INVESTIGATION OF THE NOVEL CO-RECEPTOR RL-TGR IN THE STUDY OF AVERSIVE CHEMORECEPTION IN PREDATORY FISH

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INVESTIGATION OF THE NOVEL CO-RECEPTOR RL-TGR IN THE STUDY OF AVERSIVE CHEMORECEPTION IN PREDATORY FISH

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LIST OF SYMBOLS AND ABBREVIATIONS

β_2AR	Beta-2 adrenergic receptor
cAMP	Cyclic adenosine monophosphate
E. coli	Escherichia coli
DNA	Deoxyribonucleic acid
GPCR	G-protein-coupled receptor
HEK293	Human embryonic 293 cells
LB	Lysogeny broth
RAMP	Receptor activity modifying protein
RL-TGR	(RAMP)-like triterpene glycoside receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction

SUMMARY

Chemical defenses are crucial elements in marine ecological environments. This kind of defense is especially important to sessile and soft-bodied organisms that have limited other options for protecting themselves. The identity of chemoreceptors in predators that react to chemical defenses has been an understudied area with many questions still unanswered. Previous work has determined a 97 amino acid co-receptor named RL-TGR that responds to aversive compounds found in prey organisms. In this study, mutants of the RL-TGR protein were created in order to test its mechanism of ligand binding. A gene expression assay also showed that RL-TGR is expressed at the larval stage of development in zebrafish and is found in all major tissues of the fish. By studying this novel co-receptor, the first of its kind found to respond to marine chemical defenses, a greater understanding of the physiological responses of predatory animals to chemical defenses can be gained.

CHAPTER 1

INTRODUCTION

One of the ways in which prey animals have evolved to resist predation is through chemical defenses.[1] These chemical defenses are particularly useful to soft-bodied and sessile or slow-moving organisms such as sea cucumbers and sponges.[2] These organisms contain secondary metabolites such as triterpene glycosides to protect them from predation, unlike armored or mobile marine species like squid and crustaceans, which generally lack these types of defenses.[3] However, the pathways by which these chemicals discourage predation are not completely understood.[1] It is known that predatory fish will reject food pellets that contain a natural concentration of these metabolites,[3] but the specific molecular basis for this type of chemoreception is unknown.[4]

It has been proposed that antifeedant compounds like triterpene glycosides are ligands for membrane chemoreceptors, and they trigger a signaling cascade that ultimately results in an aversive response to the compound.[5] A co-receptor called RL-TGR (RAMP-like triterpene glycoside receptor) has been implicated in triterpene glycoside chemoreception in zebrafish, specifically the triterpene glycoside called formoside.[6] This discovery is especially exciting because RL-TGR is the first receptor or co-receptor known to respond to marine chemical defenses. This co-receptor requires the co-expression of an exogenous G-protein coupled receptor (GPCR) in order to signal, but it has been found to respond in expression assays to terpene glycosides with high ligand specificity.[5] The gene for this co-receptor has been found to be expressed in the heads of adult zebrafish, but it has not yet been determined whether or not juvenile zebrafish also

express the gene in the same way. Although the existence of RL-TGR has been determined, its structure and mechanism have yet to be detailed.

Understanding how marine organisms use chemical defenses to interact with their environments is a crucial element in further understanding predator-prey interactions. Comprehending how chemical defenses fit into the overall framework of marine ecosystems also lends to further understanding species' distribution, community organization, ecological speciation, and feeding patterns. It is thus important to determine the mechanism by which RL-TGR functions, as well as its scope, in order to gain a greater understanding of chemical defenses.

CHAPTER 2

LITERATURE REVIEW

Organisms use chemical signals for a variety of functions. These signals are used to communicate feeding choices, selection of mates, habitat information, the presence of predators, and much more.[7] In recent years, considerable work has been done to study the ways in which organisms interact with chemical cues in their environments, and we are gaining more and more knowledge about the subject. It is impossible to fully understand marine environments without knowing the ways in which marine organisms interact with chemical cues. These signals play a crucial role in the maintenance of marine ecosystems.

Chemical cues can be used to defend organisms from predation. Chemical defenses are commonly found in marine environments, and these kinds of defenses can come in many forms. Phlorotannins are compounds found in some marine algae that bind to plant nutrients to make them indigestible or inactivate digestive enzymes in a predator, thus acting as a defense against grazing.[8] Some forms of chemical defenses can be toxic to predators. Tetrodotoxin (Fig. 1), the chemical that James Bond was poisoned with at the end of *From Russia with Love*, is found in the tissue of many organisms, including the pufferfish, the flatworm, and the blue-ringed octopus. Unfortunately for 007 and those who enjoy consuming pufferfish, there is no known antidote for this toxin. Tetrodotoxin works by inhibiting sodium channels and induces heart failure.[9] A recent study showed that rough-skinned newts with higher levels of tetrodotoxin were more protected that newts with lower levels of tetrodotoxin from predation by garter snakes.[10] Other forms of chemical defense do not cause the predator's death, but can disrupt normal cell function.

The marine sponge genus *Agelas* has a deterrent compound called 4,5-dibromopyrrole-2-carboxylic acid that affects the calcium homeostasis of chemoreceptive cells.[11]

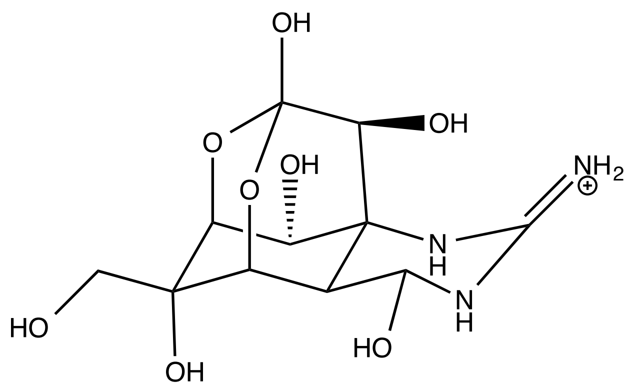

Figure 1. Structure of tetrodotoxin, a potent marine and terrestrial neurotoxin

Aversive compounds are secondary metabolites that protect marine prey organisms from predation, but may not necessarily be toxic. An everyday example of chemical defenses is the compound capsaicin that is found in many species of chili peppers (Fig. 1). The compound causes a burning sensation when it comes into contact with the tongue, deterring many mammalian would-be predators but not all spice-seeking humans and not birds, which are resistant to the hot sensation.[12] Some animals, especially slow or sessile creatures, rely on aversive compounds to act as a defense mechanism. A study of 71 Caribbean sponges showed that 69% of extracts were deterrent to a predatory fish.[13] Sponge defenses are some of the most studied of the marine chemical defenses. They have chemical defenses that include terpenes, polyketides, brominated alkaloids, and saponins, including the class of compounds known as triterpene glycosides.[14] Some marine organisms such as nudibranchs and sea hares sequester aversive compounds from

the sponges and algae they eat. These compounds are used for chemical defenses by the predator.[13]

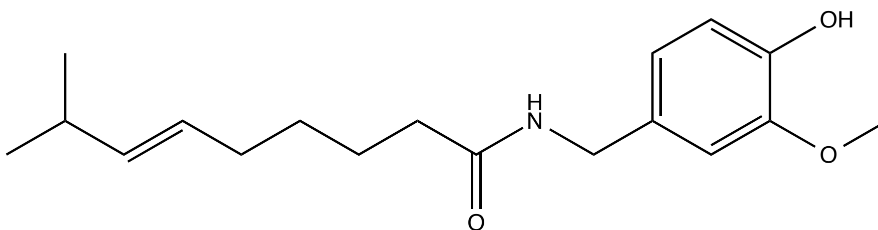

Figure 2. Structure of capsaicin, a metabolite found in chili peppers that causes an aversive response in humans

It is hypothesized that the deterrence from aversive compounds is a result of a response to the taste of the molecules. Taste is one of the five traditionally recognized senses. It is important for the distinction between beneficial and harmful foods and is the driving regulator of feeding behavior.[15] Mammals have five known types of gustatory receptors: salty, sweet, sour, bitter, and umami. Bitter receptors specifically are used to sense and respond to aversive compounds.[16] Sour taste may also play a role in aversive taste recognition, but bitter taste has been studied much more extensively.

Aversive compounds act as ligands that bind to and activate taste chemoreceptors. G-protein coupled receptors (GPCRs) are a type of receptor that transmits signals after the binding of a ligand. The binding of the ligand causes the receptor to undergo a conformational change, which induces a signaling cascade. The signal is amplified and is transmitted via neurons to the central nervous system, after which an aversive response to the compound is generated.[17] While the general system is hypothesized, not much is known about the specific signaling pathways through which the signals for aversive compounds are transmitted. Some receptors require coreceptors in order to bind ligands,

while other GPCRs function effectively without the use of these extra receptors. A co-receptor binds a signaling molecule in addition to a primary receptor and cannot work without this primary receptor. The reasons for the functionality differences between these two cases have yet to be elucidated.

The current study looks at a novel co-receptor named RL-TGR that is implicated in the aversive signaling pathways in marine predatory fish. Previous research has elucidated that RL-TGR is a 96 amino acid transmembrane co-receptor that requires the co-expression of a GPCR in order to bind a triterpene glycoside called formoside (Fig. 3). RL-TGR is predicted to have a single transmembrane domain, a short intracellular domain, and a long extracellular domain (Fig 4).[6] The extracellular region is thought to be the binding region of formoside. However, not much more is known about the protein structure besides its general structure. In order to further explore the makeup of the RL-TGR coreceptor, site-directed mutagenesis was carried out to create specific mutations of key points in the gene. The purpose of these mutations is to determine which elements of the protein's structure are crucial in its proper folding, as well as which elements are necessary to retain its functionality. By testing each of these constructs for their ability to traffic to the membrane correctly, and independently to retain the ability to transmit a signal, more information about the protein's structure can be gleaned.

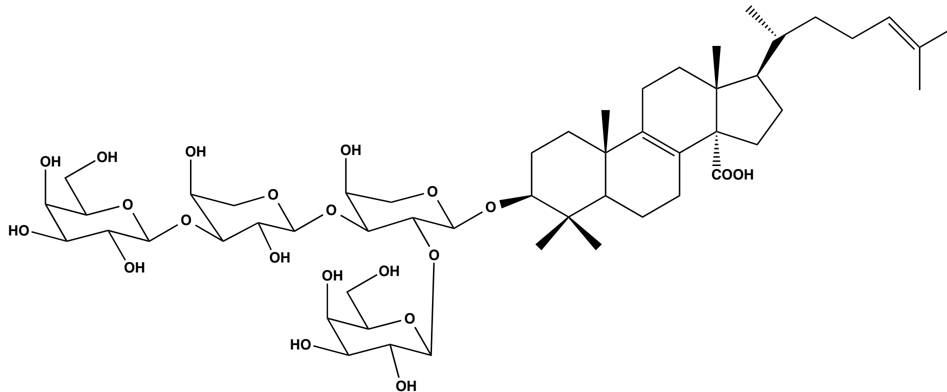

Figure 3. Structure of formoside, a triterpene glycoside that acts as a ligand with the co-receptor RL-TGR.

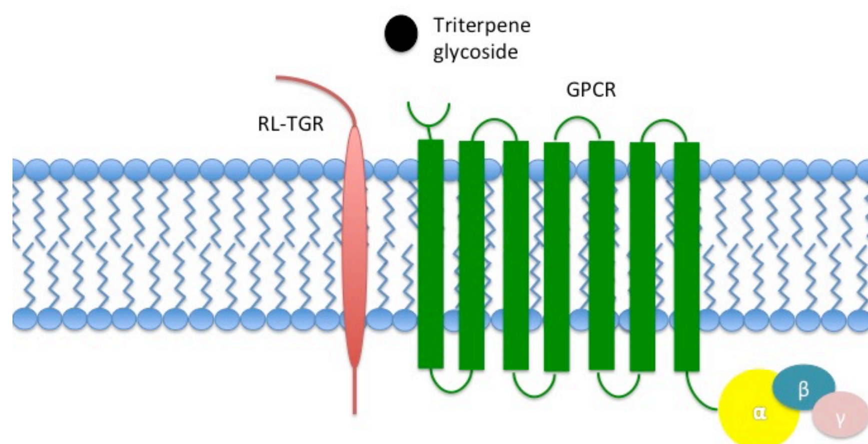

Figure 4. Predicted structure of the RL-TGR and GPCR complex. The GPCR is a seven transmembrane receptor for which RL-TGR acts as a co-receptor. RL-TGR has a single-pass transmembrane domain, a short intracellular domain, and a long extracellular domain.

The RL-TGR gene contains four cysteine codons, which have large implications for its structure (Fig. 5). Cysteine amino acids are unique due to the fact that two cysteine amino acids are able to form a disulfide bond to link with each other. This disulfide bridge affects protein folding and stability. By mutating a cysteine into an alanine, this disulfide bond cannot occur and the resulting protein will have a different shape. This is helpful in determining what parts of the protein are essential to function. It is predicted

that RL-TGR contains a disulfide bond in its extracellular domain, but which cysteine residues are involved is not yet certain.

ATG TAT CTG GAC ATT ATA GAT ATG GTT GAG ACT TGT TTA TTT AGT AGT
TTT GCT ATA TTT TCT AAG ATA TTT CCA GGA TTG CTA CTA ATA TTT TCC
CCA CAT CAT GCA TTT TGT ATT TGG GGA AAC ATG AGA GCA TTG TTA
AAA TAC ACA TGG ACA CAT TTT TTT GTT AAA TAT GAA GAT GCC TTA
AAG GTG ACA GTA TTA AAG TTT GTG AAG CAC TTA GAA CTT ATT TTT
ATA CCT GAA TTC CAA CTC TGT TTG TAT GAA CTT TGT TTT TCG ATC ACA
GAC TGT TTG AAA TAA

Figure 5. RL-TGR sequence with highlighted cysteine amino acids.

Several point mutations of RL-TGR were created in order to test which cysteine amino acid residues are making disulfide bridges. Specifically, each cysteine residue was mutated into an alanine in order to determine which residues were vital for correct protein folding, and thus protein function. The alanine codon chosen was GCG, as this is the most common codon for alanine in *Escherichia coli (E. coli)* cells.

In addition to the creation of mutants, we also sought to determine where the receptor is expressed in zebrafish. Since it is involved in aversive feeding behavior, expression of the RL-TGR gene would be expected in the taste or olfactory epithelium. In order to test this hypothesis, RNA extraction was performed on an age gradient of zebrafish ranging from 5 day-old larvae to adults. The zebrafish heads were separated from the rest of body (ROB), meaning the trunk area of the fish, in order to test whether RL-TGR transcripts would be expressed more strongly in the heads where gustatory organs are located.

CHAPTER 3

MATERIALS AND METHODS

3.1 Biological Material

Wild-type zebrafish *Danio rerio* were obtained from the Shin lab at Georgia Tech. Zebrafish ages were 5 day old, 9 day old, 14 day old, 19 day old, 45 day old, and adult (older than 90 days old) zebrafish. All fish were euthanized with an overdose of tricaine methane sulfonate and left in solution for ten minutes in accordance with NIH standard procedures.

3.2 General Procedures and Biological Materials

Plasmid maxi preps were done using E.Z.N.A Fastfilter Plasmid DNA Maxi Kit D6924-03. Plasmid mini preps were done using E.Z.N.A Plasmid Mini Kit I D6948-01. Transformed plasmids were stored long-term as glycerol stocks. For reverse-transcriptase polymerase chain reaction (RT-PCR), the AccessQuick RT-PCR system from Promega was used. All gels were run on a 1% agarose gel with 0.5µg/mL of ethidium bromide (EtBr).

3.3 Site-Directed Mutagenesis

Site-directed mutagenesis was done using the protocol from the QuikChange II XL Site-Directed Mutagenesis Kit with XL 10-Gold ultracompetent cells. *E. coli* were grown on LB containing the antibiotic ampicillin overnight at 37 °C.

3.4 RNA Extraction of Zebrafish

Zebrafish were euthanized with tricaine mesylate, preserved in RNA*later*, and then stored in a 4 °C refrigerator. Zebrafish tissues were frozen with liquid nitrogen and homogenized in TRIzol reagent (Invitrogen). Total RNA was extracted following the

manufacturer's standard protocol from the Omega Biotek E.Z.N.A Tissue RNA Kit (R6688-01). RNA was eluted with diethylpyrocarbonate (DEPC)-treated water and stored at -80 °C.

3.5 Creation of Primers for Site-Directed Mutagenesis

In order to create a mutation at the second cysteine at residue 38, primers were created according to the protocol put forth by the QuikChange II XL Site-Directed Mutagenesis Kit by Stratagene. Primers were constructed using the online software at http://bioinformatics.org/primerx/cgi-bin/DNA_1.cgi.

3.6 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using a QIAGEN OneStep RT-PCR kit in a GeneAmp PCR System 2700 (Applied Biosystems) using the following conditions: one cycle (95 °C for 2 minutes), followed by 40 cycles [95 °C for one minute, 55 °C for one minute, 72 °C for one minute], followed by a last cycle [72 °C for 7 minutes and 4 °C for infinite amount of time]. RT-PCR products were separated by agarose gel electrophoresis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Creation of RL-TGR point mutant constructs

Point mutations of each of the extracellular cysteines of RL-TGR were created to test for their functionality in ligand binding and protein-protein interactions. These mutants were created through PCR-based site-directed mutagenesis. In order to induce a mutation at the cysteine 38, appropriate primers were created (Fig. 6). The created primers MPN003 and MPN004 were 37 bases in length and had a melting temperature of 75.8 °C, which is lower than the 78 °C minimum suggested melting temperature. However, this deviation from the suggested primer characteristics did not seem to alter the effectiveness of the primers in creating the desired mutant (Fig. 7, 8). The mutant was submitted for sequencing through Eurofins Genomics.

```
MPN003: 5' CCCCACATCATGCATTTGCGATTTGGGGAAACATGAG 3'
MPN004: 5' CTCATGTTTCCCCAAATCGCAAATGCATGATGTGGGG 3'
```

Figure 6. Primers MPN003 and MPN004 were used to create the point mutation of cysteine to alanine.

```
R_L2    CACATCATGCATTTTGTATTTGGGGAAACATGAGAGCATTGTTAAAATACACATGGACAC
C_T7    CACATCATGCATTTGCGATTTGGGGAAACATGAGAGCATTGTTAAAATACACATGGACAC
        **************   *******************************************
```

Figure 7. Alignment software shows that the created sequence (C_T7) differs form the original sequence (R_L2) by the alanine base. The online service Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to create the alignment.

```
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Length=1427
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ACCCNNAGTTCGAAAAGGGTGCACTTGAAGTCCTCTTTCAGGGACCCGGGTACCAGGATCCCTTCAGA
GTTTTTATGTATCTGGACATTATAGATATGGTTGAGACTTGTTTATTTAGTAGTTTTGCTATATTTTC
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GAGCATTGTTAAAATACACATGGACACATTTTTTTGTTAAATATGAAGATGCCTTAAAGGTGACAGTA
TTAAAGTTTGTGAAGCACTTAGAACTTATTTTTATACCTGAATTCCAACTCTGTTTGTATGAACTTTG
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TCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGG
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TTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACAC
TCAACCCTATCTCGGTCTATTCTTTTGATTNNNAAGGGATTTTGCCGATTNCGGCCTATTGGTTNAAA
```

Figure 8. Full sequence of mutated RL-TGR in vector pet52b(+). The RL-TGR sequence is highlighted in yellow and the mutation is highlighted in blue.

4.2 Zebrafish Gene Expression Test show RL-TGR is expressed in all fish tissues

Zebrafish at ages 5 days, 9 days, 14 days, 19 days, and 90 days were tested for RL-TGR expression. Zebrafish at ages 4 days to 29 days are in the larval stage of development whereas adults are aged 90 days to 2 years old. The adult zebrafish was split into three parts: a head, trunk, and rest of body. The trunk consists of the area behind the head to the top fin, an area that includes the heart, kidney, spleen, and intestines. The rest of body in this case contained the rear half of the zebrafish, including parts of the intestines and the fins. The larval zebrafish were split into the head and the rest of body (ROB), which consisted of entirety of the fish except for the head. The expression of RL-TGR in the head was stronger than the rest of the body, but expression was apparent in both parts of the body (Fig. 9, 10).

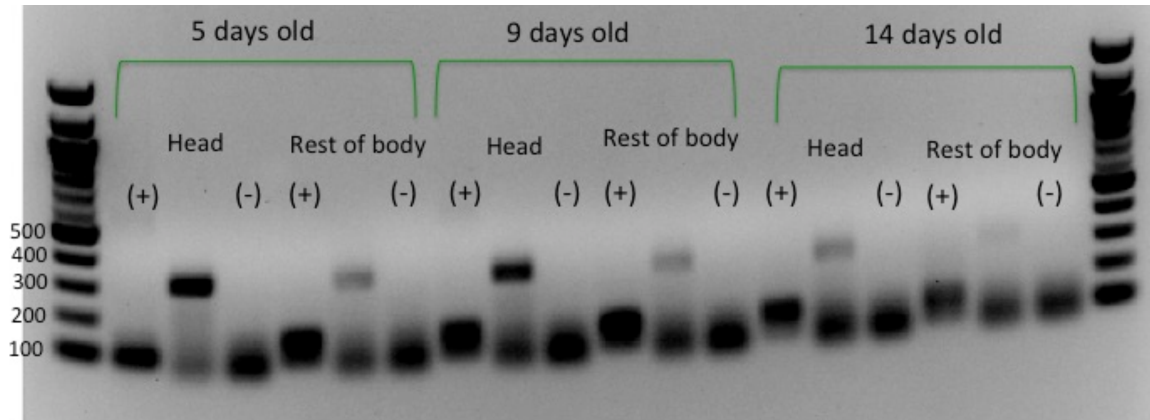

Figure 9. Gene expression of RL-TGR in the head and rest of body of a 5 day old, 9 day old, and 14 day old zebrafish. Expression of RL-TGR at ~300 bp was strongly observed in the head regions of each fish and less strongly in the rest of body.

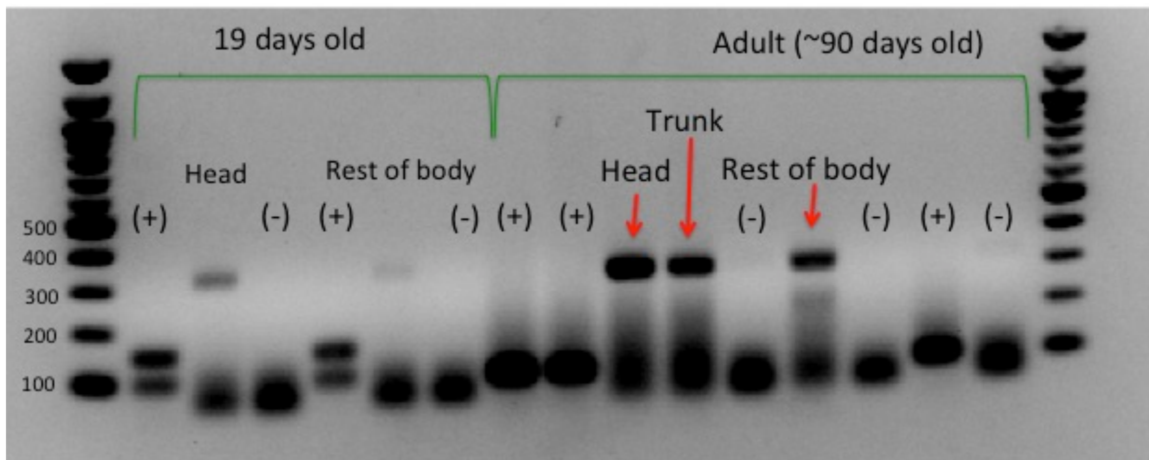

Figure 10. Gene expression of RL-TGR in the head and rest of body in a 19-day old zebrafish and an adult (64 day old) zebrafish. Expression of RL-TGR at ~300 bp was strongly observed in the head regions of the fish and less strongly in the rest of body.

When their heads and rest of body were separated in the larvae and tested for RL-TGR content, RL-TGR transcripts were expressed in all tissues (Fig. 9, 10). The heads were found to have stronger expression than the body, but expression occurred all over the fish. Expression in the gut region in particular is intriguing, as taste receptors have previously been shown to exist in the gastrointestinal system of humans and other animals.[18]

CHAPTER 6

CONCLUSION

This research will add to the growing literature on the mechanisms by which marine predators detect aversive compounds present in prey organisms. RL-TGR is an exciting find that could lead to further knowledge on chemical defense and the discovery of more co-receptors that act in aversive chemical signaling. Further research will lead to new discoveries about the mechanism and scope of how predators use chemosensory processes to interact with their environment.

Additional work is needed in order to determine the structure and localization of RL-TGR. Future work will include testing the created mutants on multiple bioassays to check for functionality. To test for signaling function, mutants will be evaluated on a cAMP immunoassay. This will confirm that RL-TGR responds to formoside in HEK293 cells co-expressing β_2AR. Through this assay, we will also be able to determine which mutants retain signaling functionality and which mutations to the gene destroy the protein's ability to function effectively. The mutants will also be evaluated on a trafficking assay that uses Strep/His-tagged RL-TGR to determine the mutant's ability to traffic to the plasma membrane of HEK293 cells co-expressing β_2AR. This assay will determine whether each mutant retains the ability to be properly transported into the cell membrane, or if the mutation will interfere with its ability to be transported. We can also identify regions of RL-TGR that interact with β_2AR from this assay.

The testing of the mutants will show if RL-TGR acts in a similar way to RAMP protein in using the cysteines on its extracellular tail for protein-protein interactions with the extracellular tail of a GPCR.[19] It is still unclear if triterpene glycosides bind to the

extracellular portion of RL-TGR, the GPCR, or some pocket created between the co-receptor and larger protein.

The presence of gene expression in the gut area of zebrafish is an interesting finding, as a taste receptor would normally be expected to be expressed very strongly in the head region and not found in other areas of the tissue. Although it has been found that taste receptors can be found in the gut area,[18] previous fish-feeding assays with zebrafish show that zebrafish will not swallow food pellets laced with triterpene glycosides like formoside.[6] The presence of gene expression within the gut does not necessarily equate to a large quantity of RL-TGR protein in the gut. Further experimentation is necessary to discern the level of RL-TGR protein within the gut. *In vivo* protein immunostaining using a RL-TGR-specific antibody could answer this question.

With the results from these tests, the study of RL-TGR will allow for a greater understanding of chemosensory pathways. Although RL-TGR is the first receptor to be discovered that responds to marine chemical defenses, it is likely to not be the last. The discovery of a co-receptor that works in conjunction with a larger GPCR for chemical reception is exciting, as it suggests that fish may be combining different combinations of co-receptors and GPCRs to bind an assortment of ligands, rather than synthesizing a new GPCR for each class of ligand. More work is needed to understand the complex relationships between marine prey and predators and the role that chemical defenses play in shaping marine ecosystems.

REFERENCES

1. Derby, C. D.; Sorensen, P. W., Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans. *Journal of Chemical Ecology* **2008,** *34* (7), 898-914.
2. Hay, M. E., Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Ann Rev Mar Sci* **2009,** *1*, 193-212.
3. Cohen, S. A.; Hatt, H.; Kubanek, J.; McCarty, N. A., Reconstitution of a chemical defense signaling pathway in a heterologous system. *The Journal of Experimental Biology* **2008,** *211* (Pt 4), 599-605.
4. Lunceford, B. E.; Kubanek, J., Reception of aversive taste. *Integr Comp Biol* **2015,** *55* (3), 507-17.
5. Cohen, S. P.; Haack, K. K.; Halstead-Nussloch, G. E.; Bernard, K. F.; Hatt, H.; Kubanek, J.; McCarty, N. A., Identification of RL-TGR, a coreceptor involved in aversive chemical signaling. *Proceedings of the National Academy of Sciences of the United States of America* **2010,** *107* (27), 12339-44.
6. Cohen SP. Functional identification and initial characterization of a fish co-receptor involved in aversive signaling. PhD thesis, Georgia Institute of Technology, Atlanta, GA, 2009.
7. Cohen, S. A.; Hatt, H.; Kubanek, J.; McCarty, N. A., Reconstitution of a chemical defense signaling pathway in a heterologous system. *The Journal of Experimental Biology* **2008,** *211* (Pt 4), 599-605.
8. Pawlik, J.; Chanas, B.; Toonen, R. J.; Fenical, W., Defenses of caribbean sponges against predatory reef fish. I. Chemical deterrancy. *Marine Ecology Progress Series* **1995,** *127*, 183-194.
9. Lago, J.; Rodriguez, L. P.; Blanco, L.; Vieites, J. M.; Cabado, A. G., Tetrodotoxin, an extremely potent marine neurotoxin: distribution, toxicity, origin and therapeutical uses. *Marine Drugs* **2015,** *13* (10), 6384-406.
10. Williams, B. L.; Hanifin, C. T.; Brodie, E. D.; Brodie Iii, E. D., Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*). *Chemoecology* **2010,** *20* (4), 285-290.
11. Bickmeyer, U.; Assmann, M.; Kock, M.; Schutt, C., A secondary metabolite, 4,5-dibromopyrrole-2-carboxylic acid, from marine sponges of the genus *Agelas* alters cellular calcium signals. *Environ Toxicol Pharmacol* **2005,** *19* (3), 423-7.
12. Jordt, S.; Julius, D., Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell* **2002,** *108*, 421-430.
13. Pawlik, J. R., Marine Invertebrate Chemical Defenses. *Chem Rev.* **1993,** *93*, 1911-1922.
14. Puglisi, M. P.; Sneed, J. M.; Sharp, K. H.; Ritson-Williams, R.; Paul, V. J., Marine chemical ecology in benthic environments. *Natural Product Reports* **2014,** *31* (11), 1510-53.
15. Yarmolinsky, D. A.; Zuker, C. S.; Ryba, N. J., Common sense about taste: from mammals to insects. *Cell* **2009,** *139* (2), 234-44.
16. Adler, P.; Taylor, J.; Charlton, J., The domineering non-autonomy of frizzled and Van Gogh clones in the *Drosophila* wing is a consequence of a disruption in local

signaling. *Elsevier* **2000,** *96* (2), 197-207.
17. Chandrashekar, J.; Hoon, M. A.; Ryba, N. J.; Zuker, C. S., The receptors and cells for mammalian taste. *Nature* **2006,** *444* (7117), 288-94.
18. Gabriel, A., Taste receptors in the gastrointestinal system. *Flavour* **2015,** *4* (14).
19. Flahaut, M.; Pfister, C.; Rossier, B. C.; Firsove, D. N-glycosylation and conserved cysteine residues in RAMP3 play a critical role for the functional expression of CRLR/RAMP3 adrenornedullin receptor. *Biochemistry* **2003**, 42 (34), 10333-10341.

SUPPLEMENTARY

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TCCCTTCAGAGTTTTTATGTATCTGGACATTATAGATATGGTTGCGACTGCGTTATTTAGTAGTTTTG
CTATATTTTCTAAGATATTTCCAGGATTGCTACTAATATTTTCCCCACATCATGCATTTTGTATTTGG
GGAAACATGAGAGCATTGTTAAAATACACATGGACACATTTTTTTGTTAAATATGAAGATGCCTTAAA
GGTGACAGTATTAAAGTTTGTGAAGCACTTAGAACTTATTTTTATACCTGAATTCCAACTCTGTTTGT
ATGAACTTTGTTTTTCGATCACAGACTGTTTGAAAGCGGCCGCAGAGCTCGCTCTGGTGCCACGCGGT
AGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAATTAACCTAGGCTGCTGCCACCGCTGA
GCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAA
CTATATCCGGATTGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTA
CGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTT
CTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAG
TGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCT
```

Figure 11. Sequence of pBEL09 in pet52b(+) vector, which has a cysteine-to-alanine mutation at the first cysteine. The RL-TGR sequence is highlighted in yellow and the mutation is highlighted in blue.

```
>pBEL11_T7 Sample_Name=pBEL11_T7 Chromat_id=3056922 Read_id=3019529
Version=1 Length=1197
NNNNNNNNNNNNNNNNNNNNTCNNNCTNNNNNNATTTTGTTTAACTTTAAGAAGGAGATATACCATGG
CAAGCTGGAGCCACCCGNNGTTCGAAAAGGGTGCACTTGAAGTCCTCTTTCAGGGACCCGGGTACCAG
GATCCCTTCAGAGTTTTTATGTATCTGGACATTATAGATATGGTTGAGACTTGTTTATTTAGTAGTTT
TGCTATATTTTCTAAGATATTTCCAGGATTGCTACTAATATTTTCCCCACATCATGCATTTTGTATTT
GGGGAAACATGAGAGCATTGTTAAAATACACATGGACACATTTTTTTGTTAAATATGAAGATGCCTTA
AAGGTGACAGTATTAAAGTTTGTGAAGCACTTAGAACTTATTTTTATACCTGAATTCCAACTCGCGTT
GTATGAACTTTGTTTTTCGATCACAGACTGTTTGAAAGCGGCCGCAGAGCTCGCTCTGGTGCCACGCG
GTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAATTAACCTAGGCTGCTGCCACCGCT
GAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGG
AACTATATCCGGATTGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGT
TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCT
TTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTT
AGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCC
```

Figure 12. Sequence of pBEL11 in pet52b(+) vector, which has a cysteine-to-alanine mutation at the third cysteine. The RL-TGR sequence is highlighted in yellow and the mutation is highlighted in blue.

```
>pBEL12_T7 Sample_Name=pBEL12_T7 Chromat_id=2883670 Read_id=2856860
Version=1 Length=1310
NNNNNNNNNNNNANNNNNNNNNTCTNNNNNNATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAA
GCTGGAGCCACCCGCAGTTCGAAAAGGGTGCACTTGAAGTCCTCTTTCAGGGACCCGGGTACCAGGAT
CCCTTCAGAGTTTTTATGTATCTGGACATTATAGATATGGTTGAGACTTGTTTATTTAGTAGTTTTGC
TATATTTTCTAAGATATTTCCAGGATTGCTACTAATATTTTCCCCACATCATGCATTTTGTATTTGGG
GAAACATGAGAGCATTGTTAAAATACACATGGACACATTTTTTTGTTAAATATGAAGATGCCTTAAAG
GTGACAGTATTAAAGTTTGTGAAGCACTTAGAACTTATTTTTATACCTGAATTCCAACTCTGTTTGTA
TGAACTTTGTTTTTCGATCACAGACGCTTTGAAAGCGGCCGCAGAGCTCGCTCTGGTGCCACGCGGTA
GTTCCGCTCATCACCACCATCATCACCATCACCACCACTAATTAACCTAGGCTGCTGCCACCGCTGAG
CAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAAC
TATATCCGGATTGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTAC
GCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTC
TCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGT
GCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTG
ATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCNTTAATAGTGGACTCTTGTTCCAACTGG
AACACACTCAANCCTATCTCGGNNATTCTTTTGATTNTAAGGGATTTTGCCGATTTCGGNNNTTGGTT
```

Figure 13. Sequence of pBEL12 in pet52b(+) vector, which has a cysteine-to-alanine mutation at the fourth cysteine. The RL-TGR sequence is highlighted in yellow and the mutation is highlighted in blue.