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RAPID COMMUNICATION

# Knockdown of a Mosquito Odorant-binding Protein Involved in the Sensitive Detection of Oviposition Attractants

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**Abstract** Odorant-binding proteins (OBPs) were discovered almost three decades ago, but there is still considerable debate regarding their role(s) in insect olfaction, particularly due to our inability to knockdown OBPs and demonstrate their direct phenotypic effects. By using RNA interference (RNAi), we reduced transcription of a major OBP gene, *CquiOBP1*, in the antennae of the Southern house mosquito, *Culex quinquefasciatus*. Previously, we had demonstrated that the mosquito oviposition pheromone (MOP) binds to CquiOBP1, which is expressed in MOP-sensitive sensilla. Antennae of RNAi-treated mosquitoes showed significantly lower electrophysiological responses to known mosquito oviposition attractants than the antennae of water-injected, control mosquitoes. While electroantennogram (EAG) responses to MOP, skatole, and indole were reduced in the knockdowns, there was no significant difference in the EAG responses from RNAi-treated and water-injected mosquito antennae to nonanal at all doses tested. These data suggest that CquiOBP1 is involved in the reception of some oviposition attractants, and that high levels of OBPs expression are essential for the sensitivity of the insect's olfactory system.

**Keywords** RNA interference · *Culex quinquefasciatus* antennae · CquiOBP1 · EAG · Oviposition attractants · MOP · Skatole · Indole

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## Introduction

Odorant binding proteins (OBPs) were identified almost three decades ago (Vogt and Riddiford 1981), but their roles in insect olfaction are still a matter of considerable debate. That OBPs are involved in odorant reception was disputed after odorant receptors (ORs) were demonstrated to respond to semiochemicals when expressed in heterologous systems These expression systems, however, have limitations in addressing the role(s) of OBPs in olfaction. The heterologous expression system that uses *Drosophila* empty neurons (Dobritsa et al. 2003) includes surrogate OBPs, i.e., OBPs expressed in the ab3 sensilla, whereas in non-insect cell systems[1] (Forstner et al. 2009) odorants are solubilized with organic solvent or with the addition of recombinant OBPs. Thus, ultimately the role(s) of OBPs in insect olfaction must be addressed by examining insects with reduced levels (knockdowns) or devoid of a test OBP (knockouts). In *Drosophila*, analysis of a mutant defective for expression of an OBP revealed that DmelOBP76a (aka LUSH) is required for the activation of pheromone sensitive neurons by (*E*)-11-vaccenyl acetate and associated behavior (Xu et al. 2005), but other insect species are not amenable to this type of genetic manipulation. Previously, we employed the empty neuron system of *Drosophila* to express the pheromone receptor from the silkworm moth, *Bombyx mori*, BmorOR1 alone or co-expressed with a pheromone-binding protein, BmorPBP1 (Syed et al. 2006). Despite the low levels of BmorPBP1 expression in this heterologous system, we demonstrated clearly that PBPs enhance the sensitivity of the insect olfactory system (Syed

[1] We apologize for not being able to cite all the relevant literature due to reference limitations of a rapid communication.

et al. 2006). Recently, it was shown that addition of a recombinant PBP to a heterologous system that expresses a pheromone receptor from *Antheraea polyphemus* increases both sensitivity and selectivity (Forstner et al. 2009).

Given that our previous attempts to knockdown PBP expression in the silkworm moth were unsuccessful (Leal and Ishida, unpublished data), we explored knocking down OBP expression in mosquitoes. We then focused on CquiOBP1, which is highly expressed in the antennae of the Southern house mosquito *Culex pipiens quinquefasciatus* (=*Cx. quinquefasciatus*) (Ishida et al. 2002). Recently, CquiOBP1 was shown to bind a mosquito oviposition pheromone (MOP) (Laurence and Pickett 1982) in a pH dependent manner and to be expressed in antennal sensilla sensitive to this pheromone (Leal et al. 2008). In the present study, we used CquiOBP1 as a target in RNA interference (RNAi) experiments to examine its function in the reception of oviposition attractants. Mosquitoes injected with double strand RNA (dsRNA) showed reduced levels of *CquiOBP1* transcripts as well as reduced antennal responses to MOP, skatole, and indole when compared to water-injected controls. Interestingly, antennal response to nonanal, a major host cue detected with extremely high sensitivity by *Cx. quinquefasciatus* antennae (Syed and Leal 2009), was not significantly affected. These findings suggest that CquiOBP1 is involved in the detection of multiple oviposition attractants and plays a key role in the sensitivity of the mosquito olfactory system.

## Methods and Materials

*CquiOBP1 RNA Interference* Full-length CquiOBP1 dsRNA was synthesized by in vitro transcription from purified PCR product that contained T7 promoter sequences in inverted orientations and purified by using RNeasy MinElute Cleanup Kit (Qiagen). Approximately 100 nl (350 ng) of dsRNA were injected through the intersegmental thorax membranes into 1-to-48 h-old *Cx. quinquefasciatus* female mosquitoes with a microINJECTOR™ System MINJ-1 (Tritech Research, Los Angeles, CA, USA). dsRNA-injected, water-injected, and non-injected mosquitoes were generated. Individual female heads were dissected in liquid nitrogen 4 d post-injection, RNA from each head was extracted with RNeasy Mini Kit (Qiagen), and individual cDNAs were synthesized from 0.1 µg of RNA using 100u SuperScript® II reverse transcriptase (Invitrogen). Real-time quantitative PCR (qPCR) was carried out by using EXPRESS SYBR® GreenER™ qPCR Super-Mix Universal (Invitrogen) in a final volume of 20 µl. Reactions were run with a standard cycling program, 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min, on an AB7300 real-time PCR system (Applied Biosystems). Determination of transcripts abundance was based on two independent replicates for each sample. CquiOBP1 expression was normalized to the expression levels of an endogenous control, the ribosomal protein that encodes gene S7 (CquiRpS7). Relative quantification analysis based on the comparative $C_t$ method ($\Delta\Delta C_t$) was performed using AB7300 system SDS software (Applied Biosystems). Non-injected mosquitoes were used for calibration purposes. Non quantitative PCR was carried out from the same cDNAs by using 2u GoTaq® DNA polymerase (Promega) in a final volume of 25 µl. CquiRpL8 amplification was used as a control of cDNA integrity.

*Electrophysiological Recordings* An excised head of an adult *Cx. quinquefasciatus* female was mounted on a Syntech EAG platform equipped with micromanipulator-12 and a high-impedance AC/DC preamplifier (Syntech, Germany). Chloridized silver wires in drawn-out glass capillaries filled with 0.1% KCl and 0.5% polyvinylpyrrolidone (PVP) were used for reference and recording electrodes. The recording electrode accommodated the two antennae of the excised head after the tips of the antennae were clipped to provide a better contact. Preparation was bathed in a high humidity air stream flowing at 20 ml/s to which a stimulus pulse of 2 ml/s was added for 500 ms. Any change in antennal deflection induced by the stimuli or control puffs was recoded for 10 s. Indole and 3-methyl indole (skatole) were purchased from Acros (USA) and were 95% pure; nonanal (99%) was from Sigma-Aldrich; racemic 6-acetoxy-5-hexadecanolide (MOP) was a gift from Bedoukian Research Incorporated, USA. Chemicals were dissolved in dichloromethane (DCM), wt/vol, to make a stock solution of 10 µg/µl and decadic dilutions were made. An aliquot (10 µl) of a stimulus was loaded onto a filter paper strip, the solvent was evaporated for 30 s, and the strip was placed in a 5 ml polypropylene syringe from which various volumes were dispensed. Solvent alone served as control. Data presented are from a pool of mosquitoes injected and tested in three different batches on different days. In each session, EAG responses of at least three of RNAi-treated and water-injected mosquitoes were recorded.

## Results and Discussion

We employed a combination of RT-PCR and real-time quantitative PCR (qPCR) to examine mRNA levels of *CquiOBP1* in heads of RNAi (dsRNA-injected) and control (water-injected, non-injected) mosquitoes using *CquiRpS7* as a control gene. RT-PCR analysis showed a clear reduction of *CquiOBP1* transcript levels in dsRNA-injected mosquitoes, as compared to water-injected and non-injected mosquitoes (Fig. 1a). We then examined by

electroantennogram (EAG) the responses of sham-and RNAi-treated female mosquitoes to oviposition attractants. Silencing the *CquiOBP1* gene clearly affected antennal responses to MOP and indole, a putative oviposition attractant (Millar et al. 1992) (Fig. 1b), but the response to nonanal was not significantly affected. Next, we quantified the reduction of transcripts by qPCR (Fig. 1c), which confirmed the trend observed by a semi-quantitative method (Fig. 1a). dsRNA-injected mosquitoes displayed reduction of *CquiOBP1* transcript levels (average 59.9%) when compared to both water-injected (sham-treated) mosquitoes (average 97.3%) and non-injected controls (normalized to 100%). dsRNA-Injected individuals displayed significant reduction of *CquiOBP1* transcripts (47% to 65%) (Fig. 1c). Furthermore, water-injected and non-injected mosquitoes displayed almost equivalent levels of *CquiOBP1* transcripts, thus demonstrating that RNAi treatment is responsible for the observed reduction of *CquiOBP1* mRNA levels (Fig. 1c). This partial silencing of *CquiOBP1* shown by qPCR analysis demonstrates the feasibility of significantly reducing even highly expressed olfactory genes like OBPs by using the RNAi approach.

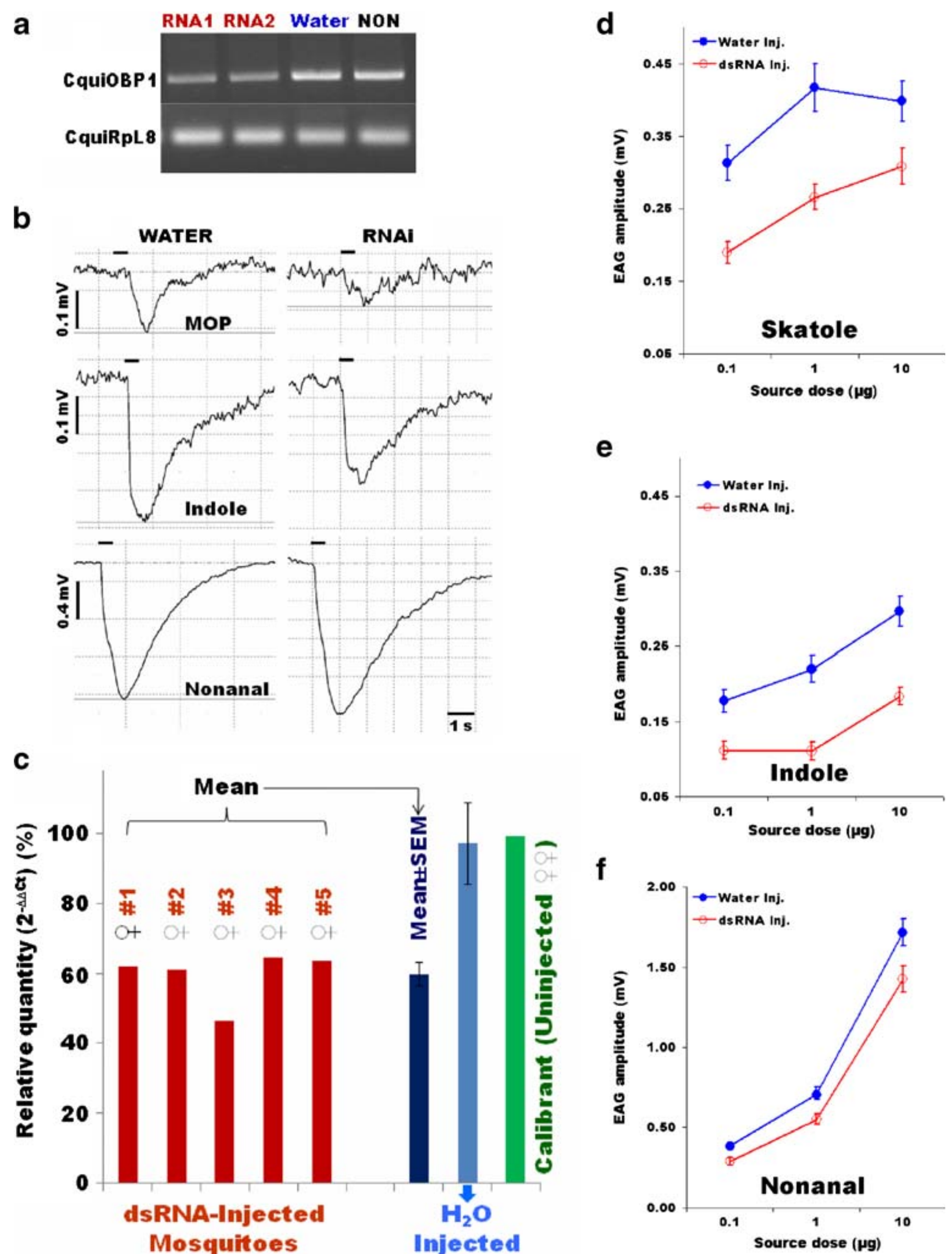


**Fig. 1** PCR and EAG data. **a** RT-PCR analysis indicating that *CquiOBP1* transcripts were reduced in RNAi-treated females (RNA1 & RNA2) when compared to the transcript levels in water-injected (Water) and non-injected (NON) females. CquiRpL8, control gene. **b** EAG traces recorded from antennae of water- and RNAi-treated female mosquitoes challenged with MOP (100 µg), indole (10 µg), and nonanal (10 µg). Bars on the top of traces indicate the duration of the 500 ms stimulus. **c** Relative expression of *CquiOBP1* by qPCR using EXPRESS SYBR® Green ER™. RNAi-treated, water-injected, and non-injected mosquitoes (each $N=5$). **d**, **e**, **f** Dose-response EAG curves for skatole, indole, and nonanal, respectively ($N \geq 10$). The scale for skatole (**d**) and indole (**e**) graphics is the same, but the high sensitivity of nonanal (**f**) required a different scale

Correlation with EAG data (Fig. 1b) also suggests that ~50% transcripts reduction is enough to generate reduced responses to several semiochemicals.

Finally, we compared the responses of sham- and RNAi-treated female mosquitoes to various doses of these oviposition-related compounds. EAG responses of RNAi-treated females to MOP were below the detection limit, but the dose required to generate consistent EAG signals with water-treated or untreated mosquitoes was high (100 µg). In contrast, reduction of *CquiOBP1* transcripts led to a significantly reduced response to skatole ($N=10$, $P<0.05$) at all doses tested (Fig. 1d). Likewise, EAG responses to indole by RNAi-treated females were significantly lower than the responses recorded from water-treated female mosquitoes at all doses tested (Fig. 1e). Lastly, we observed an apparent trend towards smaller EAG responses to nonanal by RNAi-treated compared water-treated female mosquitoes, but the differences were not significant (Fig. 1f).

The simplest explanation for these findings is that OBPs play an important role for the sensitivity of the insect's olfactory system. Although we were not able to completely silence *CquiOBP1*, probably because of the high level of transcription, the partial knockdown clearly affected antennal response to physiologically relevant compounds. Previously, we demonstrated by in vitro assays that CquiOPB1 binds MOP in a pH-dependent manner, and we showed its expression in antennal sensilla sensitive to this oviposition attractant (Leal et al. 2008). These RNAi experiments are the first evidence in vivo that CquiOBP1 is involved in the reception of *Culex* mosquito oviposition attractants. Although it is tempting to speculate that CquiOBP1 is selective because responses to nonanal were not significantly different in sham- and RNAi-treated mosquitoes (Fig. 1f), the level of transcript reduction achieved by our RNAi treatments may not be high enough to affect EAG responses of semiochemicals such as nonanal for which the olfactory system responds with remarkable sensitivity (Syed and Leal 2009). By contrast, the reduced levels of *CquiOBP1* transcripts affected the responses of compounds with higher thresholds, thus allowing us to conclude that CquiOBP1 is indeed involved in the detection of oviposition attractants, and that high levels of OBPs expression are essential for the sensitivity of the insect's olfactory system.

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**Note added in proof:** Since this manuscript was accepted for publication, the authors became aware of a report by Biessmann et al. describing that RNAi is effective in knocking down accumulation of OBP1 transcripts in the antenna of *Anopheles gambiae* mosquitoes. Their results will be reported in the following paper in PLoS ONE: Harald Biessmann, Evi Andronopoulou, Max R. Biessmann, Vassilis Dourisb, Spiros D. Dimitratos, Elias Eliopoulos, Patrick M. Guerin, Kostas Iatrou, Robin W. Justice, Thomas Kröber, Osvaldo Marinotti, Panagiota Tsitoura, Daniel F. Woods, Marika F. Walter. The *Anopheles gambiae* Odorant Binding Protein 1 (AgamOBP1) mediates indole recognition in the antennae of female mosquitoes. IN PRESS.

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