Gene Expression Profiles of Odorant Binding Proteins in *Glossina brevipalpis* (Diptera: Glossinidae)[1]

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Abstract *Glossina* spp. (Diptera: Glossinidae) vector trypanosomes, the causal pathogens of trypanosomiasis in humans and animals. *Glossina* spp. chemosensory mechanism is relevant to understanding the behavioral responses of these insects to environmental cues. Odorant binding proteins (OBPs) are involved in either olfactory or nonolfactory cue sensation depending on their spatial and temporal expression. In most insects, the OBPs are ubiquitously expressed, although the levels are higher in the antennae. This study was aimed at determining the differential gene expression patterns of OBP genes in the developmental stages (larval, pupal, teneral) and differentially starved adults of *Glossina brevipalpis* (Newstead) collected from Shimba Hills National Park, Kenya. Five OBP genes, *GbrOBP2*, *GbrOBP6*, *GbrOBP7*, *GbrOBP8*, and *GbrOBP13*, were selected for quantitative PCR expression analysis as representatives of the three OBP classes in *Glossina* spp. (classic, minus-C, classic-dimers). Antennal samples from the newly emerged insects (tenerals) served as the controls. *GbrOBP2* was highly expressed in both larval and pupal stages, whereas *GbrOBP7* was expressed in the pupal stage. *GbrOBP8*, *GbrOBP13*, and *GbrOBP6* showed high expression in adult antennas. Expression of OBPs in the immature stages suggested OBP involvement in nonolfactory chemical sensation, whereas that in adult antennae was attributed to olfaction.

Key Words *Glossina brevipalpis*, larvae, pupae, tenerals, odorant binding proteins, qPCR

Glossina spp. (Diptera: Glossinidae) are the only known biological vectors of African trypanosomes, the pathogens responsible for human African trypanosomiasis, or sleeping sickness, and animal African trypanosomiasis, or nagana, in livestock (Bouyer et al. 2006). The disease puts approximately 70 million people and 60 million cattle within 37 countries of sub-Saharan Africa at risk of infection (Cecchi and Mattioli 2009, WHO 2013). Additionally, nagana transmitted by *Glossina* spp. lowers agricultural and livestock production, resulting in annual losses estimated at US$5 billion (Samdi et al. 2011). All *Glossina* spp. are

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considered potential vectors of trypanosomes. Control strategies targeting the vector such as sterile insect technique, use of odor-baited traps, and insecticide-treated targets are suitable to reduce the transmission of trypanosomes (Omolo et al. 2009, Abda-Alla et al. 2013). However, the molecular mechanism by which different *Glossina* spp. respond to various hosts is not clearly understood. For instance, Gikonyo et al. (2002) reported a preferential feeding of *Glossina pallidipes* (Austen) toward water buffalo and oxen as opposed to waterbuck.

The study reported herein focused on profiling the expression of putative odorant binding protein (OBP) genes in *Glossina brevipalpis* (Newstead), a species of *Glossina* associated with forested habitats that is known to transmit animal trypanosomiasis (Krafsur 2009). The species occurs mostly in Central and West Africa but is also widely found in Southern and Eastern Africa, with discontinuous occurrence in Mozambique, northern Tanzania, and southeastern Kenya (Krafsur 2009, Cecchi et al. 2008).

Olfactory organs in *Drosophila melanogaster* (Meigen) are the antennae and the maxillary palps, which house about 1,200 and 120 olfactory receptor neurons (ORNs), respectively. The ORNs have sensilla that are divided morphologically into basiconic, trichoid, and coeloconic, and they function in the detection of fruit odors, pheromones, organic acids, and amines, respectively. Once stimulated, the ORNs generate action potential relative to the quality and intensity of the stimulus that activates the higher centers in the brain. The dendrites of up to four ORNs are found in each sensillum (Hallem et al. 2004, Hallem and Carlson 2006). OBPs occur inside the sensilla with their expression varying across the different sensilla classes and cell types. The occurrence of OBPs within the sensilla is abundant and diverse. More so, their expression is sensillum specific. The basiconic, coeloconic, and trichoid sensilla in *Drosophila* were found to express at least one abundant OBP, signifying their relevance in sensilla information coding (Larter et al. 2016).

OBPs are small globular proteins of 120–150 amino acids with molecular weights of 14–16 kDa and contain a signal peptide (Zhou et al. 2008). They are water soluble and extracellular proteins that are secreted by nonneuronal support cells into the sensilla lymph (Kim and Smith 2001). In insects, OBPs are mainly classified into four subfamilies defined by the occurrence of conserved cysteine residues, which influence the tertiary folding (Hekmat-Scafe et al. 2002). These include classic (conserved six cysteine signature), plus-C (more than six cysteines plus a conserved proline), minus-C (lacking the second and the fifth cysteine residues), and classic-dimers (two classical OBPs) (Fan et al. 2011). Zhou et al. (2008) reported that most OBPs belong to the classic subfamily. No plus-C OBPs were identified in *Glossina* spp. (Macharia et al. 2016). Insect OBPs consist of residues that are very divergent within and across species (Pelosi et al. 2017) but with a conserved tertiary structure suggesting a conserved function.

During olfaction, the odorants from the environmental cues (i.e., sex, alarm, or aggregation pheromones) enter into the sensilla through the cuticle pores. While inside, they bind OBPs that transport them to the receptors on the ORNs. Action potential is generated and the signal is carried to the antennal lobe, the primary olfactory center in the brain. The signal is then transmitted to higher brain centers, which include the mushroom bodies and the lateral protocerebrum (Gadenne et al. 2016). This causes them to elicit olfactory-mediated behaviors, (i.e., identification of hosts and mates, evading predators, and location of suitable resting sites) (Vieira

and Rozas 2011, Masiga et al. 2014). The OBPs may also be involved in binding lipophilic odorant molecules or in deactivation and clearing of odors after stimulation (Gong et al. 2009).

Genome annotations have revealed that OBPs vary in number among insects. For instance, reports on other dipterans indicate that there are 51 OBPs in *D. melanogaster* (Hekmat-Scafe et al. 2002), 69 in *Anopheles gambiae* (Say), 111 in *Aedes aegypti* (L.) and 109 in *Culex quinquefasciatus* (Say) (Manoharan et al. 2013), 44 in *Bombyx mori* (L.) (Gong et al. 2009), and 46 in *Tribolium castaneum* (Herbst) and 21 in *Apis mellifera* (L.) (Forêt and Maleszka 2006). In *Glossina* spp., the number of OBPs are as follows: 30 in *G. morsitans morsitans* (Westwood), *G. fuscipes* (Newstead) as well as in *G. pallidipes*, and 29 in *G. austeni* (Newstead) and 28 in *G. brevipalpis* (Macharia et al. 2016). The low number of OBPs in *Glossina* compared with other insects could be attributed to the fact that they exclusively feed on blood and with some degree of host preferences, which may have resulted to streamline their chemoreception (International Glossina Genome Initiative 2014).

The expression of OBPs in nonolfactory organs of insects, such as legs, wings, fat body, ovaries, and pupae, has been attributed to possible involvement in nonolfactory functions (Pelosi et al. 2017). For instance, significant expression of OBP45 in *Ae. aegypti* female ovaries at 48 h post-blood meal (pbm) suggested its probable role in egg maturation during reproduction (Costa-da-Silva et al. 2013).

This study presents, for the first time to best of our knowledge, the expression analysis of five OBP genes at different developmental stages and differentially starved adults of *G. brevipalpis*. The findings provide useful insights into the *Glossina* spp. biology of chemoreception that may be exploited in designing specific vector control strategies.

Materials and Methods

Sample collection. Adult *G. brevipalpis* were collected from Shimba Hills National Park, located in Kwale County, Kenya (E 04°14′, S 039°23′, elevation 445 m above sea level) in December 2014 by using biconical traps baited with acetone, 1-octen-3-ol, and 4-methylphenol (Challier and Laveissiere 1973). The *G. brevipalpis* specimens were identified morphologically from other *Glossina* spp. The insects were maintained exclusively on rabbit blood in accordance with standard procedures for animal care in the insectary at the International Center of Insect Physiology and Ecology (ICIPE) from where the larva and pupa were obtained. Antennae from female tenerals and adults starved at periods of 2, 24, 48, 72, and 96 h were dissected on ice, and the samples were preserved in liquid nitrogen until used for RNA extraction.

Total RNA extraction. Total RNA from the larval, pupal, and antennal samples was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The presence of any contaminating genomic DNA was digested with RNase free-DNAse1 (Fermentas, Life Sciences, Waltham, MA, USA). The purity of the RNA obtained was assessed using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA) and integrity was

Table 1. Primers for the five putative *G. brevipalpis* OBP genes that were quantified using qRT-PCR.

	Nucleotide Sequence (5′ to 3′)	
Primer Name	**Forward**	**Reverse**
GAPDH*	AGATGGAAGCATGTTGCGTA	TATTGGGCCTCCTGCAGATA
*Gbr*OBP2	ATGGCAGTATCCCGAAGATG	CGCACTTTGCAATCTTTTCA
*Gbr*OBP6	CTGGCAAGGGAATGCACTAT	CTTTTTGCATGTTCCACAGC
*Gbr*OBP7	TGGACCAGTGTTTAGCACCA	TTATCAAAGCCCTGCGTCTC
*Gbr*OBP8	AAGACCGGTACCACAGAGGA	CAATGTGTTGAACGGTTTCC
*Gbr*OBP13	ACTTAACTCATCGCCCAACG	CGTGAACTGCTGCAAACATT

* Positive control gene.

determined by running the RNA samples on 1.2% formaldehyde agarose gel electrophoresis.

Synthesis of cDNA. Total RNA from each sample was reverse transcribed in a 20-μl reaction to a cDNA concentration of 20 ng/μl by using the RevertAid First Strand cDNA synthesis kit (Fermentas, Thermo Scientific) following the manufacturer's instructions. The negative control consisted of all the components minus the enzyme reverse transcriptase (RT).

Conventional PCR. Sequences of the putative *G. brevipalpis* OBP genes were downloaded from VectorBase (Giraldo-Calderon et al. 2014) and used in designing primers with the Primer3Plus tool (Untergasser et al. 2012). Amplification of the 28 putative *G. brevipalpis* OBPs was conducted using conventional PCR in 10-μl reactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *G. brevipalpis* was used as the internal reference gene. The thermocycling conditions comprised of initial denaturation at 98°C for 30 s, 40 cycles of; denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by final extension at 72°C for 5 min. The reactions were conducted using a Proflex thermocycler (Applied Biosystems; Thermo Fischer Scientific, Waltham, UK).

Analysis of amplified putative OBPs by using quantitative real-time PCR (qRT-PCR). Quantitative real-time PCR analysis of the PCR-positive putative OBP genes was conducted using a Stratagene Mx3000P qPCR system (Agilent Technologies UK Ltd, Cheshire, UK) in reaction volumes of 10 μl. The *G. brevipalpis* GAPDH gene was used as the endogenous reference gene. Each reaction contained final concentrations of the following: 1× Maxima SYBR Green/ROX master mix (Fermentas, Thermo Scientific, UK), 2 ng/μl of cDNA template, 0.3 mM of forward and reverse primers of the putative OBP genes (Table 1), and nuclease-free water. The thermocycling conditions were programmed as follows: one cycle at 95°C for 10 mins, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. A dissociation curve analysis generated at the end of the PCR was used to test for the specificity of the amplification to target genes. All the experimental tests were carried out in triplicate

and a no template control included as a negative control for each set up. The expression changes of each putative OBP gene across the developmental stages and in the differentially starved adults relative to GAPDH were calculated by the $2^{-\Delta\Delta CT}$ (Livak) method (Livak and Schmittgen 2001). Tests of significance of the OBP expression were performed using a single factor analysis of variance (ANOVA), followed by Tukey's test with a threshold of $P < 0.05$.

Results

Expression patterns of five putative OBP genes in the developmental stages of *G. brevipalpis*. Five OBPs were selected for expression analysis based on their linear quantification amplification curve and as representatives of the three OBP classes in *Glossina* spp., which include minus-C OBPs (*Gbr*OBP2), dimer OBPs (*Gbr*OBP7), and classic OBPs (*Gbr*OBP6, *Gbr*OBP8 and *Gbr*OBP13). Of the five genes tested, the gene expression levels of *Gbr*OBP2 relative to its expression in the control (teneral) were highest in the larval stage, followed by *Gbr*OBP7 in the pupal stage. The other three, *Gbr*OBP6, *Gbr*OBP8, and *Gbr*OBP13, had minimal expression levels less than that of the control (Fig. 1). The ANOVA *P* values of *Gbr*OBP2, *Gbr*OBP6, *Gbr*OBP7, *Gbr*OBP8, and *Gbr*OBP13 were 1.59E−06, 8.32E−07, 1.13E−07, 3.59E−07, and 5.37E−07 respectively.

Expression patterns of five putative OBP genes in tenerals and adult female *G. brevipalpis* starved at different time periods. Out of the five genes, *Gbr*OBP8 and *Gbr*OBP13 exhibited the highest expression levels relative to the control (tenerals). Expression of *Gbr*OBP13 peaked at the 72 and 96 h, whereas that of *Gbr*OBP8 peaked at 24-h postfeeding. Next in abundance were *Gbr*OBP2 and *Gbr*OBP6, with peaked expressions between 24 and 72 h for the former and 24-h postfeeding for the latter. Generally, *Gbr*OBP7 had the least expression, with lower levels than that of the tenerals. Furthermore, the levels remained low for the remainder of the period up to the 96 h. The patterns observed for *Gbr*OBP6, *Gbr*OBP8, and *Gbr*OBP13 were similar. Soon after the insects were fed (2-h postfeeding), the expression of the three genes decreased relative to teneral levels, and later in the 24-h period, their expressions increased, followed by a decrease at 48 h and, consequently, a gradual increase from 72 to 96 h (Fig. 2). The ANOVA *P* values of *Gbr*OBP2, *Gbr*OBP6, *Gbr*OBP7, *Gbr*OBP8, and *Gbr*OBP13 were 7.43E−08, 2.42E−09, 1.83E−11, 3.08E−10, and 2.15E−12, respectively.

Discussion

An insect's age influences odor coding. Therefore, there is a variation in their response toward odors emanating from various sources. In its early life, an insect is more attracted to food odors, and then later, it may respond to oviposition-site odors and other odors that are important for its biology. For instance, immediately after emergence, a young female mosquito will seek sugars and later will start searching for vertebrates for blood sucking (Gadenne et al. 2016). Therefore, chemical sensing influences responses in both adults and juvenile insects. Despite this, expression analysis of OBP genes in most insects have only focused on the adults. For instance, information on adult OBPs is available on the lepidopterans

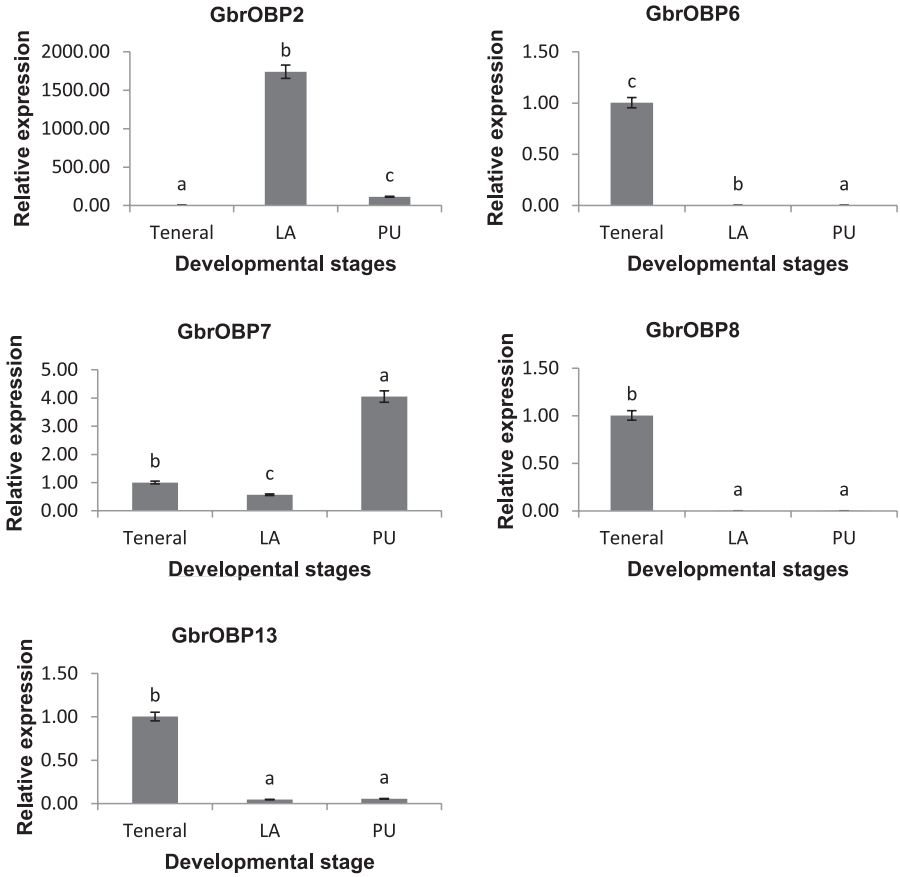

Fig. 1. Expression profiles of five putative OBPs in the larval, pupal ($n = 1$), and teneral (control, $n = 10$) stages of *G. brevipalpis*. All data represent mean values and error bars indicate standard error of the means at 95% confidence. The letters on the bars indicate statistically different expression levels as calculated by the Tukey's test.

Helicoverpa armigera (Hübner) and *H. assulta* (Guenée) (Zhang et al. 2015) and dipterans *Bactrocera dorsalis* (Hendel) (Zheng et al. 2013), *Cx. pipiens* (L.), and *Cx. quinquefasciatus* (Peiletier and Leal 2009)). However, it is also evident that OBP expression in the insect juvenile stage is important, as reported in some studies. Studies on *Chrysomya megacephala* (F.) identified one OBP, *Cmeg33593_c0*, to be highly expressed in the larval stage and was suggested to be involved in larval aging, nutrient accumulation, and regulation of the larval feeding behavior (Wang et al. 2015). In another study with the wheat midge *Sitodiplosis mosellana* (Gehin), the high expression of *SmosOBP1* and *SmosOBP10* in larvae suggested their involvement in larval-specific chemical sensing (Gong et al. 2014). Also, high

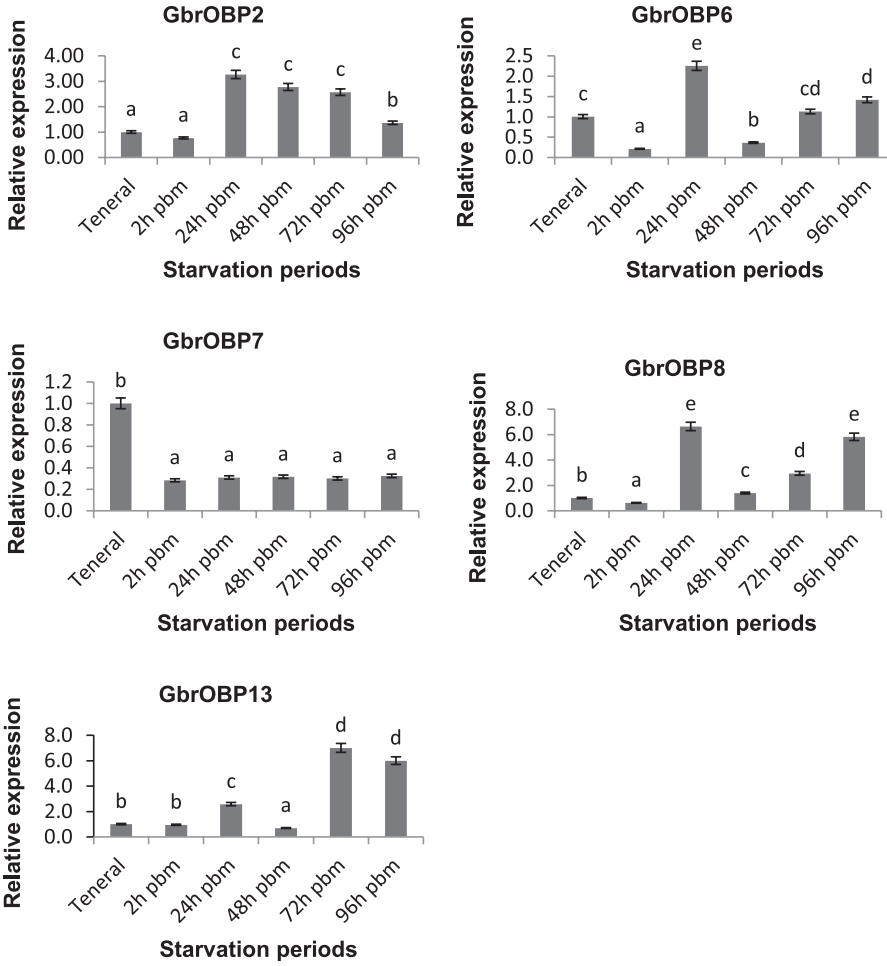

Fig. 2. Expression profiles of five putative OBPs in the antenna of *G. brevipalpis* tenerals (control, $n = 10$) and differentially starved adults ($n = 10$). All data represent mean values and error bars indicate standard error of the means at 95% confidence. The letters on the bars indicate statistically different expression levels as calculated by the Tukey's test.

expression of *SexiOBP13* in the larvae of *Spodoptera exigua* (Hübner) suggested its involvement in larval olfaction (Jin et al. 2015).

Unlike most other insects whose larvae are free living, those of *Glossina* spp. are characterized by adenotrophic viviparity (larvae develop *in utero*) whereby the larva obtains its food from the mother's milk glands. On the other hand, the pupal stage, which develops minutes after the larvae have been larviposited, is mainly dormant and is nourished by the fat body (International Glossina Genome Initiative 2014).

The high expression of *Gbr*OBP2 and *Gbr*OBP7 in the larval and pupal stages, respectively, in *G. brevipalpis* was similar to that of their orthologs in *G. morsitans morsitans*. These OBPs may, therefore, be suggested to be involved in nonolfactory chemical sensation in the juvenile stages of *Glossina* spp., owing to the fact that the larval and pupal stages have no developed antennae (Liu et al. 2010,. 2012). Elsewhere, high expression of OBP1 in *Anopheles stephensi* (Liston) (*Aste*OBP1) at the pupal stage was attributed to the physiological development of the chemosensory tissue (Sengul and Tu 2010). In yet another study with *A. mellifera* (L.), two OBPs (OBP10 and OBP13) were highly expressed throughout the pupal stages and suggested to be involved in its development while preparing to emerge (Forêt and Maleszka 2006).

Olfaction in adult insects occurs via the well-structured antennae (Jin et al. 2015). Therefore, the expression of OBPs in the antennae of *G. brevipalpis* adults as well as in other insects suggests their involvement in olfaction (Liu et al. 2010, Zheng et al. 2013). Insect feeding state influences their attraction to food whereby, when starved, they become easily attracted to food odors and, after feeding, the insects carry on with other activities relevant for their survival. Therefore, odor-guided behavior, which is driven by peripheral odor-detection proteins, such as OBPs, is driven by the nutritional state of insects. For instance, starved *D. melanogaster* were found to be easily attracted to food odor sources (Gadenne et al. 2016). The nutritional status in *G. moristans morsitans* was found to affect the expression of antennal OBPs (Otter et al. 2017).

The high expression of *Gbr*OBP8 in the adults agreed with the findings of Liu et al. (2010) who observed the orthologue gene in *G. morsitans morsitans* (*Gmm*OBP8) to be highly expressed in adults and suggested its involvement in olfaction. Differences were also noted. For instance, although *Gbr*OBP13 was relatively highly expressed in *G. brevipalpis*, this did not correspond with the findings in *G. morsitans morsitans* where the orthologous gene *Gmm*OBP13 was not among the 10 expressed genes in the antenna. This difference may be attributed to the difference in species host preference. Functional studies of the orthologue of *Gbr*OBP13 in *D. melanogaster* (*Dmel*OBP56h) showed its role in clearing bitter tastes and improving feeding (Swarup et al. 2014). The *Gbr*OBP6 elevated expression at 24-h pbm and decreased expression at 48-h pbm resembled that of *Gmm*OBP6 in *G. morsitans morsitans* (Liu et al. 2010). In functional studies of the *Gbr*OBP6 orthologous gene in *Drosophila*, *Dmel*OBP28a showed its involvement in shuttling bitter tastes to odorant receptors, hence discouraging their feeding on bitter foods (Swarup et al. 2014). Therefore, *Dmel*OBP56h and *Dmel*OBP28a in *D. melanogaster* have been suggested to have opposing functions. Minimal expression of *Gbr*OBP7 in the antennae of the differentially starved insects agreed with the findings of Liu et al. (2010) whereby the orthologous genes *Gmm*OBP7 subsequently had low expression levels in the adult antennae of *G. morsitans morsitans*. The variation in the expression of the OBP genes may be due to the different physiological needs of *Glossina* spp. However, it was difficult to pin point the specific roles, as the insects used were field collected and posed a challenge of different variations. However, the findings highlight the pattern of the expression of OBP genes that might be used as a basis in functional characterization. Also, although the insect olfactory system is better studied in adults compared with early developmental stages, this study extends the growing

recognition of the involvement of OBPs in nonolfactory chemical sensing, as depicted in the larval and pupal stages of *Glossina* spp.

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