OR2*, and *OR93*) expressed at higher levels in female antennae

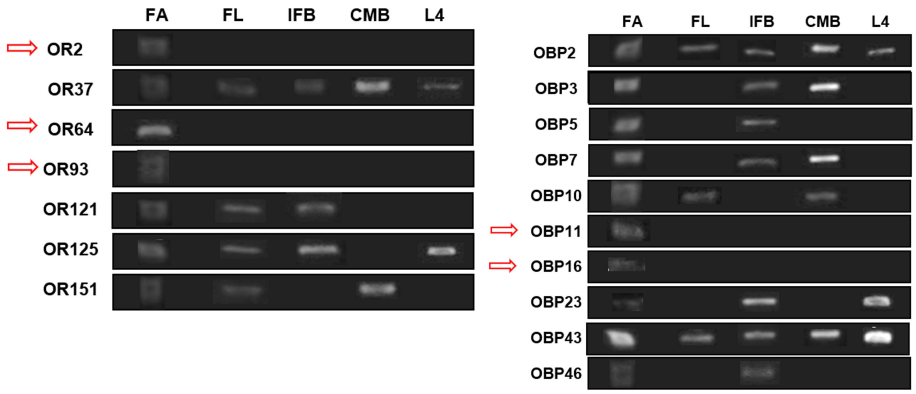

FIGURE 1
Overview of *OR* and *OBP* genes transcripts, observed through RT-PCR. The image consists of the clipping of all agarose gels performed, showing the bands related to the amplified genes in each tissue sample evaluated. FA, female body; FL, female leg; IFB, incomplete female body; CMB, complete male body; L4, fourth instar larvae. The red arrows indicate the genes with transcripts exclusively in the antennae of female *Cx. quinquefasciatus*.

from the post-mating stage in field samples, one gene at post-emergence (*OBP11*), and one gene (*OBP10*) at post-blood feeding (Figure 2). There was no significant difference of expression among the three physiological stages for both laboratory and field samples, with the exception of two genes (*OBP5* and *OBP10*), which were expressed at significantly higher levels ($p < 0.05$) in female antennae at the post-blood feeding stage, compared with the post-mating stage in laboratory colony (Figure 3C). In addition, when comparing CqSLab collected after emergence with after mating, *OBP10* was down regulated, and *OBP5* was upregulated after blood meal (Figures 3A, B, respectively).

Discussion

In the present study, we investigated a subset of olfactory genes in *Cx. quinquefasciatus* that may be associated with important traits such as host-seeking behavior. Our results showed that, in general, genes were differentially expressed in field caught mosquitoes compared with the laboratory colony in at least one of the physiological states studied, except for the *OBP5* gene, showing that the colonization process when founding a colony in the insectary impacted on the mechanisms regulating its expression.

The results of RT-PCR showed a great variety of expression pattern for the 17 genes, with only three being ubiquitously expressed in all tissues analyzed. All the remaining genes were specific to few tissues, but all of them were expressed in antennae, corroborating with Leal et al. (13) results, although they have used a colony of *Cx. quinquefasciatus* collected in the USA and colonized in a laboratory in 1950. They have observed differential expression of olfactive genes using RNA-seq in this same mosquito species, with great expression in antennae samples. These data highlight the specialization of function in the members of this important gene family.

Like other mosquito species, *Cx. quinquefasciatus* females acquire a blood meal after mating, and this behavior is directly related to the variation in the gene expression of their olfactory system, between one physiological state and another, stimulating the behavior of searching for a host to complete its gonadotropic cycle (1, 5, 30). In this study, in addition to observe the expression of *OR* and *OBP* genes at different tissues, we have investigated the expression of six genes by RT-qPCR at three different moments of adult female's life. Here, we were able to detect variation in gene expression among the different life stages for *OBP5* and *OBP10* genes in the laboratory colony, providing information for future studies, which will be required for demonstrating the ecological significance of such variation in transcript levels. Studying the differences of gene expression profile along life development and over adult life is important to better understand vectorial capacity in mosquito populations. Analysis of microarray studies in *Anopheles gambiae* revealed that specific genes are differentially expressed, displaying an age-dependent transcriptional level, which could be used to determine the age of mosquitoes (31). These changes in mosquito females' adult life could interfere in the blood-feeding pattern or the interaction with repellent products. Indeed, we have previously demonstrated that the age of *Aedes aegypti* females affect mosquito responses to DEET repellent (32). A previous study showed that *OBP5* was involved with transportation of a human-derived attractant nonanal and the insect repellent picaridin, suggesting the link of this *OBP* with the detection of blood source. There was also affinity between OBP5 and the oviposition pheromone MOP (24).

Most studies with the *OR* and *OBP* genes in mosquito species are limited to identification and genomic

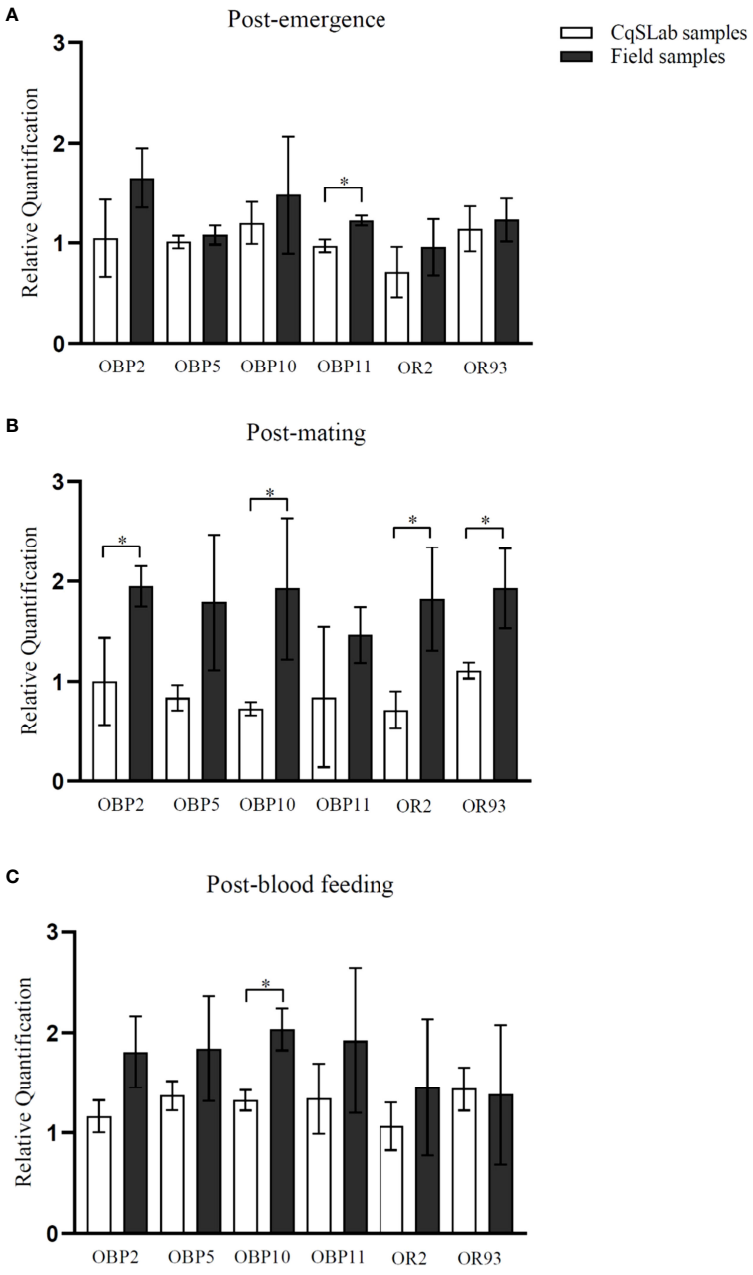

FIGURE 2
Graphs per physiological state generated from RT-qPCR assays with genes *CquiOBP2*, *CquiOBP5*, *CquiOBP10*, *CquiOBP11*, *CquiOR2*, and *CquiOR93*: **(A)** Post-emergence; **(B)** Post-mating; **(C)** Post-blood feeding. Increased expression is noted in most genes. Five genes showed a significant increase in expression ($p < 0.05$) in antennae samples from field females compared with laboratory samples, four (*CquiOBP2*, *CquiOBP10*, *CquiOR2*, and *CquiOR93*) in the post-mating physiological state, and one (*CquiOBP11*) in the post-emergence and *OBP10* again in the post-blood feeding. Bars indicate standard deviation. (*) Statistically significant ($p < 0.05$).

characterization (33–36). More recently, some studies are now focused on characterizing its function in mosquito biology. Taparia etal. (1) have demonstrated that members of the *OR8* gene subfamily are regulated in response to blood-feeding and are possibly involved with host-seeking behavior in the *Cx. quinquefasciatus* species. Likewise, Wu et al. (2) found a positive correlation between the expression of the two genes *CquiOR114/117* and the blood-feeding behavior. They found variation of the expression level of both genes at different days after emergence of mosquitoes and the correlation was demonstrated by silencing the genes using the RNA interference technique. Similarly, a study using

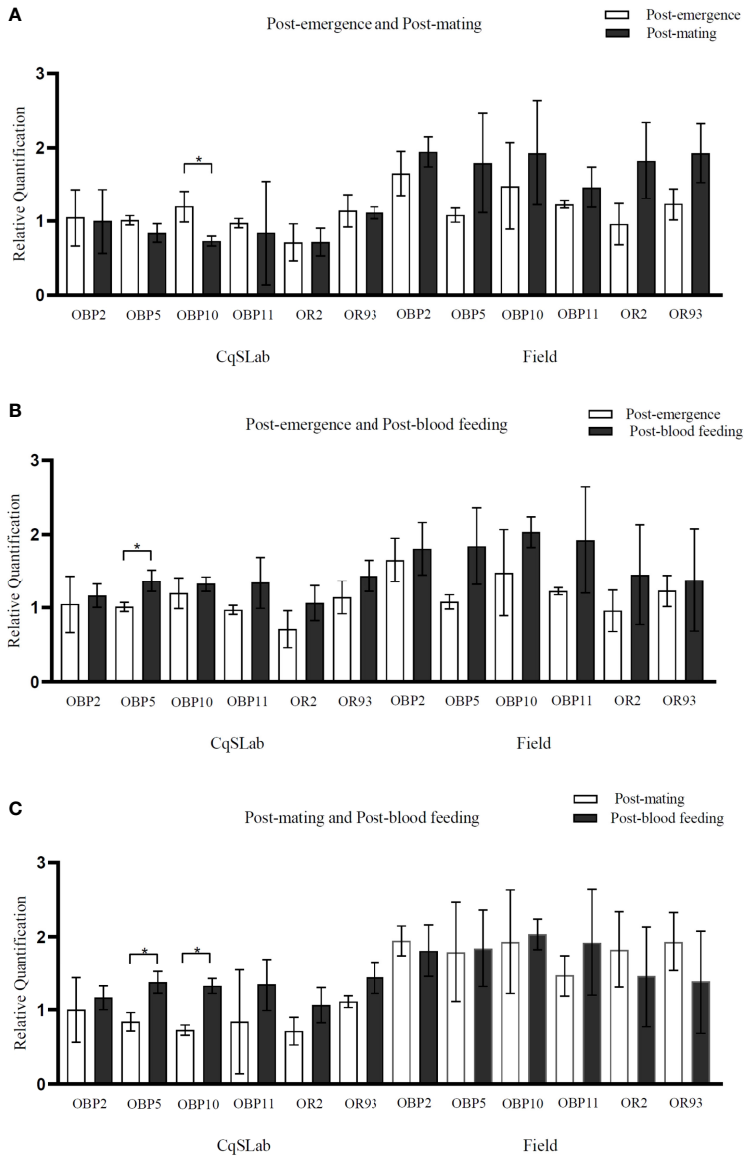

FIGURE 3
Graphs generated from RT-qPCR assays with genes *CquiOBP2*, *CquiOBP5*, *CquiOBP10*, *CquiOBP11*, *CquiOR2*, and CquiOR93 comparing the physiological stages post-emergence and post-mating **(A)**, post-emergence and post-blood feeding **(B)**, and post-mating and post-blood feeding **(C)** in laboratory colony and field population.The genes *OBP5* and *OBP10* were expressed at significantly higher levels ($p < 0.05$) in CqSLab female antennae at the post blood feeding stage, in comparison with the post-mating stage **(C)**. The gene *OBP10* was expressed at significantly lower level ($p < 0.05$) in CqSLab female antennae at the post-mating stage in comparison with the post-emergence stage (A). The differential expression of genes analysed comparing the physiological states in the field colony was not statistically significant. Bars indicate standard deviation. (*) Statistically significant ($p < 0.05$).

CRISPR/Cas9-mediated silencing of two genes in *Ae. aegypti* (*OBP10* and *OBP22*) showed its involvement in the host-seeking behavior (26). Interestingly, the authors also demonstrated that those OBPs have a pleiotropic effect on several processes important for mosquito vectorial capacity, such as reproduction and arbovirus transmission.

Recently, the higher expression of an *OBP* gene in a *Culex quinquefasciatus* resistant colony was, for the first time, associated to insecticide resistance (37). This suggests a new mechanism of insecticide resistance, and therefore, more studies should be conducted to identify OBP role in the development of insecticide resistance. In the present study, we have identified

eight OBPs that are expressed in the legs or female bodies and could be further investigated regarding its possible interaction with insecticides.

Culex quinquefasciatus has 109 OBP genes and 180 OR genes annotated (13, 35), which represent an expansion in comparison with the 131 OR in the other domestic mosquito *Ae. aegypti* (38). Regarding the OBP genes, 111 were found in *Ae. aegypti*; however, only 19 direct orthologs were observed between these two species (35). Although both species are domestic mosquitoes, *Cx. quinquefasciatus* has special features that are probably associated to its great diversity of olfactory genes, such as egg laying in a wide variety of polluted and non-polluted larval habitats and the ability to feed on diverse animal species, including human and birds (39). Considering its great vectorial capacity for transmitting several pathogens such as nematodes, virus, and protozoan and that it is a cosmopolitan species, in contrast to *Ae. aegypti* distribution (present only in tropical and sub-tropical areas), studying the functional characterization of olfactory genes is a big challenge. Divergence of gene expression pattern for olfactory genes has been observed even for subspecies. In a very recent paper, Gu et al. (18) identified genes (*OR5* and *OR65*) that were expressed in a subspecies-specific way and demonstrated through RNA interference that two olfactory genes could inhibit the blood-feeding behavior of *Cx. quinquefasciatus*. Our data show that studies for the functional characterization of olfactory genes in mosquitoes using laboratory strains should analyze the data with caution, as the results for natural populations may differ considerably. Furthermore, the expression pattern of the OR and OBP gene repertoire may vary according to each ecological niche.

We are all witnessing the impact of global warming on the environment and on the lives of different species of organisms, particularly on mosquitoes; it will probably increase its density and distribution (40–42). The current mosquito control measures have not been successful enough to reduce the number of cases of vector-borne diseases in endemic areas; in this sense, personal protection measures emerge as an important tool to prevent the increase in disease transmission. The present study opens up new research possibilities to better understand the genetic basis of mosquito olfaction.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SS: conception of the study, data acquisition, analysis of data, and manuscript writing; TR: conception of the study, experimental execution support, data analyses, and manuscript writing and revision; EH: acquisition of data, experimental execution support, and data analyses; RB: experimental execution support; WL: data analysis, manuscript revision, and intellectual contributions. CJ: intellectual contributions, manuscript writing and revision, and financial support. All authors reviewed the paper and agreed with the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2022.874727/full#supplementary-material

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