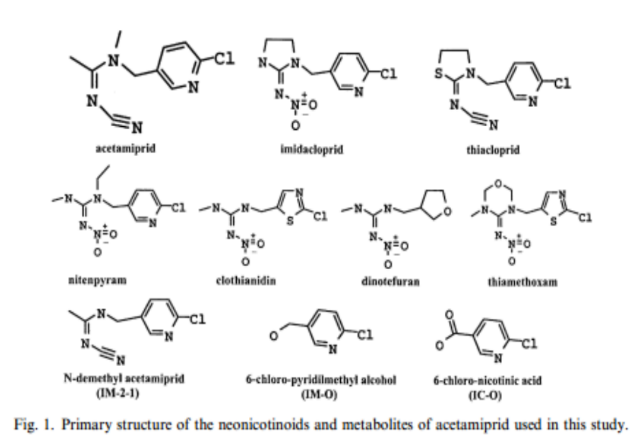
How do Neonicotinoids bind to the alpha subunit of the nicotinic acetylcholine receptor *Apis mellifera*?

1. Neonicotinoid’s and Their Role in Colony Collapse Disorder

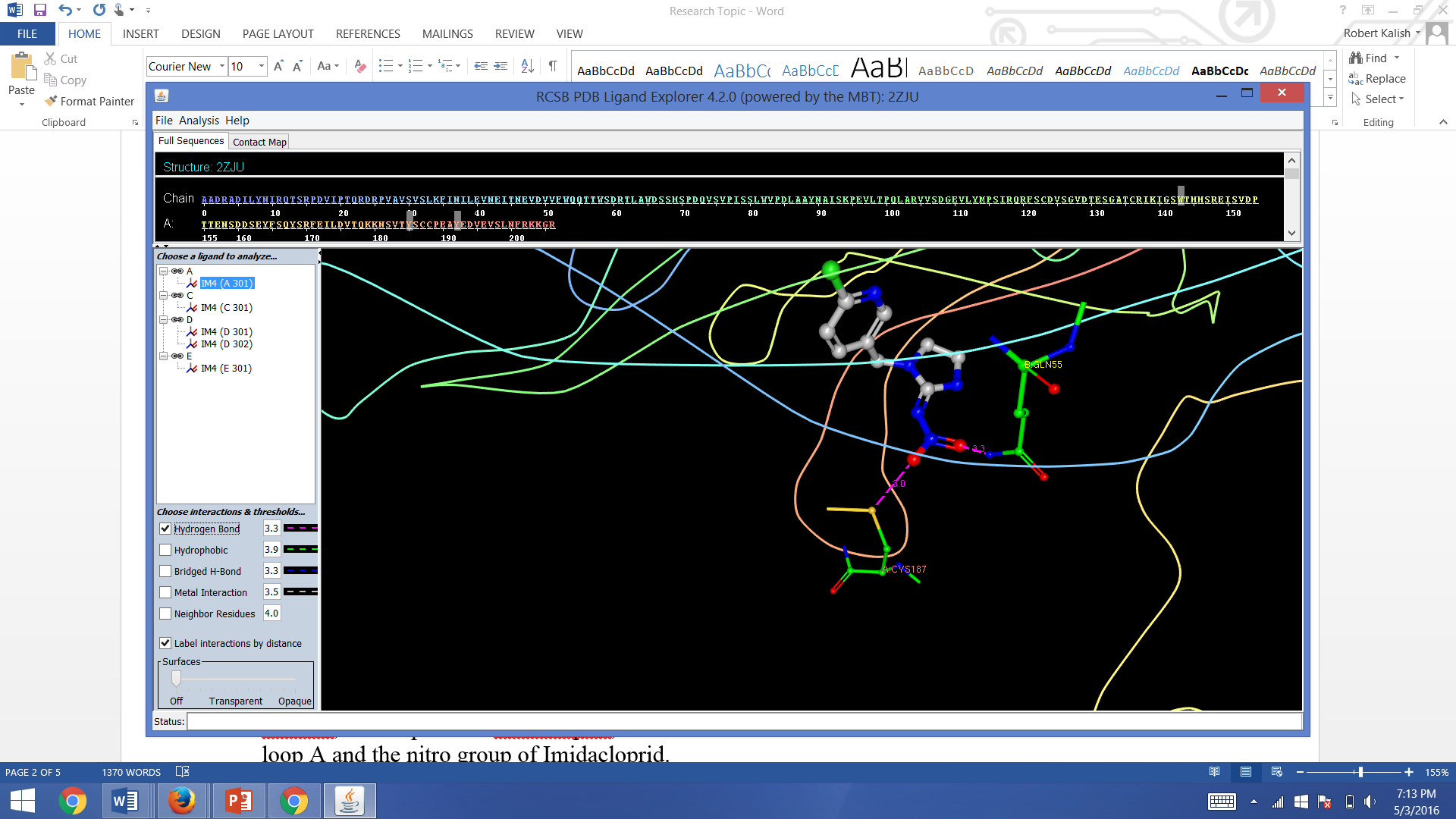
Neonicotinoid pesticides are an agonist for the Nicotinic Acetylcholine receptors (nAChRs) in insects. Their use has been very effective at combatting common agricultural pests, however they have had the unintended effect of harming populations of *Apis mellifera* (honey bee) through mortality in large doses, and impaired motor function and memory in smaller doses (Gauthier, 2010). This is because nAChR’s are receptors involved in the chemical juncture between nerve cells. They trigger the open of an ion channel that facilitates movement of Ca2+, K+, and Na+ across the cellular membrane, and then through the synaptic cleft to continue transmission of an action potential (Thany et al, 2007). Currently, through industrial scale agriculture many bees are exposed to sub-lethal amounts of these pesticides that are inadvertently spread to nearby food sources. As workers are exposed, they begin to experience impaired motor function and memory. They bring contaminated food to the hive, and it is spread throughout the population (Suchail et al, 2001). In their paper, “Honey Bee Navigation”, Dyer and Could argue that workers often rely on remembered landmarks when returning to the hive from a food source, especially when the sun is unavailable for geolocation (1983). When enough workers’ memory is too impaired for them to return, the colony collapses; neonicotinoid pesticides are one of the leading causes of colony collapse disorder (CCD). Although these chemicals are always going to unintentionally affect insects that they come in contact with; if the mechanism of toxicity is understood, we can optimize the insecticides to only effect the target species.

1. Cyano vs. Nitro-substituted Neonicotinoid’s Effect on *Apis mellifera*

One of the most common neonicotinoid pesticides used today is Imidacloprid. In their experiment “Mechanism for the Differential Toxicity of Neonicotinoid Insecticides in the Honey Bee, *Apis mellifera*”, Iwasa et al found that other neonicotinoids, Acetamiprid (LD50 = 7.07) and Thiacloprid (LD50 = 14.6), were only lethal when administered at a much higher dose than Imidacloprid (LD50 = 0.0179). A main structural difference conserved in Acetamiprid and Thiacloprid, but not Imidacloprid is the change of the N-terminus end from a nitro-substituted group to a cyano-substituted.



**Figure 1.** Chemical Structures of Acetamiprid, Imidacloprid, and Thiacloprid.

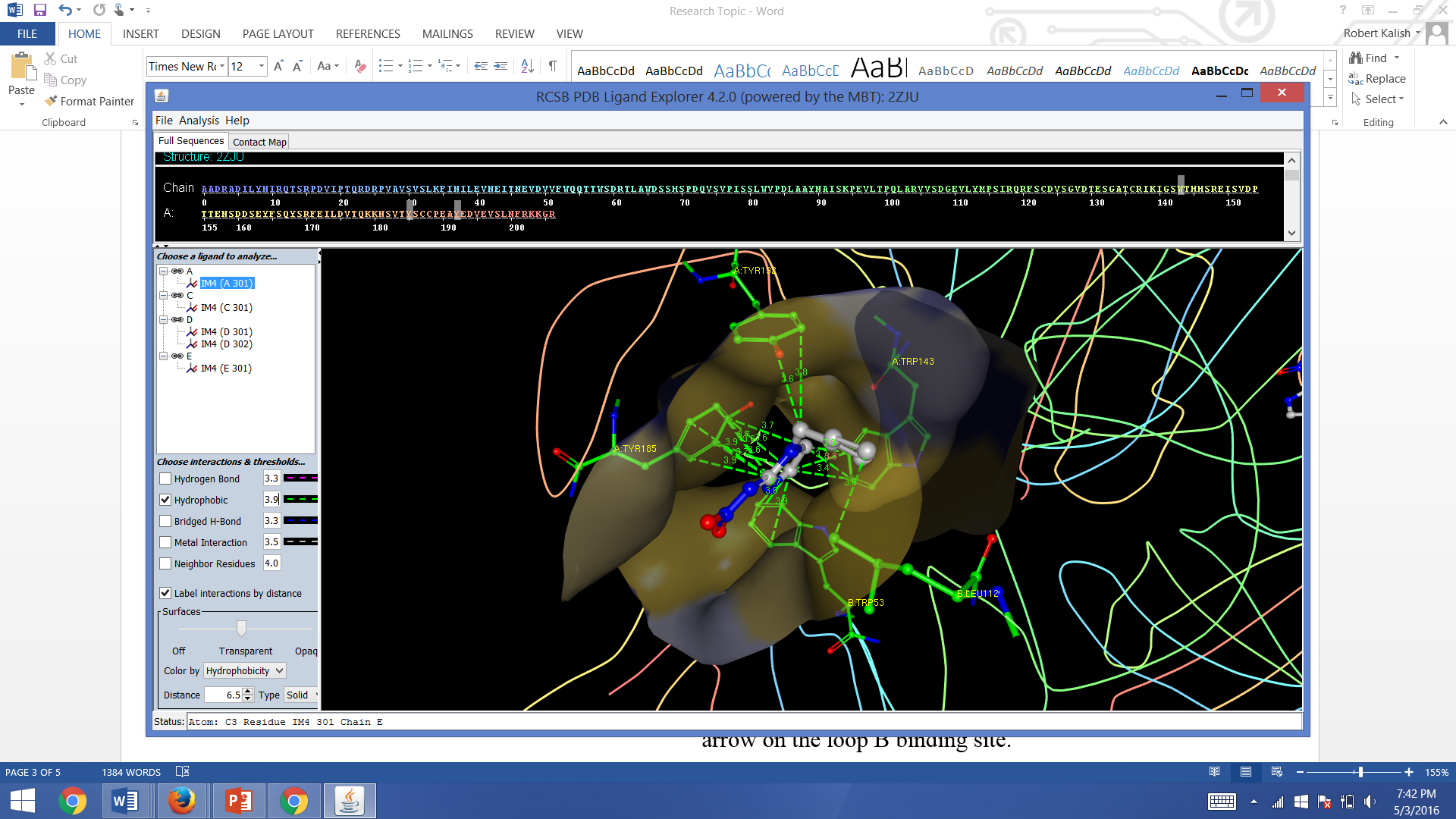
Although the structure of the nAChR in *Apis meliffera* is rather undocumented the full crystal structure of the acetylcholine receptor in the mollusk Lymnaea Stagnalis is modeled. In Lymnaea Stagnalis the Acetylcholine Binding Protein (AChBP) has high homology with the N-terminal region (23.9% identity) of the a7 subunit and forms a hompentamer (Smit et al, 2001). When binding was simulated in Ligand Explorer, Q55 and C187 of Lymnaea AChBP were both shown to bond to the nitro group of Imidacloprid. C187 corresponds to G189 in the chicken a7 subunit (Matsuda et al., 2002). Matsuda et al. tested receptors containing the mutations G189E and G189D on the chicken a7 subunit for affinity to the agonists Imidacloprid, Nicotine, and Acetylcholine. They found a statistically significant reduction in affinity of the receptor to Imidacloprid, but no change in response when the other agonists were introduced. This led them to conclude that this reduction in affinity is a result of the loss of electrostatic interactions between the nitro group of Imidacloprid and G189.

**Figure 2.** The structure of the agonist binding domain of AChBP from the mollusk *Lymnaea stagnalis* when bonded with Imidacloprid. The figure was generated based on the PDB file 2ZJU in RSCB-Ligand Explorer. Q55 and C187 of AChBP, which are colored green, correspond to Q79 and G189, respectively, of the chicken α7 subunit (Shimomura et al., 2002).

The alpha subunit of the insect nAChR is comprised of 5 loops denoted A 🡪 E each with its own specific binding site, Q55 is at the center of the loop D binding site. In *Apis mellifera* a7 subunit the loop D binding site is comprised of the residues; TNLWLKLEWND, K77 is the center of this binding site and corresponds to Q79 in the chicken a7 subunit, and therefore Q55 of *Lymnaea stargnalis*. In insects this residue is normally basic, either Lysine or Arginine. I propose that the primary mechanism for the increased affinity of Imidacloprid to the honey bee nAChR, as compared to Acetamiprid, is an electrostatic interaction that creates hydrogen bonds between the basic residues in loop D and the nitro group of Imidacloprid. If K77 is substituted for a less-basic residue; the result should decrease the affinity of the receptor to Imidacloprid, but not affect any other agonist’s potency.

1. nAChR Affinity Due to Alpha Subunit Loop C

In their experiment, "Exploring the Pharmacological Properties of Insect Nicotinic Acetylcholine Receptors.", Thany et al. isolated nAChR alpha subunits from 6 species of insects and then paired with well understood Chicken B2 subunits and vice-versa (2007). The chicken receptors were not affected by the agonist as Neonicotinoids are specific to insect nAChR’s. In this way the hybrid receptors allowed Thany et al. to focus solely on the affect of the agonist on the alpha subunit. Over a dozen different mutant and wild-type insect-alpha hybrid receptors were then exposed to various receptor agonists to see which parts of the alpha subunit’s protein determines molecular attraction. The importance of the YXCC motif of the loop C binding site in the alpha subunit has been studied in many organisms as it relates to acetylcholine affinity, but not as extensively for neonicotinoids. Thany et al. studied its effect on Imidacloprid resistance in Drosophila alpha 2 subunits by specifically looking at the mutation P242E. They found that in a majority of vertebrate subunits this residue is acidic, the mutation to glutamic acid resulted in a noticeable decrease in Imidacloprid affinity for the receptor. Loop C bonds to the acidic imidazoline ring of Imidacloprid (Figure 3), this acid to acid interaction might explain the decreased binding potential found. Mutations that introduce more acidic residues in the YXCC motif should not affect the binding affinity of agonists other than Imidacloprid as they do not contain the acidic ring.



**Figure 3.** The structure of the agonist binding domain of AChBP from the mollusk Lymnaea stagnalis when bound to Imidacloprid. The figure was generated based on the PDB file 2ZJU in RSCB-Ligand Explorer. Y192 and Y185 (of YXCC) are major structures in determining loop C binding site, W143 is a catalyst for Loop B binding.

|  |  |  |  |
| --- | --- | --- | --- |
| Table 1 | Loop D | Loop B | Loop C |
| Lymnaea stagnalis AChBP | VVFWQQTTWSD | IGSWTHHS | TYSCCPEAY |
| Gallus Gallus Domesticus a7 | TNIWLQMYWTD | FGSWTYGG | FYECCKEPY |
| Drosophila Melanogaster a2 | SNVWLRLVWYD | FGSWTYDG | YYPCCEEPY |
| Apis Mellifera a7 | TNLWLKLEWND | FGSWTYDG | YYNCCPEPY |

YXCC motif: green

Fig. 2 residues: orange

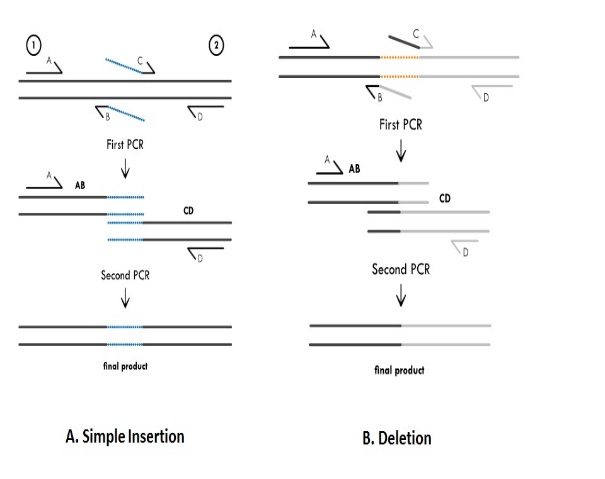
Fig. 3 residues: red and Y of YXCC

Location of mutations to be studied:

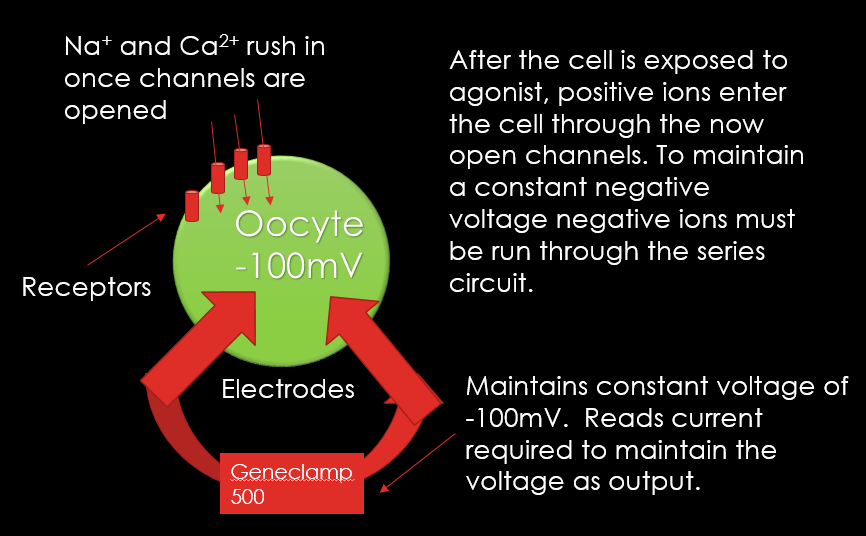
1. Experimental Procedure

The goal of my experiment is to test the differential affinities of Imidacloprid, Acetamiprid, and Thiacloprid on multiple mutated forms of Nicotinic Acetylcholine Receptors from *Apis mellifera.* Acetamiprid and Thiacloprid affinity should not be effected by mutations that cause Imidacloprid resistance as it does not contain the Imidazoline binding site or the nitro substituted group. I will prepare mutants of the a7 subunit in *Apis mellifera* by PCR following the procedure laid out by Shimomura et al. *Apis mellifera* nAChR subunit a7 (genbank: NM\_001011621) in the pMT3 vector will be used as a template, below are the primers that will be used to induce mutation.

|  |  |  |
| --- | --- | --- |
| Mutant | Forward Strand (C) | Reverse Strand (B) |
| N212E | 5’-TATTACGAGTGCTGCCCAGAACCG-3’ | 3’-CGGTTCTGGGCAGCACTCGTAATA-5’ |
| K77Q | 5’-ATCTCTGGTTACAATTGGAATGG-3’ | 3’-CCATTCCAATTGTAACCAGAGAT-5’ |
| Edge Primers | 5’-ATGAGACGTTGGACTCTCATGGC-3’  (A) | 3’-TATTTCCTTTGTATTCCTTTTCTC-5’  (D) |

15 copies of each wild-type and mutant receptor must be made so that they can undergo 3 trials of exposure to each of the 5 agonists. To induce mutation 2 standard primers that contain the start and end of the gene (A and D) will be needed to isolate the target sequence. Primers B and C contain the specific mutation that we will be inserting into the sequence. A 50 µl solution will be prepared with: 0.3 µM of all 4 primers, 0.4 mM dNTP mixture, 100 ng of pMT3-a7 as template, and 1.25 U of LA-Taq. Once prepared, the solution must now undergo 30 cycles of treatment at 98°C 30 s, 50°C 30 s, 72°C 60 s. The solution now contains the AB and CD strands of DNA. Now we must perform a 2nd round of PCR. Another 50 µl solution will be prepared with: 0.3 µM of the edge primers (A and D), 20 ng each of the first round PCR products (AB and CD), and 1.25 U of LA-Taq. Once prepared, the solution must now undergo 30 cycles of treatment at 98°C 30 s, 60°C 30 s, 72°C 90 s to yield a single strand that contains the desired mutation.

After PCR, the products of each individual PCR reaction must be taken, combined with DNA encoding a Beta subunit, and then injected into multiple Xenopus Laevis eggs, this will be done using the procedure laid out by Ihara et al. 2002. Mature Xenopus Laevis Females will be anaesthetized by immersion in 1.5g I-1 tricaine for 30 minutes. Afterwards they will be cut open and larger Oocytes (stage IV and V) will be removed. The follicle cell layer of each Oocyte must be removed manually by fine forceps. The nucleus of each Oocyte will then be injected with 20 nL of a single form of mutant DNA submersed in distilled water, they will then be incubated at 16°C in saline solution.



The final step of my project will be to test the affinity of the receptor using Voltage Clamp Electrophysiology as laid out by Ihara et al., 2002. Electrophysiology will be performed after 3-6 days of incubation to give time for the receptor DNA to be expressed. 2 2.0 M KCl-filled electrodes will be attached to form a series current. A constant voltage will be maintained at -100mV by a Geneclamp 500 clamped to the series circuit. Beforehand, .3 M Solutions of Ligands will be prepared using Acetylcholine, Nicotine (controls), Imidacloprid, Thiacloprid and Acetamiprid. Oocytes will be submerged in a single ligand solution for no longer than 2 seconds. The current required to maintain the constant voltage will be measured over the next ~5 minutes, until the current returns to its starting value.

The concentration of agonist that gives half the normalized response (EC50) will be calculated using the equation:

Ψ is the normalized response of the receptor to ligand concentration [A], Imin and Imax are the minimum and maximum responses. nH is the Hill Coefficient. EC50 and Imax values of the wild-type and mutant nAChR’s will be statistically compared through a Randomized Block Design Anova test using the agonists as treatments and each mutant receptor as a block.

Bibliography:

Dyer, Fred C., and James L. Could. “Honey Bee Navigation: The Honey Bee's Ability to Find Its Way Depends on a Hierarchy of Sophisticated Orientation Mechanisms”. *American Scientist* 71.6 (1983): 587–597. Web. <http://www.jstor.org/stable/27852344?seq=1#page_scan_tab_contents>.

Gauthier, Monique. "State of the Art on Insect Nicotinic Acetylcholine Receptor Function in Learning and Memory." *Advances in Experimental Medicine and Biology Insect Nicotinic Acetylcholine Receptors* (2010): 97-115. *Springer*. Web. <https://www.researchgate.net/profile/Monique_Gauthier/publication/45950074_State_of_the_art_on_insect_nicotinic_acetylcholine_receptor_function_in_learning_and_memory/links/0c96053a5918baeabf000000.pdf>.

Ihara, Makoto, Masaru Shimomura, Morihiko Kuwamura, Miki Akamatsu, David B. Sattelle, Kazuhiko Matsuda, Maiko Otake, and Valerie Raymond. "A Single Amino Acid Polymorphism in the Drosophila Melanogaster Dα1 (ALS) Subunit Enhances Neonicotinoid Efficacy at Dα1-chicken β2 Hybrid Nicotinic Acetylcholine Receptor Expressed in Xenopus Laevis Oocytes." *Neuropharmachology* 45.1 (2003): 133-44. Web. <http://www.sciencedirect.com/science/article/pii/S0028390803001345>.

Iwasa, Takao, Naoki Motoyama, John T. Ambrose, and R.michael Roe. "Mechanism for the Differential Toxicity of Neonicotinoid Insecticides in the Honey Bee, Apis Mellifera." *Crop Protection* 23.5 (2004): 371-78.*Elsevier*. Web. <http://www.farmlandbirds.net/sites/default/files/Iwasa2004.pdf>.

Shimomura, Masaru, Hiroshi Okuda, Kazuhiko Matsuda, Koichiro Komai, Miki Akamatsu, and David B. Sattelle. "Effects of Mutations of a Glutamine Residue in Loop D of the α7 Nicotinic Acetylcholine Receptor on Agonist Profiles for Neonicotinoid Insecticides and Related Ligands."*British Journal of Pharmacology* 137.2 (2002): 162-69. Web. <http://onlinelibrary.wiley.com/doi/10.1038/sj.bjp.0704848/full>.

Suchail, Severine, David Guez, and Luc P. Belzunces. "Discrepancy between Acute and Chronic Toxicity Induced by Imidacloprid and Its Metabolites in Apis Mellifera." *Environmental Toxicology and Chemistry*20.11 (2001): 2482-486. *Wiley Online Library*. Web. <http://onlinelibrary.wiley.com/doi/10.1002/etc.5620201113/abstract>.

Thany, Steeve H., Guy Lenaers, Valérie Raymond-Delpech, David B. Sattelle, and Bruno Lapied. "Exploring the Pharmacological Properties of Insect Nicotinic Acetylcholine Receptors." *Trends in Pharmacological Sciences* 28.1 (2007): 14-22. *Clinical Key*. Web. <https://www.clinicalkey.com/#!/content/playTesContent/1-s2.0-S0165614706002719?returnurl=null&referrer=null>.

Yamada,T.,Takahashi,H.,Hatano,R.,1999. A novel insecticide, acetamiprid. In: Yamamoto,I.,Casida,J.E. (Eds.),Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer, Tokyo,pp. 149–176.