Author

Jonathan Cheah became interested in undergraduate research after hearing positive experiences from older students *(continued on page 40)*.

Not too long ago, Diana Le changed her major from Mathematics to Biological Sciences because she decided that she wanted to become a pharmacist *(continued on page 41)*.

Key Terms

- *Anopheles gambiae*
- Gene Expression
- Malaria Prevention
- Microarray
- Odor Binding Proteins (OBPs)
- Olfaction

Odor-Binding Protein Expression in Female and Male Antennae of the Mosquito *Anopheles gambiae*

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Abstract

To better understand the olfactory process of the *Anopheles gambiae*, we investigated the differences of gene expression in the antennae of female and male mosquitoes. While both females and males feed on nectar as their main source of food, only the females blood feed in order to reproduce. Differences in expression of olfaction-related proteins between males and females were analyzed by a custom microarray to identify genes that are essential factors contributing to female blood-feeding behavior. Analysis of odorant-binding proteins was conducted to determine crucial intermediate agents in olfaction.

Faculty Mentor

We are using molecular techniques to understand the olfactory processes that control host-finding behavior of female *Anopheles gambiae* mosquitoes vs. sugar-feeding males. This will provide the basis for a promising approach to controlling transmission of the malaria parasite by blood-feeding mosquitoes. It is aimed to reduce the frequency of insect-host interaction by new and efficient insect repellents. We use microarray studies that became feasible with the recently published *A. gambiae* genome sequence to compare gene expression levels in female vs. male antennae. Our efforts have identified some genes encoding odor-binding proteins, which are most likely involved in female host seeking. Based on these findings and a better understanding of the mosquito odor recognition process, new insect repellents can be designed.

Harald Biessmann
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Introduction

Blood-feeding female mosquitoes are vectors for many global diseases, including malaria, yellow fever, and dengue fever. In the process of extracting blood from human hosts, female mosquitoes transmit parasites and viruses. For example, the mosquito species *Anopheles gambiae* are the primary vectors for the transmission of the malaria-causing parasite, *Plasmodium falciparum*, in sub-Saharan Africa. Over 400 million people are affected worldwide, and one to three million infected individuals, mostly children under five years of age, die each year of malaria (Marshall, 2000). Anti-malarial drugs and pesticides are being used to control malaria transmission, but they do not offer a permanent solution to the eradication of malaria. Parasites have developed resistance to anti-malarial drugs and mosquitoes have become resistant to pesticides. Therefore, millions of people worldwide in areas that were previously under control are being reinfected. A promising way to reduce the chance of parasite infection and reinfection is to reduce the frequency of mosquito bites. Therefore, we are studying the olfaction pathway to identify molecules responsible for mosquito host-finding.

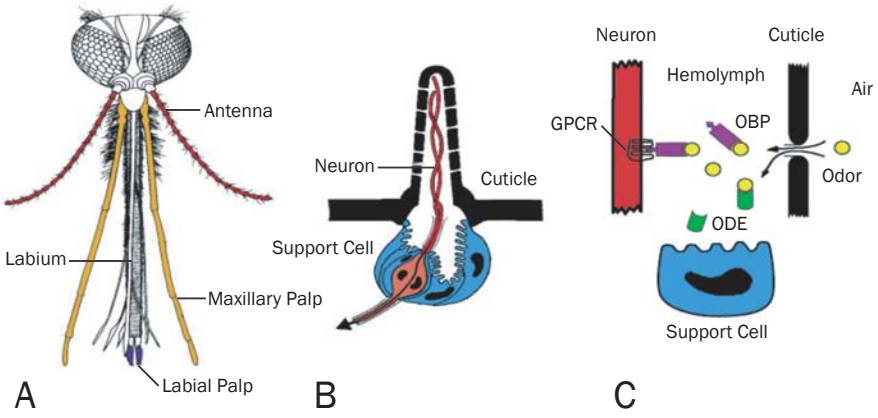


Figure 1
Insect olfactory system from Justice *et al.*, 2003: A) Mosquito head with the antennae (red), labial palps (blue) and the maxillary palps (orange). B) An enlargement of a typical olfactory sensillum found on the antennae. C) OBPs are shown as the intermediate molecule that transports odor molecules that enter through the pores of the cuticle through the sensillar lymph to the neuron for recognition.

Similar to other mosquito species, *Anopheles gambiae* females are both nectar and blood feeders, whereas male mosquitoes are solely nectar-feeders (Foster, 1995). The human-mosquito interaction is vital to the reproduction of female mosquitoes and is a crucial part of the plasmodium life cycle. These mosquitoes transmit the protozoa, *Plasmodium falciparum*, to humans. High occurrences of mosquito-human interactions are direct results of host odors that stimulate the mosquito's olfactory system to facilitate their homing and feeding. These olfactory responses are gender-specific, and previous behavioral and electrophysiological experiments have shown that olfaction plays a major role in mosquito-host recognition (Takken and Knols, 1999; Takken and Zwiebel, 2004).

The main olfactory structure responsible for recognition is the olfactory sensillum, located on the antennae of mosquitoes (Figure 1A). In the olfactory sensillum, neurons are stimulated by odor molecules, which are transported by the odor-binding proteins (OBPs). OBPs serve as intermediate molecules that carry odor molecules from the surface cuticle to the neuron (Figure 1B and 1C). Also shown are odor-degrading enzymes (ODEs), which break down the odor molecules after detection to allow the OBPs to recognize more stimuli. Due to the important role of OBPs in the olfaction process, it is assumed that female mosquitoes that seek humans, unlike their male counterparts, will produce a higher amount of OBPs that are specific to human odor molecules.

By identifying differences between male and female gene expression of OBPs, we hope to distinguish genes that could be involved in the host-seeking process of female mosquitoes. We hypothesize that the OBPs expressed higher in females than in males are used to sense hosts such as humans, since only female mosquitoes need to seek out humans to initiate egg production. Using the microarray technique, we can determine the levels of gene expression for various genes that we believe to be used in the olfactory system. Once these genes are identified, odor blockers can be developed to inhibit the detection of humans by the mosquito olfactory system. Such a treatment may lead to a reduction in mosquito-human interactions and disease transmission.

Materials and Methods

A custom microarray containing approximately 350 olfactory genes was used. The genes selected for testing were identified and established from previous cloning data

(Biessmann et al., 2002; Justice et al., 2003) and from the sequence data of the *Anopheles gambiae* genome (Ensemble Mosquito Genome Browser). The expression results of the microarray can be analyzed to distinguish different levels of gene expression in female mosquitoes, thereby identifying those that could be essential to their host-seeking ability.

Principle of Microarray: Specific 70 base pair oligonucleotides were synthesized by Qiagen (Valencia, CA), which hybridized to the unique RNA sequences of the genes to be analyzed. These oligonucleotides were printed from a microtiter plate onto a glass slide (Microarray Facility, Department of Biology, UCI). To analyze the differences between female and male gene expression, female cDNAs were labeled with Cy3 fluorescence, while male cDNAs were labeled with Cy5 fluorescence. Following hybridization, the slides were washed to remove non-homologous cDNA. The amount of transcripts bound to the corresponding oligonucleotides is representative of the levels of gene expression. Using a laser scan, we measured the intensity of the fluorescence given off by the labeled cDNA.

RNA Isolation: To extract RNA from mosquito heads, 60 male and 60 female heads were ground in Trizol (Invitrogen, Carlsbad, CA) to yield 30-40 µg of RNA. To extract RNA from antennae, 100 mosquito antennae were ground in 100 µl of Trizol, followed by an additional 400 µl of Trizol. A total of 400 male pairs and 800 female pairs of antennae were used to obtain 8 µg of total RNA from these preparations.

cDNA Synthesis: 19 µl of total RNA were mixed with 2 µl oligodT18 and 1 µl of a random hexamer. This mixture was then placed in the PCR machine for 10 min at 70 ˚C, followed by cooling in ice. A mixture of 8 µl water, Superscript buffer, 4 µl DTT, 1 µl of amino allyl dUTP/dNTP mix, and 2 µl of Superscript III buffer was added to the RNA and left at room temperature (RT) for 10 min. Following this, the mixture was incubated at 42 ˚C for 5 hr and then frozen.

Indirect Labeling: After cDNA synthesis, 13 µl of 1 M NaOH and 5 µl of 5M EDTA were added and the tube was incubated at 65 ˚C for 20 min to degrade the RNA. To neutralize the solution, 33 µl of 1 M Tris with a pH of 7.5 was added. The amino allyl cDNA was purified and concentrated by running the solution through a Microcon YM-30 concentrator. To the resulting 8.5 µl of cDNA, we added 4.5 µl of 1 M $NaHCO_3$, and this solution was added to the dried Cy3 or Cy5 mono-reactive dyes (Amersham, Piscataway, NJ) and incubated for one hr in the dark at RT. After this coupling reaction, we quenched the labeled cDNA and purified it through a Qiagen PCR Purification Kit column.

Spectroscopy Measurements: Spectroscopy was used to measure the amount of DNA from the Cy3 and Cy5 labeled cDNA at 260 nm. The maximum absorption of Cy3 was observed at 550 nm wavelengths, while the maximum absorption of Cy5 was at 650 nm. From these absorptions, the concentration of fluorescence incorporated into the cDNA was calculated. This concentration was used to calculate the frequency of incorporation, which is the amount of fluorescence that was successfully incorporated into the cDNA.

Hybridization to the Slides: The Cy3- and Cy5-labeled male and female cDNA were pooled and dissolved in 25 µl of 35% formamide buffer. Slides were pretreated with 95 ml of BSA blocking solution, heated for one hr at 50 ˚C, and then mixed with 10.5 ml of 10% SDS. The slide was immersed into the blocking solution for 5 min and then washed with water. The labeled cDNA solution was then pipetted onto the array and covered with a Grace Biolab HybriSlip plastic cover strip (22 x 40 mm). The slide was placed into a hybridization chamber (Corning, Corning, NY) and incubated in a humid chamber overnight at 42 ˚C to prevent dessication. After hybridization, the slides underwent many washes to reduce background readings on the arrays. Slides underwent Wash-1 with 2 x SSC and 0.5% SDS at 42 ˚C, followed by Wash-2 with 0.5 x SSC and 0.2% SDS at RT. Wash-3 consisted of 0.2 x SSC at RT, which was followed by shaking the slide up and down within Wash-4 solution of 0.01 x SSC. After the slides were dried, they were placed into a slide scanner that emitted wavelengths of light that corresponded to the fluorophones used. Intensities of both female and male fluorescence were measured and compared to find a significant ratio of expression.

Analysis: From the slide scanner, we obtained the numerical value of incorporation intensities of the genes, which was made up of gene expression and the background intensity. The average background intensity was then calculated and subtracted to obtain the true gene expression value. These values were subjected to a visual inspection to ensure that there were no erroneous readings from flaws in the array. Based on the numerical expression of each gene, we ranked OBP expression from highest to lowest. In addition to this, a female/male expression ratio was calculated to observe which sex exhibited a higher expression of each OBP. The OBPs were also classified into groups by their similarity to *Drosophila melanogaster* OBPs, in order to further identify each OBP's function. Additional visual inspections of OBP expression from arrays with mosquito palps and head RNA were also performed.

Results

Preliminary Experiments with Mosquito Heads

Previous experiments on male and female mosquito heads showed different intensity levels between various genes, which are seen as spots in Figure 2. These preliminary trials demonstrated the feasibility of our approach and further improved the technique. Within an array, there are eight sub-arrays, as shown by the white boxes (Figure 2).

Each spot shown represents a particular gene with its level of expression corresponding to the intensity of the spot. From analysis of these arrays, we confirmed the accuracy of the technique by testing certain housekeeping genes. These housekeeping genes such as actin, tubulin, and ribosomal proteins are all essential to normal growth and survival of both males and females. Visual analysis of expression levels of OBPs and the control genes was done on sub-array 2-2.

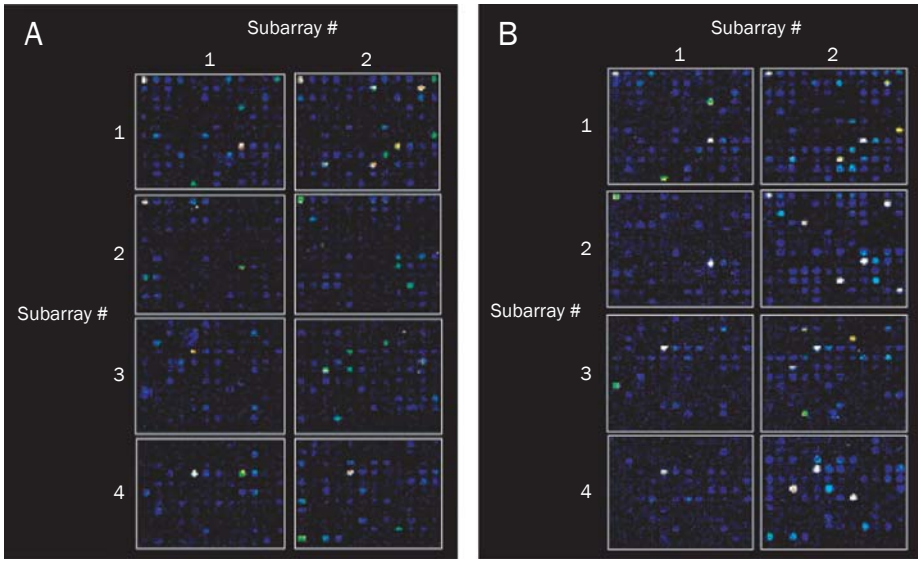
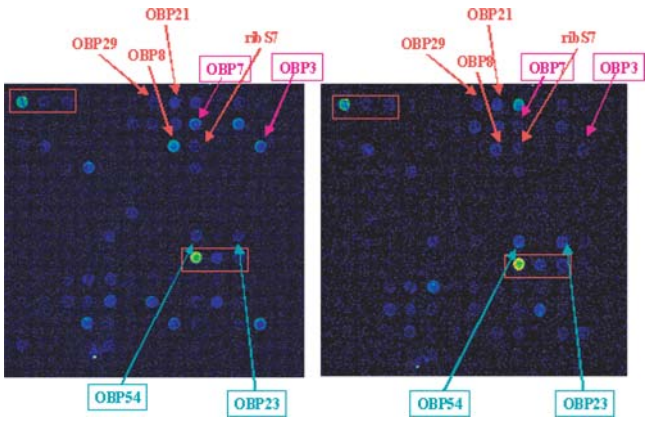


Figure 2

A) Array hybridized with female head RNA labeled with Cy3. B) Array hybridized to male head RNA labeled with Cy5. White boxes show the sub-arrays with their corresponding column and row labels.

Gene Expression in Antennae

To investigate the differences between males and females in olfaction-related proteins, antennal RNA was isolated from males or females and used for hybridization to the array. The expression levels shown in Figure 3 are from one sub-array (2-2) of the entire microarray plate. In these sub-arrays, there is detectable variation between the males and females. Thus, each oligonucleotide was printed three times to ensure an accurate average expression value for each gene. The numerical expression values were also visually inspected to ensure that there were no extraneous flaws in the array. In order to focus on olfaction, analysis was concentrated upon the OBPs and the controls. A total of 57 genes in *Anopheles gambiae* encode OBPs (Ensemble Mosquito Genome Browser).



Female Antennae RNA Male Antennae RNA

Figure 3

Sub-array 2-2 hybridized to female and male antennae RNA. The spots in the red box are the *Arabidopsis* genes, which are used as a control. Rib7 gene encodes the ribosomal protein S7, which is a housekeeping gene used as a control. The OBPs listed in pink appear at a higher intensity in females than in males, while the green-labeled OBPs have a higher visual intensity in males than in females.

Figure 3 shows a sub-array (2-2) hybridized to male and female RNA with labeled gene names, in order to compare

Table 1

Analysis of OBPs Represented in Sub-Array 2-2.

GENE	Expression in male antennae vs. female antennae	Visual expression level in female antennae
OBP 21	Same	Med
OBP 10	Same	Low
OBP 7	**Up in female**	High
OBP 8	**Up in females**	High
Rib protein S7	Same	Low
OBP 3	**Up in female**	High
OBP 54	**Up in male**	Med
OBP 23	**Up in male**	Med

the intensities by visual inspection. Table 1 lists the gene names and specifies which gender expresses a particular gene at a higher intensity. By visual determination of the expression of gene levels, we categorized the expression as high, medium or low in female antennae. As shown in bold, OBP-3 and OBP-7 appear to be expressed higher in females, indicated by higher fluorescent intensity. The Ribosomal protein S7 gene is essential to all mosquitoes, and thus acts as a normalizing control since equal expression between males and females is expected. This was confirmed by visually identifying the relatively equal levels of intensity of such genes on the arrays. The red box of the array shows the *Arabidopsis thaliana* spikes, which are plant cDNAs. The corresponding RNAs of the *Arabidopsis* spikes were added in three different concentrations to the mosquito RNA preparations as an additional control. The other bright spots are Arrestins and other housekeeping genes, which are abundantly expressed in both sexes.

This analysis was extended to the entire array and expression intensities were measured using a laser scanner. By collecting and analyzing all the OBPs tested in the entire array, we found 14 OBPs that were strongly expressed, visible, and present at a greater degree in females than in males (Table 2). OBP-8 was identified to be the highest expressed among the female OBPs, which was expressed six times higher than in males. OBP-7 had the highest female/male expression ratio by being expressed eleven times more in females. These 14 OBPs were cross-referenced and classified to previous research performed on *Drosophila* in order to provide a higher level of identification. The mosquito OBPs have been classified into various groups, such as the classical, atypical, and C+ groups (Xu and Galindo, 2003). The classical group is identified by six cysteines, resembling pheromone-binding proteins of moths. The atypical group is classified by having two tandem OBPs joined, resembling dual OBPs. The C+ group is similar to the classical group, except that it is longer and has 12 cysteines, six of which are in the same relative positions as in the classical OBPs. A visual inspection of these OBPs was also extended to palps and heads. Only OBP-2 and OBP-7 were found to be present in palps, while the majority of the proteins were expressed only in antennae. By performing a visual inspection of OBP expression in heads, we were able to see if there were other head tissues expressing a certain OBP.

When extending this analysis to the entire antennal array, genes that were expressed higher in males were also noted. OBP-54 was expressed four times higher in males than in females (Table 3). It was also found that OBP-5 was the highest expresser of OBPs in males; it was expressed 2.7 times greater in males than in females. A majority of the OBPs expressed highly in males were also expressed in the palps.

Table 2

Higher female OBP expression in antennae with nearest *Drosophila* homolog, female/male ratio, expression rank, and comparison to male and female head expression. The OBPs listed exhibit a higher expression in females than the males, as shown by the female/male ratio. The *Drosophila* homologs were identified in previous studies (Gallindo and Smith, 2001). These OBPs were also visually examined in palps and heads.

Name (OBP#)	Class (Xu, Smith)	Nearest *Drosophila* homolog	Fem/male antennae ratio	Rank in female antennae	Antennae, palps or both	Visible in male heads	Visible in female heads
8	C+	-	6.02	2	Both	Med	Med
47	C+	-	9.52	6	Antennae	Low	Low
17	Classic	83ab (OSE/F)	5.98	4	Antennae	Low	Low
3	Classic	83ab (OS-E/F)	10.0	7	Antennae	Med-low	Low
1	Classic	83ab (OS-E/F)	3.83	3	Antennae	Med	Med
7	Classic	69a	11.63	8	Antennae	Low	Low
4	Classic	76a (Lush)	3.35	9	Antennae	Low	Low
20	Classic	19a	3.78	10	Antennae	---	---
2	Classic	83ab	1.52	12	Antennae	Very low	---
12	Classic	99ab	3.27	19	Antennae	---	---
42	Atypical	-	2.51	18	Antennae	---	---
8	Classic	19c	1.66	17	Antennae	---	---
57	C+	-	1.26	16	Both	Low	Low
22	Classic	99ab	1.25	22	Antennae	---	---

Discussion

When performing our analysis, a visual check was used to inspect all OBPs for false positives, which are caused by the presence of a white speck that falsely increased the expression intensity. The presence of these white specks could possibly alter data by increasing the intensity of surrounding spots. In addition, the subtraction of the average background intensity could cause a variance in certain spots, since certain areas of the array had a higher degree of background intensity than others.

By performing the microarray experiment with male and female antennae, we identified a number of OBPs that are expressed more strongly in females than in males.

Table 3
Higher male OBP expression in antennae with nearest *Drosophila* homolog, male/female ratio, expression rank, and comparison to male and female head expression. All OBPs listed exhibit a higher expression in males than in females, as shown by the male/female ratio. The *Drosophila* homologs were identified in previous studies (Gallindo and Smith, 2001). Expression of these OBPs was also examined in heads and palps.

Name (OBP#)	Class	Nearest *Drosophila* homolog	Male/female antennae ratio	Rank in male antennae	Antennae, palps or both	Visible in male heads	Visible in female heads
5	Classic	76a (Lush)	2.712	1	Both	High	High
54	C+	--	4.504	2-3	Both	Med	Med
9	Classic	99ab	2.454	2	Both	High	High
28	Classic	56ab (?)	3.922	3	Both	Med	Med
23	Classic	56ab (?)	2.702	4-5	Both	Med	Med
13	Classic	99ab	3.941	9	Both	Med	Med
21	Classic	56ab (?)	1.601	4	Both	Med	Med
41	Atypical	-	---	18	Antennae	Very low	---
38	Atypical	-	---	17	Antennae	Low	Very low
24	Classic	56ab (?)	4.030	15	Antennae	Very low	Very low
10	Classic	19c	1.538	8	Both	Med	Med
19	Classic	19a	4.230	19/20	Both	Low	Low
45	Atypical	-	2.279	20	Antennae	Very low	Very low
44	Atypical	-	1.094	24	Antennae	Very low	---
29	Classic	57c	1.340	13	Both	Med	Med

Such high expressions of an almost ten-fold difference, as found in OBP-47 and OBP-7, could signify genes that are important to female mosquitoes. The high expression of genes like OBP-8, shown by the corresponding ranks, indicates that such genes are essential in female antennae. Our data support the hypothesis that there are specific genes that are expressed higher in females than in males. These genes' protein products can then be analyzed to identify which OBPs are specific to human odor molecules.

The genes expressed higher in males, such as OBP-5 and OBP-28, are assumed to be used primarily in nectar feeding. Such genes can also be analyzed to further test which are for sugar feeding and which are for other activities.

We did not find any genes that were expressed uniquely in females or in males. The lack of unique OBP gene expression by males is probably because both females and males perform nectar feeding. Thus, the genes expressed in males are also needed in females. The lack of OBPs expressed only in females is most likely because males may also use the same OBPs to sense humans. This is necessary because male mosquitoes can then find female mosquitoes that have taken a blood meal from a vertebrate and are ready to mate.

The genes that were expressed in both antennae and palps could signify that these OBPs are also used for gustatory functions. Thus, in close contact the OBPs expressed higher in females in both antennae and palps could be used to probe a human's skin to determine if the mosquito will blood feed. The OBPs expressed in both antennae and palps of males are most likely used for close-range sensing during nectar feeding.

The classification of the OBPs that were included from previous studies in *Drosophila* is based on structural differences and not on function. Until the specific protein of each gene is analyzed and tested, these classifications do not encompass the OBPs' possible functions.

Conclusion

This study revealed that female *Anopheles gambiae* express certain genes that code for odor-binding proteins at high levels, while their male counterparts express these genes at much lower levels. These genes are assumed to have female-specific functions, such as detection of human hosts for blood-feeding. By further studying these gene products, specifically the OBPs, we can identify the type of human odor molecules that bind by these OBPs. Once this is known, repellents can be synthesized to inhibit this interaction in the olfactory system of female mosquitoes, thereby reducing the ability of female mosquitoes to detect human targets. These repellents may lead to lower incidents of bites and parasite transmissions in order to reduce malaria transmissions. Since these repellents are specific to the physiology of the mosquito, they should prove to be more effective than current repellents and be environmentally safe. Efficient attractants can also be synthesized in order to lure female mosquitoes away from dense areas of human population, thus reducing the potential for transmission.

Jonathan Cheah Biography

(continued from page 35) During his sophomore year, he learned of a lab looking for undergraduate researchers from one of his biology teachers. Jonathan followed up on the opportunity and was soon engaged in a research project. For him, the most enjoyable part of this experience is the one-on-one communication with his faculty mentor. In

addition to his research activities, Jonathan is a LARC tutor, a private tutor, and a member of the National Society of Collegiate Scholars. He enjoys playing sports in his leisure time and is particularly fond of his latest new hobby, surfing at nearby beaches.

Diana Le Biography

(continued from page 35) However, she says that her research in Dr. Biessmann's lab is causing her to reconsider her plan again. She may decide instead to pursue graduate study in Biology so she can continue to observe the molecular effects expressed in the morphology or physiology of an organism. Diana is grateful that her research contributes to eradicating the problems of mosquito-borne diseases.

Acknowledgements

We would like to thank our faculty advisors, Dr. Harald Biessmann and Dr. Peter Bryant, for giving us the opportunity to work in their lab and participate in the interactive process of research. We would also like to thank Dr. Marika Walter for training us to dissect mosquito antennae and heads. We also appreciate Patrick Williams, a member of the research team, who has passed down his knowledge to us while working in the lab.

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