Effects of Diet Manipulation on Conopeptide Profiles in Fish-Eating *Conus striatus*

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By

Elizabeth A. Andrews

Thesis Committee:

Jon-Paul Bingham, Chairperson
Qing Li
Harry Ako

Abstract

There are approximately 500 different species of cone snails (*Conus*). Each armed with a unique venom profile used to immobilize prey and to defend against predators. *Conus* toxins ("conopeptides") are comprised of small peptides approximately 10-30 amino acids in length. Individual conopeptides are capable of selectively targeting ligand-gated or voltage-gated ion channels, as well as specific isoforms of receptors. An interesting facet to conopeptides is their hypervariability in amino acid sequence. This is compounded by post-translational modifications which may play a determining role in how the conopeptides fold and hence their reactivity at K^+, Na^+, and Ca^{2+} ion channels and various protein receptors. Our research aims to investigate how the manipulation of diet will affect native Hawaiian *Conus striatus* venom profiles. This study will serve as a model system to explore the biochemical repertoire of the cone snails' venom profile. We believe that the current estimation of 100,000 conopeptides is an underestimation and we seek to broaden the present library. The cone snails will be fed capsules containing either fish food, with either human vitamin pack (w/w), a designated halide, or a combination of the two. Quantitative analysis of the resulting milked venom output will be undertaken by reverse-phase high performance liquid chromatography (RP-HPLC).

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List of Abbreviations

AAA	Amino Acid Analysis
Ala	Alanine
Arg	Arginine
Asp	Asparagine
AUC	Area Under Curve
C.	*Conus*
Ca_v	Calcium voltage-gated ion channel
cDNA	clone Deoxyribonucleic Acid
CID	Collision Induced Dissociation
Cys	Cysteine
DNA	Deoxyribonucleic Acid
ED_{50}	Effective Dose
ESI-MS	Electrospray Ionization-Mass Spectroscopy
FMOC	Fluorenylmethyloxycarbonyl Chloride
Glu	Glutamate
Gly	Glycine
His	Histidine
Hyp	Hydroxy Proline
Ile	Isoleucine
K_v	Potassium voltage-gated ion channel
LC	Liquid Chromatography
LD_{50}	Lethal Dose
Leu	Leucine
Lys	Lysine
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization -Time of Flight-Mass Spectroscopy
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectroscopy
nACh	Nicotinic Acetylcholine
Na_v	Sodium voltage-gated ion channel
NMR	Nuclear Magnetic Resonance
NSI-MS	Nanospray Ionization Mass Spectroscopy
OPA	O-Phthalaldehyde
PAL	Peptidylamido-glycolatelayse
PAM	Peptidyl α-Monooxygenase
PDI	Protein Disulfide Isomerase
Phe	Phenylalanine
PHM	Peptidyl α-Hydroxylating Monooxygenase
PITC	Phenylisothiocyanate
PPI	Peptidyl-Prolyl cis-Isomerase
Pro	Proline
PTC	Phenylthiocarbamoyl
PTM	Post Translational Modification
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
Ser	Serinea
TFA	Trifluoroacetic Acid
Thr	Threonine
TTX	Tetrodotoxin
Tyr	Tyrosine
Val	Valine
VGSC	Voltage-Gated Sodium Channel

Chapter 1: INTRODUCTION

1.1 Cone Snails

Cone snails (*Conus*) are a successful species of carnivorous marine gastropods. There are over 500 different species, each equipped with a venomous cocktail to immobilize their prey. The venom is produced in a long tubular venom duct containing secretory cells (Jakubowski et al., 2005). The three classes of *Conus* are piscivore (fish eating), vermivore (worm eating), and molluscivore (mollusk eating). Of the three species, fish-hunting cone snails are the most readily understood. Piscivore cone snails can be divided into two subtypes; "net fishing" cone snails (*Conus geographus*) and "hook and line" cone snails (*Conus striatus*). Both types use disposable radular harpoons that act like hypodermic needles to envenomate their prey (Olivera et al., 2007). The mechanisms of transport for conotoxins from the venom duct into the proboscis for envenomation are not very well understood (Jakubowski et al., 2005).

Cone snails sense the presence of their prey through chemoreceptors in their siphon. From the rostrum (mouth), the snail extends its proboscis when prey is present and fires the radula and will "reel" in the fish. Net fishing cone snails, have a large distensible rostrum to engulf prey prior to stinging. Their venom is comprised of over 100 different peptides that selectively target ligand gated and voltage gated ion channels. Conopeptides can range in size from 12 to 30 amino acids in length (Olivera, 1997). Conopeptides are highly constrained due to the formation of disulfide bridges and are capable of undergoing post-translational modifications.

The specific actions of conopeptides can be grouped into what Olivera et al., (1994) describes as "cabals." Conopeptides that inhibit neuromuscular functioning are designated as "motor cabal," while those that induce excitotoxic shock form the "lightening-strike cabal." Conopeptides are further classified into pharmacological families, which include targeted receptors and physiological activity, and is noted by a Greek symbol. All piscivorous cone snails manufacture peptides that selectively target

ligand and voltage-gated ion channels, including voltage-gated calcium (Ca_v), potassium (K_v), and sodium (Na_v) channels as well as nicotinic acetylcholine (nACh) receptors (Kelley et al., 2006). These particular subsets of conopeptides have proven to be valuable probes for neurophysiological studies (Kaas et al., 2010) in vertebrates as well as in the development of therapeutics (Kelley et al., 2006; Terlau et al., 2004).

1.1.2 Cone snail anatomy

External anatomy of the cone snail includes the siphon, foot, eye stalks, and rostrum. The rostrum and proboscis are visible during the feeding or hunting process. Cone snails have a complex system of venom production and delivery of the venom. The apparatus itself includes the venom bulb, venom duct/gland, and radula sac (Marshall et al., 2002). Venom is produced in the venom duct, which is then propelled forward when the venom bulb contracts. In the radula sac, hypodermic needle-like harpoons are made and stored until feeding (Figure 1).

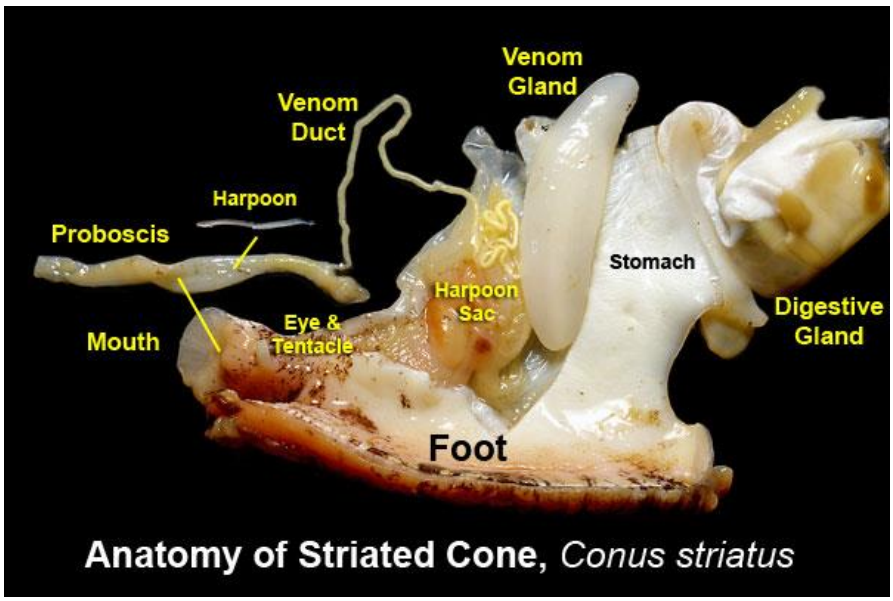


Figure 1. Picture courtesy of Keoki Stender; illustrates *C. striatus* internal anatomy. Highlighting the venom gland, venom duct, harpoon sac, and proboscis.

1.2 *Conus striatus*

The striated cone snail has a voracious appetite for fish, in which it actively and aggressively will hunt for fish, most notably at night. This particular species of *Conus* can be found buried in the sandy substrate along coral reefs. As noted above, *Conus striatus* uses the hook and line technique of prey capture. The "lightening-strike cabal" of *C. striatus* causes substantial depolarization of axons in the surrounding area of injection, inducing immediate tetanic paralysis (Olivera, 2002). Delta (δ) conopeptides are responsible in this particular cabal preventing sodium channels from closing, while other conopeptides inhibit various potassium channels. After the immobilization of its prey with the "lightening-strike cabal," conopeptides of the motor cabal cause irreversible neuromuscular blockage (Olivera, 2002).

When *C. striatus* conotoxin α-conotoxin SII was tested in frog neuromuscular junctions, the conotoxin reversibly blocked electrically evoked postsynaptic responses (Ramilo et al., 1992). ω-Conotoxins SVIA and SVIB also reversibly blocked electrically evoked postsynaptic responses. At a concentration of 10 μM, ω-conotoxin SVIA completely blocked all synatpic responses. However, at the same concentration, ω-conotoxin SVIB did not completely block all synaptic responses (Ramilo et al., 1992).

Conotoxin SO-3 is homologous to ω-conotoxin MVIIA from *Conus magus* in sequence (Wen et al., 2006) even though SO-4 and SO-6 lack the same homology to suggest their function. SO-3 was synthesized by Wen et al. (2006) using FMOC strategies for solid phase peptide synthesis. SO-3 was tested in comparison to ω-conotoxin MVIIA against a wide variety of pain models including tail-flick latency, standard hot plate, acetic acid stimulus in mice, light radiation, and mechanical tail tests in rats. When injected intrathecal or intracerebral, SO-3 demonstrated comparable to slightly improved analgesic effects when compared to identical doses of ω-conotoxin MVIIA (Wen et al., 2006). Toxicity tests of SO-3 in *Carassius carassius* at a max dose of 8.5 μg/per goldfish, did not demonstrate any associated lethality, despite ω-conotoxin MVIIA being lethal at the same dosage. LD_{50} (median lethal dose) levels in mice, following intracerebral injection, was 13.5 mg/kg. In comparison to the median effective

dose (ED_{50}) of 0.75 μg/kg, the lethal dose is 18,000 times higher, suggesting that SO-3 has an optimal safety index (Wen et al., 2006).

Voltage-gated sodium channels (VGSCs) regulate sodium levels across the membrane of neurons. μ-Conotoxins like SIIIA from *C. striatus* are utilized as research probes in studying ion channels and membrane receptors (Wang et al., 2006). Tetrodotoxin (TTX) from the puffer fish, blocks the outer portion of the VGSCs and is used as a standard in sodium channel studies (Wang et al., 2006). Neuronal VGSCs that are susceptiple to TTX are of particular interest in investigating nociception transmission. μ-Conotoxin-SIIIA has demonstrated irreversible inhibition of the TTX resistant VGSCs in neurons of receptors. At 100 nM to 3 μM, μ-conotoxin SIIIA almost completely inhibited TTX resistant sodium VGSCs within 60 mins (Wang et al., 2006). Table 1 categorizes all known striatus conotoxins and conopeptides.

Table 1. List of all known conotoxins and conopeptides isolated from *Conus striatus*.

Family	Structure	Class	Name	Amino Acid Sequence	Calc. MH	References
A	CC - - C - - C - - C - - C	κ	SIVA	ZKSLVPSVITTCCGYDOGTMCOOCRCTNSC*	3190.29	Craig, Zafaralla et al. 1998 ; Wang, Jiang et al. 2003; Santos, McIntosh et al. 2004)
			ST4082	EKSLVPSVITTCCGYDOGTMCOOCRCTNSC*		
		Genetic Derived	SIVB	ZKELVPSVITTCCGYDOGTMCOOCRCTNSCOTKOKKO*	4059.8	(Santos, McIntosh et al. 2004)
	CC - - C - - C	α	SI	ICCNPACGPKYSC*	1352.51	(Zafaralla, Ramilo et al. 1988; Benie, Whitford et al. 2000)
			SIA	YCCHPACGKNFDC*	1454.5	(Myers, Zafaralla et al. 1991)
			SII	GCCCNPACGPNYGCGTSCS	1789.51	(Ramilo, Zafaralla et al. 1992)
		Genetic Derived	S1.1	NGCCRNPACESHRC*	1543.56	(Santos, McIntosh et al. 2004)
M	CC - - C - - C - - CC	μ	SIIIA	ZNCCNGGCSSKWCRDHARCC*	2205.76	(Bulaj, West et al. 2005; Wang, Zhang et al. 2006; Schroeder, Ekberg et al. 2008)
			SIIIB	ZNCCNGGCSSKWCKGHARCC*	2119.75	(Schroeder, Ekberg et al. 2008)
I	C - - C - - CC - - CC - - C - - C	Genetic Derived	A11.2a	GCKKDRKPCSYQADCCNCCPIGTCAPSTNWILPGCSTGPFMAR	4573.91	(Buczek, Yoshikami et al. 2005)
			S11.3	CVPPSRYCTRHRPCCRGTCCSGLCRPMCNLWY	3712.52	(Kaas, Westermann et al. 2008)
O	C - - C - - CC - - C - - C	ω	SVIB	CKLKGQSCRKTSYDCCSGSCGRSGKC*	2737.14	(Ramilo, Zafaralla et al. 1992; Nielsen, Thomas et al. 1996)
			SVIA	CRSSGSOCGVTSICCGRCYRGKCT*	2493.01	(Ramilo, Zafaralla et al. 1992)
		δ	SVIE	DGCSSGGTFCGIHOGLCCSEFCFLWCITFID	3328.3	(Ramilo, Zafaralla et al. 1992; Bulaj, West et al. 2005; Kauferstein, Melaun et al. 2005; Leipold, Hansel et al. 2005)
		Genetic Derived	Conotoxin-2	AADCIEAGNYCGPTVMKLCCGFCSPYSKICMNYPKN	3887.61	(Kauferstein, Melaun et al. 2005)
			Conotoxin-3	CESYGKPCGIYNDCCNACDPAKKTCT	2780.04	(Kauferstein, Melaun et al. 2005)
			Conotoxin-9	EGCSSGGTFCGIHPGLCCSEFCFLWCITFID	3325.31	(Kauferstein, Melaun et al. 2005)
			Conotoxin-15	CRPSGSPCGVTSICCGRCSRGKCT	2410.98	(Kauferstein, Melaun et al. 2005)
			S6.1	CKAAGKSCSRIAYNCCTGSCRSGKC	2550.05	(Kaas, Westermann et al. 2008)
			S6.2	CRSSGSPCGVTGICCGRCYRGKCT	2446.98	(Kaas, Westermann et al. 2008)
			S6.6	CKGKGAPCRKTMYDCCSGSCGRRGKC	2748.14	(Pi, Liu et al. 2006)

Table 2. List of all known conopeptides isolated from *Conus striatus.*

Family	Structure	Class	Name	Amino Acid Sequence	Calc. MH	References
O	C - - C - - CC - - C -- C	Genetic Derived	S6.8	DGCSNAGGFCGIHPGLCCSEICLVWCT	2738.05	(Kaas, Westermann et al. 2008)
			S6.10	CTPDDGACAEPVQCCSTFCNPVTNMCIDWLGIGLSRSVL	4111.74	(Pi, Liu et al. 2006)
			S6.11	CRTWNAPCSFTSQCCFGKCAHHRCIAW	3109.26	(Pi, Liu et al. 2006)
			S03	CKAAGKPCSRIAYNCCTGSCRSGKC*	2559.08	(Lu, Yu et al. 1999; Yan, Tu et al. 2003; Wen, Yang et al. 2005)
			S04	ATDCIEAGNYCGPTVMKICCGFCSPYSKICMNYPKN	3917.62	(Lu, Yu et al. 1999; Kauferstein, Melaun et al. 2005)
			S05	STSCMEAGSYCGSTTRICCGYCAYFGKKCIDYPSN	3762.47	(Lu, Yu et al. 1999; Kauferstein, Melaun et al. 2005)
Unclassified			Bromocontryphan-S	GCOWEPWC*	1068.27	(Jakubowski, Kelley et al. 2006)
			Contryphan-S	GCOwEPWC*	990.36	(Nielsen, Thomas et al. 1996; Jakubowski, Kelley et al. 2006)
			Conopressin S [Arg]	CIIRNCPRG*	1027.52	(Cruz, Santos et al. 1987; Gray, Olivera et al. 1988; Walker, Jensen et al. 2009)
			Con-ikot-ikot	SGPADCCRMKECCTDRVNECLQRYSGREDKFVSFCYQ -EATVTCGSFNEIVGCCYGYQMCMIRVVKPNSLSGAHEA -CKTVSCGNPCA	9425.97	(Walker, Jensen et al. 2009)
		Genetic Derived	S18.1	AGLTVCLSENRKRLTCSGLLNMAGSVCCKVDTSCCSSQ	3930.76	(Kaas, Westermann et al. 2008)
			S4.3	QKELVPSKTTTCCGYSPGTMCPSCMCTNTCPPQK	3617.49	(Pi, Liu et al. 2006)
			S6.7	CMEAGSYCGSTTRICCGYCAYSASKNVCDYPSN	3500.3	(Pi, Liu et al. 2006)
			Conkunitzin-S1	KDRPSLCDLPADSGSGTKAEKRIYYNSARKQCLRFDYTGQGGNENNFRR TYDCQRTCLYT	6929.63	(Bayrhuber, Vijayan et al. 2005; Dy, Buczek et al. 2006)
			Conkunitzin-S2	ARPKDRPSYCNLPADSGSGTKPEQRIYYNSAKKQCVTFTYNGKGGNGNN FSRTNDCRQTCQYPVG	7207.9	(Korukottu, Bayrhuber et al. 2007)

1.3 Post-Translational Modifications

Post-translational modifications (PTMs) extend or enhance the biological functioning of the peptide by binding to other functional groups. PTMs to proteins are essential for the functioning of a variety of cellular pathways due to their unique ability to alter the localization, conformation, and/or binding partners of a given protein (Molecular Cell, 2013). Such as phosphates, lipids, acetates, and carbohydrates, which can change the physical structure of the peptide, altering disulfide bridge formation and hence, functionality. Common PTMs are γ-carboxylation of glutamate, C-terminal amidation, sulfonation of tyrosine, pyroglutamylation, hydroxyproline residues, glycosylation of serine and threonine residues, as well as bromination of tryptophan (Jakubowski et al., 2004). Which is reported by Craig et al., (1997) in the bromination of tryptophan in peptides from *Conus imperialis* and *Conus radiatus*.

Posttranslational modifications within conopeptides are known to perform a variety of functions. For example in conantokin-G, γ-carboxylation affects activity (Tayo et al., 2010). Another thought is that PTMs serve to stabilize the conopeptide from enzymatic degradation. As with the racemization of L- to D- tryptophan in contryphans and the cyclization of N-terminal glutamate to pyroglutamate in *C. striatus* and *C. geographus* (Tayo et al., 2010). However, many of these modifications are unobservable through current proteomic and genomic methodologies, such as amino acid epimerization (Buczek et al., 2008).

Hydroxylation of proline has been most characterized in collagen. Hydroxyproline (Hyp) in the Pro-Hyp-Gly repeats stabilizes the collagen triple helix (Lopez-Vera et al., 2008) and contributes protein stability. There are studies that suggest Hyp may affect the biological activity of conotoxins. Lopez-Vera et al. (2008) investigated the role hydroxyproline in μ-conotoxin GIIIA, ω-conotoxin MVIIC, α-conotoxins GI and ImI. The peptides were chemically synthesized with either proline or hydroxyproline, and their biological activity and oxidative folding were studied *in vitro*.

Table 3. Illustrates post-translational modifications identified in *Conus striatus* conotoxins.

PTM	Modification	Toxin	Sequence	Reference
C-terminal modification	Amidation	α-SI α-SIA ω-SO3	ICCNPACGPKYSC* YCCHPACGKNFDC* CKAAGKPCSRIAYNCCTGSCRSGKC*	Ramilo et al. (1992) Myers et al. (1991) Wen et al. (2005)
Hydroxylation	Proline	κ-SIVA ω-SVIA	ZKSLVPSVITTCCGYD**O**GTMC**OO**CRCTNSC* CRSSGS**O**CGVTSICCGRCYRGKCT*	Wang et al. (2003) Ramilo et al. (1992)
Bromination	Bromotryptophan	Bromocontryphan-S	GCO(**BTr)**EPWC*	Jakubowski et al. (2006)
Pyroglutamation	Glutamate	μ-SIIIA μ-SIIIB	**Z**NCCNGGCSSKWCRDHARCC* **Z**NCCNGGCSSKWCKGHARCC*	Bulaj et al. (2005) Schroeder et al. (2012)
Glycosylation	Serine	κ-SIVA	ZKSLVP(**Gser)**VITTCCGYDOGTMCOOCRCTNSC*	Craig et al. (1998)

Post translational modifications within the amino acid sequences are noted as such: C-terminal modification (*****), Hydroxylation of Proline (**O**), Brominated tryptophan (**BTr**), Pyroglutamation of Glutamate (**Z**), Glycosylation of Serine (**Gser**).

Results indicated that Hyp residues can affect the bioactivity and folding of the conotoxins. The transformation from proline to hydroxyproline is catalyzed by the enzyme proline hydroxylase (EC 1.14.11.2) (Gorres and Raines, 2010). The reaction yields two isoforms, cis- and trans-4-hydroxyproline.

Bromination of tryptophan has recently been established as a post-translational modification (Jimenez et al., 2004) and plays key role in the activity of conotoxins, styelins, macrocyclic peptides, and cathelicidins (Bittner et al., 2007). The Craig group (1997) confirmed L-6-bromotryptophan as a secondary amino acid and present in in the venoms of *C. imperialis* and *C. radiatus.* Jimenez et al. (2004) describes bromotryptophan residues in a sleep-inducing peptide. The bromo-sleeper was isolated from *C. radiatus*, causes a sleep-like state in mice. 14-day-old mice were injected with the bromo-sleeper which caused a light sleep state in which the mice were easily aroused. The level of of the light sleep state was dose dependent, however, even at the highest dosage the mice were still recovered. Currently, it is believed that bromoperoxidase (Craig et al., 1999) is the enzyme that catalyzes the transformation between tryptophan to bromotryptophan.

C-terminal amidation arises from oxidative cleave of a terminal glycine. The enzyme that catalyzes the reaction is peptidylglycine alpha-monoxygenase or PAM (EC 1.14.17.3) (ExPASY). C-terminal amidation is necessary for biological function. PAM has two functions, the first function is PHM (peptidyl α-hydroxylating monooxygenase) hydroxylates glycine at the C-terminal (Ul-Hasan et al., 2013). Peptidylamido-glycolatelayse (PAL) then cleaves the hydroxylated glycine, which results in a c-terminally amidated peptide glyoxylate. C-terminal amidation has been reported to improve the stability of peptide ligands, and receptor affinity, and hence overall biological functioning (Ul-Hasan et al., 2013).

γ-Carboxylation of glutamate residues was first characterized in prothrombin, a blood clotting factor and in proteins involved in bone metabolism (Craig et al., 1999). It was originally believed that γ-carboxylation, which is vitamin K dependent, was limited only to vertebrates. However, approximately 10% of *Conus* peptides contain γ-

carboxylated residues (Craig et al., 1999). It is believed that the γ-carboxylation plays an important role in the folding and formation of the alpha helix.

Current techniques to identify these modifications include Edman degradation, amino acid analysis, as well as mass spectrometry and cDNA sequencing (Jakubowski et al., 2004; Espiritu et al., 2013). α-Conotoxin CnIA, from the duct venom of *Conus consors*, was one of the first identified from these advanced techniques (Favreau et al., 1999). However, some of these processes, like the Edman degradation are very time consuming and require large amounts of duct venom, and do not always provide complete sequences (Jakubowski et al., 2004).

Jakubowski and colleagues (2004) utilized a combination of methods to characterize the PTMs of 3 conopeptide sequences from *Conus victoriae*. They were able to do so by amplifying the cDNA library via polymerase chain reaction, confirming the sequence and type of post-translational modification through a combination of liquid chromatography (LC) and electrospray ionization mass spectrometry (ESI-MS), matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS), as well as nanospray ionization mass spectrometry with collisionally induced dissociation (NSI-MS with CID).

Table 4. Illustrates potential post-translational modifications that may be observed in *striatus* and their detection methods.

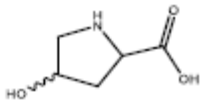
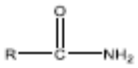
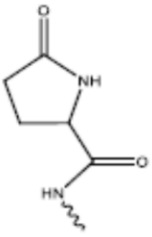
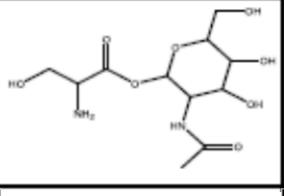
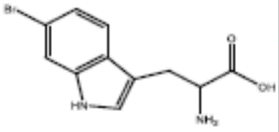
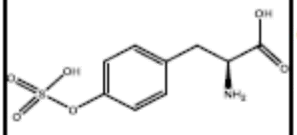
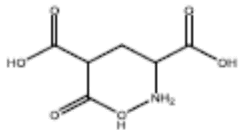
PTM	Structure	Comments	Reference
Hydroxyproline *cis-* and *trans-*		Can be characterized through the use of w- or d- ion analysis, or by hydrolysis and amino acid analysis.	Espiritu et al., 2013
C-terminal Amidation		C-terminal amidation can be detected through the utilization of oxazolone derivatization in combination with mass spectroscopy.	Espiritu et al., 2013

Table 5. Continuation of Table 4, displaying potential post-translational modifications that may be observed in *striatus* and their detection methods.

Pryroglutamate		Pyroglutamation has been identified in various conopeptides/toxins via ESI-MS, MALDI-MS, LSI-MS in combination with conventional Edman degradation.	Espiritu et al., 2013
Glycosylation of serine		Glycosylation of serine (or threonine) can be identified/characterized via NMR (nuclear magnetic resonance), 2D-electrophoresis, and MS.	Espiritu et al., 2013, Mann et al., 2003
Bromotryptophan		Bromination of tryptophan has been discovered through enzyme digestion, comparative RP-HPLC, MS, and SPPS (Solid Phase Peptide Synthesis).	Espiritu et al., 2013
Sulfotyrosine		Sulfation of tyrosine has been observed using negative charged ions in soft ionization at lower collision energies during MALDI-MS and/or ESI-MS.	Espiritu et al., 2013
γ-Carboxyglutamic acid		Carboxylation of glutamic acid has been identified through sequential enzymatic digestion and Edman degradation.	Espiritu et al., 2013

1.4 Amino Acid Analysis

Amino acid analysis (AAA) is a successful method to quantify individual amino acids in a given protein. Knowing the amino acid sequence and composition is important to understanding its mechanism of action, what is three-dimensional structure is, and provides an evolutionary history (Berg et al., 2002). There are four prominent steps involved in AAA: (i) hydrolysis, derivatization, (ii) separation of derivatized amino acids, (iii) data interpretation and calculations. The traditional method of automated amino acid analysis of separation by ion-exchange chromatography, a method developed by Moore, Spackman, and Stein (White et al., 1986; Buckle, 1961). However, this method has been abandoned with the advancement of newer technologies, in favor of pre-column derivatization with separations based on RP-HPLC (Springer, 2000).

Pehr Edman, in 1950 developed a procedure that allows for the removal and identification of each residue, one at a time from the *N*-terminus of a protein (Horton et al., 2006). This method has become known as the Edman degradation method. The *N*-terminus residue of protein or peptide reacts with PITC to generate at PTC- derivative. The derivative is then treated with trifluoroacetic acid which releases an anilinothiazolinone derivative of the *N*-terminal amino acid residue (Horton et al., 2006).

The anilinothiazolinone is then removed and treated with aqueous acid. This stabilizes the derivative into a phenylthihydantoin derivative that can be identified chromatographically (Horton et al., 2006). The remaining polypeptide chain, that now has a new *N*-terminal residue that was previously in the second position, is now subjected to the next round of Edman degradation (Horton et al., 2006). The process continues until the sequence of the amino acid is determined, generally by HPLC.

Acid hydrolysis is the first step in amino acid analysis. There are three types of hydrolyzing methods; acid hydrolysis, alkaline hydrolysis, and enzymatic hydrolysis (Khan and Faiz, 2008). With acid hydrolysis the peptide is hydrolyzed into its constituent amino acids by heating it in 6 N HCl at 110ºC for 24 hours (Berg et al., 2002, Horton et all, 2006). Hydrochloric acid is used since it can be readily removed from the hydrolysate (Khan and Faiz, 2008). HCl hydrolysis of proteins and peptides generally yields 16 amino acids, with special hydrolysis methods being needed for phosphoamino acids and

tryptophan (West et al., 1998). Tryptophan has been recovered by using a protective agent (3,2-aminoethylindole) and 3N p-toluene sulphonic acid (Khan and Faiz, 2008). Other amino acids such as serine and threonine are partially destroyed during acid hydrolysis, as valine and isolucine are slow to cleave. Methionine is subject to oxidation. This oxidation can be minimized by adding 2-mercaptoethanol to the acid (Pickering et al., 1990). Glutamine and asparaginine are deamidated during the process to form glutamic acid and aspartic acid respectively (West et al., 1998, Pickering et al., 1990). Tryptophan is generally not recovered under acid hydrolysis, however base hydrolysis can be used to quantify this amino acid (Pickering et al., 1990).

Alkaline hydrolysis is not commonly used since the destruction of arginine, threonine, serine, cystine and cysteine occurs. It is however applicable for acid labile tryptophan. The sample can be hydrolyzed in 4N $Ba(OH)_2$ at 110ºC for 50 to 70 hours (Khan and Faiz, 2008).

Enzymatic hydrolysis offers an alternative method to acid hydrolysis and avoiding the loss of certain amino acids. The digestion of a protein by papain followed by treatment with protein prolidases and peptidases. This causes complete hydrolysis of all peptide bonds and recovery of asparagine, tryptophan, and glutamine is high (Khan and Faiz, 2008).

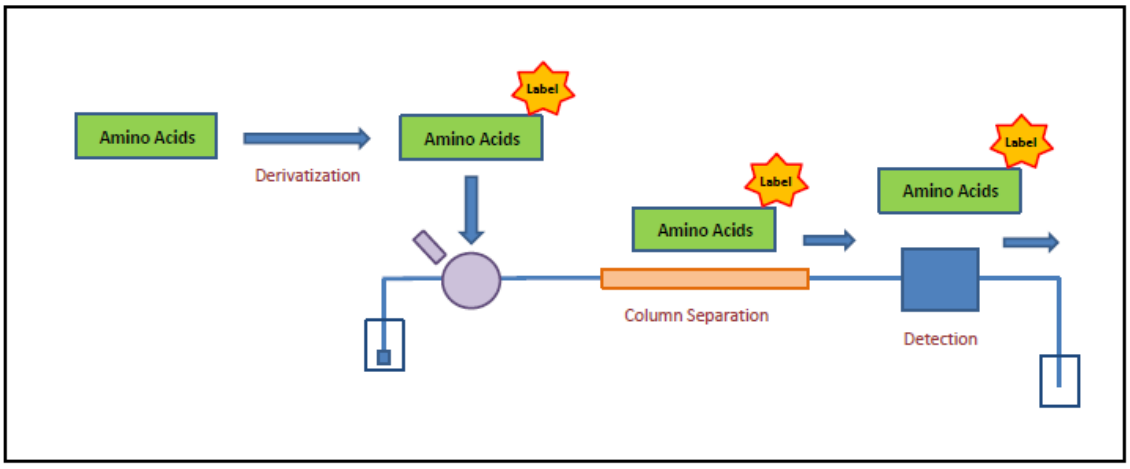


Figure 2. Illustrates the process of pre-column derivatization. Amino acids are derivatized with PITC (phenylisothiocyanate) prior to being processed by reverse phase high performance liquid chromatography. Amino acids are detected under fluorescence at approximately 254 nm at which peak absorbance is measured.

The Pre-column method of derivatization uses phenylisothiocyanate (PITC), also known as Edman reagent, requires a salt free sample, and is processed at pH 9.0 to generate phenylthiocarbamoyl (PTC) derivatives (Horton et al., 2006). Sensitivity is relatively high requiring 0.5 to 1.0 µg, with accuracy at 90-95% depending upon instrument and operator (West et al., 1998). The PTC- amino acid mixture is then processed by RP-HPLC and separated by their unique hydrophobic properties (Horton et al., 2006). As the derivatives are eluted their concentration is determined by measuring the degree of absorbance at 254 nm (peak absorbance for PTC-amino acid eluate) (Horton et al., 2006).

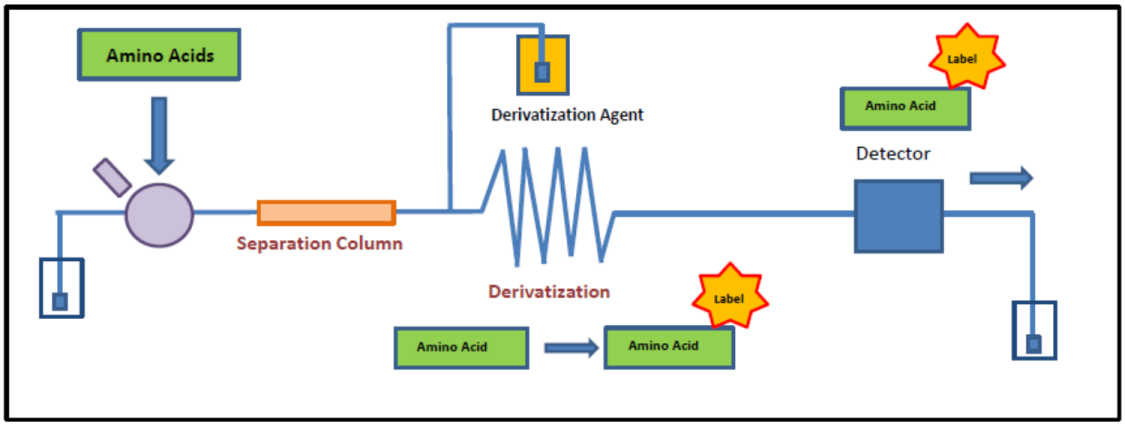


Figure 3. Illustrating the process of post-column derivatization. Amino acids are subjected to either ninhydrin and OPA (o-phthalaldehyde) after undergoing separation either by ion-exchange or reverse phase high performance liquid chromatography. After amino acids are derivatized they pass through either a UV-Vis or fluorescence detector (dependent upon derivitazing agent).

Post-column derivatization occurs after the amino acids have been separated by cation exchange chromatography. There are two primary reagents utilized, ninhydrin and o-phthalaldehyde (OPA). Ninhydrin is usable for UV-Vis absorption detection and OPA is for fluorescence detection. Since amino acids are zwitterions, it makes ion exchange a reliable tool for separation. Adjusting the pH to more acidic levels will support formation of anions, and cause faster elution. Whereas at more basic pH levels, will cause slower elution of the amino acids.

1.5 Milked venom versus duct venom, biosustainability?

With a proven track record for novel therapeutics, cone snails represent a prominent biological resource. Milked venom provides a reliable means of obtaining potential drug candidates. This can be successfully illustrated by the ω-conotoxin MVIIA or Prialt™, which is expressed in the milked venom of *Conus magus* (Chun, et al., 2012). Currently, too little is known of how cone snail venom is synthesized and expelled by the animal.

There are currently five sources available for study of cone snail venom: duct venom, milked venom, radular extract, genomic DNA, and cDNA. There have been a number of studies performed that compare milked venom to duct venom that show that milked venom provides cleaner samples even though there are fewer peptides, but with higher degree of peptide concentration (Bingham et al. 1996; Jakubowski et al., 2005; Biass et al., 2009). Milked venom offers a sustainable research method (Chun et al., 2012) to sacrificing the animals for their venom glands. Even though the techniques that are typically employed in the analysis of *Conus* venom requires very small amounts of venom, mRNA, or tissue, and it is common practice to synthesize the peptides to understand their individual functionality (Duda et al., 2004).

A comparative study performed by Biass et al. (2009) using ESI-MS analysing traditional duct venom against milked venom of *Conus consors*, demonstrated that even though the milked venom lacked the same peptides, approximately 50% of the peptides found the milked venom were not in the duct venom. Milked venom also provides researchers with biologically relevant peptides that are utilized to immobilize prey (Jakubowski et al., 2005). Genomic DNA employs the isolation of the stop and start codons directly from the cone snail genome, whereas with cDNA, mRNA is isolated. Recently, Chun et al. (2012) performed radular harpoon analysis of *Conus purpurascens* utilizing MALDI-TOF-MS which they observed yet to be identified molecular masses. Recombinant production in heterologous expression systems is also another plausible means of obtaining necessary quantities (Becker et al., 2008).

1.6 Diet and venom variation in other species

Scorpion and/or snake venom is often times used to help elucidate the functionality and specificity of *Conus* toxins at receptors (Bingham et al., 2010). One method used to understand potential function of *Conus* toxins is to compare the toxin to those identified from other venomous organisms. Albeit this information is rather controversial, studies have shown that there is geographic variation in the venom composition of snakes and scorpions, and these variations could be the result of differences in diet (Duda et al., 2009). Venom from snakes like *Viperidae* and *Elapidae* are the most widely studied (Sanz et al., 2006). The components of snake venom contain various proteins and enzymes that interfere with tissue repair, coagulation, immobilization, and digestion of prey.

Sanz et al. (2006) examined the protein composition of three subspecies of *Sistrurus catenatus* and *Sistrurus miliarius babouri* utilizing RP-HPLC, *N*-terminal sequencing, CID-MS/MS, and MALDI-TOF peptide mass fingerprinting. They propose that if venom composition is related to diet, then differences in venom composition should be detected. Snakes were obtained from various wildlife areas and venom obtained manually. Results showed a high degree of differentiation in the venom proteome of the snakes of similar species with variable diets (Sanz et al., 2006).

Saw-scaled vipers which have evolved to eat scorpions, have also evolved venom that is more lethal to scorpions. This demonstrates the importance of diet and its role in venom modulation (Phys.org, 2009). A study conducted Barlow and colleagues (2009) examined whether or not shifts in diet of saw-scaled viper (*Echis*) displayed any variation in venom composition. A great deal of diet variation has been reported with the genus (Barlow et al., 2009). They hypothesize that the diversification in diets from vertebrates to arthropods (scorpions and centipedes) is the result of adaptive evolution driven by natural selection. Arthropods are not a typical food source of vipers (Barlow et al., 2009; Shine et al., 1998). There is further evidence of natural selection on snake venom composition which derives from natural prey species that display a high level of resistance to snake venoms (Richards et al., 2012). After performing a phylogenetic analysis using Bayesian inference methods, electrophoretic venom profiles were developed from the vipers studied. The exent and diversity of arthropod feeding was

determined through dissesction of the stomach and hindgut contents (Barlow et al., 2009). Barlow et al. (2009) also conducted LD_{50} trials on scorpions to test *Echis* venom toxicity.

The results of the experiment provide strong evidence towards natural selection playing a key role in shaping venom composition amongst the genus *Echis* (Barlow et al., 2009). The variation in arthropod feedings amongst the *Echis* species examined, confirmed significant variation in the amount of prey consumed, as well as an association between venom toxicity and diet (Barlow et al., 2009).

Ontogenetic (referring to physical growth) shifts in diet are common for snakes and such shifts in diet for venomous snakes may be associated with changes in venom composition (Mackessy et al., 2003). It is proposed that if the diet of the snake changes of the course of its lifespan, then the composition of its venom should also change (Mackessy et al., 2003). Previous studies performed by Mackessy (1988, 1993, 1996) looked at ontogenetic changes in diet in to subspecies of *Crotalus*, a diet change from ectotherms to endotherms; as well as ontogenetic shift in venom compostion. Smaller more juvenile snakes exhibited higher toxicity, whereas larger adult snakes displayed greater predigestive properties.

Spiders with specialized diets have also demonstrated prey-specific venom lethality (Casewell et al., 2013). Pekár et al. (2008) provides sufficient evidence that spider *Zodarion germanicum*, has dietary and venom adaptations by which it targets certain subfamily of ants. The spiders were raised from the first instar and fed either a diet of formicine ants, myrmicine ants, or a mixture of both formicine and myrmicine ants. Life history parameters were studied as well as venom lethality on ant subtypes. Composition of ants were tested for differences in macro-nutrient content, however no differences were detailed. Results of the study showed in part that the spiders longevity and growth was greatly affected by their diet. Mean survival of spiders on myrmicine diet was 95 days, which is significantly shorter than that of those on formicine diet (203 days) and mixed-ant diet (215 days) (Pekár et al., 2008).

Little is known about what influences venom synthesis within *Conus* species. Duda et al. (2009) studied venom allelic composition in *Conus ebraeus*, examining the distribution of alleles in a polymorphic O-superfamily conotoxin locus *E1*. *C. ebraeus*

was obtained from Okinawa, Guam, and Hawaii. Geographic variation in diets of *C. ebraeus* has been noted. The primary prey of *C. ebraeus* being eunicid polychaetes. Fecal and gut examination of specimens showed that cone snails from Okinawa and Guam predominantly fed on eunicid polychaetes, however those from Hawaii preyed on nereid polychaetes (Duda et al., 2009). Results of the study showed differences in conotoxin allelic frequencies in Hawaii, Guam, and Okinawa.

Hypotheses

General Hypotheses

- Snails relegated to the pill diet will exhibit marked changes in peptide concentrations, as well as increased post-translational modifications.
- An analysis of venom components under diet manipulation may reflect snail health and productivity showing the impacts of diet on venom composition.
- A comparison between the quantitative analysis of a series of venom constituents and the diet of *Conus striatus* may advance husbandry techniques by maximizing venom production.

Specific Hypotheses

Sex

- Volume of venom will vary between males and females
- Venom composition will vary between males and females

Diet

- Snails will alter amount of venom injected
- Diversity of snail venom composition will change
- Feeding behavior will change in accordance/preference for to pill given

Chapter 2: METHODS

2.1 Housing

Cone snails were housed in Aquatic Habitat® Benchtop Systems that were modified to contain snails and added protein skimmers. The system supports 10 aquaria (10L capacity) with biological, carbon, and physical filtration (to 50 micron) pumped at a rate of six water exchanges per tank per hour.

2.2 Venom Collection

Conus striatus were collected on SCUBA from various locations around Oahu, Hawaii. Photographs were taken of each individual snail to identify each specimen by their shell pattern. Venom samples were acquired during their weekly feedings as described by Nelson (2004) for a period of 60 weeks. Utilizing two-milliliter eppendorf collection vials that have been fitted with a latex membrane (TROJAN® Condoms – Non lubricated). Vial was banded to the end of a dental tool for feeding. A swordtail (*Xyphophorus sp*.) served as food. The fin of the fish was placed flush against the latex membrane and when the snail's proboscis extended, the snail would shoot its harpoon-like tooth through the fish's fin and into the eppendorf through the membrane.

2.3 Diet Manipulation

Group 1	Group 2	Group 3	Group 4	Control
Diet: Fish Food	Diet: Fish Food + KBr	Diet: Fish Food + Human Vitamin Pack	Diet: Fish Food + Human Vitamin Pack + KBr	Diet: Fish
Animals: 2-2, 3-2, 3-3	Animals: 4-1, 4-3, 5-2	Animals: 6-1, 6-2, 7-2	Animals: 8-1, 9-1,9-2	Animals: 2-1, 4-2, 7-1, 8-2

Group 1: 2-2, 3-2, and 3-3 were fed a pill diet of fish food. Group 2: 4-1, 4-3, and 5-2 were fed a pill diet of fish food and potassium bromide. Group 3: 6-1, 6-2, 7-2 were fed a pill diet of fish food and Centrum®. Group 4: 8-1, 9-1, and 9-2 were fed a pill diet of fish food, Centrum®, and potassium bromide. The snails were fed the pill diet alternating with fish every other week for 60 weeks. Gelatin capsules for group 1 were filled with 100% crushed Silvercup™ fish feed. Group 2 capsules were filled with 100% fish food and 0.1 mg potassium bromide (J.T. Baker® Chemicals). Group 3 capsules were filled with 25% w/w human vitamin pack and 75% w/w fish food. Group 4 capsules were filled with 25% w/w human vitamin pack and 75% w/w fish food and 0.1 mg potassium bromide. Control group: 2-2, 4-2, 7-1, and 8-2 were fed a diet of fish. Capsules were either made a few days prior to feeding and stored at 5 ºC or made on the day of feeding. (See Appendix 1 for components of human vitamin pack and Silver cup fish feed).

2.4 Sample Preparation

Milked venom samples were volumetrically measured using an Eppendorf p100 pipette and dried on a speed-vac centrifuge. Two hundred microliters of 0.1% v/v trifluoroacetic acid (TFA) in distilled water were added to each eppendorf to dissolve the sample. From these samples, 110 µL were placed in a 150 µL insert within a glass vial that entered the Waters 2695 Separations Module via automated carousel.

2.5 RP-HPLC Profiling

The milked venom samples were then injected onto a Phenomenex C_{18} column (OG-4053-A0 Jupiter 5U C18 300A 250x1.00 mm 5 micron 41248-1) in an aqueous solvent (99.9% v/v distilled water, 0.1% v/v TFA). Organic solvent concentration (90% v/v acetonitrile, 9.99% v/v distilled water, 0.01% v/v TFA) was increased at a linear rate of 1% every minute for one hour to elute the entire sample from the column. Analytes were analyzed at 214 nm utilizing a Waters 996 Photodiode Detector and integrated with Waters Empower software.

2.6 MS Analysis

RP-HPLC fractionated peak peptide masses were determined on a PE Sciex API 3000 Triple Quadrupole LCMS system using Analyst software. Peptide masses were compared to known masses from *C. striatus* (Tables 1 and 2).

2.7 Amino Acid Analysis

Quantitative Amino Acid Analysis of six peptide standards was performed by the Advanced Protein Technology Centre in Toronto, Canada under the direction of Dr. Reynold Interior. A Pico-Tag System using a Waters Alliance 2690 and Waters 2487 Dual Wavelength Absorbance Detector, and Pico-Tag System using the Waters 510 Pumps, WISP 712B Cooled Autosampler, Column Heater with Temperature Control Module, and 2487 Dual Wavelength Absorbance Detector in addition to the Waters Pico-Tag Workstation for hydrolysis of samples. Data collection, instrument control, and processing were handled by Waters Millenium[32] Chromatography Software v. 4.0 on a Pentium 4 PC.

2.8 Quantitative Analysis

Area under the curve of RP-HPLC profiles of pill and control animals will be calculated from the Waters Millennium[32] (v3.2) software to quantify the presence of each isolated peptides present in each milked sample. This quantity can then be used in combination with the total volume of the sample collected at the time of the milking to determine the overall concentration of the individual peptide of interest.

2.9 Statistical Analysis

Where applicable, the data were analyzed on Microsoft Excel using two statistical comparisons; t-tests and ANOVA. Statistical significance was defined as any results returned with a p-value less than 0.05.

Chapter 3: RESULTS

3.1 Venom Volume

In comparison to previous milkings obtained by Jeffrey Milisen (MS thesis, 2012), there is an approximate decrease in venom volumes by 31% of the snails involved in the pill study. Group 1 experienced an average venom volume reduction of 34.4%, Group 2 27.9%, Group 3 25.9%, and Group 4 experienced an average reduction in venom volume of 36.7%. ANOVA analysis of each individual snail involved in the pill study was performed in comparison to non-pill venom volumes previous collected.

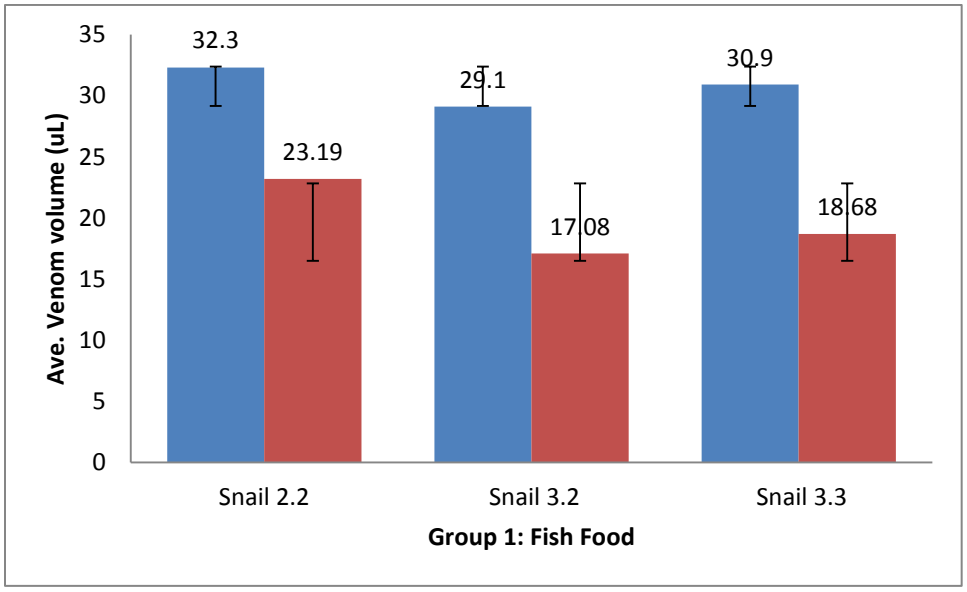


Figure 4. Depicts Group 1 (n = 3) which was fed a diet of fish food. The graph compares venom volume readings prior to the diet study (blue) and during (red). Analysis between snails within the groups shows that there is not a statistical difference in venom volume within the group. However, ANOVA analysis comparing volumes previous collected from each individual snail prior to being subjected to the pill diet and during, shows that there is a significant statistical difference. Snail 2.2 $F(1, 81) = 8.6$, $p = 0.00435$; Snail 3.2 $F(1, 83) = 21.3$, $p = 0.0000136$; Snail 3.3 $F(1,82) = 15.3$, $p = 0.000187$.

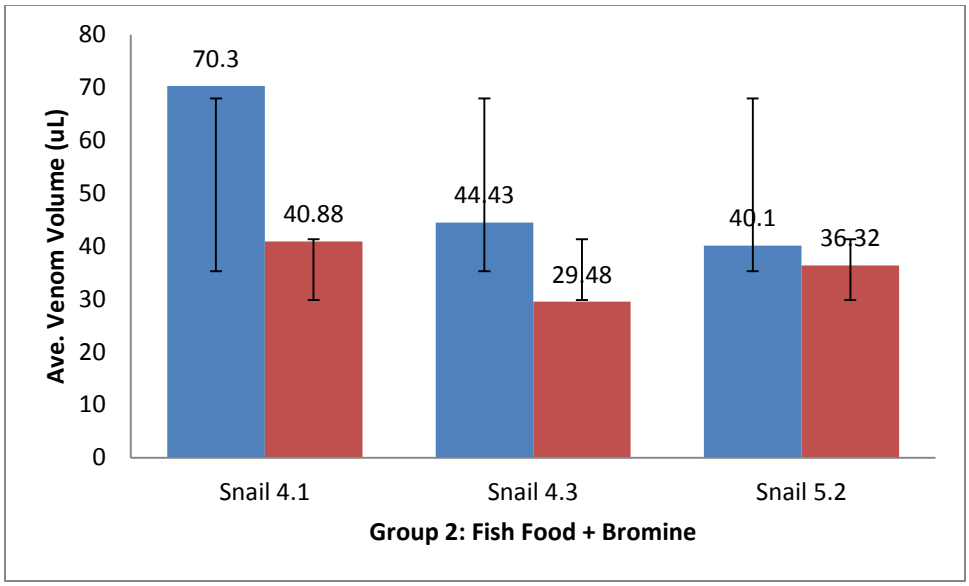


Figure 5. Shows Group 2 (n = 3) which was fed a diet of fish food and bromine. The graph compares venom volume readings prior to the diet study (blue) and during (red). Analysis between snails within the groups shows that there is not a statistical difference in venom volume within the group. However, ANOVA analysis comparing volumes previous collected from each individual snail prior to being subjected to the pill diet and during, shows that there is a significant statistical difference. Snail 4.1 F (1, 79) = 41.5, p = 8.5E-09; Snail 4.3 F (1, 79) = 15.7, p = 0.000162. However, with snail 5.2 F (1,78) = 0.8357, p = 0.3634, indicates that there is not a statistical difference between venom volumes prior to and during the diet manipulation.

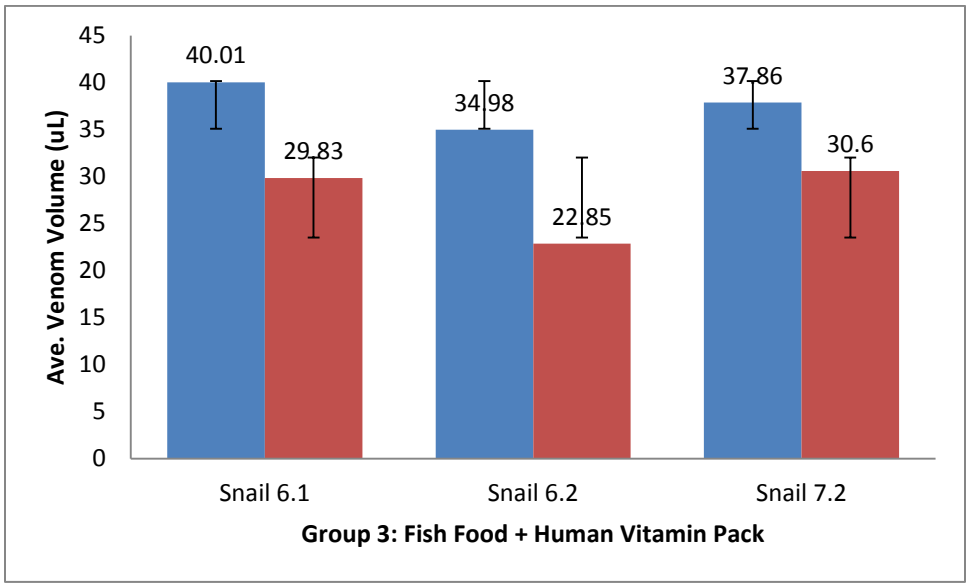


Figure 6. Shows Group 3 (n = 3) which was fed a diet of fish food and human vitamin pack. The graph compares venom volume readings prior to the diet study (blue) and during (red). Analysis between snails within the groups shows that there is not a statistical difference in venom volume within the group. However, ANOVA analysis comparing volumes previous collected from each individual snail prior to being subjected to the pill diet and during, shows that there is a significant statistical difference. Snail 6.1 F (1, 68) = 9.26, p = 0.003319; Snail 6.2 F (1, 67) = 12.7, p = 0.00067; Snail 7.2 (1, 78) = 6.56, p = 0.012352.

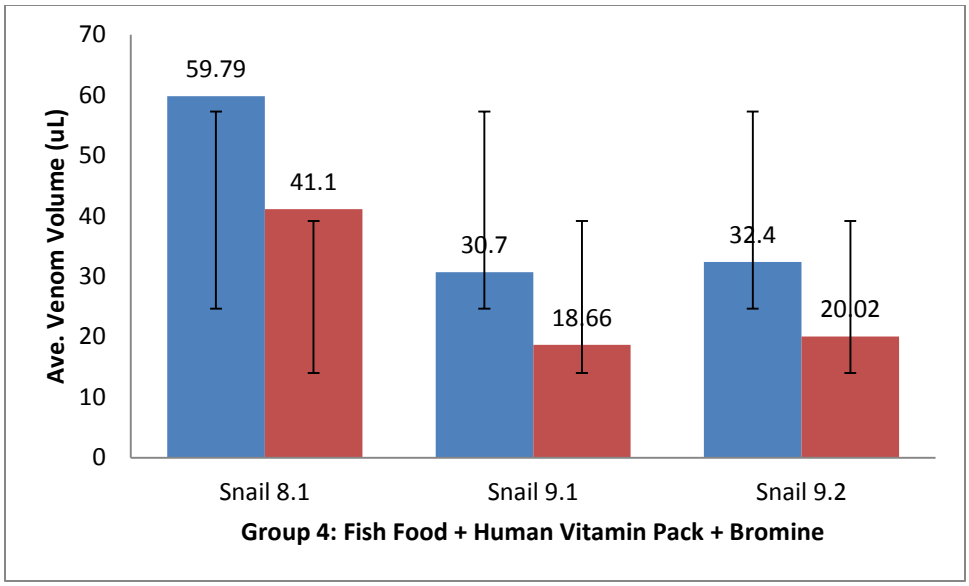


Figure 7. Shows Group 4 (n = 3) which was fed a diet of fish food, human vitamin pack, and bromine. The graph compares venom volume readings prior to the diet study (blue) and during (red). Analysis between snails within the groups shows that there is not a statistical difference in venom volume within the group. However, ANOVA analysis comparing volumes previous collected from each individual snail prior to being subjected to the pill diet and during, shows that there is a significant statistical difference. Snail 8.1 F (1, 80) = 24.9, p = 3.37E-06; Snail 9.1 F (1, 81) = 16.5, p = 0.000112; Snail 9.2 (1, 87) = 13.2, p = 0.000475.

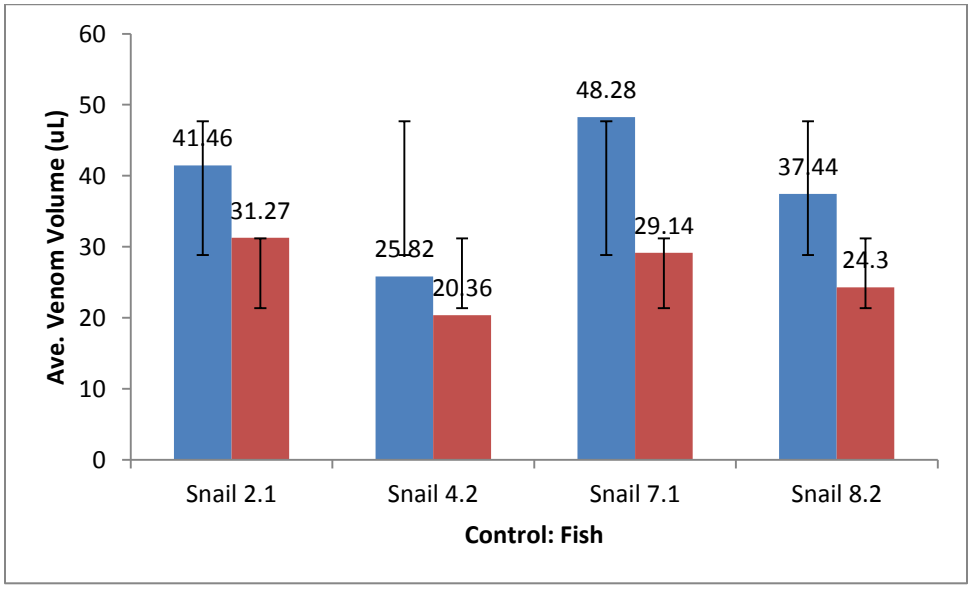


Figure 8. Shows Control Group (n = 4) which was fed a diet of fish. The graph compares venom volume readings prior to the diet study (blue) and during (red). Analysis between snails within the groups shows that there is not a statistical difference in venom volume within the group. However, ANOVA analysis comparing volumes previous collected from each individual snail prior to being subjected to the pill diet and during, shows that there is a significant statistical difference. Snail 2.1 F (1, 78) = 5.56, p = 0.020827; Snail 7.1 (1, 82) = 14.0, p = 0.00033; Snail 8.2 (1, 82) = 10.7, p = 0.001515; however with Snail 4.2 F (1, 82) = 2.47, p = 0.1195, there is not a statistical difference in venom volume.

3.1.1 Venom Volume and Sex

The sex of four out of the twelve snails involved in the study are known. Snail 2.2 (male) of Group 1 had an average venom volume of 23.19 uL ± 2.51. Snail 3.3 (female) also of Group 1 had an average venom volume of 18.68 uL ± 2.52. A two-tail t-test showed that both snails produced comparable volumes of venom (two tailed p-value = 0.21). Male and female comparison of snails from Group 3 shows snail 6.1 (female) mean venom volume 29.8 uL ± 3.63 and snail 6.2 (male) had an average venom volume 22.85 uL ± 2.67, two tailed p-value = 0.13.

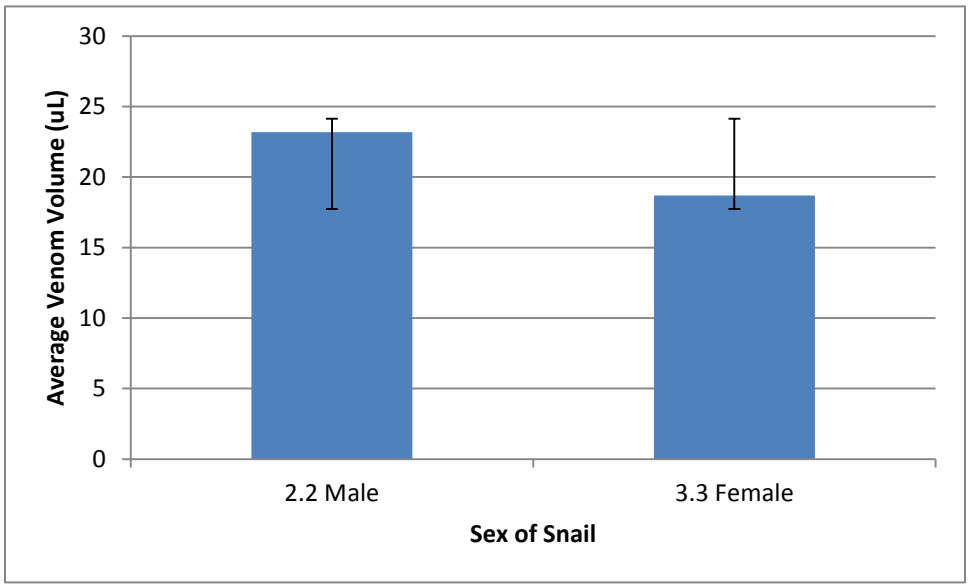


Figure 9. Venom volume in regards to sex. Illustrates the comparison of mean venom volume between male and female snails in Group 1, receiving the fish food diet. ANOVA analysis also indicates no statistical difference between mean venom production between male and female ($F(1, 76) = 1.60$, $p = 0.21$).

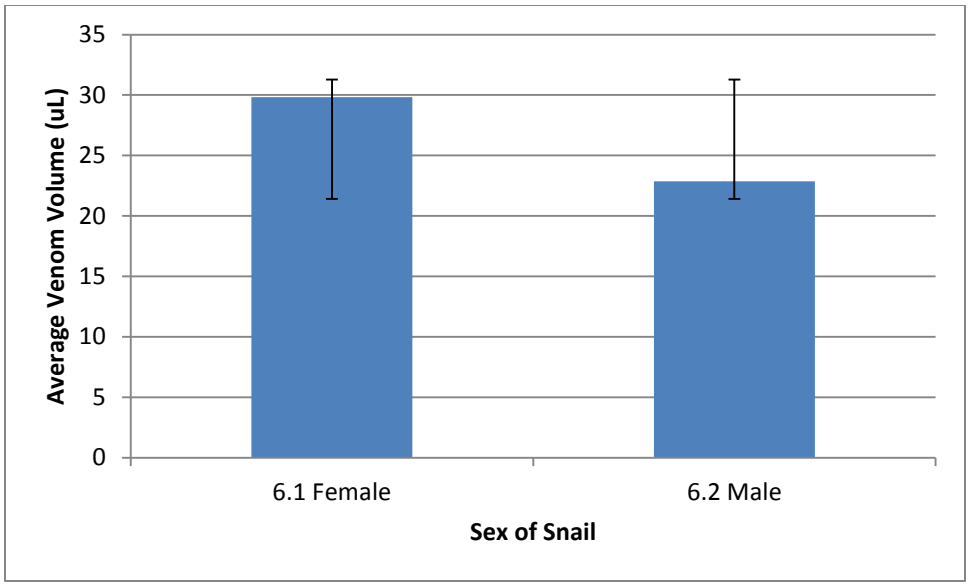


Figure 10. Venom volume in regards to sex. Illustrates the comparison of mean venom volume between male and female snails in Group 3, receiving the fish food and human vitamin pack diet. ANOVA analysis also indicates no statistical difference between mean venom production between male and female ($F(1, 53) = 2.37$, $p = 0.1296$.

3.2 Diversity of Venom Composition

The venom profiles between the four groups involved, displays the diversity between the diets and potentially sexes. Prominent peaks from each individual snail in each group were labeled and matched to isolated peptides for quantification. Each grouping of representative chromatograms illustrates the continuity of peptides obtained during each milking, as well as indicating a potential cycle of venom delivery. In comparison to typical/baseline chromatograms (not shown) from previous milkings prior to the start of the diet manipulation, Group 1 (fish food diet) all three snails show little to no deviation in venom profiles. Typically, the snails only produced one to two main peaks and a series of smaller ones during each weekly milking. In Group 2 (fish food and bromine), snail 4.1 shows increased complexity in it venom production. Snail 4.3 and 5.2 do not exhibit the same increased complexity in comparison to baseline profile. Snails in Group 3 (fish food and human vitamin pack) all display increased peptide complexity in comparison to pre-diet manipulation milkings. From Group 4 (fish food, human vitamin

pack, and bromine), snails 8.1 and 9.2 show increased venom diversity, snail 9.1 does not deviate from typical venom production even under diet manipulation.

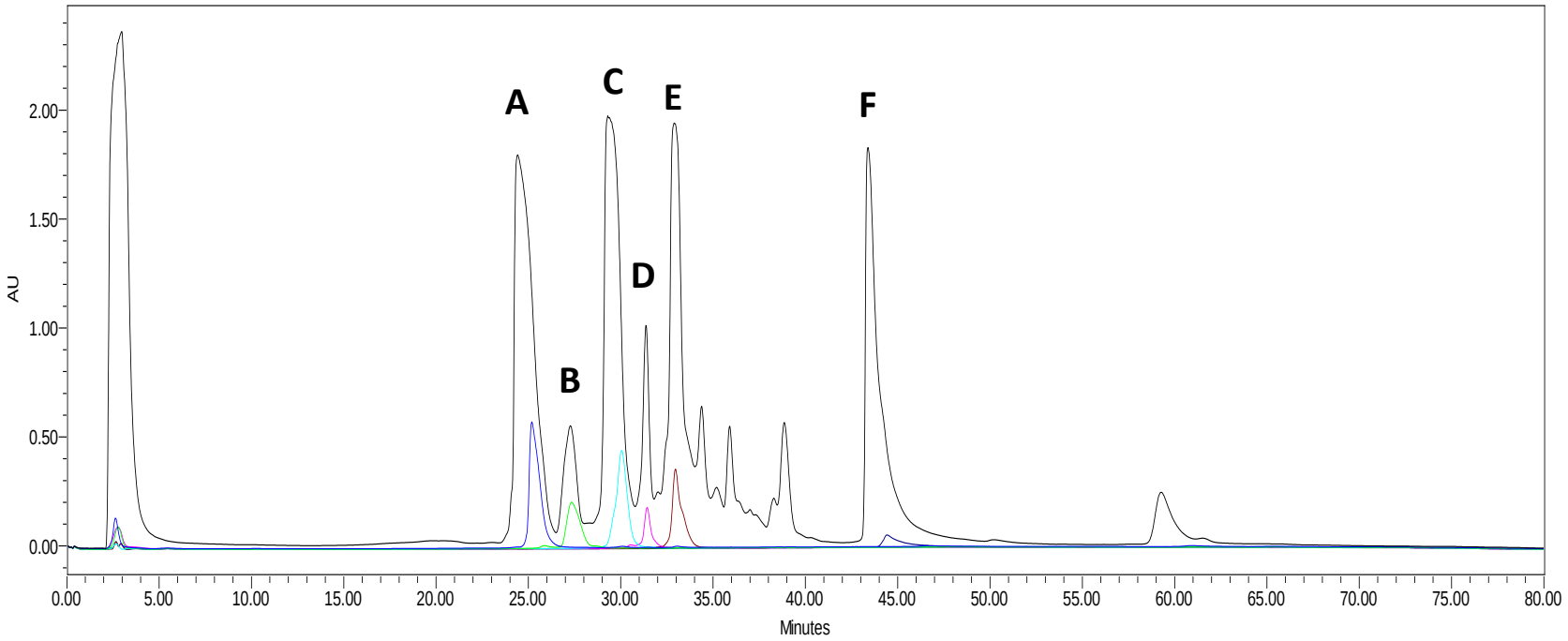


Figure 11. Pooled milked venom of *Conus striatus* and labeled isolated peaks (colored peaks). Peak A (blue) has been identified through MS and amino acid analysis as ω-conotoxin SVIA. Peak B (green) has been identified through MS and amino acid analysis as α-conotoxin SII. Peak C (aqua blue), Peak D (pink), Peak E (red), and Peak F (purple) have yet to be identified. Amino acid analysis and MS data were inconclusive, indicating that these isolated peptides may have yet to be identified.

Table 6. Amino Acid Analysis of Peak A

Peak A				
ω-SVIA	CRSSGSOCGVTSICCGRCYRGKCT			
αα		Amount (pmoles)	Calculated Residues	Actual Residues
Asp	2	230.546	-	-
Glu	2	260.933	-	-
Ser	1	3930.28	4	4
Gly	1	4236.800	4	4
His	2	51.395	-	-
Arg	1	3217.95	3	3
Thr	1	1922.81	2	2
Ala	2	190.202	-	-
Pro	2	88.384	-	-
Tyr	1	945.538	1	1
Val	1	1084.78	1	1
Cys	2	1126.14	-	-
Ile	1	992.365	1	1
Leu	2	88.880	-	-
Phe	2	127.471	-	-
Lys	1	973.074	1	1

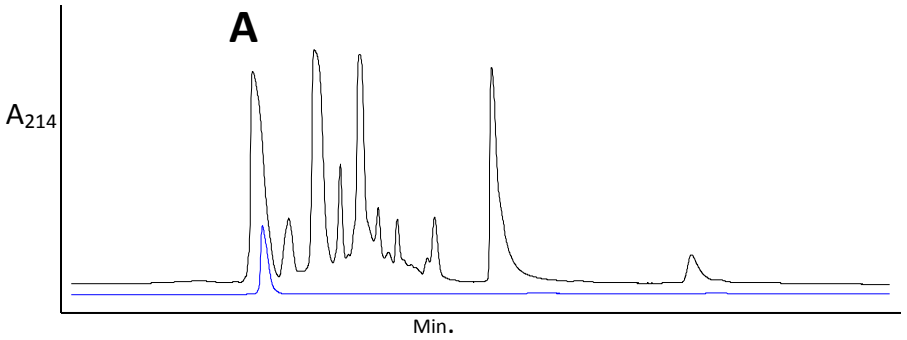


Figure 12.1. RP-HPLC of pooled crude venom and isolated peak A (blue).

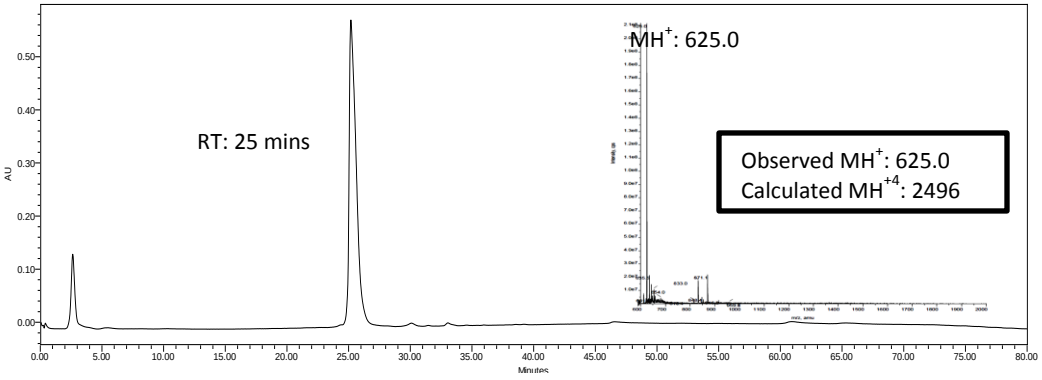


Figure 12.2. RP-HPLC and MS chromatograms of isolated peak A. Retention Time (RT) for peak A is 25 minutes.

Table 6 and Figure 12.1 – 12.2. Amino Acid Analysis (Table 6). Confirming characterization of κ-conotoxin SVIA, sequence is defined above. Numerical coding of (1) or (2) is representative of whether or not the amino acid was considered or not considered in determining the identity of the peptide. (1) - the amino acid was considered, (2) - the amino acid was not considered . The number of amino acid residues were calculated and then compared to the actual number of residues in the sequence. Figure 12.1. RP-HPLC of pooled crude *striatus* venom and isolated peak A. Figure 12.2. RP-HPLC and MS chromatograms illustrating the retention time and *m/z* ratio of peak A.

Table 7. Amino Acid Analysis of Peak B

Peak B				
α-SII	**GCCCNPACGPNYGCGTSCS**			
αα		**Amount (pmoles)**	**Calculated Residues**	**Actual Residues**
Asp	1	1064.200	2	2
Glu	2	172.895	-	-
Ser	1	957.811	2	2
Gly	1	1733.380	3	4
His	2	18.045	-	-
Thr	1	354.188	1	1
Ala	1	583.804	1	1
Pro	1	1250.420	2	2
Tyr	1	572.405	1	1
Val	2	86.184	-	-
Cys	2	384.121	-	-
Phe	2	37.980	-	-
Lys	1	258.859	-	-

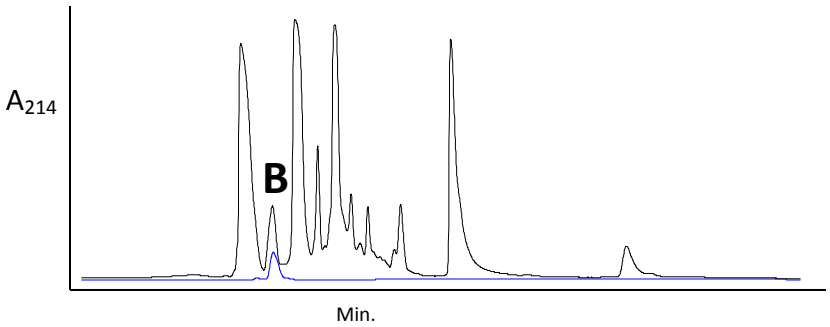


Figure 13.1. RP-HPLC of pooled crude venom and isolated peak B (blue).

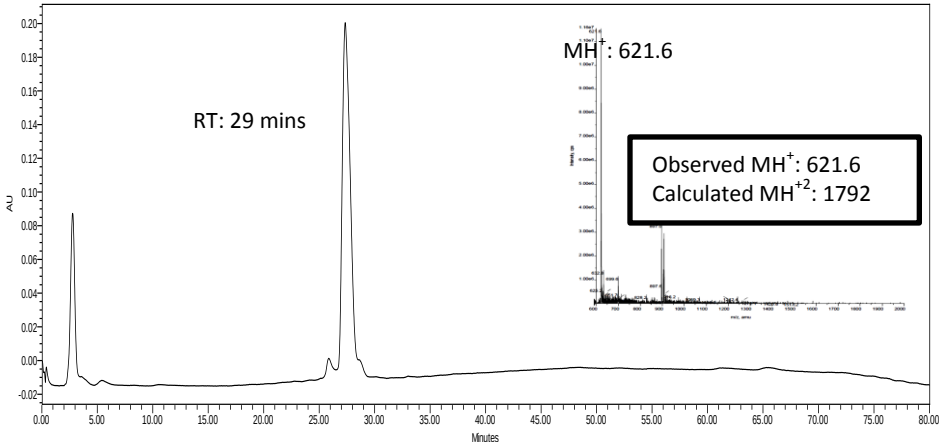


Figure 13.2. RP-HPLC and MS chromatograms of isolated peak A. Retention Time (RT) for peak B is 29 minutes.

Table 7 and Figures 13.1 – 13.2. Amino Acid Analysis (Table 7). Confirming characterization of α-conotoxin SII, sequence is defined above. Numerical coding of (1) or (2) is representative of whether or not the amino acid was considered or not considered in determining the identity of the peptide. (1) - the amino acid was considered, (2) - the amino acid was not considered . The number of amino acid residues were calculated and then compared to the actual number of residues in the sequence. Figure 13.1. RP-HPLC of pooled crude *striatus* venom and isolated peak B. Figure 13.2. RP-HPLC and MS chromatograms illustrating the retention time and *m/z* ratio of peak B.

Group 1: Fish food

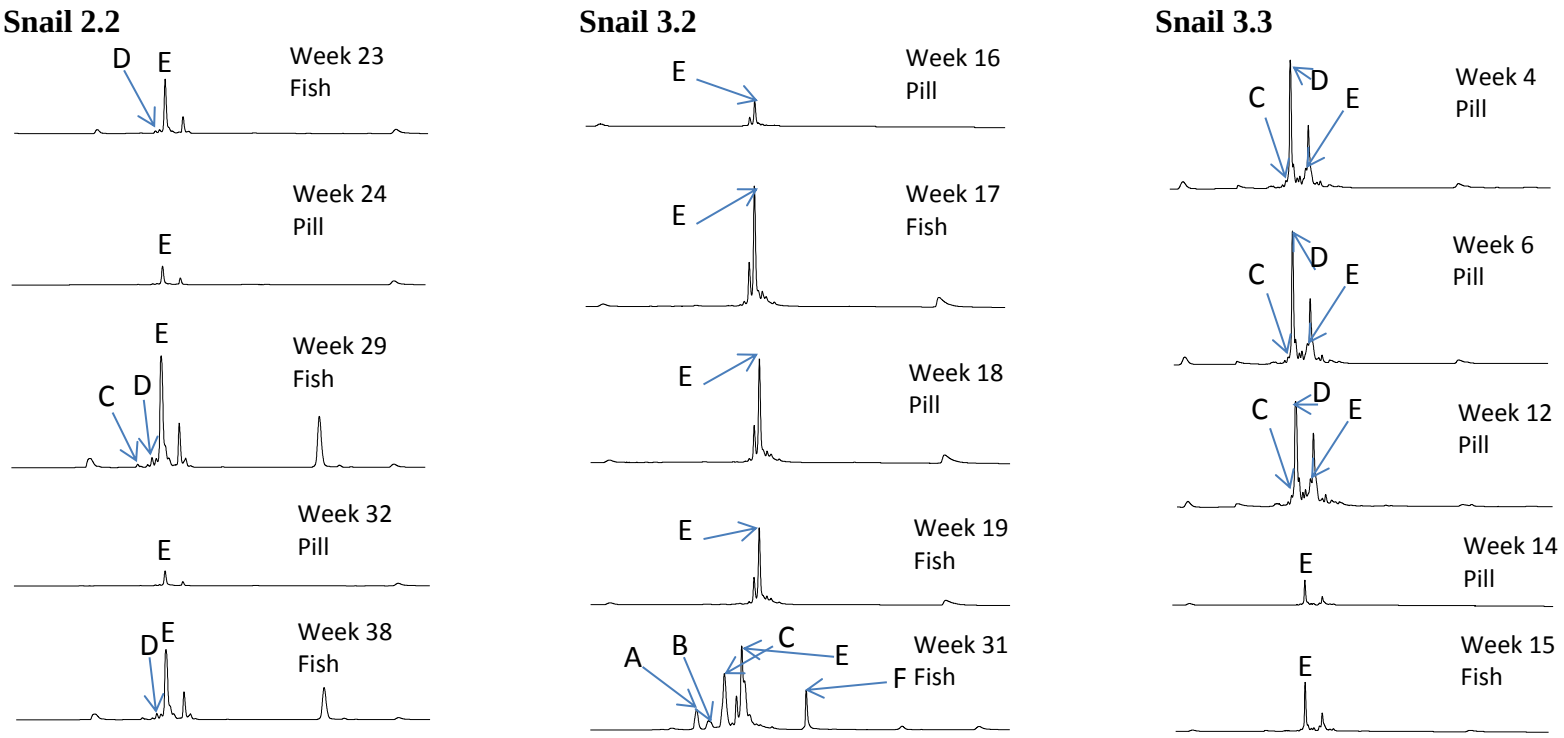


Figure 14. Illustrates representative RP-HPLC chromatographs of individual milkings of Group 1 (fish food). Chromatograms are labeled with the week the sample was collected, as well as the corresponding diet the snail received that given week. Isolated peptide peaks are identified based upon relative retention times. Half the venom volume was utilized and extracted at 214 nm to obtain each HPLC profile.

Table 8. Snail 2.2 - Diet Fish Food Pill.

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
23					1318180		Peaks E and D were identified. Peaks A, B, C and F were not produced.
24					445424		
29				979548	3776544		
32					600220		
38				5235141	75160530		

Table 8. Summary of area under curve (AUC) from representative chromatograms of the fish food pill diet (illustrated on pg. 43) and isolated peak(s).

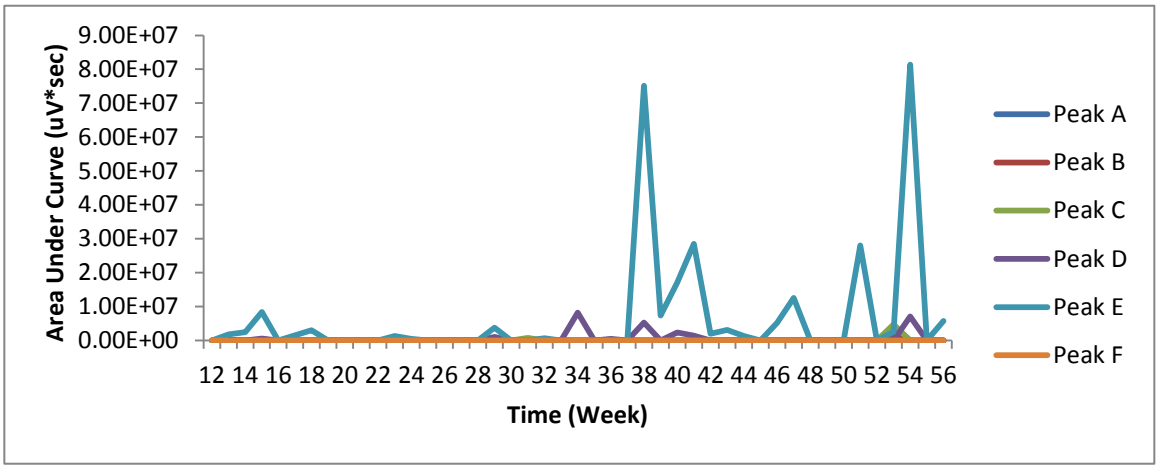


Figure 15. Snail 2.2 Peak production expressing area under curve. Diet – fish food.

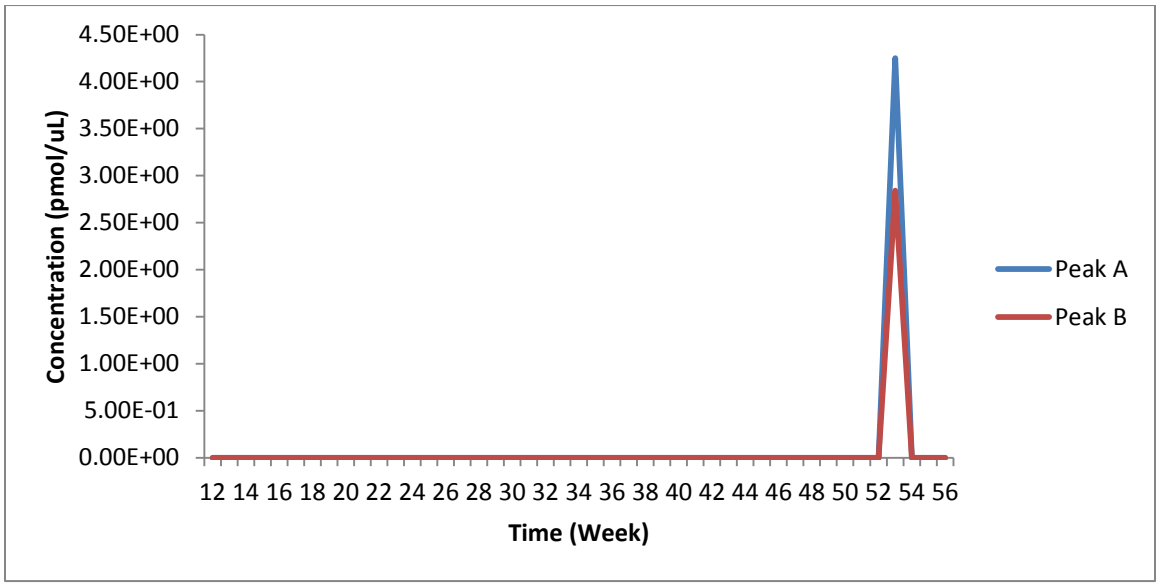


Figure 16. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 2.2; fish food pill. Study started at Week 0.

Table 9. Snail 3.2 - Diet Fish Food Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
16					1719281		Peaks A, B, C, E and F were identified. Peak D was consistently not produced.
17					1085081		
18					708553		
19					747024		
31	12877433	7819182	45460659		14666991	14335979	

Table 9. Summary of area under curve (AUC) from representative chromatograms of the fish food pill diet (illustrated on pg. 43) and isolated peak(s).

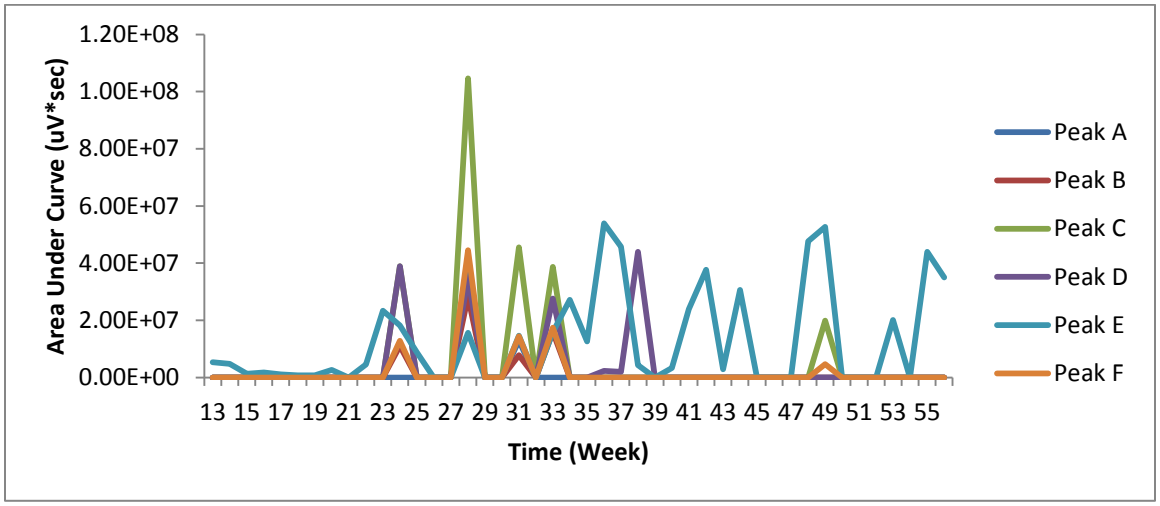


Figure 17. Snail 3.2. Peak production expressing area under curve. Diet – fish food.

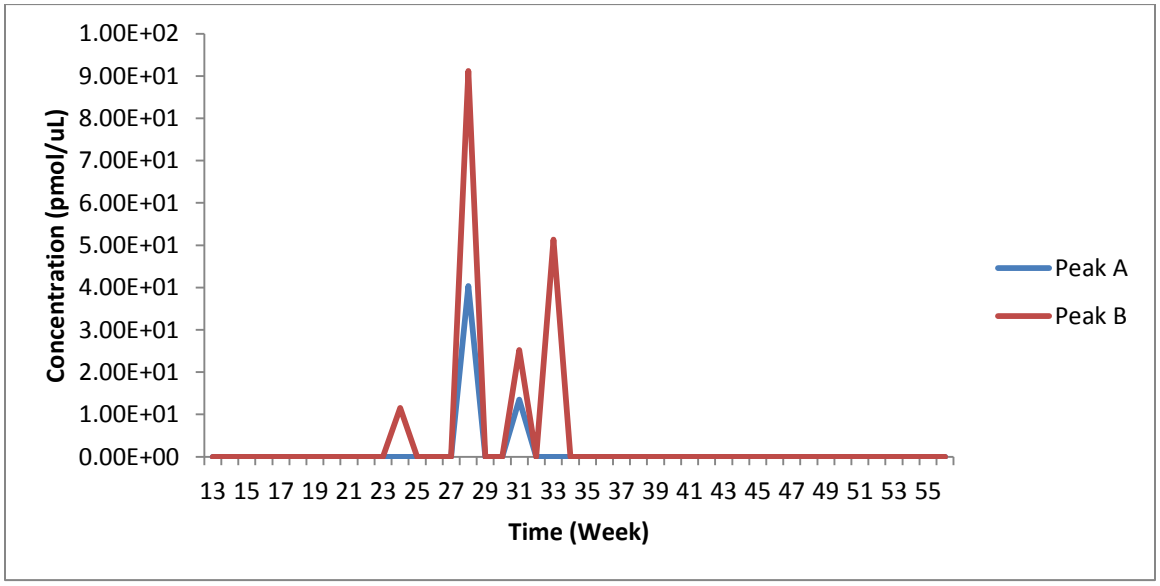


Figure 18. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 3.2; fish food pill. Study started at Week 0.

Table 10. Snail 3.3 – Diet fish food pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
4			1996923	8133162	7101575		Peaks C, D, E, and F were identified. Peaks A and B were not produced.
6			2061553	7553570	6486380		
12			1986414	7293481	7578203		
14			4452360	11550876	14856331	602640	
15					6353893		

Table 10. Summary of area under curve (AUC) from representative chromatograms of the fish food pill diet (illustrated on pg. 43) and isolated peak(s).

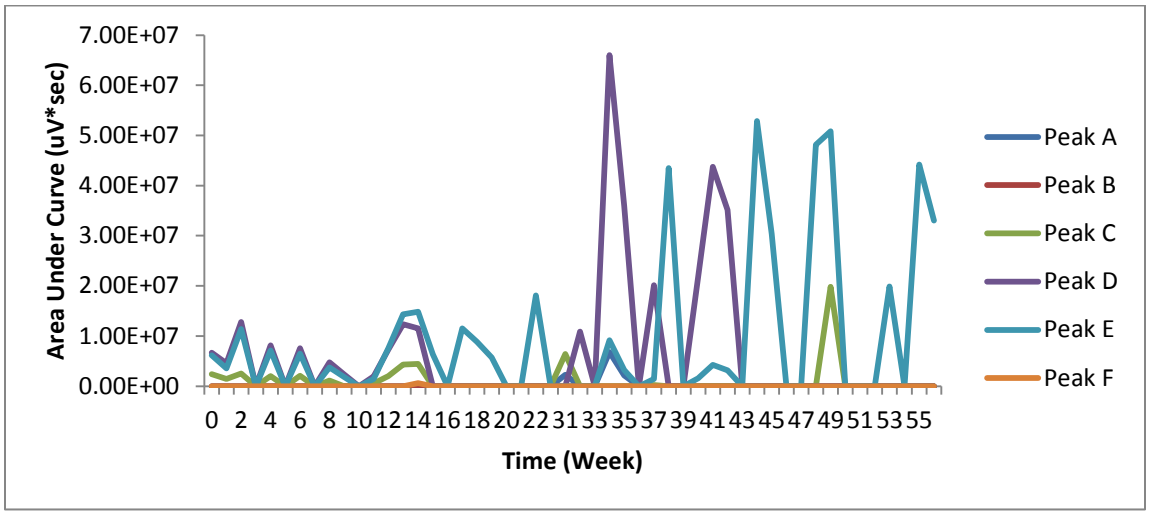


Figure 19. Snail 3.3 Peak production expressing area under curve. Diet – fish food.

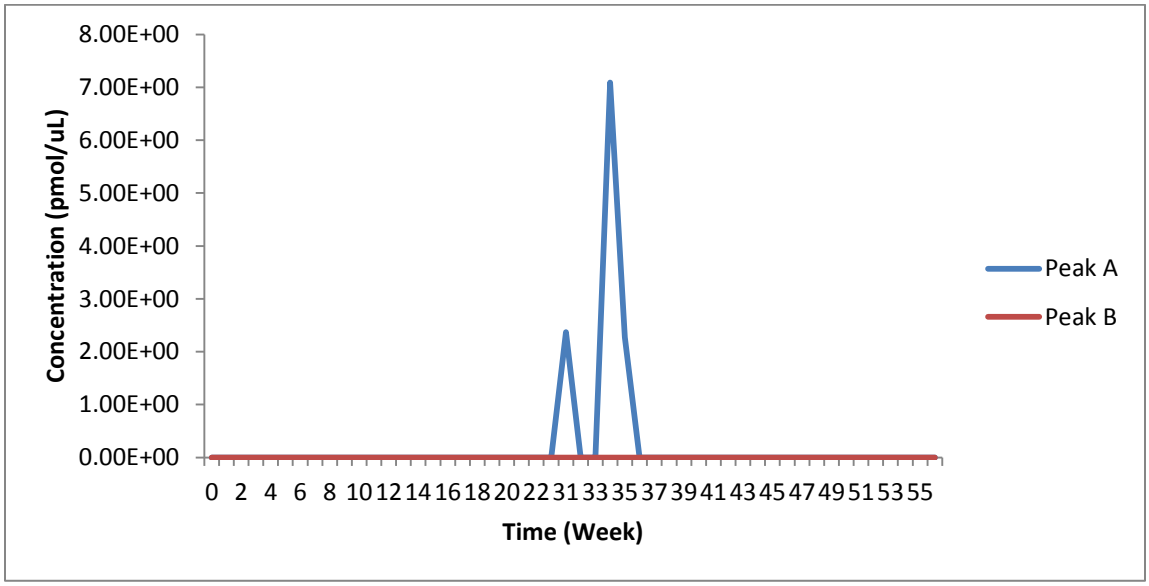


Figure 20. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 3.3; fish food pill. Study started at Week 0.

Group 2: Fish food and bromine

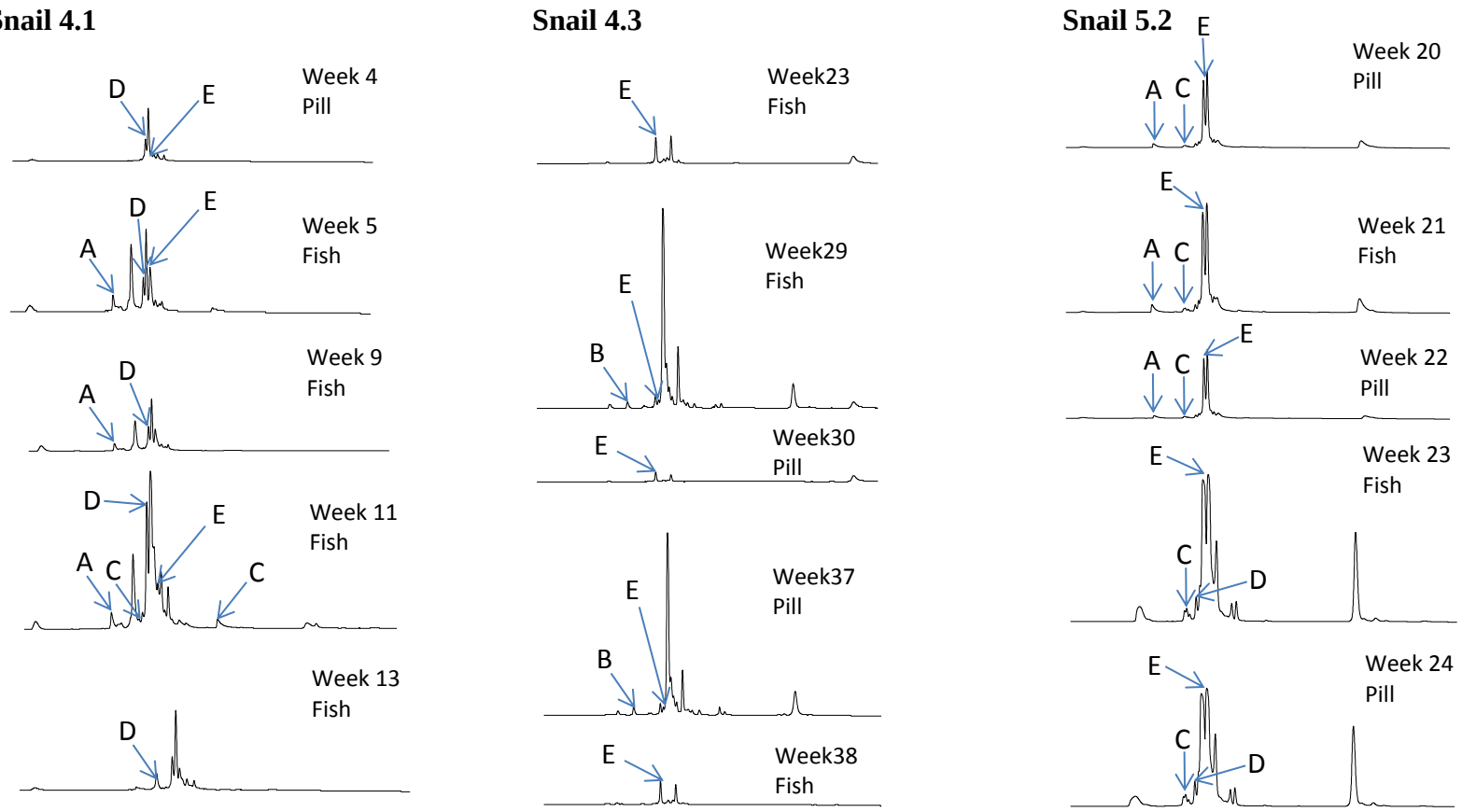


Figure 21. Illustrates representative RP-HPLC chromatographs of individual milkings from Group 2 (fish food and bromine). Chromatograms are labeled with the week the sample was collected, as well as the corresponding diet the snail received that given week. Isolated peptide peaks are also identified based upon relative retention times. Half the venom volume was utilized and extracted at 214 nm to obtain each HPLC profile.

Table 11. Snail 4.1 - Diet Fish Food + Bromine Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
4				29133489	7457310		Peak production is more random. Peaks A, C, D, E, and F were identified. Snail produced Peak D on a consistant basis.
5	15369090			24909852	6198359		
9	4739256			10878099	15581765		
11	7015739		3664065	46450070	15557808	5804529	
13				406217			

Tables 11. Summary of area under curve (AUC) from representative chromatograms of the fish food and bromine pill diet (pg. 47), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

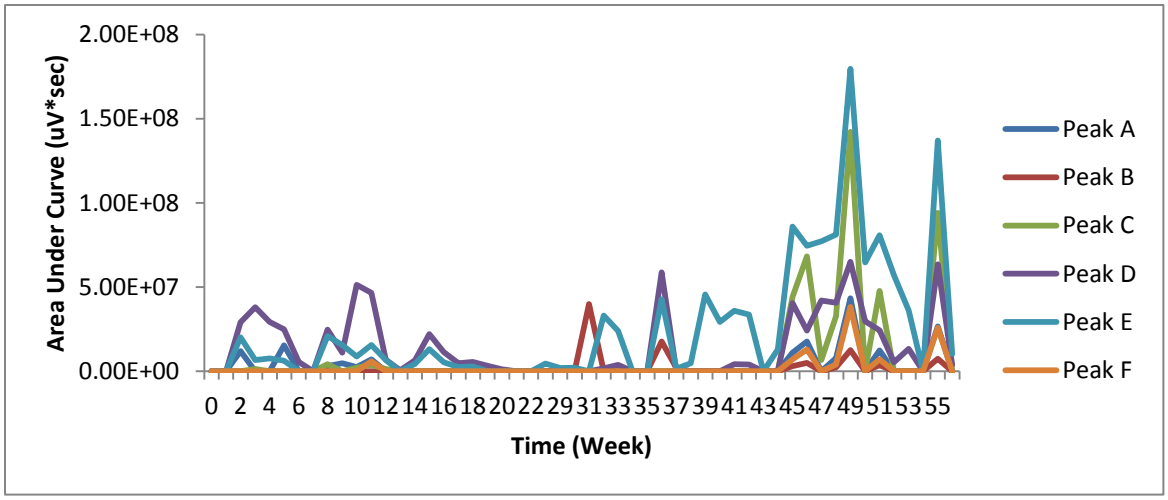


Figure 22. Snail 4.1 Peak production expressing area under curve. Diet – fish food and bromine.

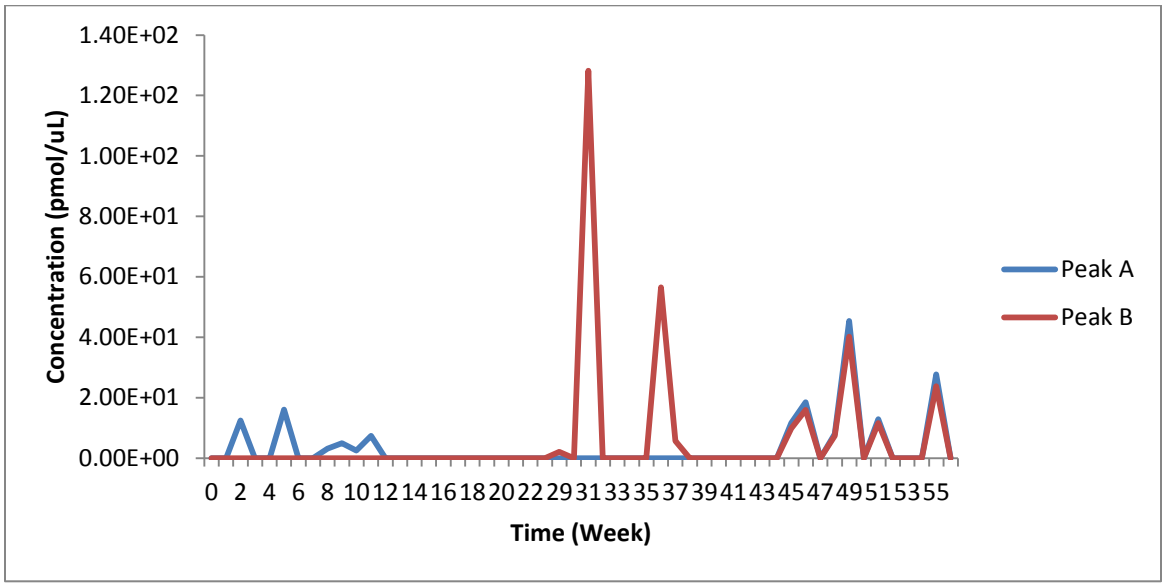


Figure 23. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 4.1; fish food and bromine pill. Study started at Week 0.

Table 12. Snail 4.3 - Diet Fish Food + Bromine Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
23					4556606		Peaks B and E were identified. Peak E produced on a consistent basis. Peaks A, C, D, and F were not produced.
29		657433			1820633		
30					2094063		
37		1756151			1525266		
38					4720852		

Table 12. Summary of area under curve (AUC) from representative chromatograms of the fish food and bromine pill diet (pg. 47), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

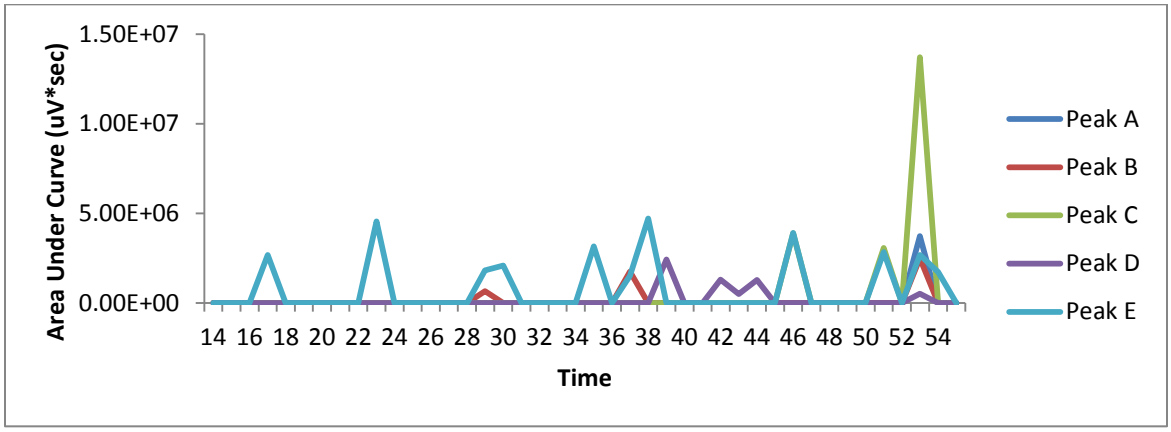


Figure 24. Snail 4.3 Peak production expressing area under curve. Diet – fish food and bromine.

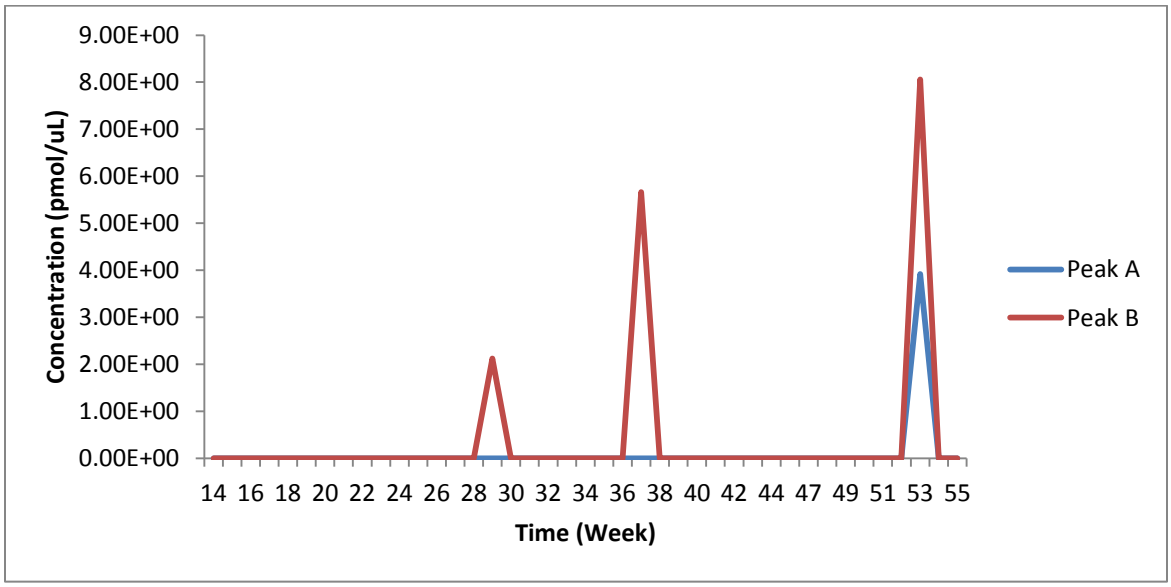


Figure 25. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 4.3; fish food and bromine pill. Study started at Week 0.

Table 13. Snail 5.2 - Diet Fish Food + Bromine Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
20	49205		1604444		34959966		Peaks A, C, D, and E were identified. Peaks B and F were not produced. Peak E was produced on a consistent basis.
21	5014112		2577304		53172693		
22	903022		1176926		24311042		
23			2288144	7840410	91922287		
24	2653348			11302146	101934206		

Tables 13 . Summary of area under curve (AUC) from representative chromatograms of the fish food and bromine pill diet (pg. 47), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

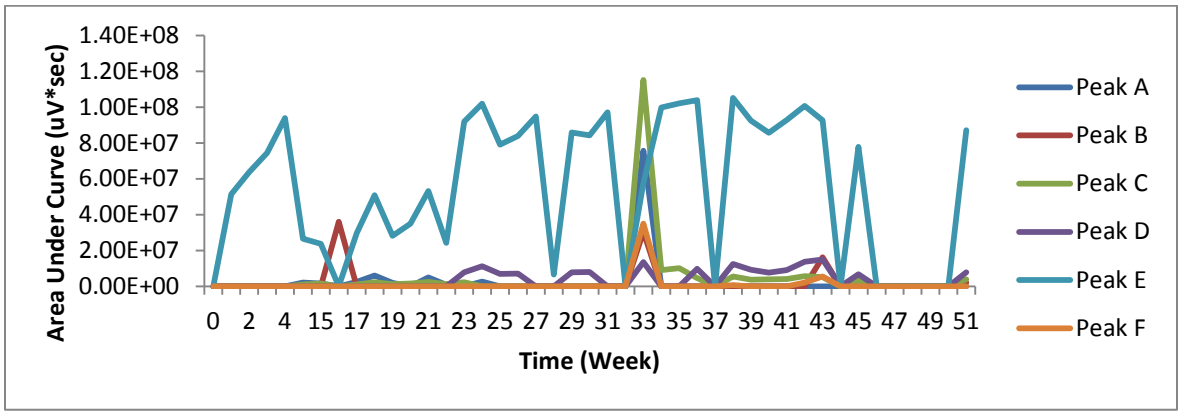


Figure 26. Snail 5.2 Peak production expressing area under curve. Diet – fish food and bromine.

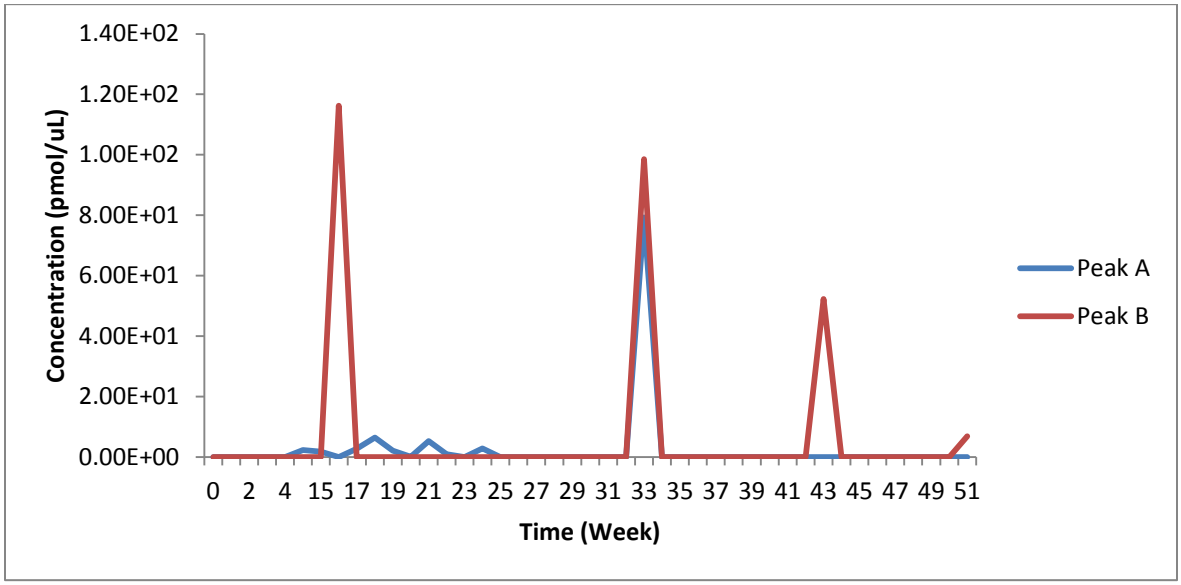


Figure 27. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 5.2; fish food and bromine pill. Study started at Week 0.

Group 3: Fish food and human vitamin pack

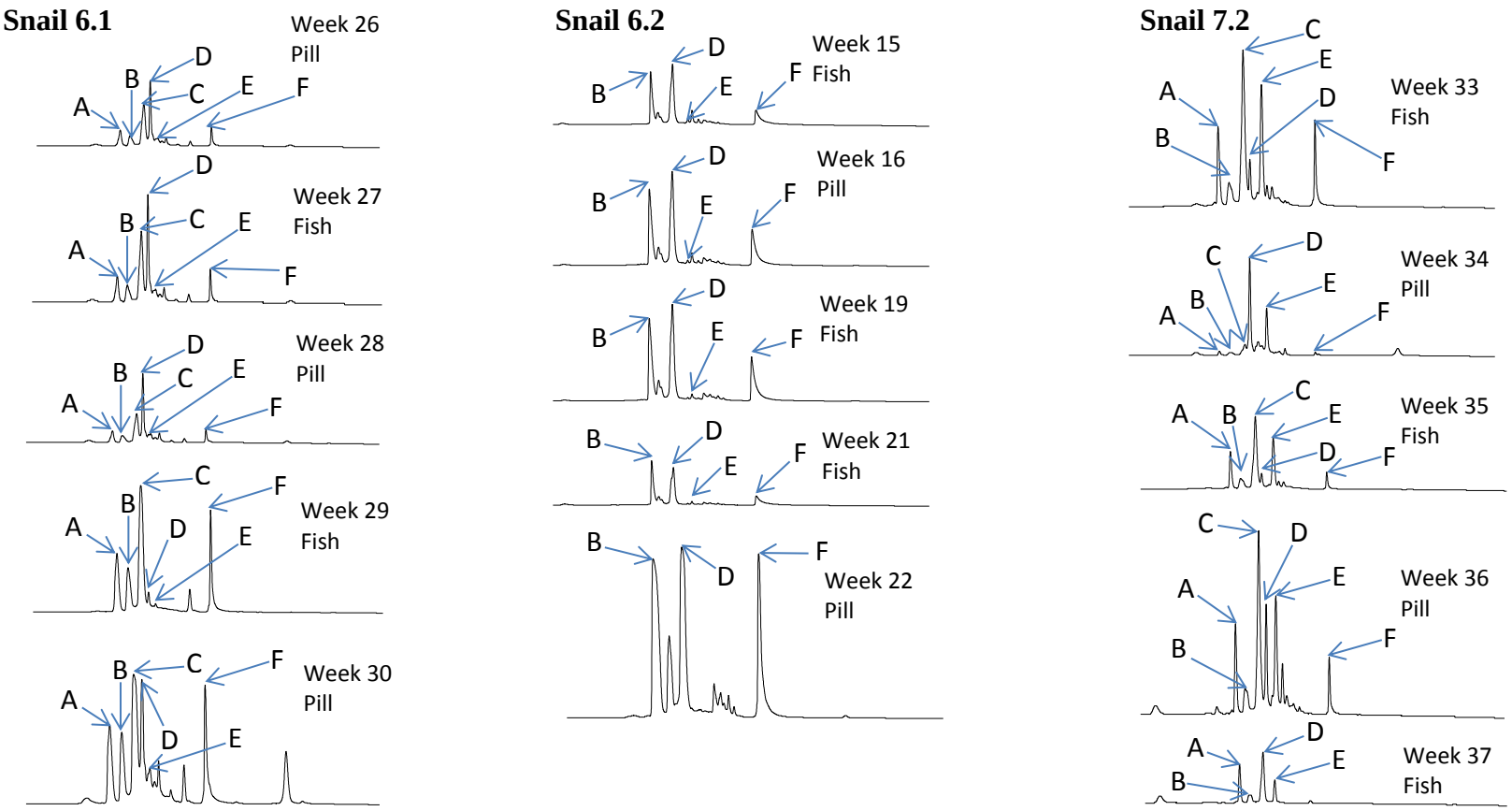


Figure 28. Illustrates representative RP-HPLC chromatographs of individual milkings from Group 3 (fish food and human vitamin pack). Chromatograms are labeled with the week the sample was collected, as well as the corresponding diet the snail received that given week. Isolated peptide peaks are also identified based upon relative retention times. Half the venom volume was utilized and extracted at 214 nm to obtain each HPLC profile.

Table 14. Snail 6.1 - Diet Fish Food + Human Vitamin Pack Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
26	34704649	28447556	98612019	38152707	11162970	36230636	Peaks A - F were identified and consistently produced.
27	7152504	5831673	23064735	17568564	2441877	4394654	
28	52587549	41935488	123417349	42033318	16245514	3204642	
29	4195173	3407786	13352889	14905312	2230978	2377366	
30	48487396	38119293	118980209	8773299	3800052	47692089	

Table 14. Summary of area under curve (AUC) from representative chromatograms of the fish food and human vitamin pack pill diet (pg. 51), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

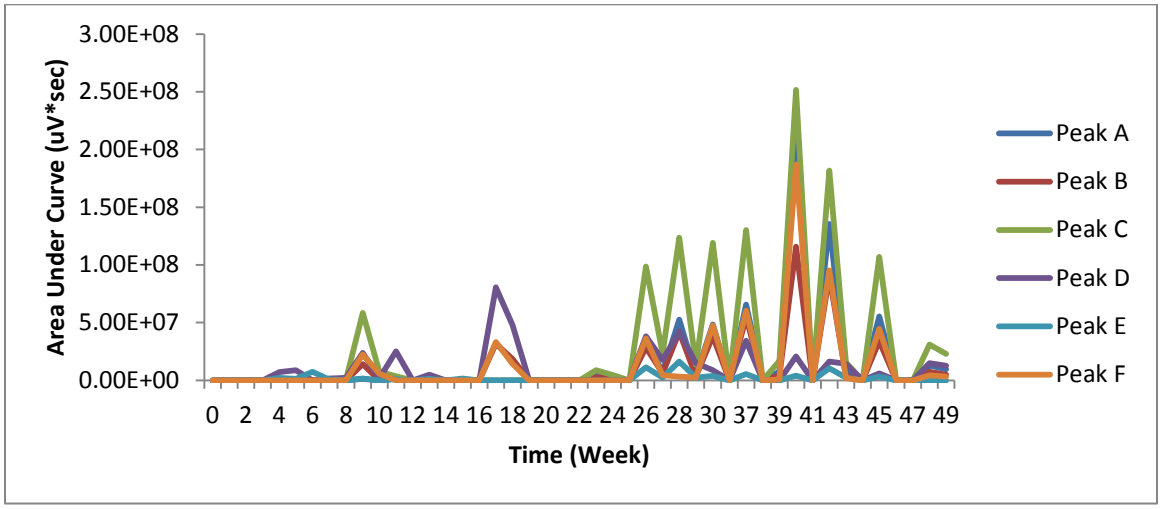


Figure 29. Snail 6.1 Peak production. Diet – fish food and human vitamin pack.

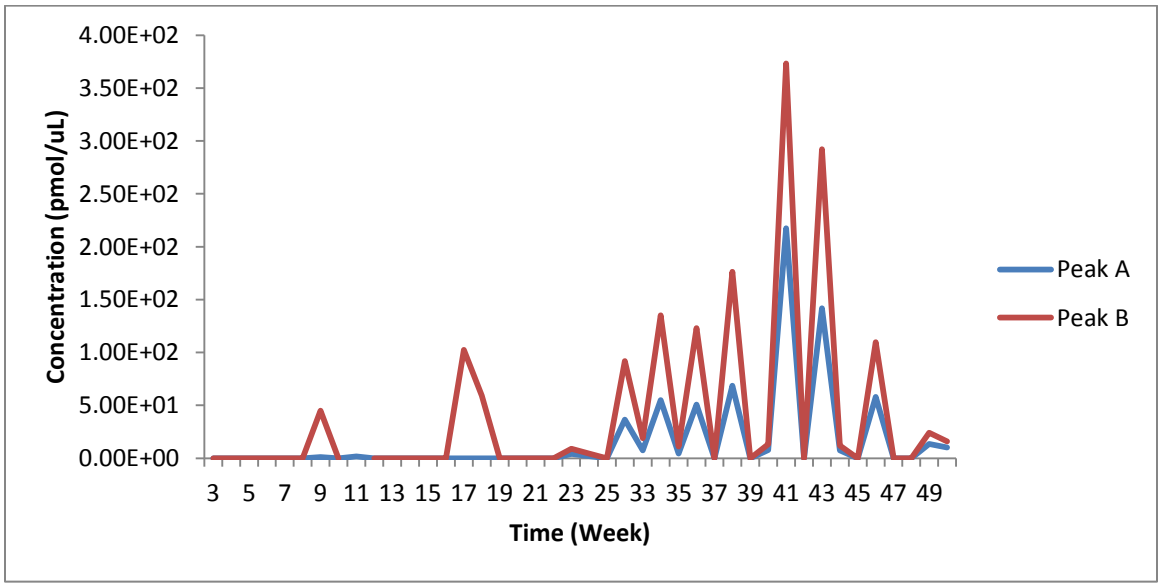


Figure 30. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 6.1; fish food and human vitamin pack. Study started at Week 0.

Table 15. Snail 6.2 - Diet Fish Food + Human Vitamin Pack Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
15		17374401	27292429		1926635	7051308	Peaks B, C, E and F were identified. Peaks B, C, and F were produced on a consistent basis. Peaks A and C were not produced.
16		17664688	27042550		1124485	7074159	
19		51739525	67748798		1686457	26974890	
21		62383147	77422002		1077797	38140029	
22		18342330	22030598			7100819	

Table 15. Summary of area under curve (AUC) from representative chromatograms of the fish food and human vitamin pack pill diet (pg. 51), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

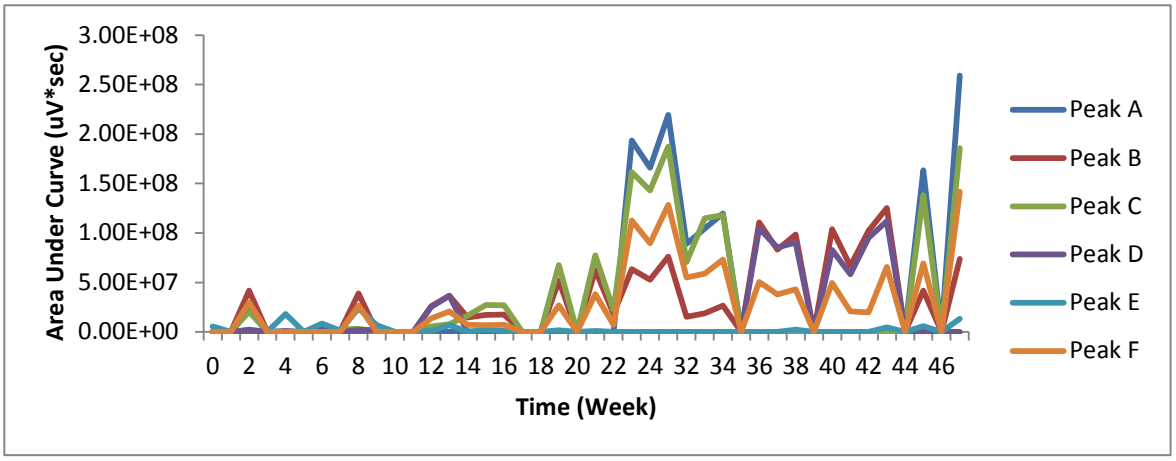


Figure 31. Snail 6.2 Peak production expressing area under curve. Diet – fish food and human vitamin pack.

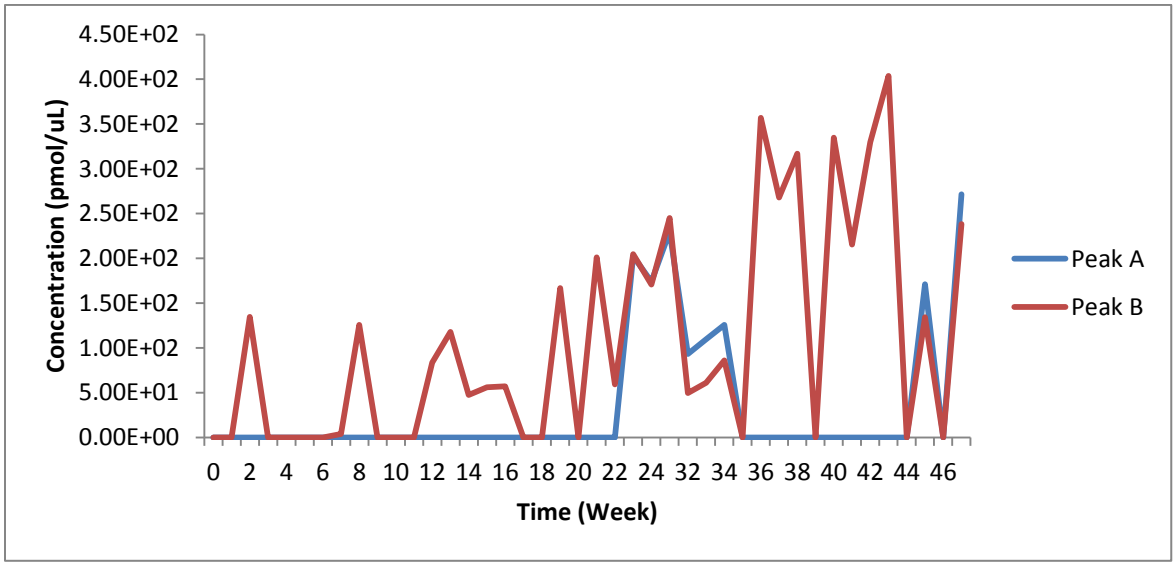


Figure 32. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 6.2; fish food and human vitamin pack. Study started at Week 0.

Table 16. Snail 7.2 - Diet Fish Food + Human Vitamin Pack Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
33	1085725	12814202	68222727	11010880	39056768	13166494	Peaks A - F were identified. Production was not consistent.
34	1101063	2097310	1864974	29237572	5255892	843828	
35	9165095	5183379	28300584	3250964		2406025	
36	12054925	7072114	36060847	13077728	18654281	4795425	
37	7348825	3151368		14554024	3892506		

Table 16. Summary of area under curve (AUC) from representative chromatograms of the fish food and human vitamin pack pill diet (pg. 51), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

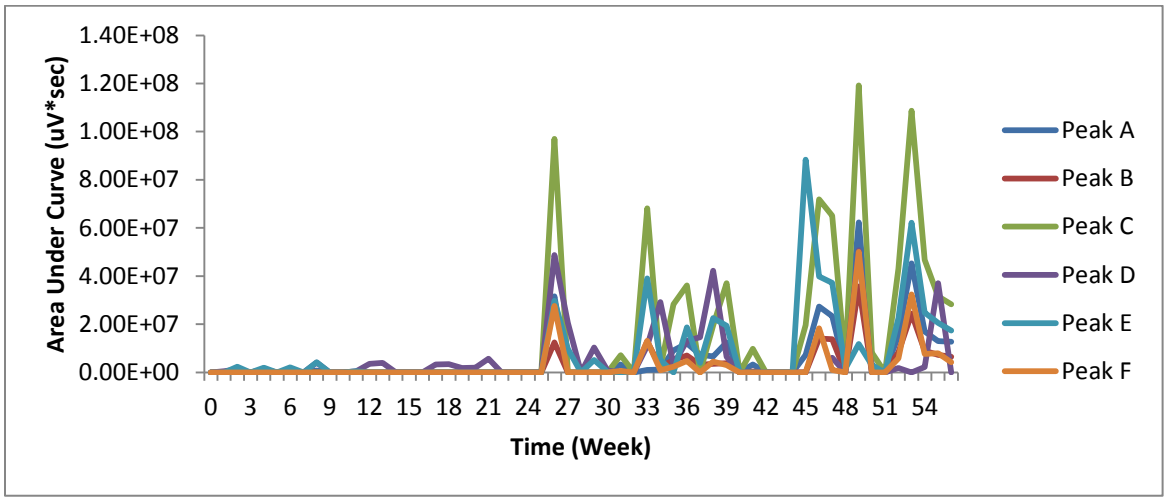


Figure 33. Snail 7.2 Peak production expressing area under curve. Diet – fish food and human vitamin pack.

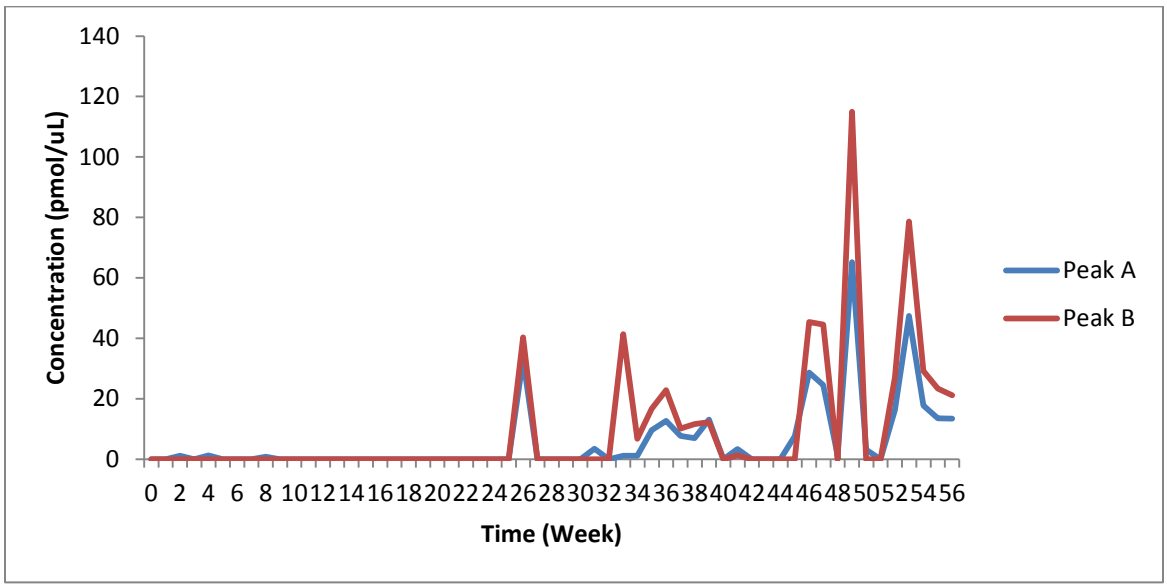


Figure 34. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 7.2; fish food and human vitamin pack. Study started at Week 0.

Group 4: Fish food, human vitamin pack, and bromine

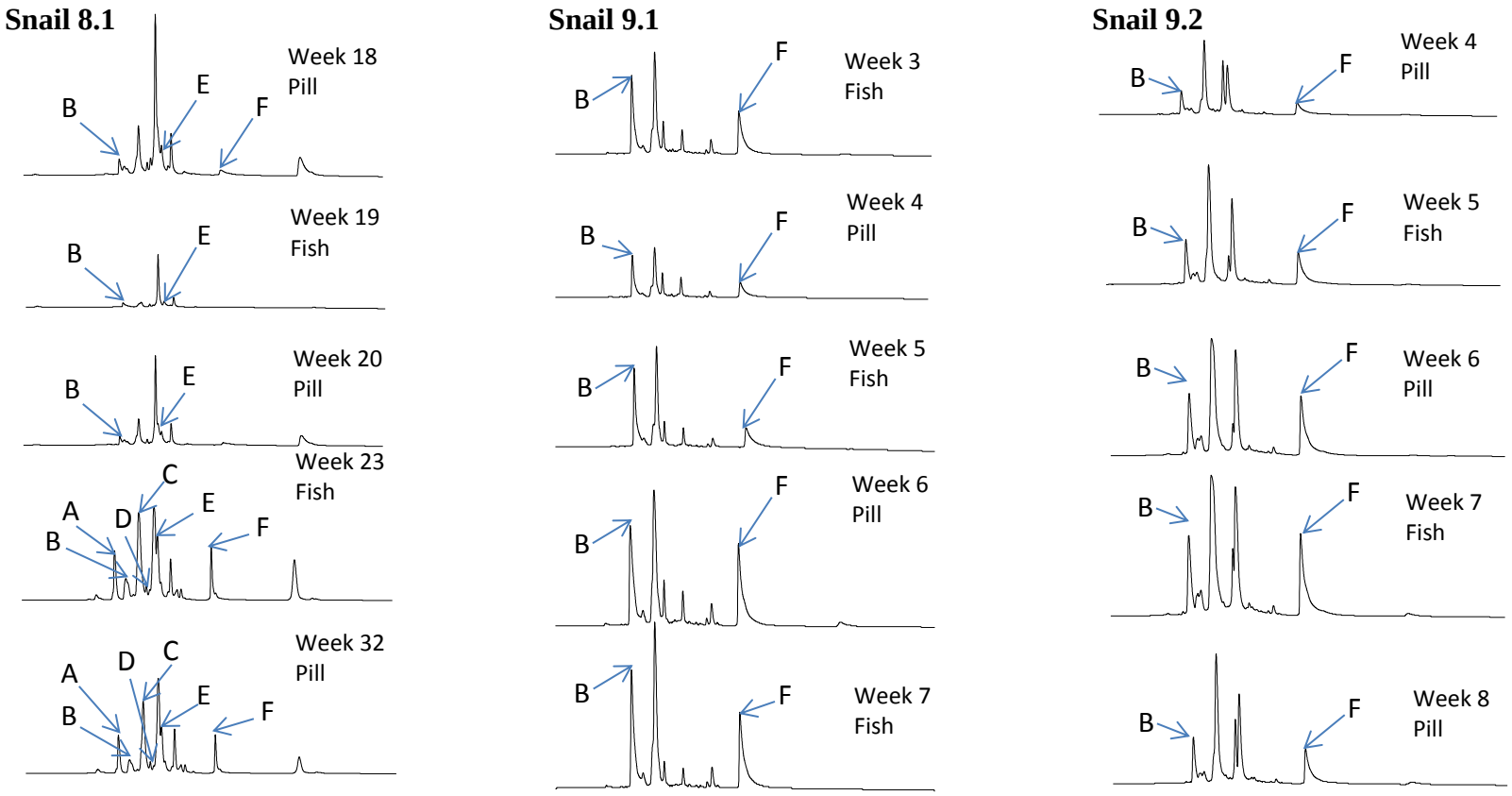


Figure 35. Illustrates representative RP-HPLC chromatographs of individual milkings from Group 4 (fish food, human vitamin pack, and bromine). Chromatograms are labeled with the week the sample was collected, as well as the corresponding diet the snail received that given week. Isolated peptide peaks are also identified based upon relative retention times. Half the venom volume was utilized and extracted at 214 nm to obtain each HPLC profile.

Table 17. Snail 8.1 - Diet Fish Food + Human Vitamin Pack + Bromine Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
18		4614432			8229291	2038020	Peaks A - F were identified. Peaks B and E were produced consistently.
19		840256			425682		
20		2713997			772140	625297	
23	31464332	26413272	94831152	7261420	43701864		
32	22865160	24011853	66567388	5543181	2726266	15478820	

Tables 17. Summary of area under curve (AUC) from representative chromatograms of the fish food, human vitamin pack, and bromine pill diet (pg. 55), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

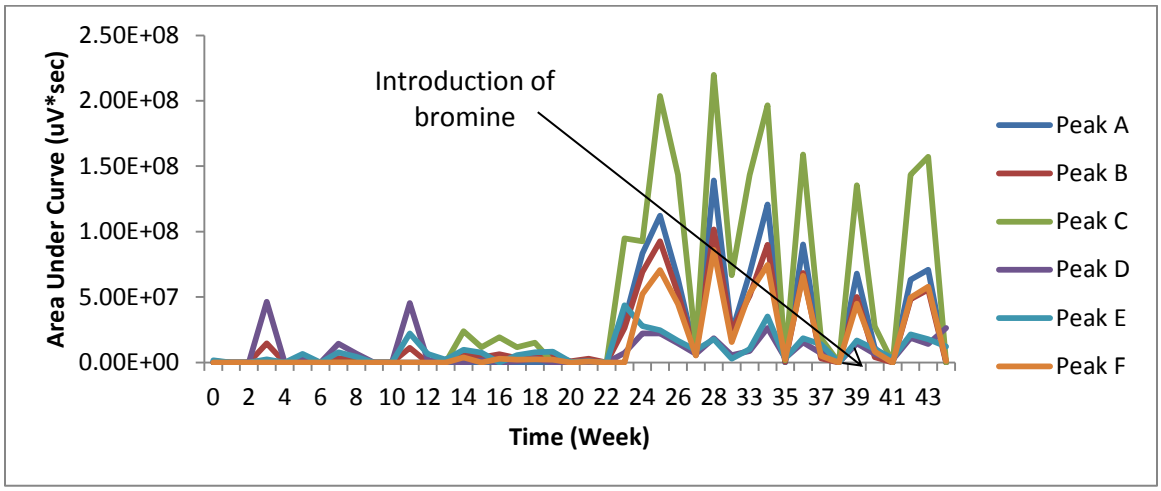


Figure 36. Snail 8.1 Peak production expressing area under curve. Diet – fish food, human vitamin pack, bromine. Arrow indicates introduction of bromine to pill at week 39.

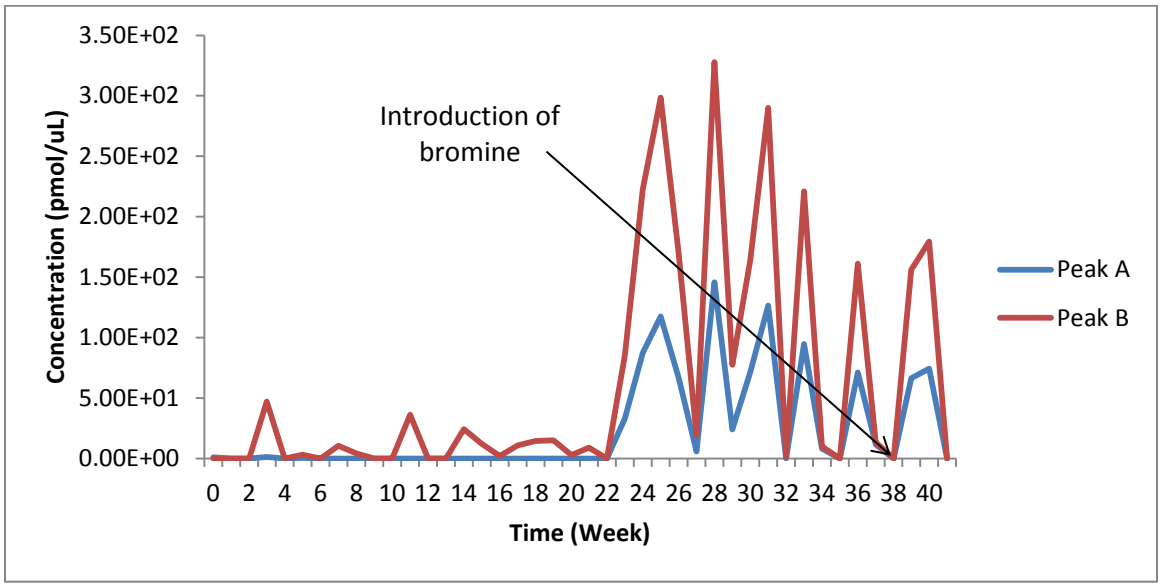


Figure 37. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 8.1; fish food, human vitamin pack, and bromine. Study started at Week 0.

Table 18. Snail 9.1 - Diet Fish Food + Human Vitamin Pack + Bromine Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
3		29716113				21587720	Peaks B and F were identified and produced consistently. Peaks A, C, D, and E were not produced.
4		14852330				11302885	
5		9430774				5764101	
6		61761508				71366925	
7		38314364				32811502	

Tables 18. Summary of area under curve (AUC) from representative chromatograms of the fish food, human vitamin pack, and bromine pill diet (pg 55), and isolated peak(s). AUC shows a potential cycling of peptide concentration.

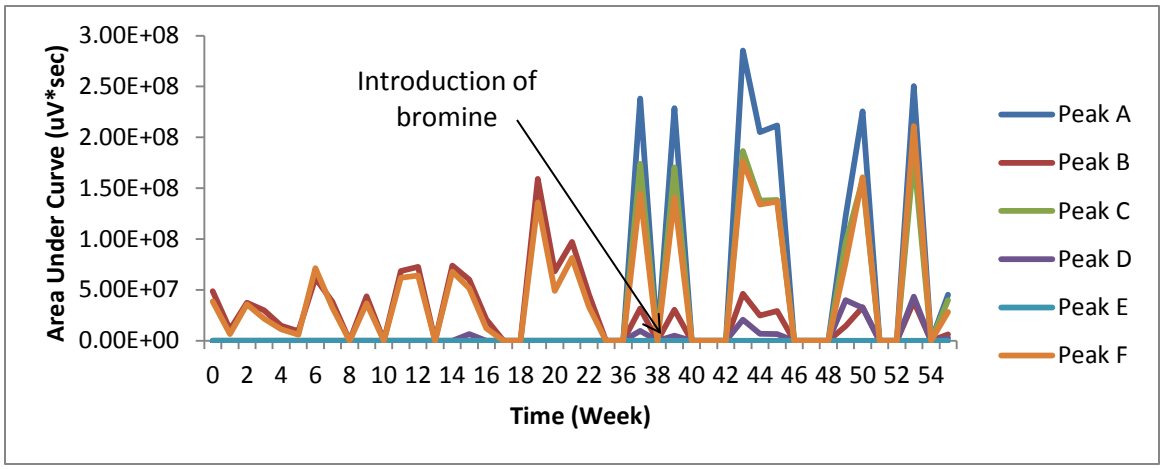


Figure 38. Snail 9.1 Peak production expressing area under curve. Diet – fish food, human vitamin pack, bromine. Arrow indicates introduction of bromine to pill at week 39.

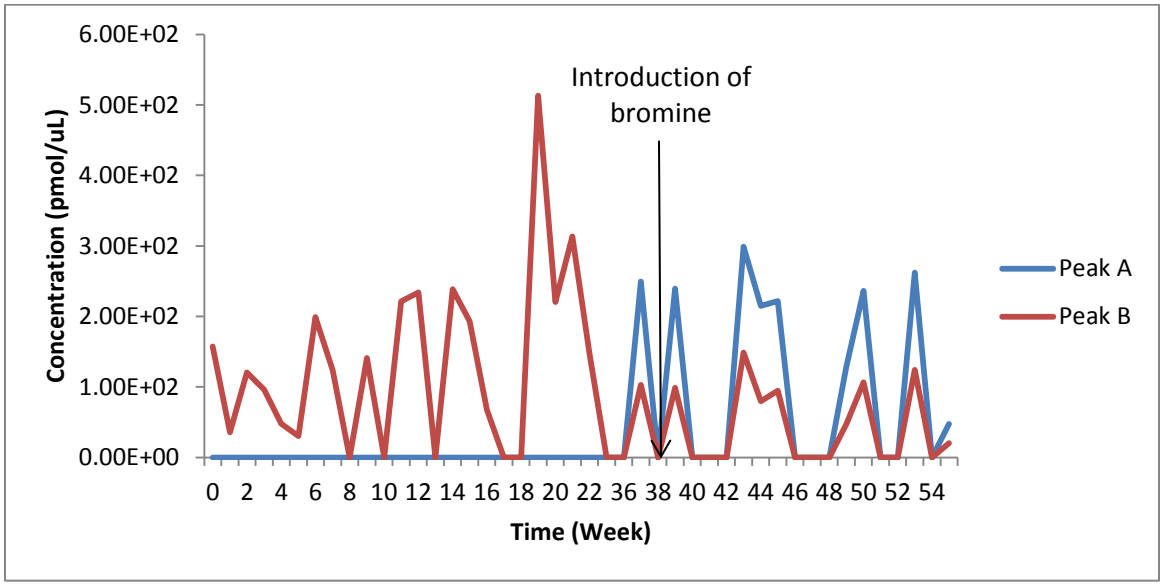


Figure 39. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 9.1; fish food, human vitamin pack, and bromine. Study started at work 0.

Table 19. Snail 9.2 - Diet Fish Food + Human Vitamin Pack + Bromine Pill

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
4		7338836				6134521	Peaks B and F were identified and produced consistently. Peak A was identified, however production was not consistent. Peaks C, D, and E were not produced.
5		21458792				26028042	
6	1194094	42364903				54341051	
7	1123392	46633168				66832730	
8		17290347				18819628	

Table 19. Summary of area under curve (AUC) from representative chromatograms of the fish food, human vitamin pack, and bromine pill diet (pg. 55), and isolated peak(s). AUC shows a potential cycling of peptide concentration

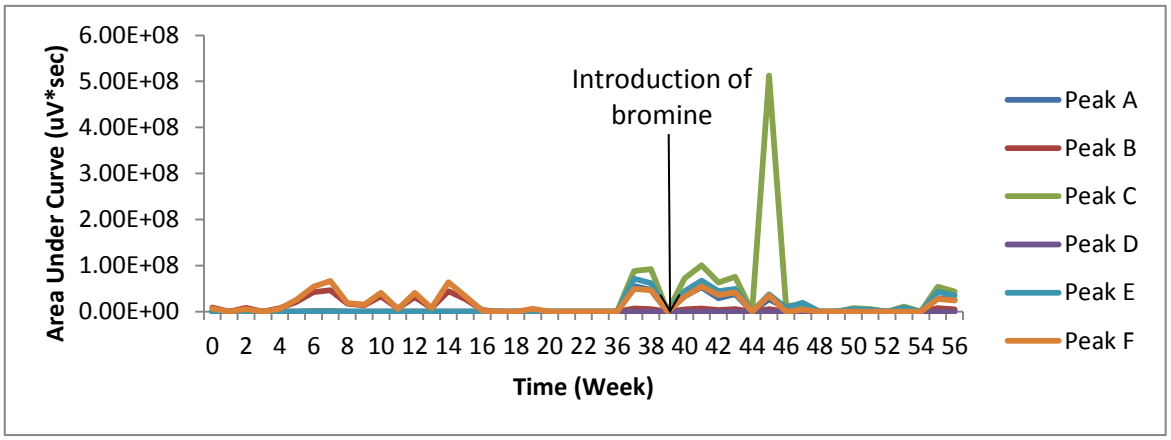


Figure 40. Snail 9.2 Peak production expressing area under curve. Diet – fish food, human vitamin pack, bromine. Arrow indicates introduction of bromine to pill at week 39.

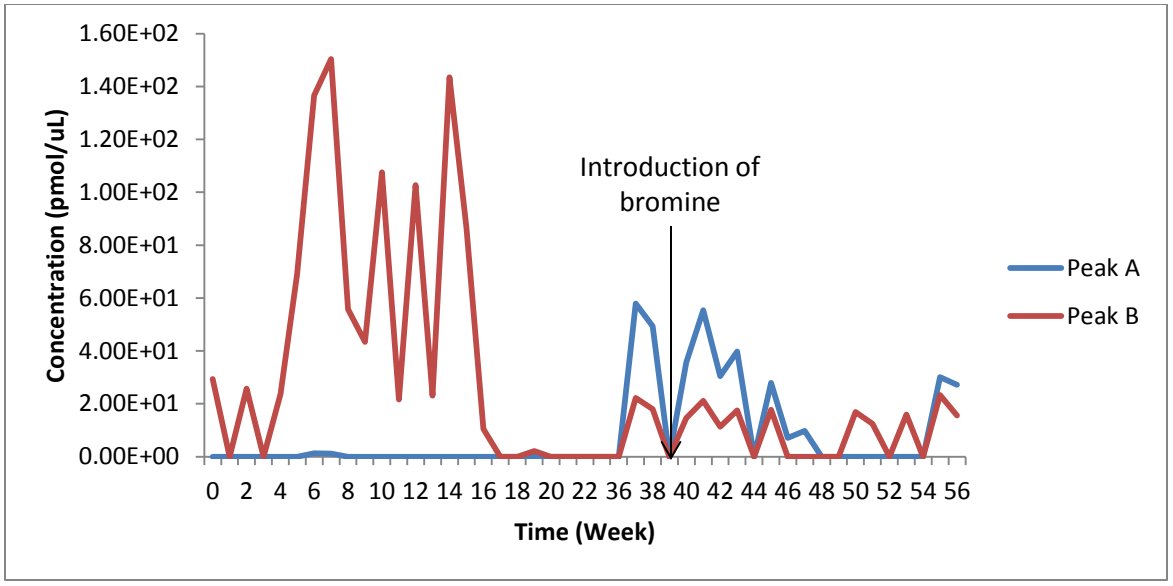


Figure 41. Comparison of Peak A and Peak B concentration (pmol/µL) of snail 9.2; fish food, human vitamin pack, and bromine. Study started at Week 0.

Control Snails (fish diet)

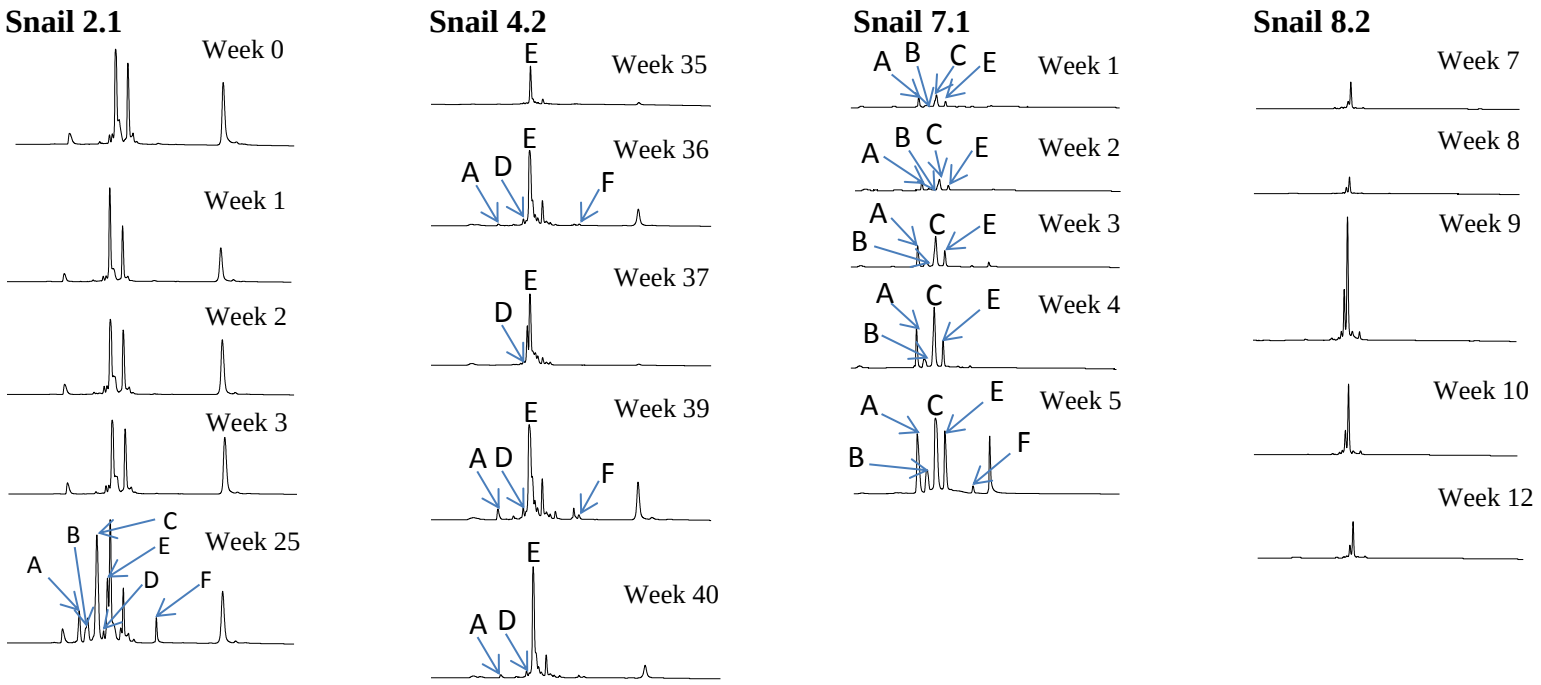


Figure 42. Illustrates representative RP-HPLC chromatographs of individual milkings from Control Group (fish). Chromatograms are labeled with the week the sample was collected. Control snails were fed fish each feeding/milking. Isolated peaks are also identified based upon relative retention times. Half the venom volume was utilized and extracted at 214 nm to obtain each HPLC profile.

Table 20. Snail 2.1 - Control Diet Fish

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
0							Peak production is not consistent. Peaks A - F were identified at week 25.
1							
2							
3							
25	15417805	12724697	71450583	3988560	54660686	7324212	

Tables 20. Summary of area under curve (AUC) from representative control chromatograms (pg. 59), and isolated peak(s).

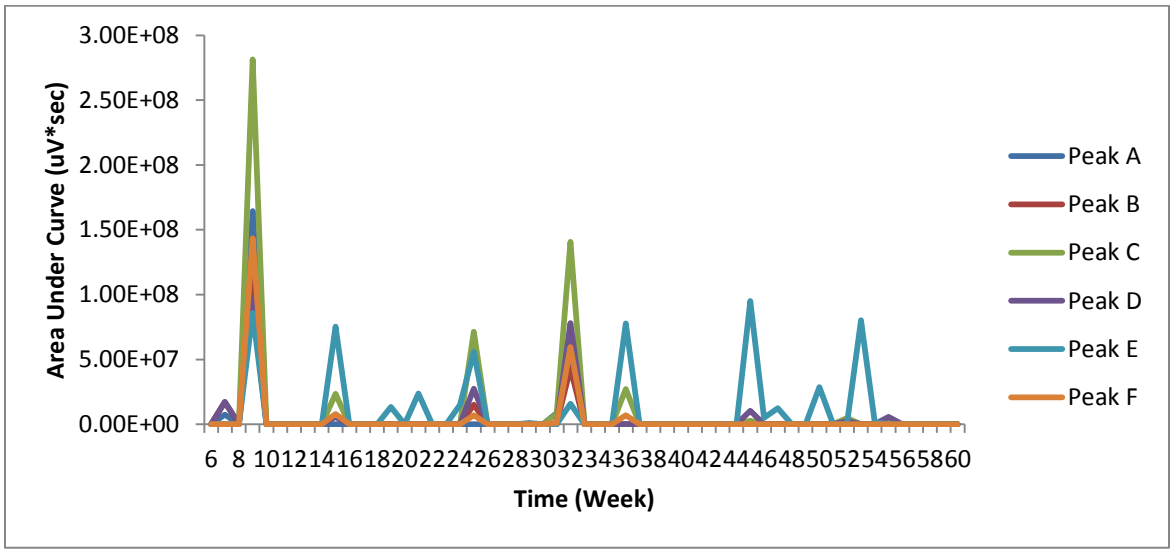


Figure 43. Control Snail 2.1 Peak production expressing area under curve. Diet – fish.

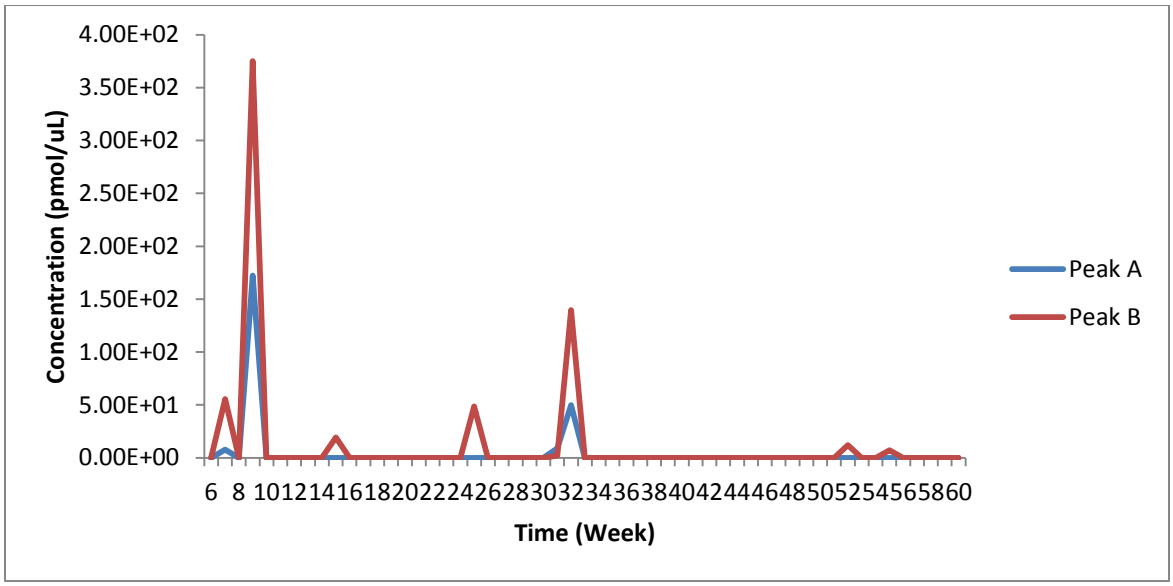


Figure 44. Comparison of Peak A and Peak B concentration (pmol/µL) of control snail 2.1; fish. Study started at Week 0.

Table 21. Snail 4.2 - Control Diet Fish

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
35					11864528		Peaks A, D, E, and F were identified. Peaks B and C were not produced. Peaks D and E were produced on a consistent basis.
36	958128			4692971	84896655	923779	
37				2763973	57070993		
39	7678206			6669678	95023823	2337758	
40	1219644			3031395	63583690	357097	

Tables 21. Summary of area under curve (AUC) from representative control chromatograms (pg. 59), and isolated peak(s).

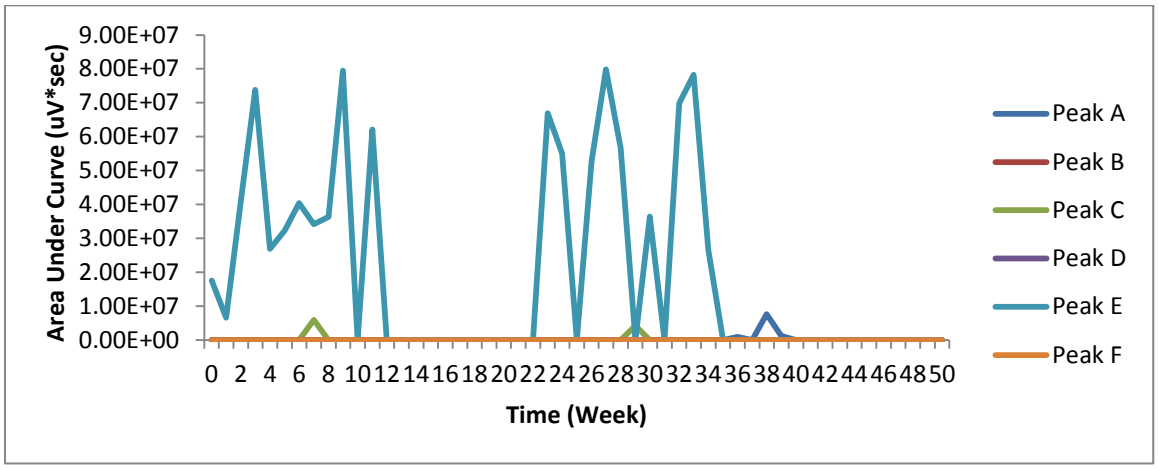


Figure 45. Control Snail 4.2 Peak production expressing area under curve. Diet – fish.

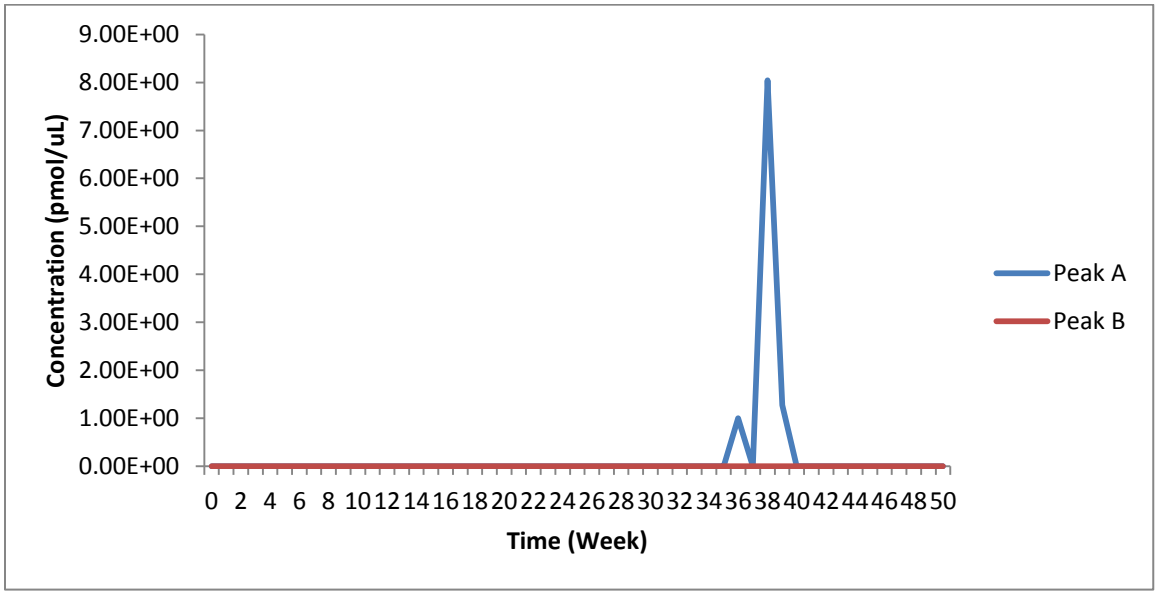


Figure 46. Comparison of Peak A and Peak B concentration (pmol/µL) of control snail 4.2; fish. Study started at Week 0.

Table 22. Snail 7.1 - Control Diet Fish

Peak (area under curve; µV*sec)							Comments
Week	A	B	C	D	E	F	
1					3925857		Peaks C, E, and F were identified, but not produced on a consistent basis. Peaks A, B, and D were not produced.
2					1421013		
3					8031014		
4					6555424		
5			115235014			33842525	

Table 22. Summary of area under curve (AUC) from representative control chromatograms (pg. 59), and isolated peak(s).

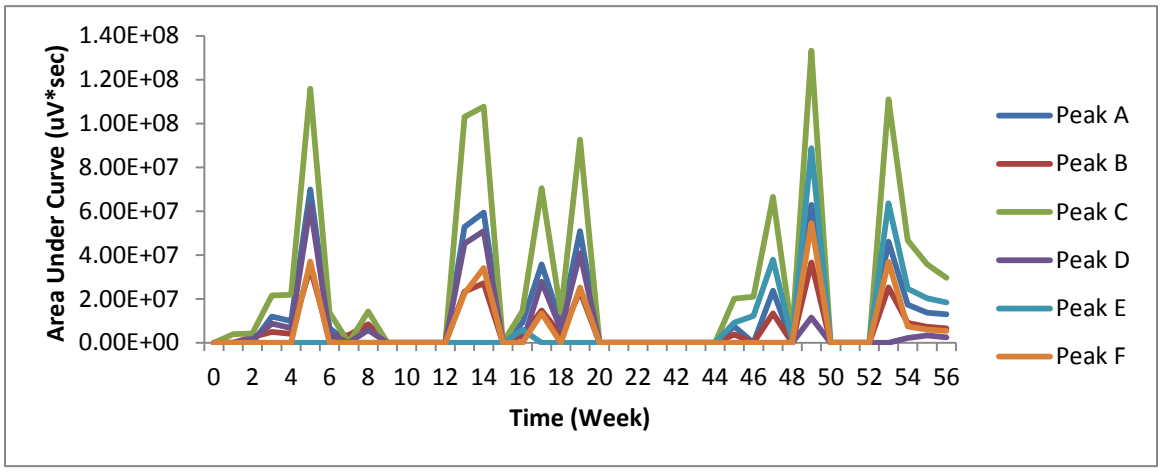


Figure 47. Control Snail 7.1 Peak production expressing area under curve. Diet – fish.

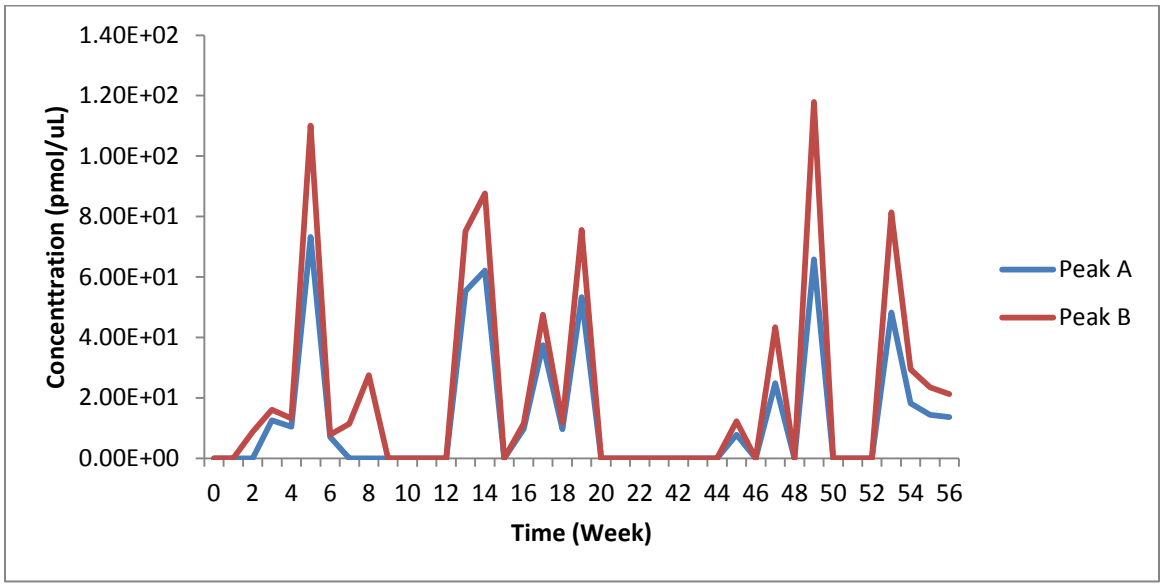


Figure 48. Comparison of Peak A and Peak B concentration (pmol/µL) control snail 7.1; fish. Study started at Week 0.

***Control Snail 8.2 is has been excluded having not produced any of the isolated peaks during the study.**

3.2.1 Venom Composition and Sex

Venom composition in comparison between male and female snails indicates a difference. Snail 2.2 (male) from Group 1 (fish food) produced two out of the six isolated peptides, primarily Peptide E and Peptide D. Snail 3.3 (female) also from Group 1, in contrast produced four out of the six isolated peptides, primarily Peptides C, D, E and F. See tables 8 and 10 (located on pgs. 45 and 46).

A similar trend can be seen with snails from Group 3 (fish food and human vitamin pack). Snail 6.2 (male) produced four out of the six isolated peptides, mainly Peptides B, C, E, and F. This is in contrast to snail 6.1 (female) that produced all six of the isolated peaks; see Tables 14 and 15 (located on pgs. 52 and 53). However, snail 7.2 (male; Table 16), also from Group 3, in comparison to snail 6.1 (female), also produced all of the isolated peptides as is represented in Figure 28 (pg. 51) in the representative chromatograms.

3.2.2 Venom Composition in Comparison to Pre-Diet Milkings

Overall the venom composition of the snails prior to the diet study (data not shown) to diet manipulation milkings indicates increased variety of peptides produced. This is opposite to venom volume, which is has significantly decreased over the course of the diet manipulation study in comparison to pre-diet manipulation milking (see Figures 4 through 8).

Chapter 4: DISCUSSION

4.1 Venom Synthesis

Synthesis and delivery of venom involves three basic steps. Step (1) involves the synthesis, processing, and packaging of the peptides; step (2) development and storage of radula teeth and transfer of the radula to tip of proboscis; the final step (3) is envenomation (Marshall et al., 2002). Venom synthesis occurs in the long, serpentine venom duct (see Figure 1, pg. 13). The proximal and distal ends of the venom duct have been shown to differ in venom content as well as structure (Marshall et al., 2002). After synthesis in the secretory cells, the venom is then packaged into granules and secreted into the lumen of the gland (Safavi-Hemami et al., 2011).

The radular teeth are synthesized in the long arm of the radular sac and mature radula are stored in the short arm (see Figure 1, pg. 13) (Marshall et al., 2002). The mechanism at which a single radula is transported into the pharynx and to the tip of the proboscis is yet to be elucidated.

Previous thought has been that the venom bulb has little to no involvement in the secretion of venom (Marshall et al., 2002). However, recent findings of the Safavi-Hemami group (2010) indicate that the venom bulb is actually a highly specialized organ of venom movement. It is the sudden contraction of the venom bulb that rapidly pushes the venom through the radula into the given prey (Safavi-Hemami et al., 2010). By sequencing and characterizing an integral protein involved in rapid muscular movement in invertebrates, arginine kinase, Safavi-Hemami et al. (2010) discovered high concentrations of the enzyme in the venom bulb in comparision to the foot muscle and the venom gland.

Proteomic investigation into the venom gland of *Conus* has led to the identification of novel glandular proteins. These glandular proteins may be of potential importance in conotoxin synthesis and secretion (Safavi-Hemami et al., 2011). To date, little is known about the glandular proteins involved in conotoxin maturation, processing, and packaging. Glandular proteins that have been identified are PDI (protein disulfide isomerase), which is one of the most abundant glandular proteins in *Conus* (Safavi-

Hemami et al., 2011). PDI has shown to catalyze the isomerization and oxidation of native disulfide bonds *in vitro*. Additional venom gland specific enzymes involved in *in vitro* modification, folding and processing of toxins include peptidyl-prolyl cis-trans isomerase (PPI), γ-carboxylase, and cysteine-rich protease Tex31 (Safavi-Hemami et al., 2011).

From the data collected there definitely appears to be a correlation between diet and venom synthesis in the experimental cone snails. Not only does diet manipulation affect synthesis, but affects peak diversity and concentration (discussed below).

4.2 Diversity of Venom Composition

Visual inspection of RP-HPLC chromatograms pre-diet manipulation (data not shown) in comparison to diet manipulated chromatograms shows a change in venom composition. The change does not occur across all snails. This could be due to a variety of factors, one of course being the alteration from the normal diet of fish. Little is known about cone snail venom synthesis and what predisposes the snail(s) to synthesize certain peptides. All the snails expressed varying concentrations of the isolated peaks.

The composition and concentration of each milking does not stay the same, but appears to go through a cycling. This cycling can be due to the mechanism of which the venom is expelled during each envenomation. The mechanism at which the venom is expelled from the cone snails is still poorly understood. The cycling of peptides also appears to mirror peptide concentration, which can be observed by the AUC.

Research conducted by Modahl et al. (2010), looked to understand the relationship between long-term captivity on venom composition and how it affects antiserum production. They analyzed venom from 15 long-term captive *Naja naja* (Indian Cobra). The cobra venom was analyzed by liquid chromatography and electrophoresis in which it was found that the profiles of each snake differed. Further analysis of the composition showed that the cobras produced the same peptides, but they varied in concentration (Modahl et al., 2010).

Snails receiving the fish food diet (Group 1) showed little to no deviation in venom profiles. Typically, the snails only produced one to two main peaks and a series of

smaller ones during each weekly milking. These two main peaks are suspected to be the isolated Peptides D and E. Group 2 (fish food and bromine), snail 4.1 displayed increased complexity in it venom profiles. However, snails 4.3 and 5.2 did not exhibit the same increased complexity in comparison to baseline profile, as their counterpart. Group 3 (fish food and human vitamin pack) all displayed increased peptide complexity in comparison to pre-diet manipulation milkings. From Group 4 (fish food, human vitamin pack, and bromine), snails 8.1 and 9.2 show increased venom diversity, snail 9.1 did not deviate from typical venom production even under diet manipulation. The stress of captivity alongside the stress of an altered diet could be a possible explanation for decreased venom yield and increase in venom diversity.

We know that venom yield decreases with prolonged captivity. The snails utilized in the study have been in captivity for 182+ weeks. Figures 43, 47, 51, 55, and 59 illustrate the increase in venom complexity comparison to baseline milkings obtained from Jeffrey Milisen (Master's Thesis, 2012; data not shown). This in conjunction with decreased venom volume indicates that peptide concentration increases with captivity.

Neither of the control snails' profiles differed from baseline milkings prior to the start of the diet manipulation. The control venom profiles also appear to experience a similar level of cycling when it comes to peptide production. This suggests that the cycling seen in both the control group and the diet manipulated groups, that it is more a mechanism inherent to the snails, rather than a side-effect of the diet manipulation. When observing individual peak profiles for each animal, some interesting trends can be seen. Some of the snails experience a huge spike in concentration at either week 24 or 37 respectively. Some of the peak profiles for the snails also appear to mirror each other even though the area under the curve for individual peaks differs. In some cases we observe that while the AUC increases for one peak during a particular time frame, another decreases during that time as can be seen with snail 9.2 (individual peak profiles located in Appendix O). This may be a mechanism for metering in response to the stresses of the diet or captivity; further investigation is needed.

4.2.1 Concentration and stability of Peaks A and B

Amino acid analysis and ESI-MS confirmed the identity of Peak A as ω-conotoxin SVIA (αα sequence CRSSGSOCGVTSICCGRCYRGKCT). The same methods were used to confirm the identity of Peak B as α-conotoxin SII (αα sequence GCCCNPACGPNYGCGTSCS). α-Conotoxin SII is an acetylcholine receptor blocker and ω-conotoxin SVIA (a paralytic) targets calcium channels. ω-Conotoxins act upon presynaptic membranes, to which they attach and block voltage-gated calcium channels.

Table 23. Analysis of peak concentration in A and B.

	A (ω-SVIA)	B (α-SII)	Comments
Group 1 (2.2, 3.2, 3.3)	-	-	Fish food pill did not have a positive effect. Minimal production of Peaks A and B.
Group 2 (4.1, 4.3, 5.2)	-	-	Fish food pill did not have a positive effect. Minimal production of Peaks A and B.
Group 3 (6.1, 6.2, 7.2)	+/-	+/-	Fish food and human vitamin pack had a positive effect on production of Peaks A and B. Production of B was most notable in Snail 6.1 and was slightly reversed with 6.2.
Group 4 (8.1, 9.1, 9.2)	+/-	+/-	Fish food and human vitamin pack (and bromine) had a positive and negative effect on production of Peaks A and B. While Peak B increased, Peak A decreased in concentration. Introduction of bromine is inconclusive as to its overall effect. Additional study is needed.

With the amino acid analysis and area under curve values I was able to calculate the concentration of Peaks A and B in each sample. Concentrations of Peaks A and B were calculated from the chromatograms area under the curve values. Amino acid analysis and MS data for Peaks C through F was inconclusive as to the identity of these peaks.

The concentrations of A and B in Groups 1 and 2 are very low in comparison to Groups 3 and 4. The highest concentration for A (Groups 1 and 2) was 79.26 pmol/µL, from snail 5.2 (pg.50). The highest concentration for B (Groups 1 and 2) was 128.18 pmol/µL (snail 4.1, pg. 48). Peak production of all peaks (A-F) is random and sporadic. Production of Peaks A and B is more sporadic with Groups 1 and 2 (see pgs. 41-43), than it is with Groups 3 and 4 (see pgs. 52-54 and 56-58). Peak stability is not achieved on the fish food (Group 1) and fish food and bromine diet (Group 2). The snails from Groups 3 and 4 display more continuity in production of all six isolated peaks. This continuity can also be seen in Peaks A and B (pgs.49-51) of Groups 3 and 4. Production of Peaks A and B in Groups 1 and 2 are very similar to the Controls (pgs.60-62). Therefore, it can be said that the fish food (Group 1) and fish food and bromine (Group 2) diets did not have a positive effect on the synthesis of Peaks A (ω-conotoxin SVIA) and B (α-conotoxin SII).

However, the opposite can be said for Groups 3 (fish food and human vitamin pack) and 4 (fish food, human vitamin pack, and bromine). The first approximate 20 weeks of the study, snails 6.1, 6.2, and 7.2 synthesize very little of the isolated peaks (pgs. 52-54). This could be attributed to as an adjustment phase for the snails, for afterwards the snails start producing increasing amounts of isolated peaks. Synthesis is controlled and lacks the randomness of Groups 1 and 2. A clear pattern of cycling can be seen with snails 6.1 and 6.2 (Group 3). Looking at Figures 30 and 32 (pgs. 52 and 53), all peaks will increase to various concentrations, but at the following milking, concentrations decrease and this pattern repeats. This same type of trend can also be seen with snails 8.1 and 9.1 (Group 4). The key difference between Groups 1 and 2, and Groups 3 and 4 is the addition of the human vitamin pack. Analysis of concentration of Peaks A and B (of groups 3 and 4) confirm the trend of cycling. Interestingly, very seldom does the concentration of B drop below A. When Peak B is being produced, the concentration of A drops or A is not produced at all. The highest concentration for Peak B was 403.69 pmol/µL (Snail 6.2) and the lowest concentration of B was 3.7 pmol/µL. The highest for A amongst Group 3 was 271.34 pmol/µL (Snail 6.2) and the lowest concentration calculated for A was 0.78 pmol/µL (Snail 7.2).

From the data for Group 3 (pgs. 52-54) I can conclusively say that the fish food and human vitamin pack had both a positive and negative effect on the production of

Peaks A and B. This may be the result of a synergistic role between the two conotoxins, ω-conotoxin SVIA and α-conotoxin SII. Groups 3 and 4 display a greater level of stability and consistent peak production of Peaks A and B, as well as additional isolated peaks. An interesting trend seen in Group 4 with snails 9.1 and 9.2 is that approximately around week 36, production of Peak B drops. Almost in response to the drop in production of Peak B, Peak A concentration increases. Peak B is predominantly produced for about 22 weeks in snails 9.1 and 9.2. Production drops for several weeks, then approximately around week 36 Peak A appears and production of Peak B is suppressed.

There is peak mimicry present with A and B (and also observed with other isolated peaks). Snails 8.1, 9.1, and 9.2 (Group 4 – fish food, human vitamin pack, and bromine) demonstrate this mimicry (pgs. 56-58). The same type of peak mimicry can be observed with snails 6.1, 6.2, and 7.2 (Group 3 – fish food and human vitamin pack).

The addition of the human vitamin pack (Centrum®) has acted as a stabilizing agent and has encouraged more consistent production of peaks, as well as an increase in concentration. The addition of bromine to the pills in Group 4 did not have effect on the snails overall health or peak profiles. Even though a direct effect was not seen, I do believe it deserves further study.

4.3 Venom Volume

Results of the diet manipulation study indicate a significant reduction in venom volume. This may be a result of the altered diet. However, control snails (2.1, 4.2, 7.1, 8.2), that received a diet of fish, also showed a significant drop in venom volume. This alteration in venom volume could therefore be a result of time in captivity and not due to the diet manipulation itself.

Tare and colleagues (1985) investigated venom yield in *Naja naja*, as well as *Vipera russelli* and *Bungarus caeruleus*, comparing snakes from the open field to those in captivity, as well as the effects of various milking methods. They found that snakes maintained in open farm yielded more venom than those maintained in captivity (Tare et al., 1985). The reduction in venom yield could be a matter of insufficient nutrition, even with the combined nutrients of the pill, as well as poor husbandry techniques. In

captivity, the snails are fed and milked once a week. If the snails feed more often in the wild, then the decrease in venom volume could be a matter of nutrition.

Milisen (2012) did a brief pill experiment with three *Conus striatus*, snails 1.1, 1.2, and 1.3 (data not shown). The snails were fed the same Silvercup™ fish feed inside a gelatin capsule for 12 consecutive feedings/milkings. The snails were brought back to their regular diet of fish after the 12 milking. Milisen (2012) found an increase in venom composition, but an overall decrease in venom yield. Milisen (2012) sites that this could be an adaptive issue (the snails adjusting to captive environment) or just natural anomaly. Comparisons of venom volume to past milkings and those obtained during the study, I cannot definitively say that the decrease in venom volume is solely due to the diet change.

4.3.1 Venom Volume and Sex

As mentioned previously, there was a significant reduction in milked venom volume during the diet manipulation to pre-diet manipulation volumes. Statistical comparison of sexed snails milked venom volumes during the study shows no indication of difference between males and females. This is different in comparison other venomous animals, such as scorpions. De Sousa and colleagues (2010) compared venom volumes of male and female *Tityus nororientalis*. Female scorpions yielded 0.98 mg/individual, which is significantly lower than males scorpions which yielded 2.39 mg/individual (De Sousa et al., 2010). It is important to note that the scorpions used in the study were wild and immediately used in the course of the study.

Similar results can be seen with spiders. Typically, female spiders yield more venom than their male counterparts of the same species (Herzig, 2010). Electrostimulation was utilized to acquire venom from *Vitalius dubius*, found in southeastern Brazil. Male spiders yielded significantly less venom (12.5±0.7 mg of liquid/spider) than their female counterparts (25.5±2.0 mg of liquid/spider) (Rocha-e-Silva et al., 2009). Herzig also comments that size plays an important factor in venom yield.

Snail 2.2 (male) of Group 1 (fish food) had an average venom volume of 23.19 uL ± 2.51. Snail 3.3 (female) also of Group 1 had an average venom volume of 18.68 uL ± 2.52. Male and female comparison of snails from Group 3 (fish food and human vitamin pack) shows snail 6.1 (female) mean venom volume 29.8 uL ± 3.63 and snail 6.2 (male) had an average venom volume 22.85 uL ± 2.67, two tailed p-value = 0.13. The lack of variation in milked venom volumes between male and female *C. striatus* could be due to captivity, lack of individual housing, or more likely due to a very small population to sample from.

4.4 Pill Diet

One of the primary goals of this project was to see if the diet manipulation could enhance the cone snails' biochemical arsenal and increase the amount of post-translational modifications. For this reason we decided to use bromine and a human vitamin pack paired with fish food in hopes of accomplishing these goals.

The decision to use the halide bromine comes from previous discovery of brominated tryptophan in the conopeptide bromocontryphan (Jakubowski et al.,2006), as well as other findings such the bromosleeper (Jimenez et al., 2004) in *C. radiatus*. Bromine is already naturally present in ocean water in its ion form. By increasing the snails intake of bromine it was hoped that it might increase production of bromocontryphan and/or cause the synthesis of new brominated peptides.

The metabolism of cone snails is not well understood and deserves further investigation. It has been investigated amongst snakes and scorpions that they regulate venom expenditure because of the high metabolic cost of venom synthesis (McCue et al., 2006; Nisani et al., 2011). It is not known if cone snails meter their venom in a similar manner.

Group 4 (snails 8.1, 9.1 and 9.2) diet was fish food, human vitamin pack, and bromine. For safety of the snails, bromine was omitted from their pill until I could determine the dosage of bromine was safe. Group 2 (snails 4.1, 4.3, and 5.2) were on the fish food and bromine diet (0.1 mg dosage of bromine per pill) and were the trial group. After determining that the dosage of bromine was not overtly harmful to the snails health,

bromine was incorporated into Group 4's diet at week 39. As stated previously, I can conclusively say based up on the data, that bromine did not have a positive effect.

4.5 Feeding Behavior

The snails were fed the pill diet on an alternating schedule for 60 weeks. This was done to maintain their overall health. The snails would be fed the pill diet one week, the following they would be fed fish. This schedule continued throughout the length of the study.

The feeding behavior of the snails did not drastically change over the course of the diet manipulation study. Most of the snails readily consumed the pill as they would a fish. On occasion some of the snails would try to refuse intake or regurgitate the pill. If this occurred, the snail was prevented from expulsing the pill by gentle pressure with a pair of tweezers. When faced with refusal of the pill, a fish was used to entice the snail to extend and open its rostrum, at which time the pill was inserted in place of the fish.

4.6 Deaths

Two deaths occurred during the study. Snails 6.1 and 6.2 died. However, this was due to accidental closure of aerating valves to the cage and is not due to the diet they were receiving.

4.7 Pregnancy

Reproduction typically occurs between the months of May and August (as observed in lab). A gravid cone snail will lay anywhere between 3 and 78 egg sacs on a hard surface. The egg sacs can contain up to 11,000 eggs (Milisen, 2012).

At the start of the experiment in April, 2012, it was at the cusp of their mating season. There was nothing remarkable to note about this particular breeding season. However, some oddities occurred several months after the normal breeding months. In

November 2012, female snail 3.3 (Group 1) laid eggs. During the breeding season of 2013, no eggs were laid by any of the identified females. This disruption suggests that in some manner, the diet manipulation was having a negative effect on their reproduction. This may be why we see a drop in some peak production and a gain in others, as a self-preservation/armament response to the stress of the diet.

4.8 General Comments and Future Directions

Effects of captivity are an important factor that must be taken into consideration when looking at the entirety of the experiment. The snails involved in the diet manipulation study, including controls, have been in captivity for +182 weeks. The snails chosen to undergo diet manipulation, was based upon the recommendation of Milisen (2012) to utilize snails whose venom yield was consistent. Venom yield across all snails decreased by 31% over the course of the 60-week study. The decrease in venom yield is less likely to be in direct response to the diet manipulation, but more as a result of time in captivity.

I can conclusively say that the diet manipulation (for certain groups) was a success in increasing peak diversity, concentration, and stability. The addition of the human vitamin pack or Centrum® is an integral component to increasing venom composition and obtaining stability. Bromine has proven to be ineffectual at increasing peak concentration and diversity. However, this could be a dosage issue. Perhaps increasing the dosage or changing the type of the halide, would elicit more of a response.

Another avenue would be to see how the profiles would change in response to diet of only the human vitamin pack or increasing the dosage of a singular vitamin, such as vitamin K or vitamin C. Vitamin K is involved in gamma-carboxylation, which is a prominent post-translational modification among *Conus*. Vitamin K is a co-factor for the enzyme gamma-glutamyl carboxylase. In humans, the enzyme activates blood clotting factors through carboxylation of the N-terminal glutamic acid residues (Cornell University). By increasing the amount of vitamin K present in the snails diet, we may increase the frequency of post-translational γ-carboxylation.

Vitamin C (ascorbate) is a co-factor for prolyl hydroxylase. The enzyme responsible for catalyzing the formation of hydroxyproline. The post-translational modification of procollagen is essential for the development of mature collagen molecules. When cells are lacking ascorbate, the procollagen chains are not sufficiently hydroxylated to for stable triple helices (Lodish et al., 2000). Lacking the structural support of collagen, skin, blood vessels, and tendons become fragile.

Blocking or inhibiting co-factors is also another possibility. Cone snails are the only nonvertebrate animal for which γ-carboxyglutamate-containing proteins have been physiologically and biochemically characterized (Bandyopadhyay et al., 2002). Warfarin (Coumadin), which in humans, interferes with how the body uses vitamin K. Inhibiting vitamin K usage in the cone snails may stimulate a reaction in venom peptide synthesis.

An improvement to this experiment would be to completely isolate each experimental and control groups in their own environmental system. This would eliminate the possible effects of snail byproducts affecting the entire system and influencing venom yields, and composition.

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Appendix A : Components of human vitamin pack (Centrum®)

	Approximated amount used in pill
Vitamin A	518 IU
Vitamin C	8.88 mg
Vitamin D	59.2 IU
Vitamin E	4.44 IU
Vitamin K	3.7 mcg
Thiamin	0.222 mg
Riboflavin	0.2516 mg
Niacin	2.96 mg
Vitamin B6	0.296 mg
Folic Acid	59.2 mcg
Vitamin B12	0.888 mcg
Biotin	4.44 mcg
Pantothenic Acid	1.48 mg
Calcium	29.6 mg
Iron	2.664 mg
Phosphorous	2.96 mg
Iodine	22.2 mcg
Magnesium	7.4 mg
Zinc	1.628 mg
Selenium	8.14 mcg
Copper	0.074 mg
Manganese	0.3404 mg
Chromium	5.18 mcg
Molybdenum	6.66 mcg
Chloride	10.656 mg
Potassium	11.84 mg
Boron	11.1 mcg
Nickel	0.74 mcg
Silicon	0.296 mg
Tin	1.48 mcg
Vanadium	1.48 mcg

Appendix B : Components of Silvercup Fish Feed – Extruded Floating

Manufactured by Nelson & Sons Inc., Murray, Utah
(proprietary portions unknown)

Fish meal
Soybean meal
Wheat flour
Stabilized fish oil
Wheat middlings
Poultry by-product meal
Blood meal
Hydrolyzed feather meal
Corn gluten meal
Poultry oil
Vitamin A acetate
D-Acytylated animal-sterol
Vitamin B12 supplement
Riboflavin supplement
Niacin
Folic acid
Menadione sodium bisulphite complex
Calcium pantothenate
Pyridoxine hydrochloride
Thiamine
Biotin
Vitamin E
Vitamin C
Betaine
Zinc sulfate
Copper sulfate
Ferrous sulfate
Manganese sulfate
Ethylenediamine dihydroiodide
Ethoxyquin

Appendix C : Amino Acid Analysis of Peptides C through F

Table 24. Amino Acid Analysis of Peak C

Peak C				
Sequence Unknown				
αα		Amount (pmoles)	Calculated Residues	Actual Residues
Asp		1398.995	-	-
Glu		171.891	-	-
Ser		1239.425	-	-
Gly		2122.402	-	-
His		26.433	-	-
Thr		374.213	-	-
Ala		934.159	-	-
Pro		1984.487	-	-
Tyr		920.975	-	-
Val		83.886	-	-
Cys		718.117	-	-
Ile		573.849	-	-
Phe		66.411	-	-
Lys		498.935	-	-

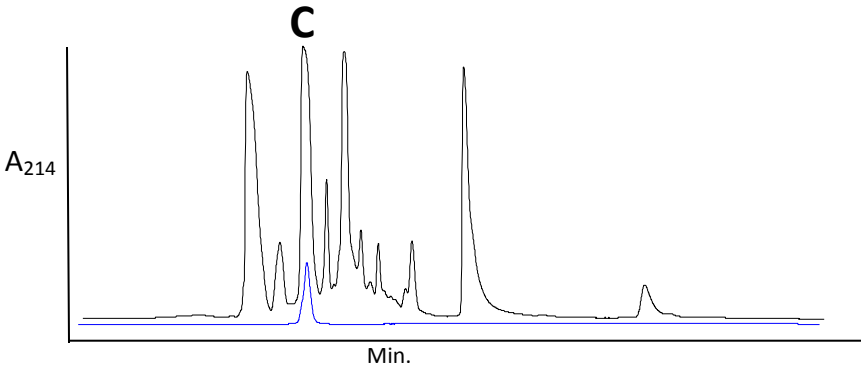

Figure 49.1. RP-HPLC of pooled crude venom and isolated peak C (blue).

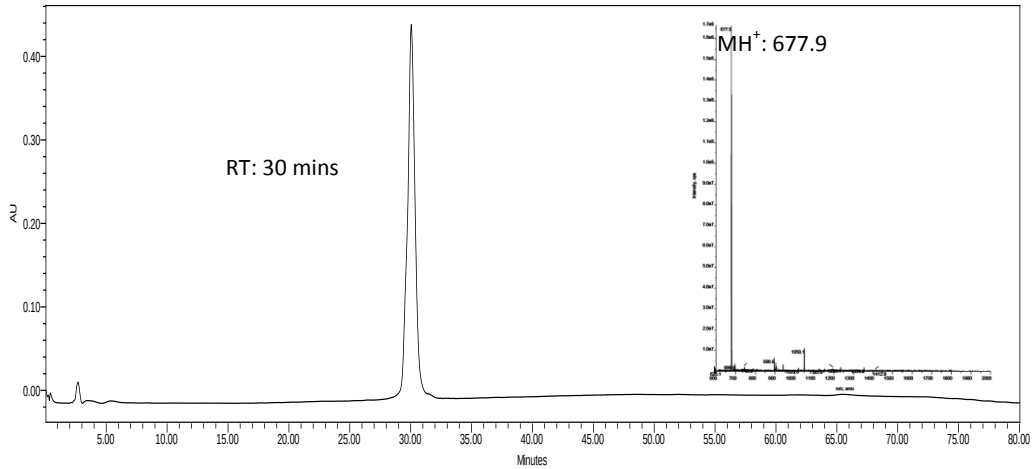

Figure 49.2. RP-HPLC and MS chromatograms of isolated peak C. Retention Time (RT) for peak C is 30 minutes.

Amino Acid Analysis of Peak C was not confirmed. Amount (pmoles) of each residue was determined via amino acid analysis. RP-HPLC of pooled crude *C. striatus* venom and isolated peak C (blue). RP-HPLC and MS chromatograms illustrating the retention time (30 minutes) and *m/z* ratio of peak C (obs. m/z 677.9).

Table 25. Amino Acid Analysis of Peak D

Peptide D				
Sequence Unknown				
αα		Amount (pmoles)	Calculated Residues	Actual Residues
Asp		443.331	-	-
Glu		317.776	-	-
Ser		614.373	-	-
Gly		501.564	-	-
His		26.048	-	-
Arg		203.302	-	-
Thr		674.885	-	-
Ala		93.585	-	-
Pro		66.801	-	-
Tyr		167.055	-	-
Val		365.585	-	-
Met		167.452	-	-
Cys		115.228	-	-
Ile		175.623	-	-
Leu		185.122	-	-
Phe		35.079	-	-
Lys		181.673	-	-

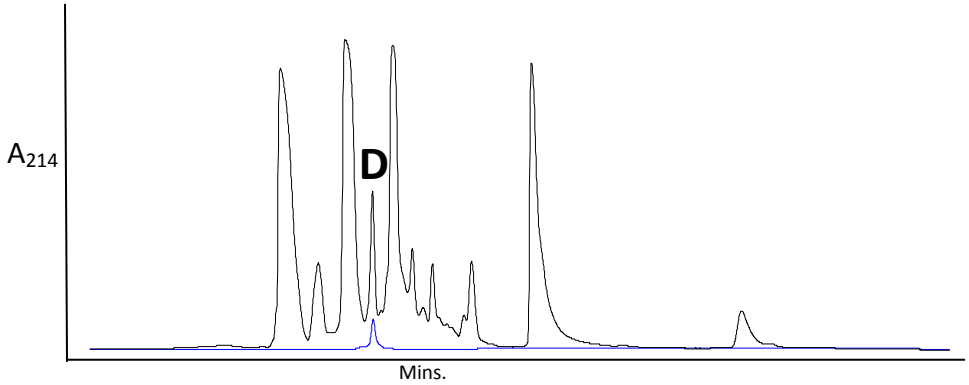

Figure 50.1 RP-HPLC of pooled crude venom and isolated peak D (blue).

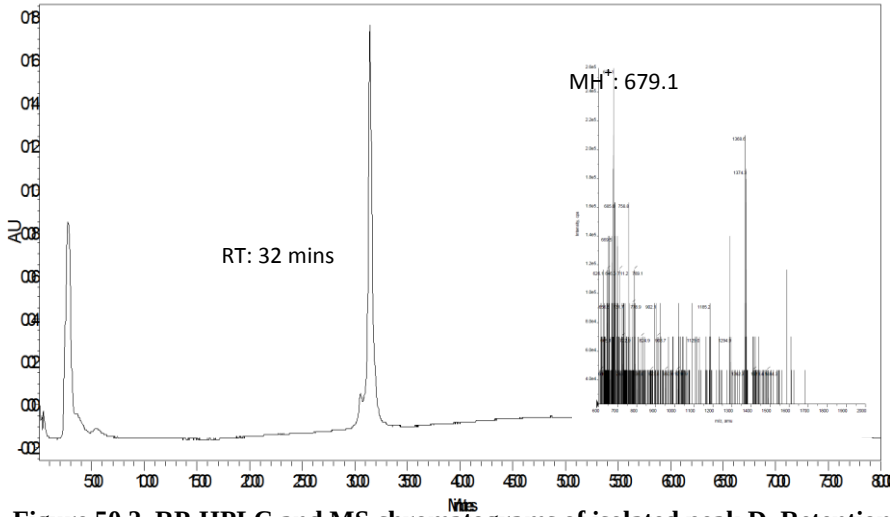

Figure 50.2. RP-HPLC and MS chromatograms of isolated peak D. Retention Time (RT) for peak D is 32 minutes.

Amino Acid Analysis of Peak D was not confirmed. Amount (pmoles) of each residue was determined via amino acid analysis. RP-HPLC of pooled crude *C. striatus* venom and isolated peak D (blue). RP-HPLC and MS chromatograms illustrating the retention time (32 minutes) and *m/z* ratio of peak D (obs. *m/z* 679.1).

Table 26. Amino Acid Analysis of Peak E

Peak E				
Sequence Unknown				
αα		Amount (pmoles)	Calculated Residues	Actual Residues
Asp		977.619	-	-
Glu		641.392	-	-
Ser		1200.806	-	-
Gly		1110.562	-	-
His		123.101	-	-
Arg		424.756	-	-
Thr		1210.393	-	-
Ala		245.163	-	-
Pro		592.527	-	-
Tyr		414.9773	-	-
Val		662.689	-	-
Met		345.554	-	-
Cys		167.291	-	-
Ile		434.62	-	-
Leu		523.157	-	-
Phe		287.509	-	-
Lys		365.749	-	-

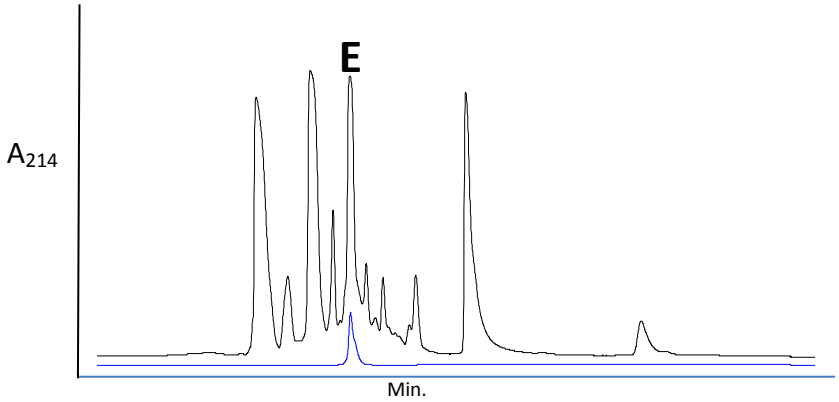

Figure 51.1. RP-HPLC of pooled crude venom and isolated peak E (blue).

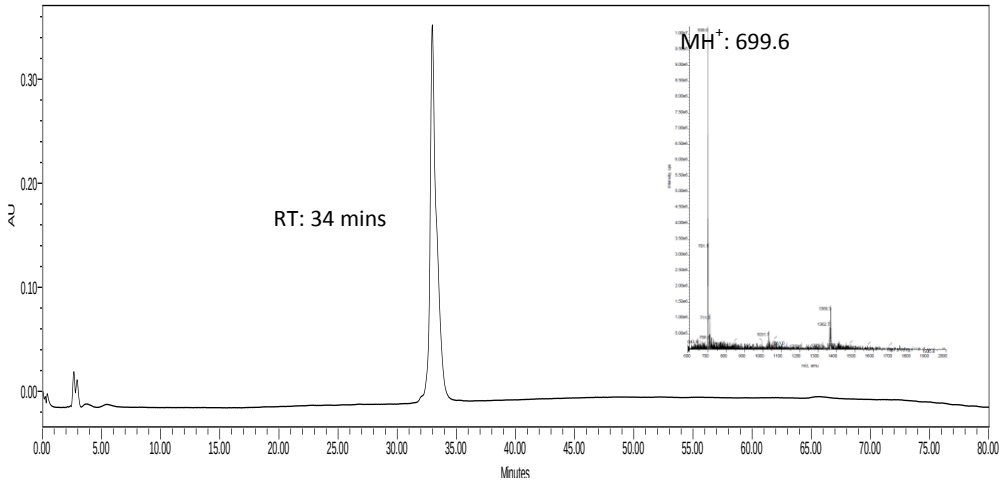

Figure 51.2. RP-HPLC and MS chromatograms of isolated peak E. Retention Time (RT) for peak E is 34 minutes.

Amino Acid Analysis of Peak E was not confirmed. Amount (pmoles) of each residue was determined via amino acid analysis. RP-HPLC of pooled crude *C. striatus* venom and isolated peak E (blue). RP-HPLC and MS chromatograms illustrating the retention time (34 minutes) and *m/z* ratio of peak E (obs. m/z 699.6).

Table 27. Amino Acid Analysis of Peak F

Peak F				
Sequence Unknown				
αα		Amount (pmoles)	Calculated Residues	Actual Residues
Asp		346.879	-	-
Glu		464.783	-	-
Ser		328.959	-	-
Gly		373.801	-	-
His		45.192	-	-
Arg		211.251	-	-
Thr		158.103	-	-
Ala		209.868	-	-
Pro		139.938	-	-
Tyr		152.279	-	-
Val		302.836	-	-
Met		115.916	-	-
Ile		133.151	-	-
Leu		95.874	-	-
Phe		156.941	-	-
Lys		130.147	-	-

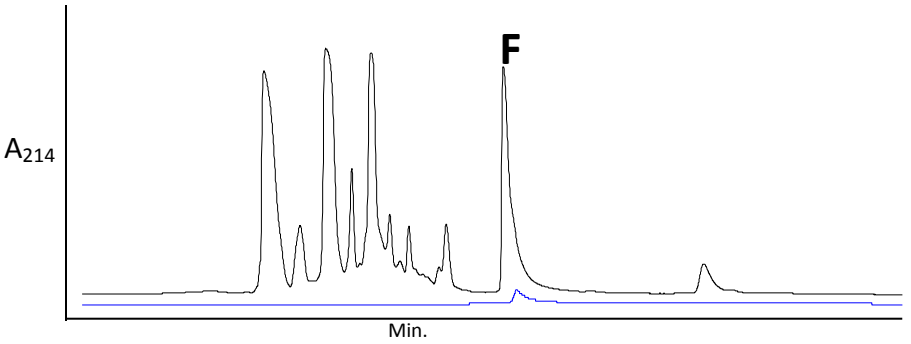

Figure 52.1. RP-HPLC of pooled crude venom and isolated peak F (blue).

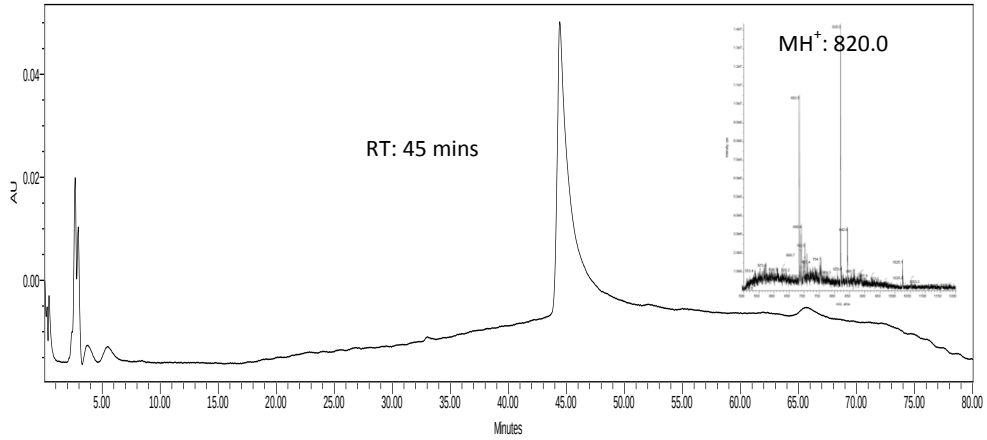

Figure 52.2. RP-HPLC and MS chromatograms of isolated peak F. Retention Time (RT) for peak F is 45 minutes.

Amino Acid Analysis of Peak F was not confirmed. Amount (pmoles) of each residue was determined via amino acid analysis. RP-HPLC of pooled crude *C. striatus* venom and isolated peak F (blue). RP-HPLC and MS chromatograms illustrating the retention time (45 minutes) and *m/z* ratio of peak F (obs. m/z 820.0).

Appendix D : Snail 2.2 – Predominant Isolated Peaks

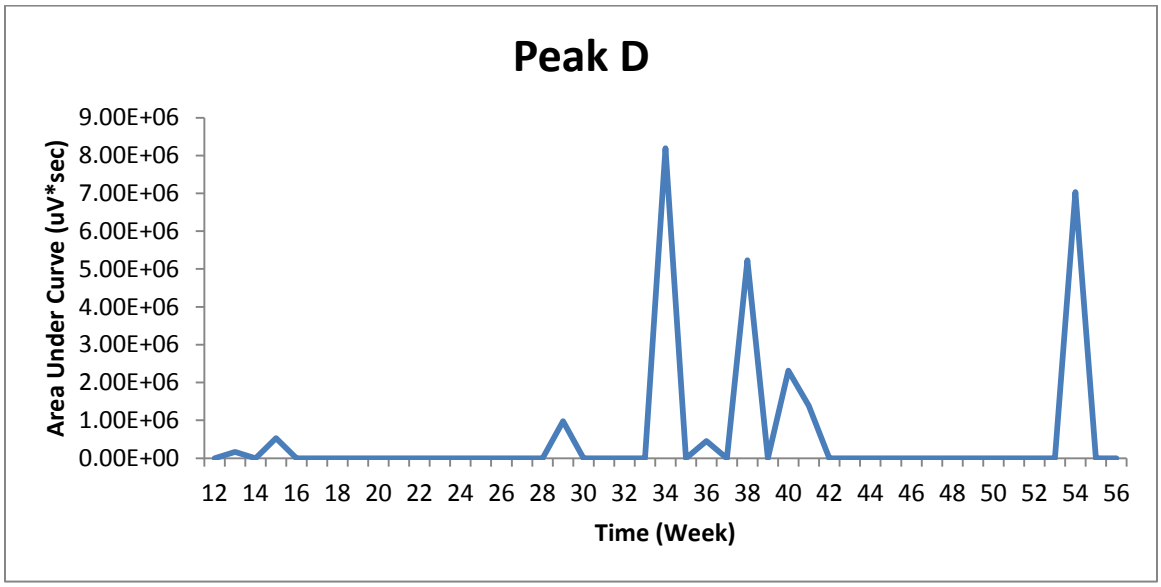

Figure 53. Illustrating area under curve for isolated Peak D.

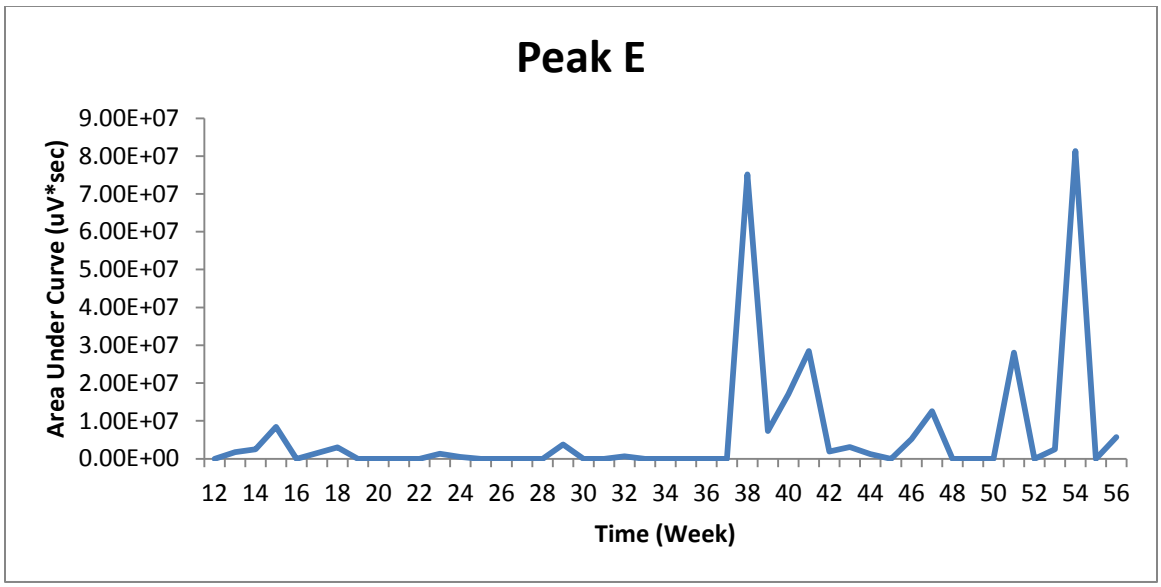

Figure 54. Illustrating area under curve for isolated Peak E.

Snail 2.2 regularly produced Peaks D and E. Around weeks 34 and 37 respectively, we see a spike in peak AUC.

Table 28. Snail 2.2 (Fish Food) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
12	0	0	0	0	0	0	Pill
13	0	0	0	163437	1751513	0	Fish
14	0	0	0	0	2456058	0	Pill
15	0	0	298083	529046	8424993	0	Fish
16	0	0	0	0	0	0	Pill
17	0	0	0	0	1479157	0	Fish
18	0	0	0	0	3046971	0	Pill
19	0	0	0	0	0	0	Fish
20	0	0	0	0	0	0	Pill
21	0	0	0	0	0	0	Fish
22	0	0	0	0	0	0	Pill
23	0	0	0	0	1318180	0	Fish
24	0	0	0	0	445424	0	Pill
25	0	0	0	0	0	0	Fish
26	0	0	0	0	0	0	Pill
27	0	0	0	0	0	0	Fish
28	0	0	0	0	0	0	Pill
29	0	0	0	979548	3776544	0	Fish
30	0	0	0	0	0	0	Pill
31	0	0	690791	0	0	0	Fish
32	0	0	0	0	600220	0	Pill
33	0	0	0	0	0	0	Fish
34	0	0	0	8194393	0	0	Fish
35	0	0	0	0	0	0	Pill
36	0	0	0	451803	0	0	Fish
37	0	0	0	0	0	0	Pill
38	0	0	0	5235141	75160530	0	Fish
39	0	0	0	0	7312068	0	Pill
40	0	0	0	2315624	17049440	0	Fish
41	0	0	0	1383629	28451848	0	Pill
42	0	0	0	0	1933579	0	Fish
43	0	0	0	0	3109400	0	Pill
44	0	0	0	0	1262525	0	Fish
45	0	0	0	0	0	0	Pill
46	0	0	0	0	5168048	0	Fish
47	0	0	0	0	12539143	0	Pill
48	0	0	0	0	0	0	Fish
49	0	0	0	0	0	0	Pill
50	0	0	0	0	0	0	Fish

Table 29. Snail 2.2 (Fish Food) Area Under Curve Data and schedule of diet

51	0	0	0	0	28044863	0	Pill
52	0	0	0	0	0	0	Fish
53	4064081	891173	4715112	0	2516631	0	Pill
54	0	0	0	7038098	81379262	0	Fish
55	0	0	0	0	0	0	Pill
56	0	0	0	0	5751307	0	Fish

Appendix E : Snail 3.2 – Predominant Isolated Peaks

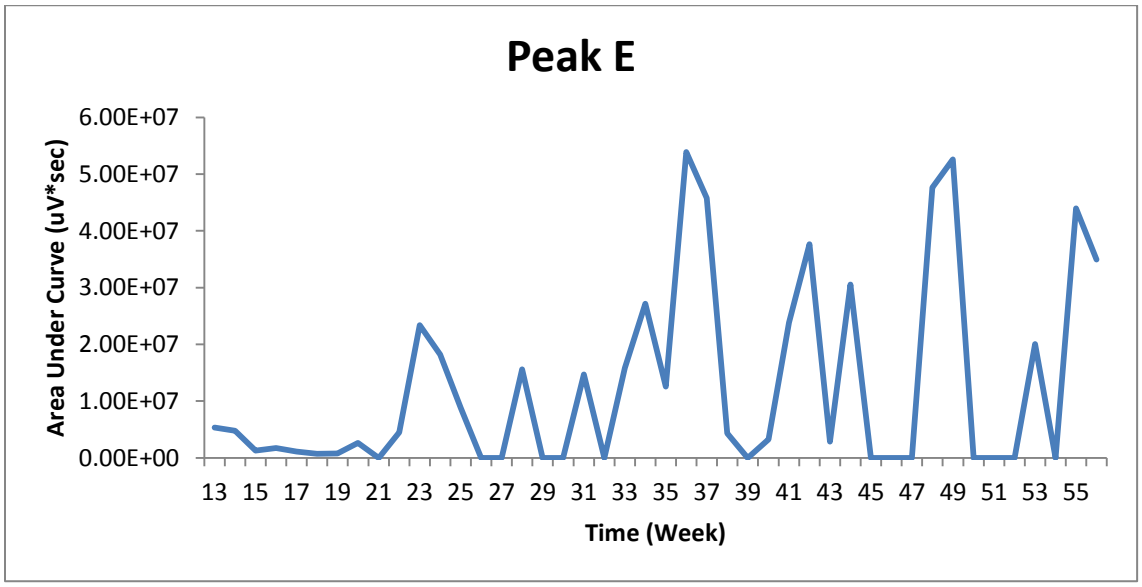

Figure 55. Illustrating area under curve for isolated Peak E.

Snail 3.2 regularly produced Peak E. We see a spike in peak AUC approximately around week 36.

Table 30. Snail 3.2 (Fish Food) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
13	0	0	0	0	5344751	0	Fish
14	0	0	0	0	4768254	0	Pill
15	0	0	0	0	1247233	0	Fish
16	0	0	0	0	1719281	0	Pill
17	0	0	0	0	1085081	0	Fish
18	0	0	0	0	708553	0	Pill

Table 31. Snail 3.2 (Fish Food) Area Under Curve Data and schedule of diet

19	0	0	0	0	747024	0	Fish
20	0	0	0	0	2632893	0	Pill
21	0	0	0	0	0	0	Fish
22	0	0	0	0	4488201	0	Pill
23	0	0	0	0	23376843	0	Fish
24	0	11028045	38940561	38931641	18195301	12847496	Pill
25	0	0	0	0	8832328	0	Fish
26	0	0	0	0	0	0	Pill
27	0	0	0	0	0	0	Fish
28	38528065	28294276	1.05E+08	34610851	15575587	44483469	Pill
29	0	0	0	0	0	0	Fish
30	0	0	0	0	0	0	Pill
31	12877433	7819182	45460659		14666991	14335979	Fish
32	0	0	0	0	0	0	Pill
33	0	15934261	38656755	27583938	15808808	17422481	Fish
34	0	0	0	0	27174851	0	Fish
35	0	0	0	0	12558650	0	Pill
36	0	0	0	2348940	53886328	0	Fish
37	0	0	0	1963581	45767004	0	Pill
38	0	0	0	43903244	4315312	0	Fish
39	0	0	0	0	0	0	Pill
40	0	0	0	0	3261561	0	Fish
41	0	0	0	0	23848415	0	Pill
42	0	0	0	0	37639427	0	Fish
43	0	0	0	0	2852499	0	Pill
44	0	0	0	0	30565421	0	Fish
45	0	0	0	0	0	0	Pill
46	0	0	0	0	0	0	Fish
47	0	0	0	0	0	0	Pill
48	0	0	0	0	47648897	0	Fish
49	0	0	19924753	0	52618564	4682517	Pill
50	0	0	0	0	0	0	Fish
51	0	0	0	0	0	0	Pill
52	0	0	0	0	0	0	Fish
53	0	0	0	0	20049150	0	Pill
54	0	0	0	0	0	0	Fish
55	0	0	0	0	43974816	0	Pill
56	0	0	0	0	34937191	0	Fish

Appendix F : Snail 3.3 – Predominant Isolated Peaks

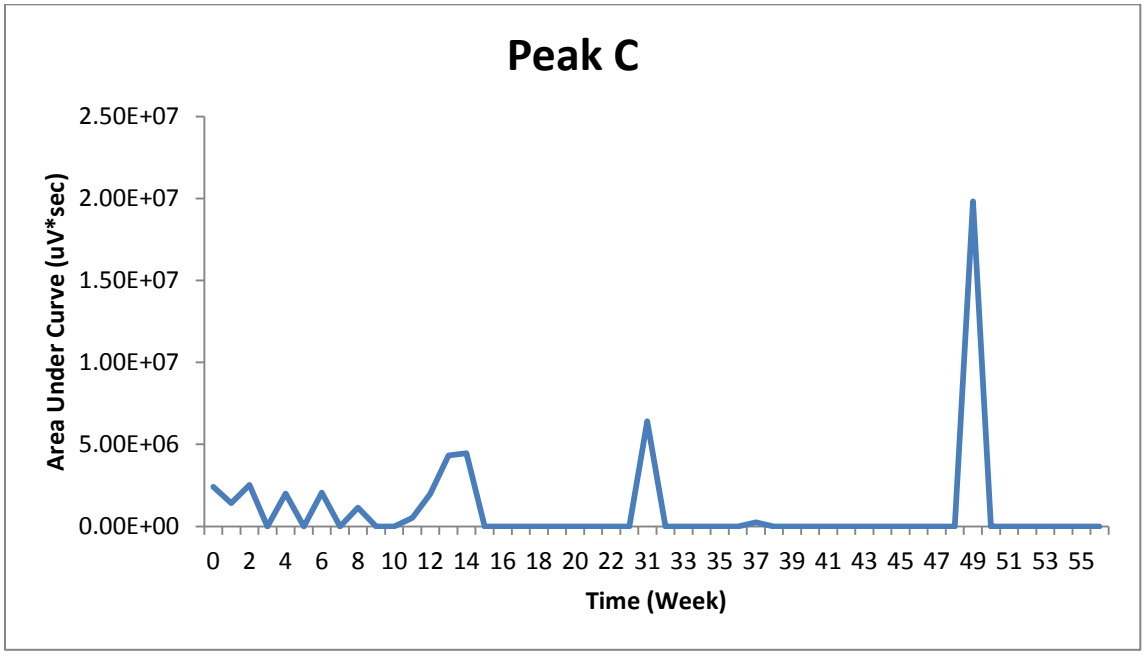

Figure 56. Illustrating area under curve for isolated Peak C.

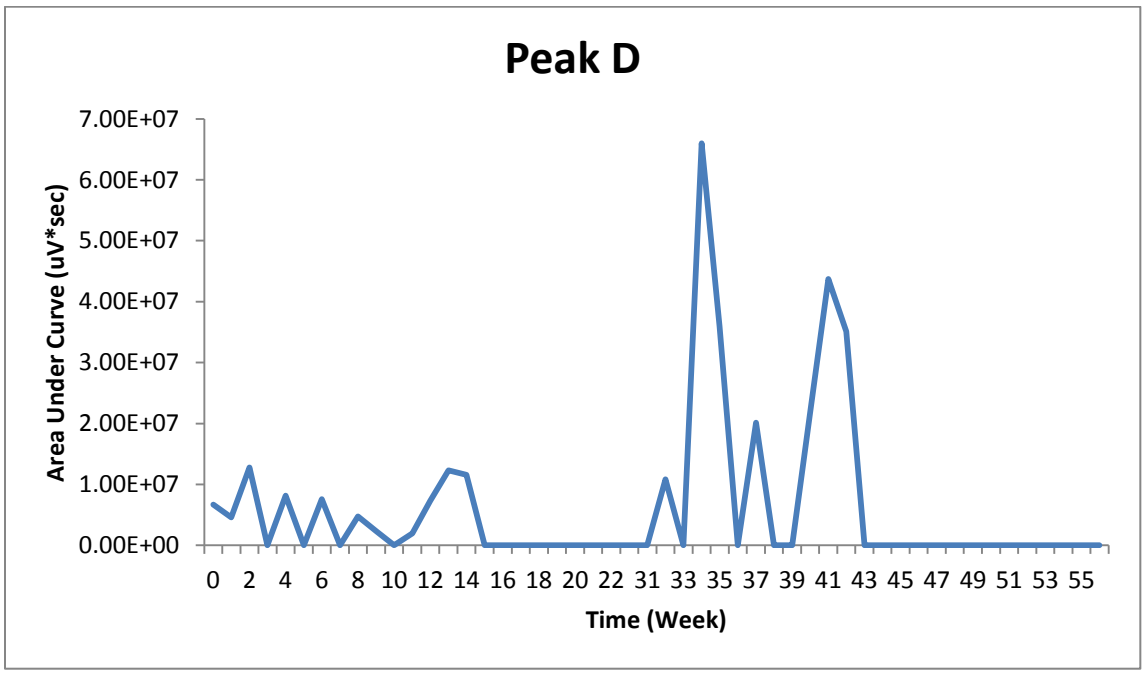

Figure 57. Illustrating area under curve for isolated Peak D.

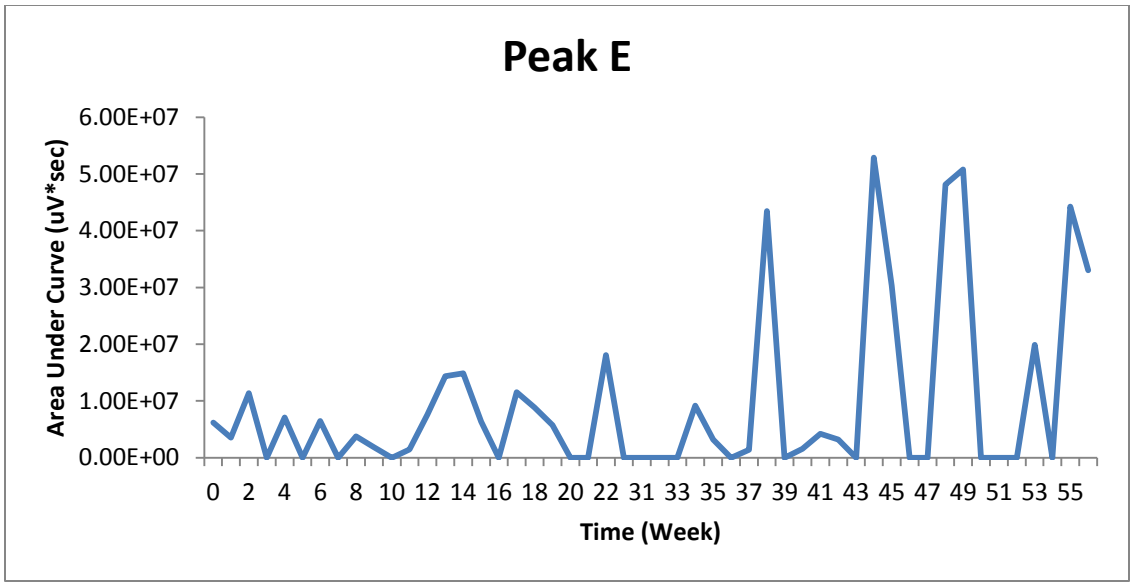

Figure 58. Illustrating area under curve for isolated Peak E.

Snail 3.3 predominantly produced Peaks C, D, and E. However, it is only Peak E that was the most consistently produced. For both Peaks D and E we see an increase in AUC around week 37.

Table 32. Snail 3.3 (Fish Food) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	0	2420718	6683311	6160109	0	Pill
1	0	0	1416493	4569594	3524669	0	Fish
2	0	0	2536813	12766440	11391196	0	Pill
3	0	0	0	0	0	0	Fish
4	0	0	1996923	8133162	7101575	0	Pill
5	0	0	0	0	0	0	Fish
6	0	0	2061553	7553570	6486380	0	Pill
7	0	0	0	0	0	0	Fish
8	0	0	1146928	4736933	3762439	0	Pill
9	0	0	0	2302328	1814907	0	Fish
10	0	0	0	0	0	0	Pill
11	0	0	525281	1969596	1462060	0	Fish
12	0	0	1986414	7293481	7578203	0	Pill
13	0	0	4315977	12321475	14341851	0	Fish
14	0	0	4452360	11550876	14856331	602640	Pill
15	0	0	0	0	6353893	0	Fish
16	0	0	0	0	0	0	Pill
17	0	0	0	0	11517593	0	Fish
18	0	0	0	0	8815857	0	Pill

Table 33. Snail 3.3 (Fish Food) Area Under Curve Data and schedule of diet

19	0	0	0	0	5697857	0	Fish
20	0	0	0	0	0	0	Pill
21	0	0	0	0	0	0	Fish
22	0	0	0	0	18093100	0	Pill
30	0	0	0	0	0	0	Pill
31	2264813	0	6415510	0	0	0	Fish
32	0	0	0	10853655	0	0	Pill
33	0	0	0	0	0	0	Fish
34	6770734	0	0	66003572	9177346	0	Fish
35	2166238	0	0	35966293	3198239	0	Pill
36	0	0	0	0	0	0	Fish
37	0	0	244463	20107688	1409094	0	Pill
38	0	0	0	0	43478672	0	Fish
39	0	0	0	0	0	0	Pill
40	0	0	0	21739386	1548976	0	Fish
41	0	0	0	43734073	4224220	0	Pill
42	0	0	0	35104443	3163553	0	Fish
43	0	0	0	0	0	0	Pill
44	0	0	0	0	52850968	0	Fish
45	0	0	0	0	30321148	0	Pill
46	0	0	0	0	0	0	Fish
47	0	0	0	0	0	0	Pill
48	0	0	0	0	48122244	0	Fish
49	0	0	19813123	0	50806139	0	Pill
50	0	0	0	0	0	0	Fish
51	0	0	0	0	0	0	Pill
52	0	0	0	0	0	0	Fish
53	0	0	0	0	19895626	0	Pill
54	0	0	0	0	0	0	Fish
55	0	0	0	0	44221355	0	Pill
56	0	0	0	0	33026614	0	Fish

Appendix G : Snail 4.1 – Predominant Isolated Peaks

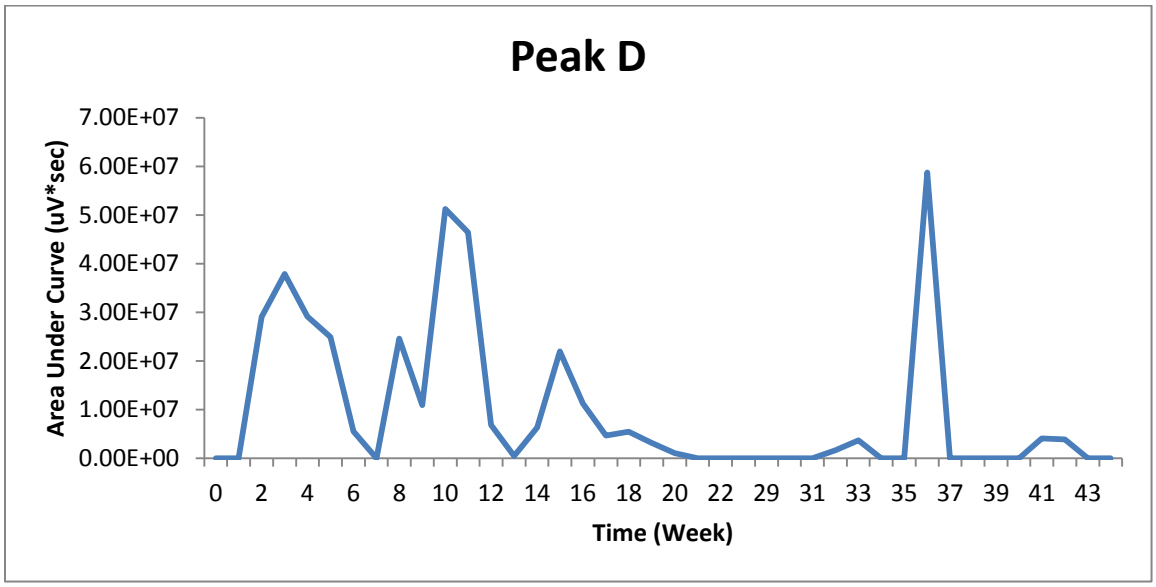

Figure 59. Illustrating area under curve for isolated Peak D.

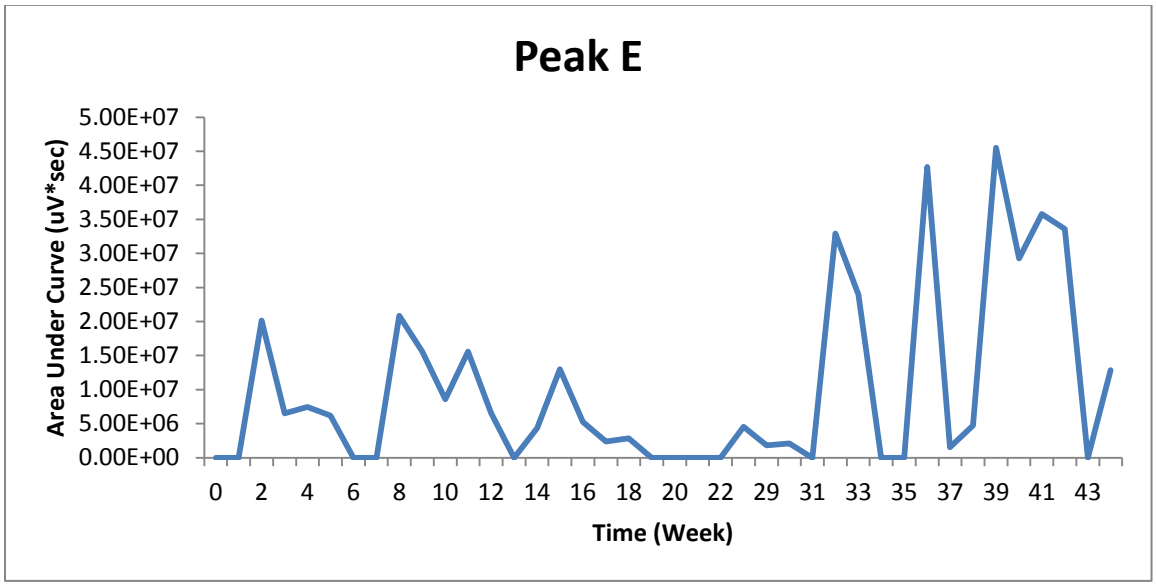

Figure 60. Illustrating area under curve for isolated Peak E.

Snail 4.1 produced Peaks D and E with regularity. Interestingly, even though AUCs differ, at approximately 36 weeks we see the same sharp spike in peak AUCs.

Table 34. Snail 4.1 (Fish Food + Bromine) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	0	0	0	0	0	Pill
1	0	0	0	0	0	0	Fish
2	11929107	0	0	29048780	20141838	0	Pill
3	0	0	1157588	37868401	6511375	0	Fish
4	0	0	0	29133489	7457310	0	Pill
5	15369090	0	0	24909852	6198359	0	Fish
6	0	0	0	5490729	0	0	Pill
7	0	0	0	0	0	0	Fish
8	3028435	0	4100845	24579769	20866346	0	Pill
9	4739256	0	0	10878099	15581765	0	Fish
10	2363321	0	2014125	51234989	8589106	0	Pill
11	7015739	0	3664065	46450070	15557808	5804529	Fish
12	0	0	1243094	6804053	6569076	0	Pill
13	0	0	0	406217	0	0	Fish
14	0	0	0	6280768	4348536	0	Pill
15	0	0	0	21917829	13007791	0	Fish
16	0	0	0	11248863	5260309	0	Pill
17	0	0	0	4629990	2376903	0	Fish
18	0	0	0	5442715	2826486	0	Pill
19	0	0	0	3146439	0	0	Fish
20	0	0	0	1051631	0	0	Pill
21	0	0	0	0	0	0	Fish
22	0	0	0	0	0	0	Pill
23	0	0	0	0	4556606	0	Fish
29	0	657433	0	0	1820633	0	Fish
30	0	0	0	0	2094063	0	Pill
31	0	39762699	0	0	0	0	Fish
32	0	0	0	1639488	32901491	0	Pill
33	0	0	0	3654566	23942486	0	Fish
34	0	0	0	0	0	0	Fish
35	0	0	0	0	0	0	Pill
36	0	17520325	0	58721100	42698470	0	Fish
37	0	1756151	0	0	1525266	0	Pill
38	0	0	0	0	4720852	0	Fish
39	0	0	0	0	45522733	0	Pill
40	0	0	0	0	29278524	0	Fish
41	0	0	0	4058467	35788518	0	Pill

Table 35. Snail 4.1 (Fish Food + Bromine) Area Under Curve Data and schedule of diet

42	0	0	0	3883774	33604114	0	Fish
43	0	0	0	0	0	0	Pill
44	0	0	0	0	12855635	0	Fish
45	11026576	3030682	43942036	40444149	85912025	6883201	Pill
46	17613925	4934009	68188863	24050150	74482820	12800172	Fish
47	0	0	6482948	41886644	77034685	0	Pill
48	7665861	2280569	32575956	40615864	81086679	3948110	Fish
49	43340972	12453203	142106032	65023987	179503672	38142135	Pill
50	0	0	0	29543578	64626729	0	Fish
51	12310480	3601536	47592280	24228137	80797027	6989481	Pill
52	0	0	0	5141990	56988207	0	Fish
53	0	0	0	13257192	36058696	0	Pill
54	0	0	0	0	0	0	Fish
55	26440822	7360997	93953011	63494207	136888200	25680739	Pill
56	0	0	0	3627287	10207428	0	Fish

Appendix H : Snail 4.3 – Predominant Isolated Peaks

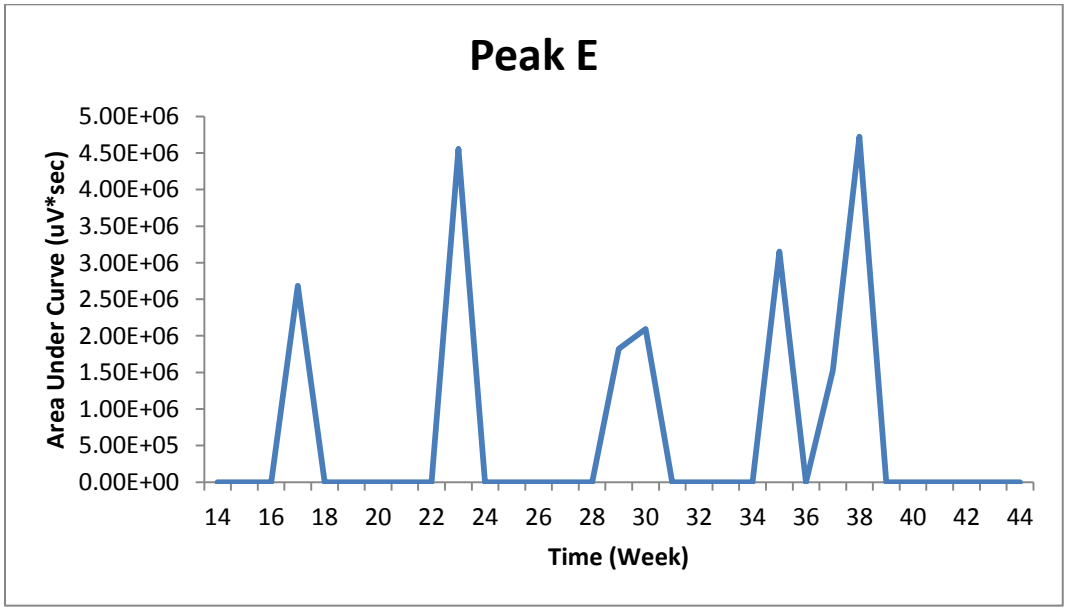

Figure 61. Illustrating area under curve for isolated Peak E.

Snail 4.3 produced Peak E the most regularly.

Table 36. Snail 4.3 (Fish Food + Bromine) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
14	0	0	0	0	0	0	Pill
15	0	0	0	0	0	0	Fish
16	0	0	0	0	0	0	Pill
17	0	0	0	0	2683539	0	Fish
18	0	0	0	0	0	0	Pill
19	0	0	0	0	0	0	Fish
20	0	0	0	0	0	0	Pill
21	0	0	0	0	0	0	Fish
22	0	0	0	0	0	0	Pill
23	0	0	0	0	4556606	0	Fish
24	0	0	0	0	0	0	Pill
25	0	0	0	0	0	0	Fish
26	0	0	0	0	0	0	Pill
27	0	0	0	0	0	0	Fish
28	0	0	0	0	0	0	Pill
29	0	657433	0	0	1820633	0	Fish
30	0	0	0	0	2094063	0	Pill
31	0	0	0	0	0	0	Fish
32	0	0	0	0	0	0	Pill
33	0	0	0	0	0	0	Fish
34	0	0	0	0	0	0	Fish
35	0	0	0	0	3154376	0	Pill
36	0	0	0	0	0	0	Fish
37	0	1756151	0	0	1525266	0	Pill
38	0	0	0	0	4720852	0	Fish
39	0	0	0	2435081	0	0	Pill
40	0	0	0	0	0	0	Fish
41	0	0	0	0	0	0	Pill
42	0	0	0	1307762	0	0	Fish
43	0	0	0	509281	0	0	Pill
44	0	0	0	1286876	0	0	Fish
45	0	0	0	0	0	0	Pill
46	0	0	3896917	0	3913033	0	Fish
47	0	0	0	0	0	0	Fish
48	0	0	0	0	0	0	Pill
49	0	0	0	0	0	0	Fish
50	0	0	0	0	0	0	Pill

Table 37. Snail 4.3 (Fish Food + Bromine) Area Under Curve Data and schedule of diet

51	0	0	3076596	0	2850299	0	Fish
52	0	0	0	0	0	0	Pill
53	3740163	2498912	13708384	528164	2674396	0	Fish
54	0	0	0	0	1728307	0	Pill
55	0	0	0	0	0	0	Fish

Appendix I : Snail 5.2 – Predominant Isolated Peaks

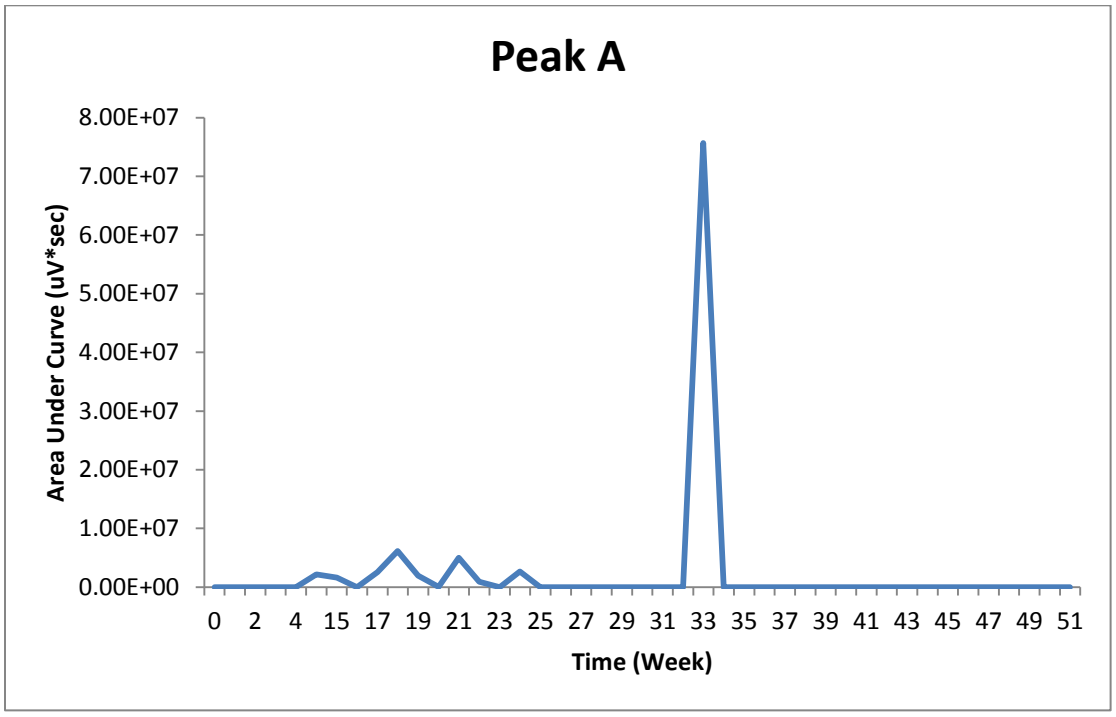

Figure 62. Illustrating area under curve for isolated Peak A.

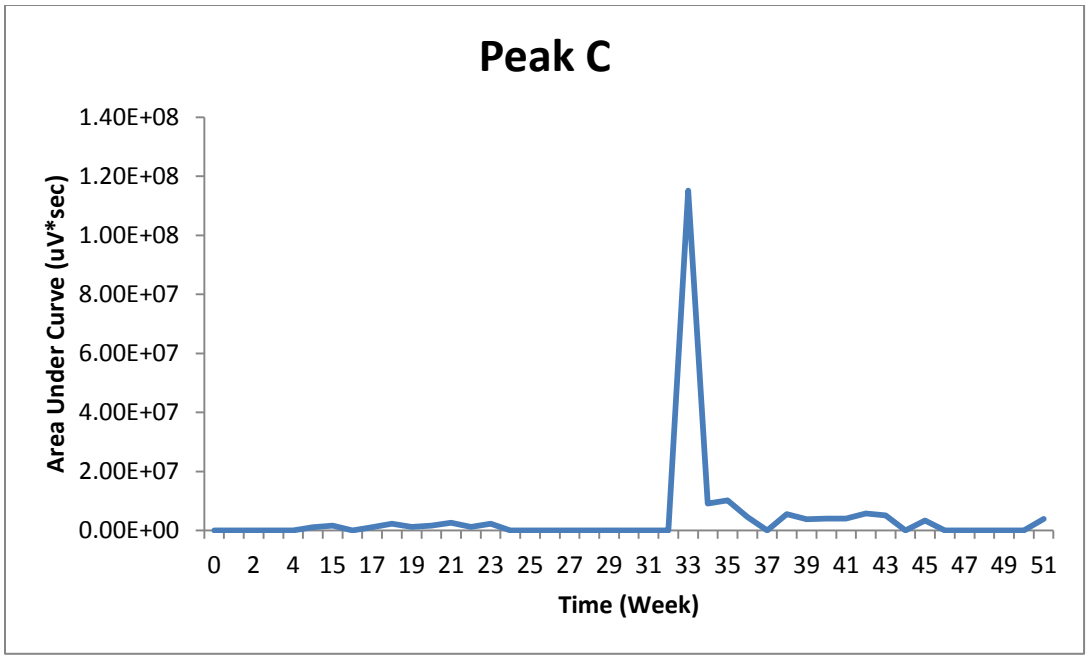

Figure 63. Illustrating area under curve for isolated Peak C.

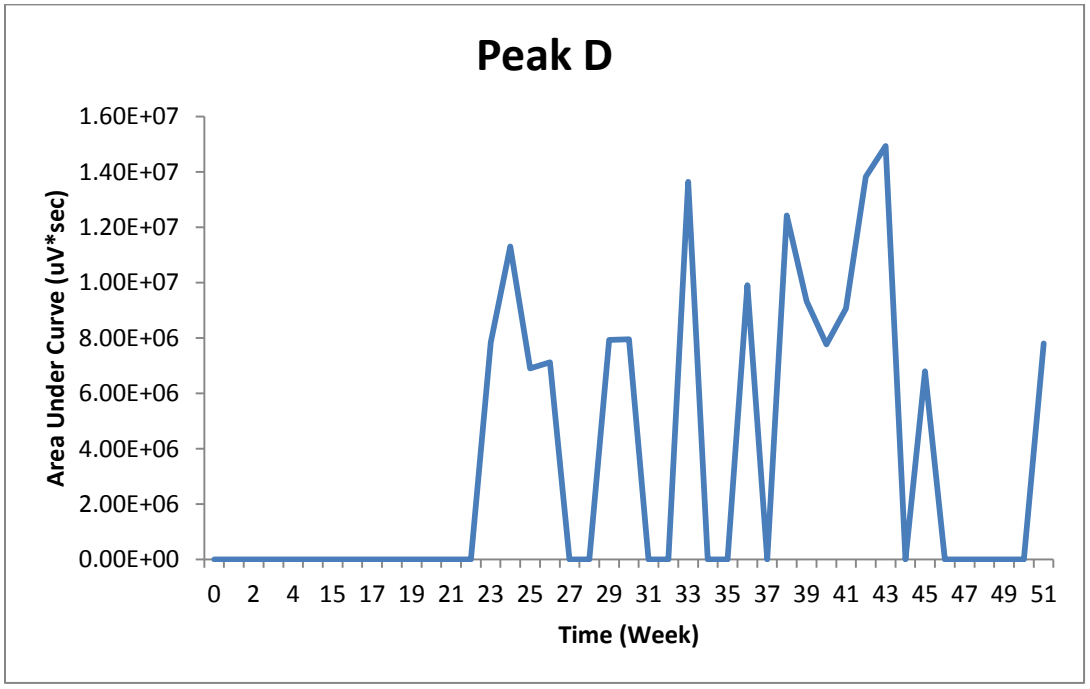

Figure 64. Illustrating area under curve for isolated Peak D.

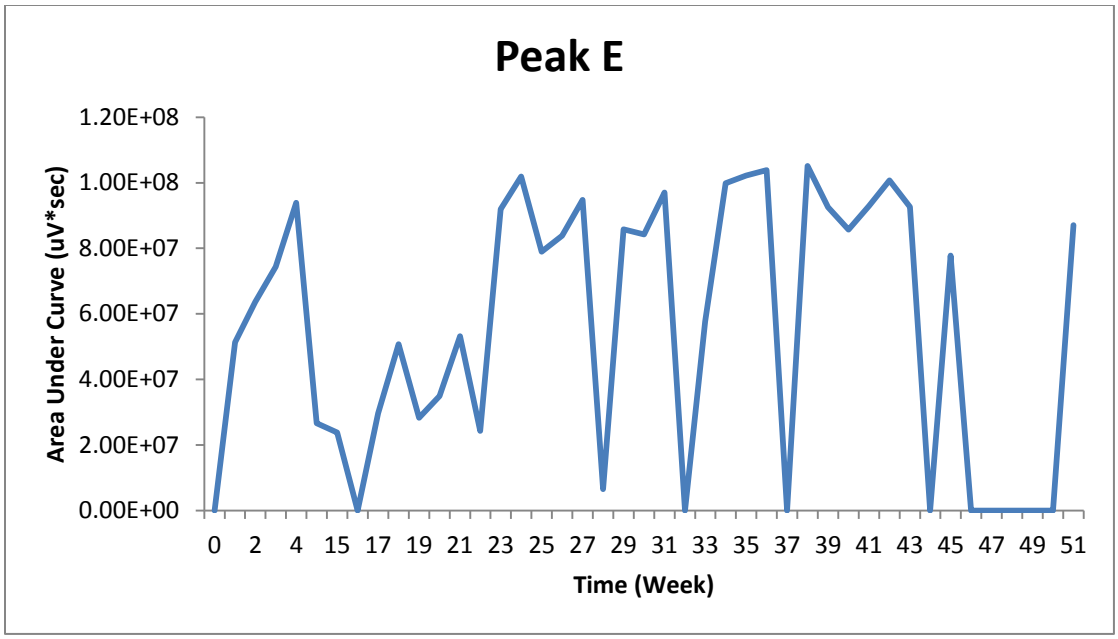

Figure 65. Illustrating area under curve for isolated Peak E.

Snail 5.2 predominant peaks were A, C, D, and E. Interesting at approximately week 33 we see a sharp spike in peak AUC with peaks A, C, and D. AUC drops soon afterwards. Peak E at week 33 we also observe a spike in AUC, however it continues to increase a little before dropping like its fellow peaks.

Table 38. Snail 5.2 (Fish Food + Bromine) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	0	0	0	0	0	Fish
1	0	0	0	0	51352011	0	Pill
2	0	0	0	0	63676405	0	Fish
3	0	0	0	0	74359444	0	Pill
4	0	0	0	0	93919335	0	Fish
14	2169944	0	1036347	0	26624103	0	Fish
15	1631357	0	1583238	0	23779872	0	Pill
16	0	36036535	0	0	0	0	Fish
17	2577438	0	1137110	0	29651819	0	Pill
18	6161353	0	2239714	0	50796791	0	Fish
19	1939688	0	1176655	0	28266669	0	Pill
20	49205	0	1604444	0	34959966	0	Fish
21	5014112	0	2577304	0	53172693	0	Pill
22	903022	0	1176926	0	24311042	0	Fish
23	0	0	2288144	7840410	91922287	0	Pill

Table 39. Snail 5.2 (Fish Food + Bromine) Area Under Curve Data and schedule of diet

24	2653348	0	0	11302146	1.02E+08	0	Fish
25	0	0	0	6900460	78986560	0	Pill
26	0	0	0	7123466	83877631	0	Fish
27	0	0	0	0	94734850	0	Pill
28	0	0	0	0	6559195	0	Fish
29	0	0	0	7929143	85780844	0	Pill
30	0	0	0	7953778	84220062	0	Fish
31	0	0	0	0	97072657	0	Pill
32	0	0	0	0	0	0	Fish
33	75688606	30557155	1.15E+08	13638132	57690591	34911105	Fish
34	0	0	9083019	0	99849438	0	Pill
35	0	0	10223584	0	1.02E+08	0	Fish
36	0	0	4600768	9905899	1.04E+08	0	Pill
37	0	0	0	0	0	0	Fish
38	0	0	5549546	12421149	1.05E+08	788981	Pill
39	0	0	3753338	9326677	92521117	0	Fish
40	0	0	3979267	7766786	85731062	0	Pill
41	0	0	4051718	9059890	92884434	0	Fish
42	0	0	5778233	13823726	1.01E+08	2113991	Pill
43	0	16219525	5142058	14930316	92626587	5506244	Fish
44	0	0	0	0	0	0	Pill
45	0	0	3313527	6794430	77807510	0	Fish
46	0	0	0	0	0	0	Pill
47	0	0	0	0	0	0	Fish
48	0	0	0	0	0	0	Pill
49	0	0	0	0	0	0	Fish
50	0	0	0	0	0	0	Pill
51	0	2123990	3958645	7804533	87061702	0	Fish

Appendix J : Snail 6.1 – Predominant Isolated Peaks

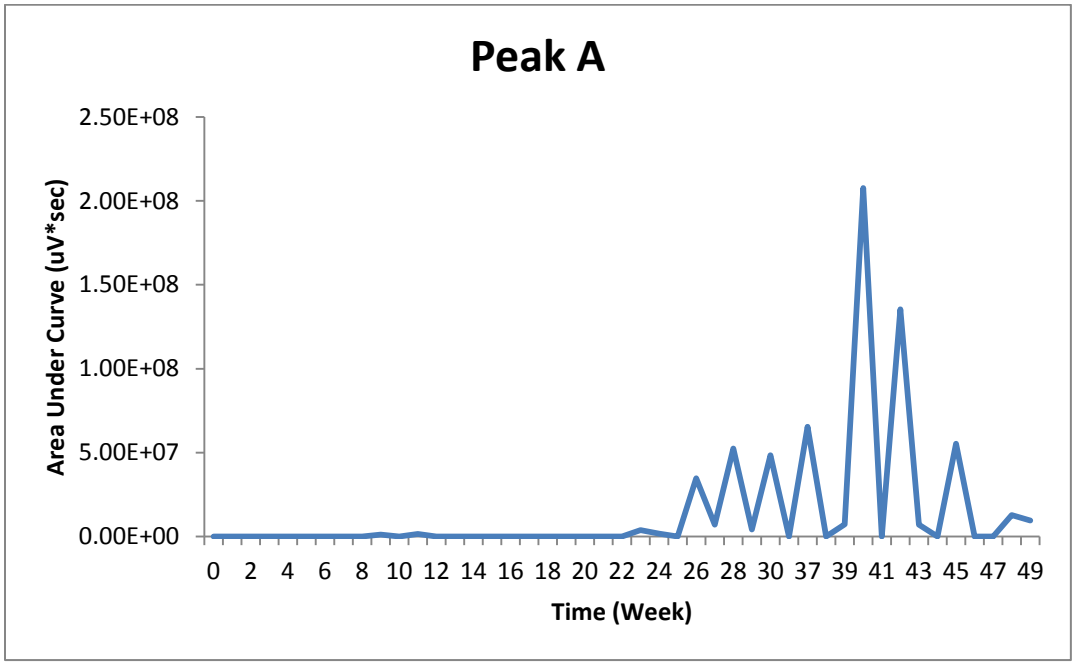

Figure 66. Illustrating area under curve for isolated Peak A.

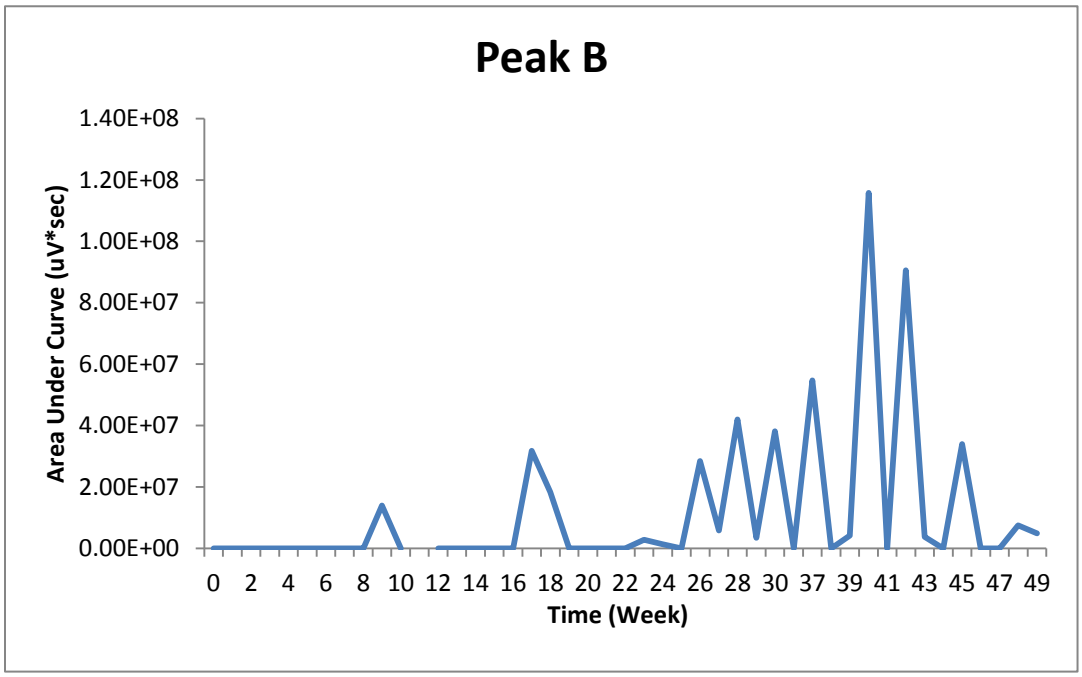

Figure 67. Illustrating area under curve for isolated Peak B.

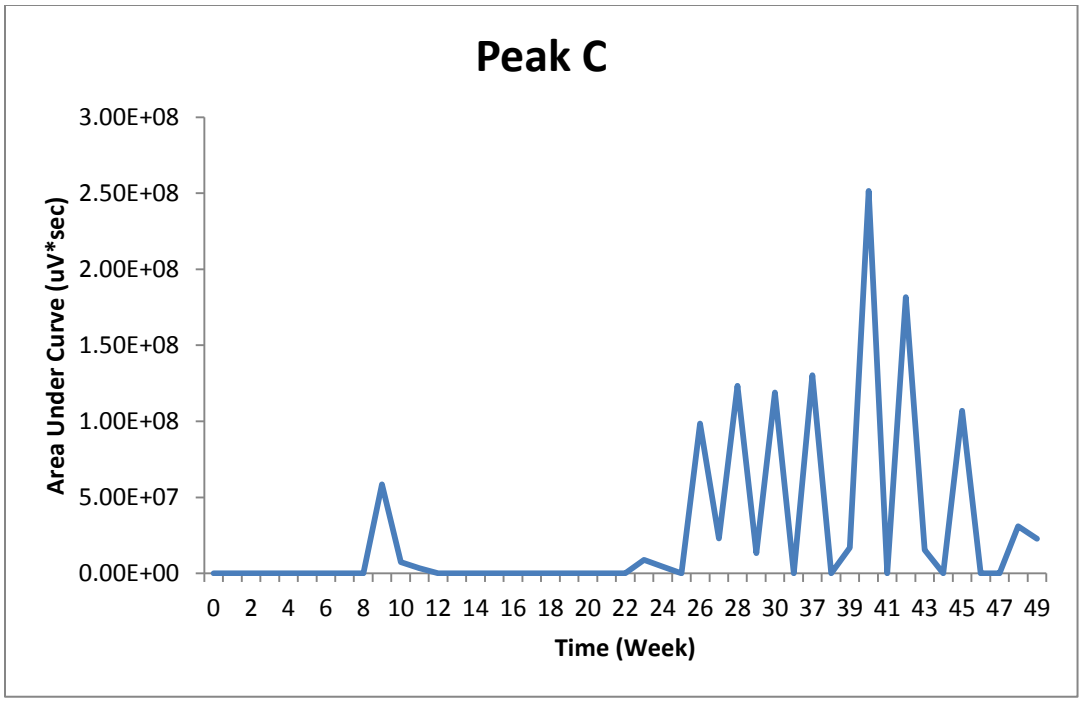

Figure 68. Illustrating area under curve for isolated Peak C.

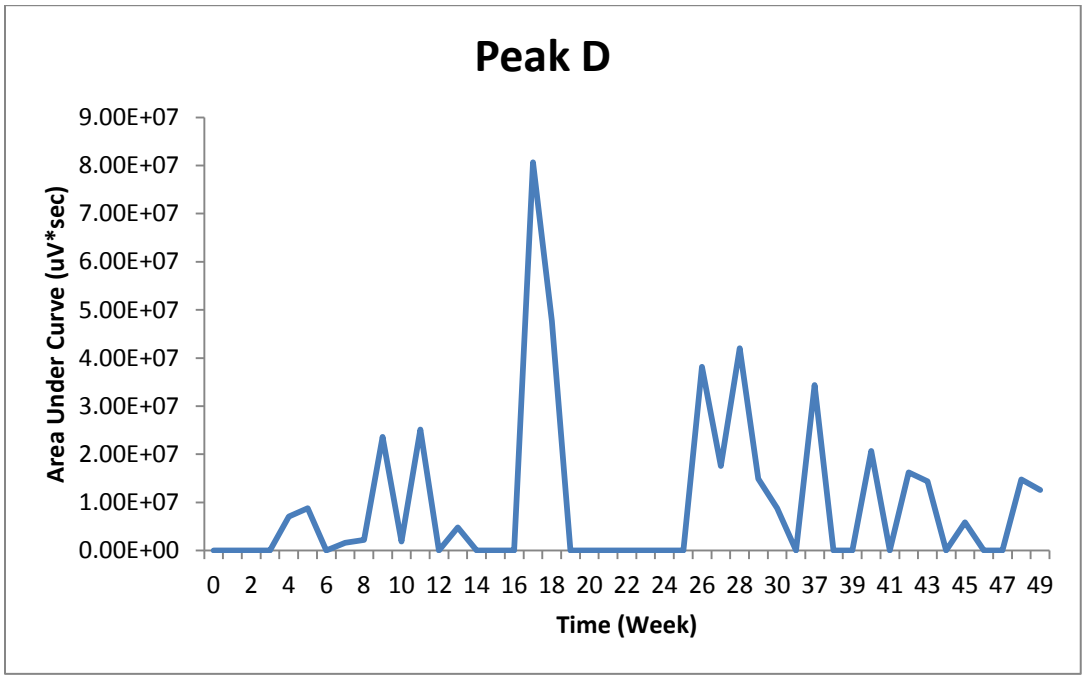

Figure 69. Illustrating area under curve for isolated Peak D.

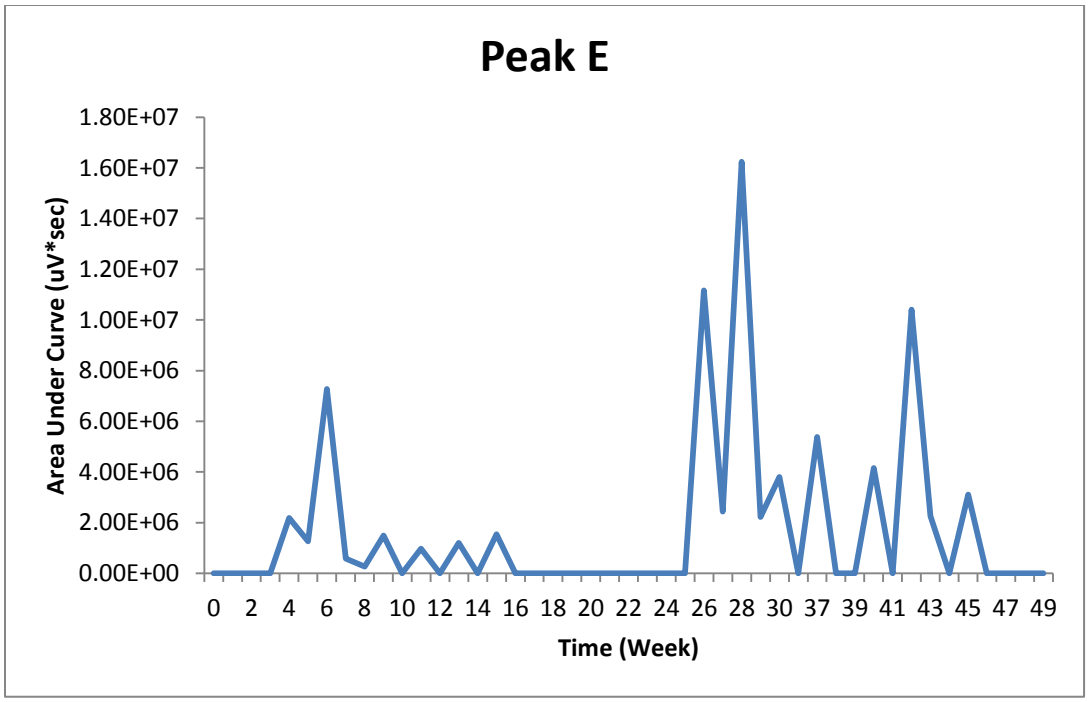

Figure 70. Illustrating area under curve for isolated Peak E.

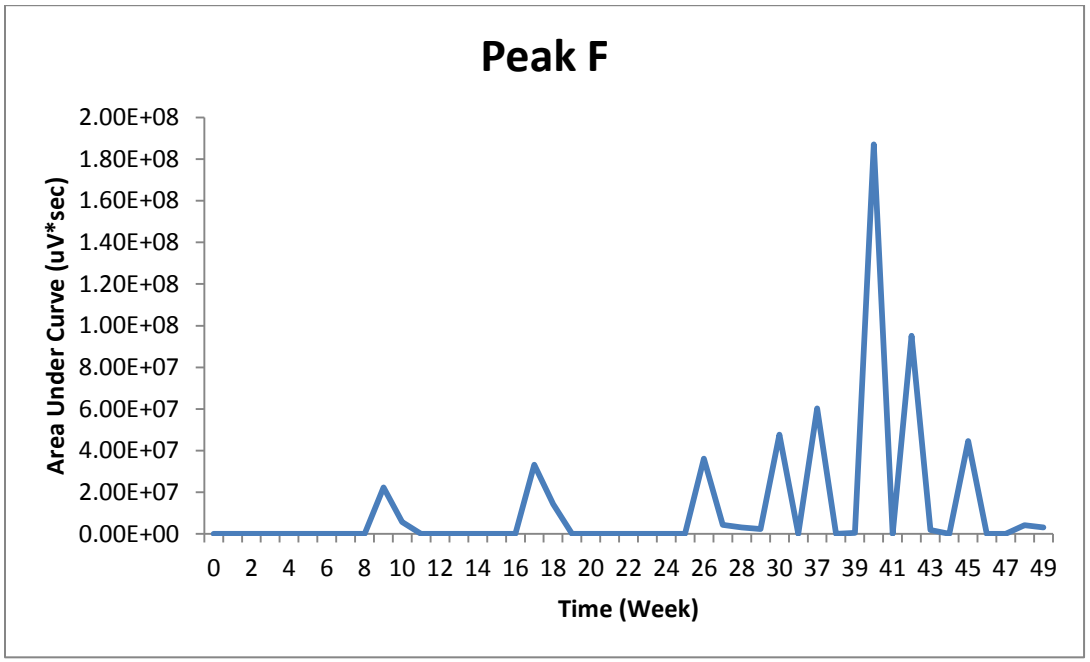

Figure 71. Illustrating area under curve for isolated Peak F.

Snail 6.1 produced all six isolated peaks. Peak AUCs almost mirror each other at starting at approximately 27 weeks. There appears to be a more "controlled" production of these peaks and a more obvious cycling of peptide AUCs.

Table 40. Snail 6.1 (Fish Food + Vitamin Pack) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	0	0	0	0	0	Pill
1	0	0	0	0	0	0	Fish
2	0	0	0	0	0	0	Pill
3	0	0	0	0	0	0	Fish
4	0	0	0	7078508	2191261	0	Pill
5	0	0	0	8774971	1263368	0	Fish
6	0	0	0	0	7272585	0	Pill
7	0	0	0	1575007	586317	0	Fish
8	0	0	0	2195642	273115	0	Pill
9	1138011	13905744	58465628	23581441	1492130	22313614	Fish
10	0	0	7470219	1839958	0	5703699	Pill
11	1646099		3379835	25120898	980702	0	Fish
12	0	0	0	0	0	0	Pill
13	0	0	0	4815221	1203223	0	Fish
14	0	0	0	0	0	0	Pill
15	0	0	0	0	1542265	0	Fish
16	0	0	0	0	0	0	Pill
17	0	31753849	0	80683761	0	33201228	Fish
18	0	18350595	0	47712411	0	14338482	Pill
19	0	0	0	0	0	0	Fish
20	0	0	0	0	0	0	Pill
21	0	0	0	0	0	0	Fish
22	0	0	0	0	0	0	Pill
23	3950865	2737502	8802683	0	0	0	Fish
24	1809869	1316749	4475506	0	0	0	Pill
25	0	0	0	0	0	0	Fish
26	34704649	28447556	98612019	38152707	11162970	36230636	Pill
27	7152504	5831673	23064735	17568564	2441877	4394654	Fish
28	52587549	41935488	1.23E+08	42033318	16245514	3204642	Fish
29	4195173	3407786	13352889	14905312	2230978	2377366	Pill
30	48487396	38119293	1.19E+08	8773299	3800052	47692089	Fish
36	0	0	0	0	0	0	Pill
37	65512614	54699318	1.3E+08	34361898	5378146	60267603	Fish
38	0	0	0	0	0	0	Pill
39	7253168	4115165	16806741	0	0	565260	Fish
40	2.08E+08	1.16E+08	2.52E+08	20720115	4155803	1.87E+08	Pill
41	0	0	0	0	0	0	Fish
42	1.35E+08	90604634	1.82E+08	16231174	10409808	95158645	Pill

Table 41. Snail 6.1 (Fish Food + Vitamin Pack) Area Under Curve Data and schedule of diet

43	7054015	3723335	15496682	14382067	2267152	1947229	Fish
44	0	0	0	0	0	0	Pill
45	55302578	33982143	1.07E+08	5850673	3102635	44573828	Fish
46	0	0	0	0	0	0	Pill
47	0	0	0	0	0	0	Fish
48	12790270	7434525	30938413	14740566	0	4260433	Pill
49	9598635	4899938	22861862	12570386	0	3185372	Fish

Appendix K : Snail 6.2 – Predominant Isolated Peaks

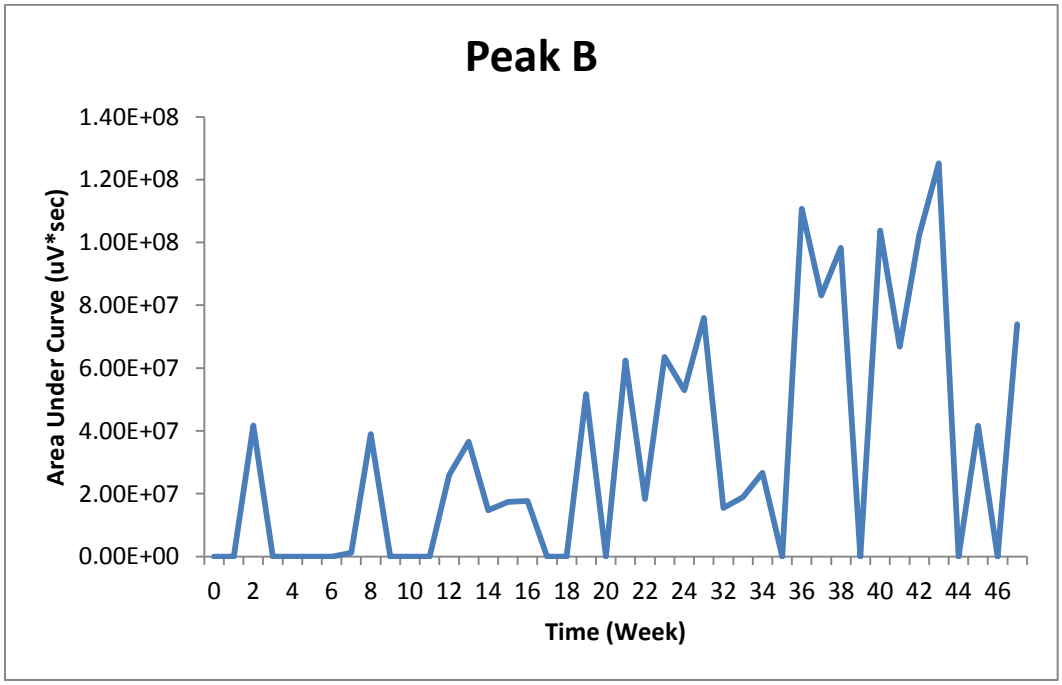

Figure 72. Illustrating area under curve for isolated Peak B.

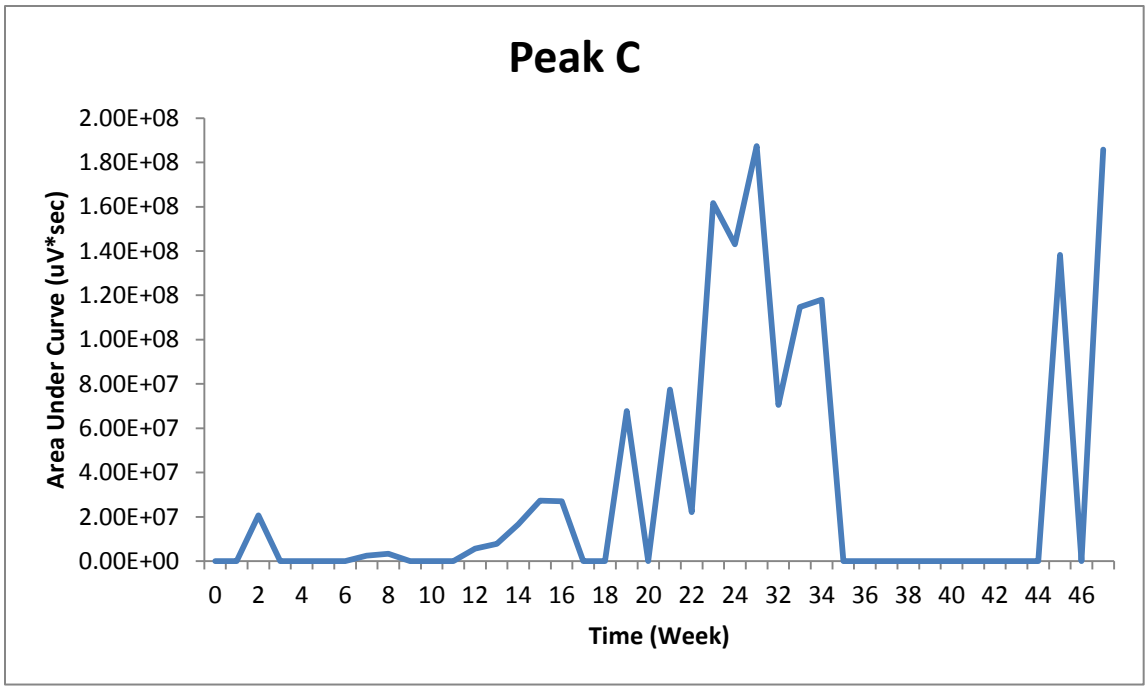

Figure 73. Illustrating area under curve for isolated Peak C.

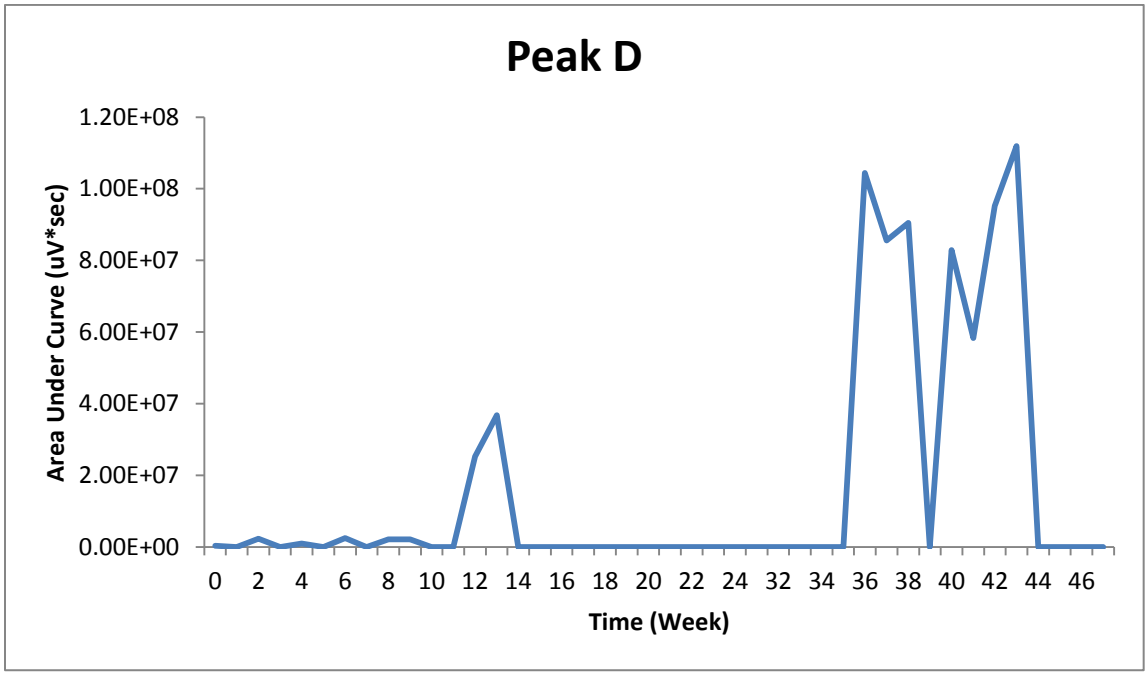

Figure 74. Illustrating area under curve for isolated Peak D.

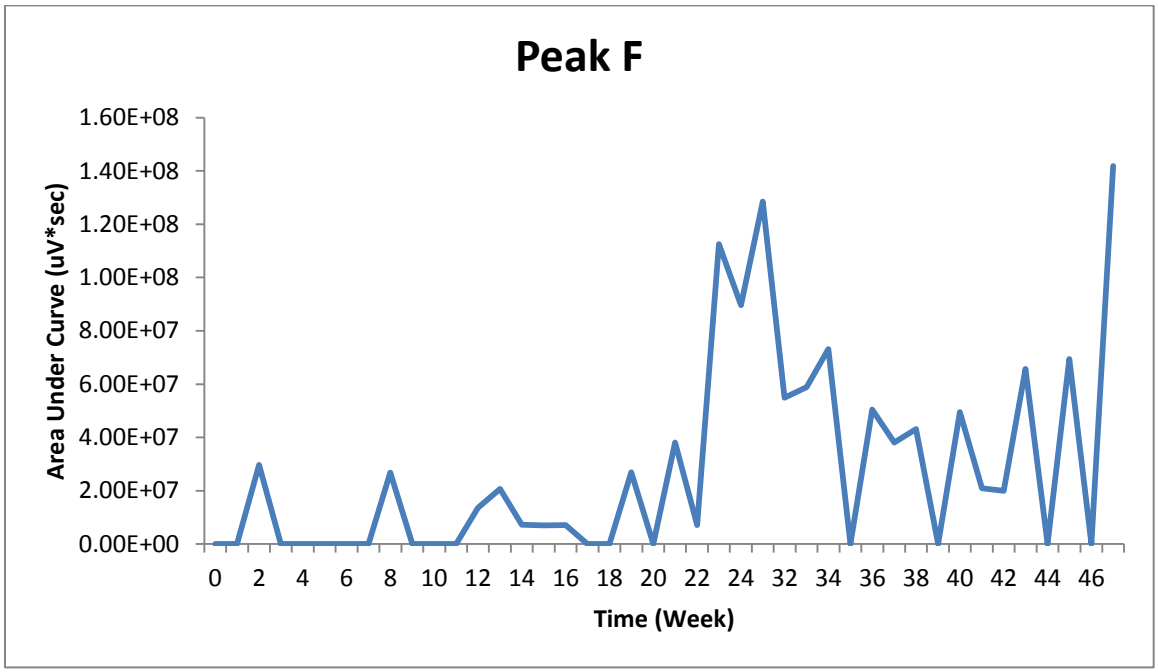

Figure 75. Illustrating area under curve for isolated Peak F.

Snail 6.2 peak production is a little more varied and does not appear as regulated as 6.1.

Table 42. Snail 6.2 (Fish Food + Vitamin Pack) Area Under Curve and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	0	0	320900	5712848	0	Pill
1	0	0	0	0	0	0	Fish
2	0	41778019	20595732	2312632	28834838	29731385	Pill
3	0	0	0	0	0	0	Fish
4	0	0	0	943727	18342852	0	Pill
5	0	0	0	0	0	0	Fish
6	0	0	0	2467888	8501721	0	Pill
7	0	1157946	2389793	0	1502949	0	Fish
8	0	38976762	3347262	2110617	23994041	26762165	Pill
9	0	0	0	2097880	6757887	0	Fish
10	0	0	0	0	0	0	Pill

Table 43. Snail 6.2 (Fish Food + Vitamin Pack) Area Under Curve Data and schedule of diet

11	0	0	0	0	0	0	Fish
12	0	25934062	5595863	25280442	1085188	13553210	Pill
13	0	36554197	7820353	36793465	7036557	20674686	Fish
14	0	14703964	16671565	0	427543	7216279	Pill
15	0	17374401	27292429	0	1926635	7051308	Fish
16	0	17664688	27042550	0	1124485	7074159	Pill
17	0	0	0	0	0	0	Fish
18	0	0	0	0	0	0	Pill
19	0	51739525	67748798	0	1686457	26974890	Fish
20	0	0	0	0	0	0	Pill
21	0	62383147	77422002	0	1077797	38140029	Fish
22	0	18342330	22030598	0	0	7100819	Pill
23	1.93E+08	63522143	1.62E+08	0	0	1.13E+08	Fish
24	1.66E+08	52956967	1.43E+08	0	0	89659808	Pill
25	2.19E+08	75970036	1.87E+08	0	0	1.28E+08	Fish
32	88978643	15414808	70477713	0	0	54942186	Pill
33	1.05E+08	18931060	1.15E+08	0	0	58851543	Fish
34	1.2E+08	26644786	1.18E+08	0	0	73098527	Fish
35	0	0	0	0	0	0	Pill
36	0	1.11E+08	0	1.04E+08	0	50435657	Fish
37	0	83076862	0	85579032	0	38102044	Pill
38	0	98293758	0	90459645	2243598	43102293	Fish
39	0	0	0	0	0	0	Pill
40	0	1.04E+08	0	82847389	0	49463194	Fish
41	0	66820691	0	58274610	0	20968516	Pill
42	0	1.02E+08	0	95226371	0	19987338	Fish
43	0	1.25E+08	0	1.12E+08	4491911	65697194	Pill
44	0	0	0	0	0	0	Fish
45	1.63E+08	41667374	1.38E+08	0	5990617	69463438	Pill
46	0	0	0	0	0	0	Fish
47	2.59E+08	73906659	1.86E+08	0	13445087	1.42E+08	Pill

Appendix L : Snail 7.2 – Predominant Isolated Peaks

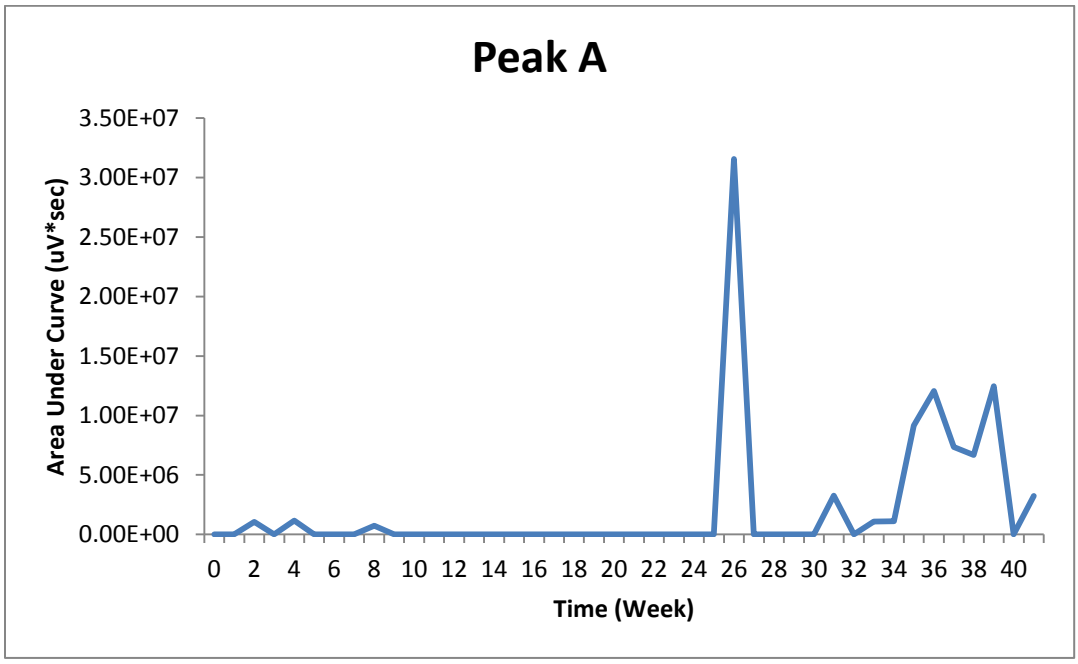

Figure 76. Illustrating area under curve for isolated Peak A.

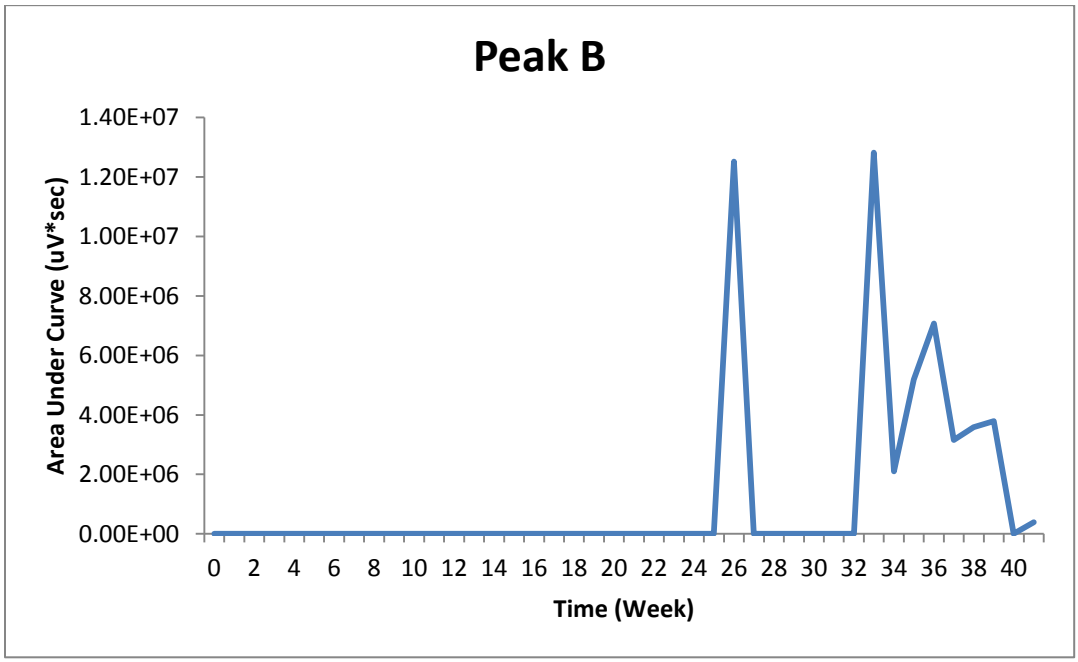

Figure 77. Illustrating area under curve for isolated Peak B.

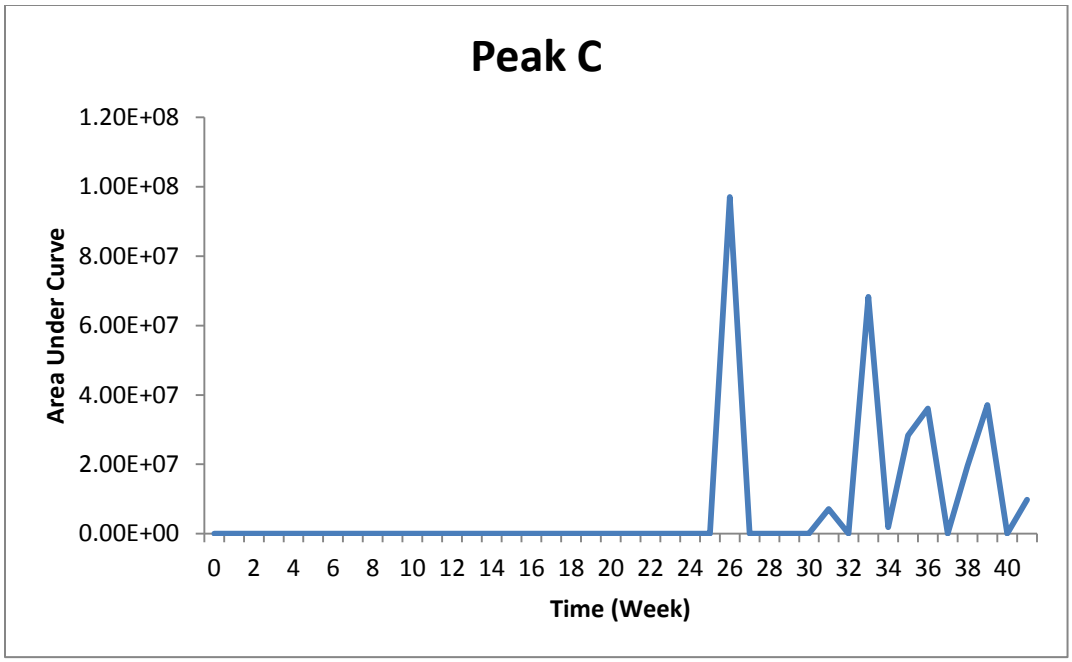

Figure 78. Illustrating area under curve for isolated Peak C.

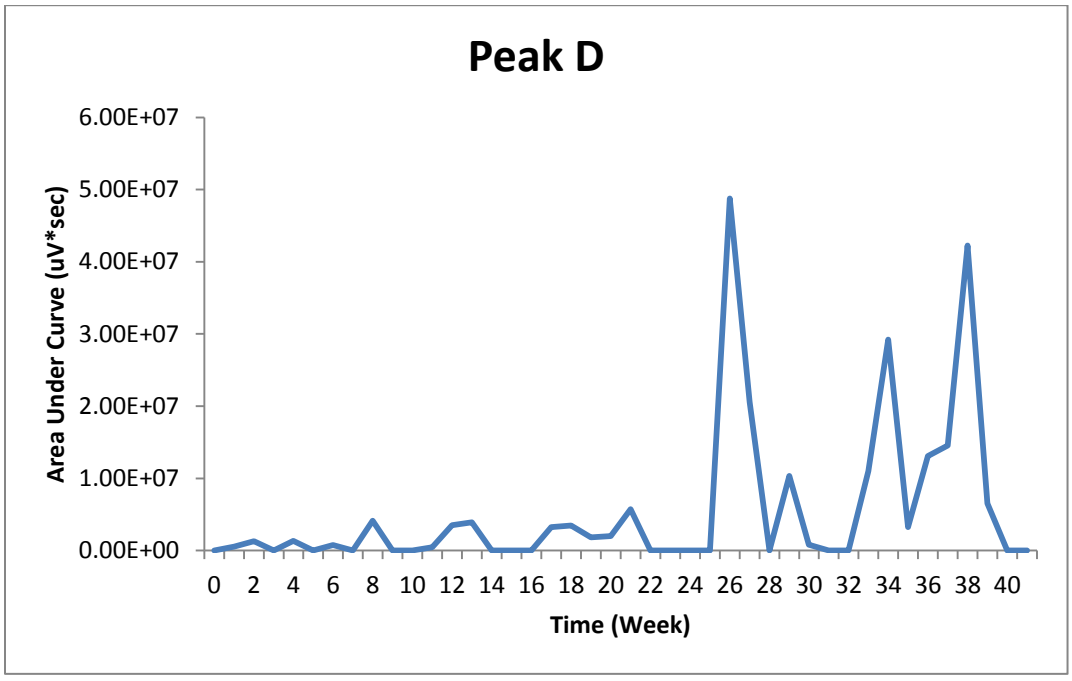

Figure 79. Illustrating area under curve for isolated Peak D.

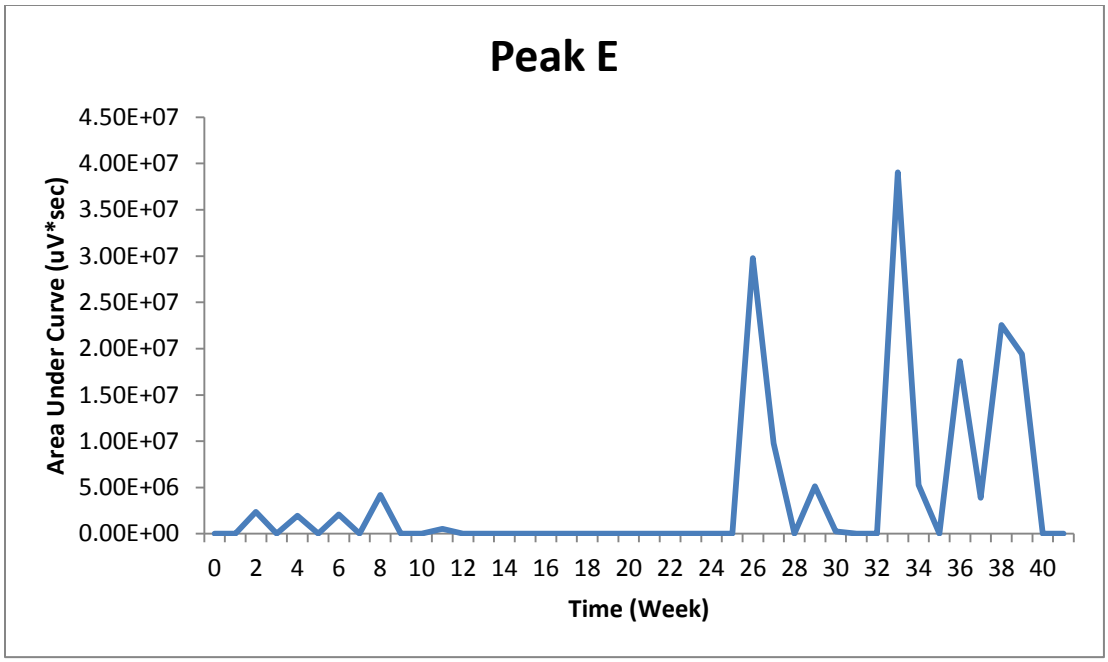

Figure 80. Illustrating area under curve for isolated Peak E.

Snail 7.2 predominantly produced isolated peaks A – E. We also observe the same spike in AUC across all peaks at approximately week 37. All three isolated peak profiles for this snail appear to mirror each in cycling.

Table 44. Snail 7.2 (Fish Food + Vitamin Pack) Area Under Curve and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	0	0	0	0	0	Pill
1	0	0	0	540887	0	0	Fish
2	1053703	0	0	1287924	2358725	0	Pill
3	0	0	0	0	0	0	Fish
4	1175860	0	0	1336515	1960616	0	Pill
5	0	0	0	0	0	0	Fish
6	0	0	0	768183	2082304	0	Pill
7	0	0	0	0	0	0	Fish
8	745802	0	0	4122387	4185804	0	Pill
9	0	0	0	0	0	0	Fish
10	0	0	0	0	0	0	Pill
11	0	0	0	440518	526724	0	Fish
12	0	0	0	3501530	0	0	Pill

Table 45. Snail 7.2 (Fish Food + Vitamin Pack) Area Under Curve and schedule of diet

13	0	0	0	3924918	0	0	Fish
14	0	0	0	0	0	0	Pill
15	0	0	0	0	0	0	Fish
16	0	0	0	0	0	0	Pill
17	0	0	0	3242173	0	0	Fish
18	0	0	0	3477346	0	0	Pill
19	0	0	0	1828511	0	0	Fish
20	0	0	0	1984680	0	0	Pill
21	0	0	0	5728503	0	0	Fish
22	0	0	0	0	0	0	Pill
23	0	0	0	0	0	0	Fish
24	0	0	0	0	0	0	Pill
25	0	0	0	0	0	0	Fish
26	31556750	12511644	97001019	48781486	29761028	27659662	Pill
27	0	0	0	20552576	9780041	0	Fish
28	0	0	0	0	0	0	Pill
29	0	0	0	10312599	5143306	0	Fish
30	0	0	0	785677	248648	0	Pill
31	3261560	0	7144695	0	0	694948	Fish
32	0	0	0	0	0	0	Pill
33	1085725	12814202	68222727	11010880	39056768	13166494	Fish
34	1101063	2097310	1864974	29237572	5255892	843828	Fish
35	9165095	5183379	28300584	3250964	0	2406025	Pill
36	12054925	7072114	36060847	13077728	18654281	4795425	Fish
37	7348825	3151368	0	14554024	3892506	0	Pill
38	6666339	3582923	19545000	42254925	22543373	4455126	Fish
39	12472796	3787040	37117580	6500231	19424670	3069324	Pill
40	0	0	0	0	0	0	Fish
41	3227853	388587	9841754	0	0	0	Pill
42	0	0	0	0	0	0	Fish
43	0	0	0	0	0	0	Pill
44	0	0	0	0	0	0	Fish
45	7274615	0	19689491	0	88330832	0	Pill
46	27397639	14083378	71832025		39803035	18337885	Fish
47	23420455	13819361	65116855	6094079	37198172	1027760	Pill
48	1139637	0	3810312	0	1535954	0	Fish
49	62246185	35661927	119139198	50767606	11860042	50143464	Pill
50	2974341	0	8240469	0	2980801	0	Fish

Table 46. Snail 7.2 (Fish Food + Vitamin Pack) Area Under Curve and schedule of diet

51	0	0	0	0	0	0	Pill
52	15569315	8380786	43191729	1840291	22551687	5782235	Fish
53	45285975	24388324	108705984	0	62163073	32446794	Pill
54	16979202	9034433	46780234	2180116	24775401	7624845	Fish
55	12941078	7228836	31634110	37066676	20631937	7907980	Pill
56	12767345	6545979	28338067	0	17325922	4277096	Fish

Appendix M : Snail 8.1 – Predominant Isolated Peaks

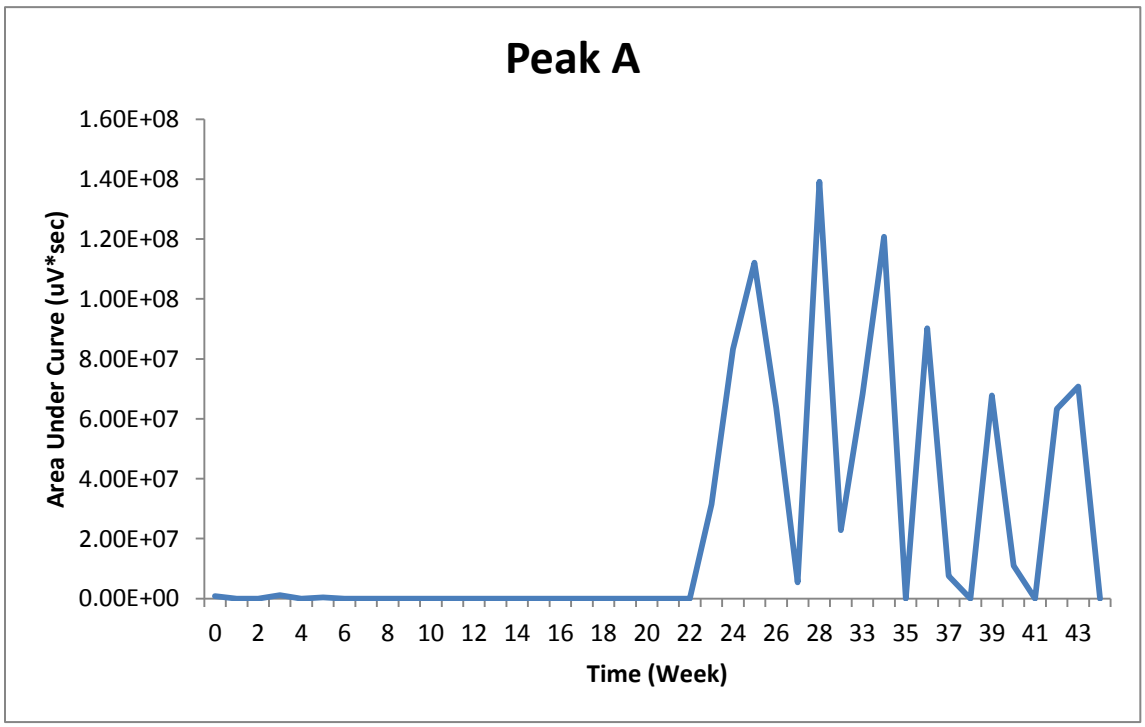

Figure 81. Illustrating area under curve for isolated Peak A.

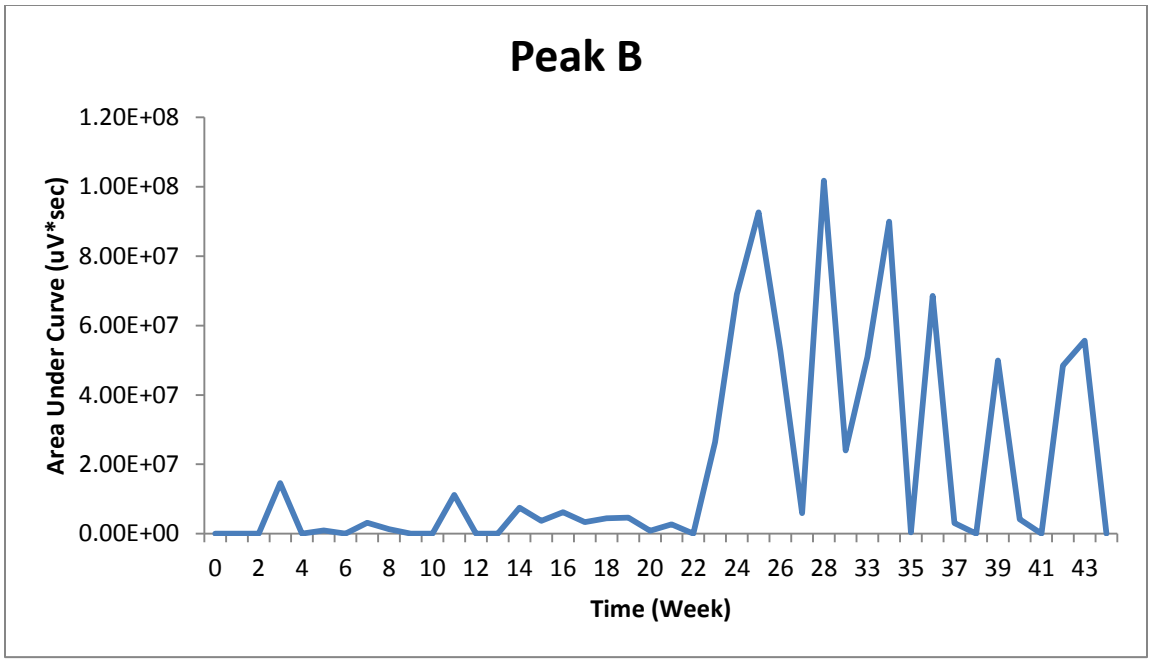

Figure 82. Illustrating area under curve for isolated Peak B.

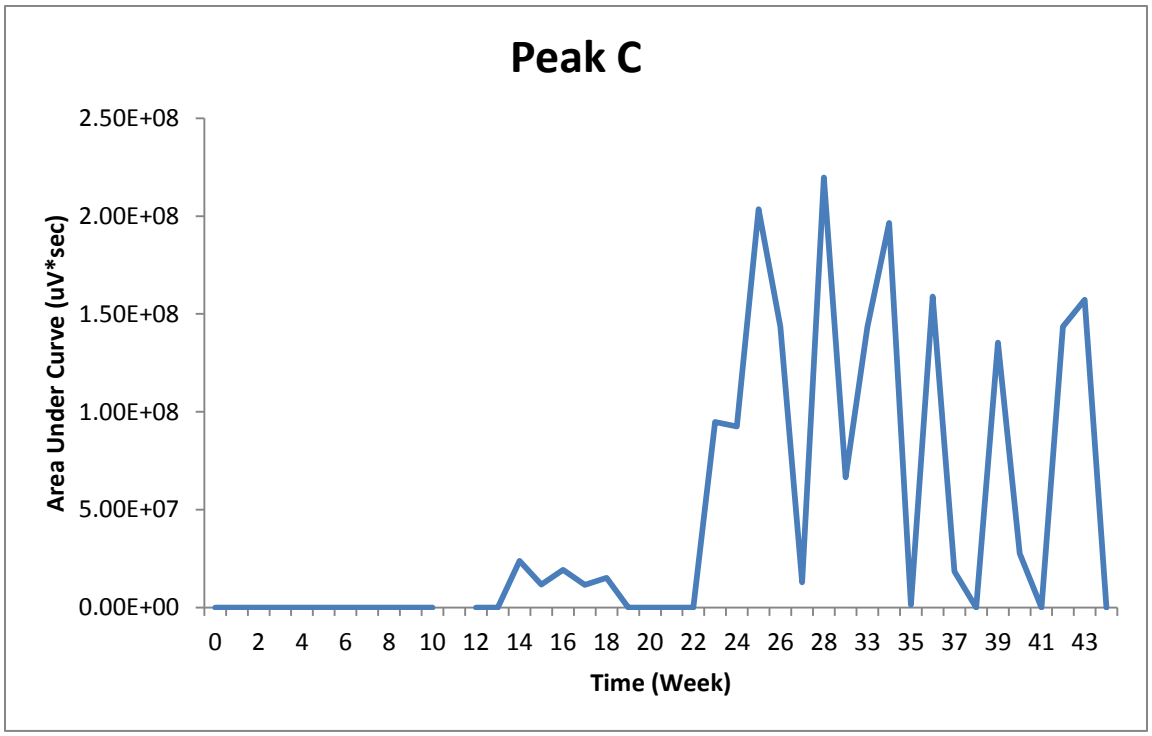

Figure 83. Illustrating area under curve for isolated Peak C.

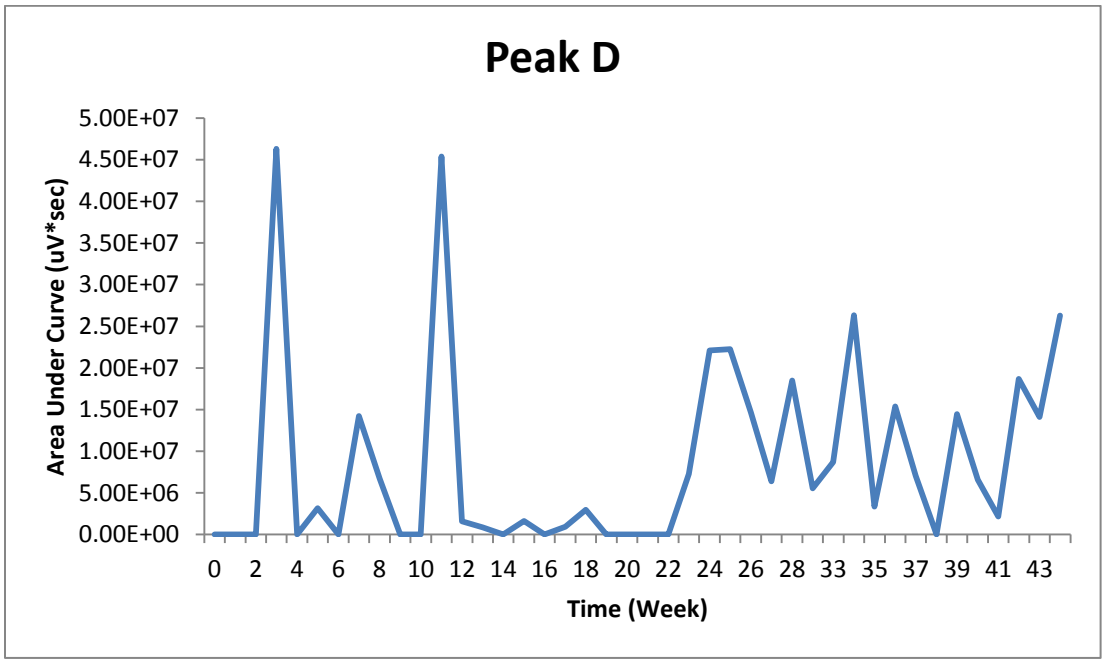

Figure 84. Illustrating area under curve for isolated Peak D.

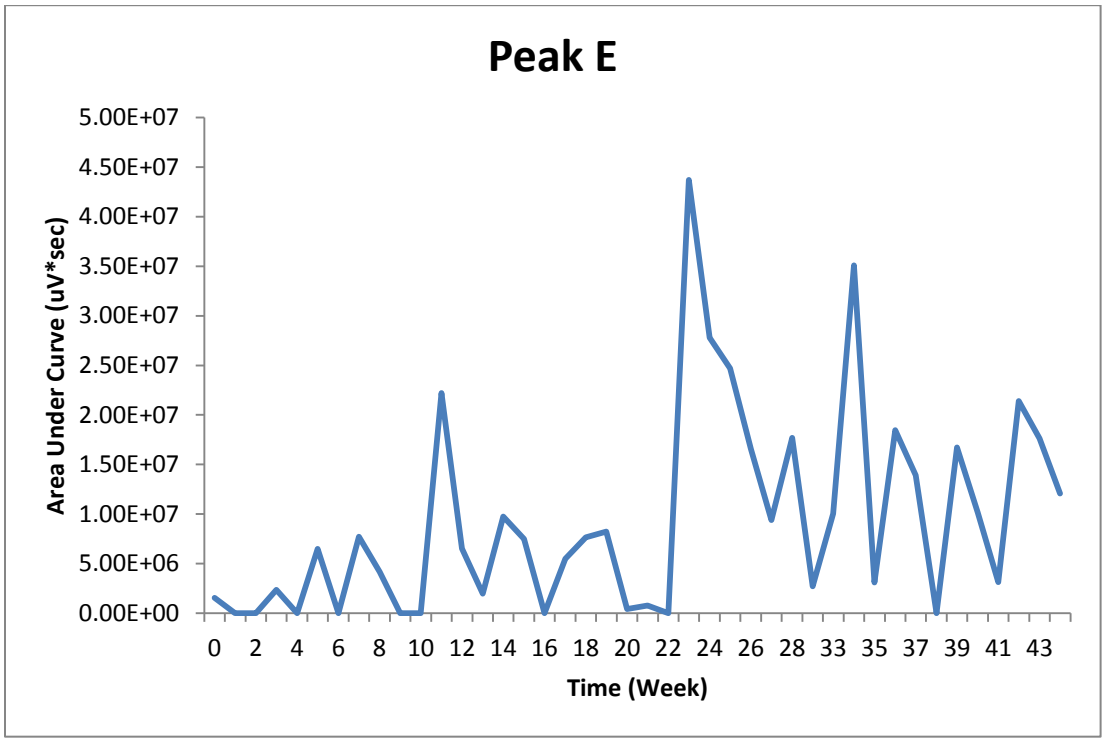

Figure 85. Illustrating area under curve for isolated Peak E.

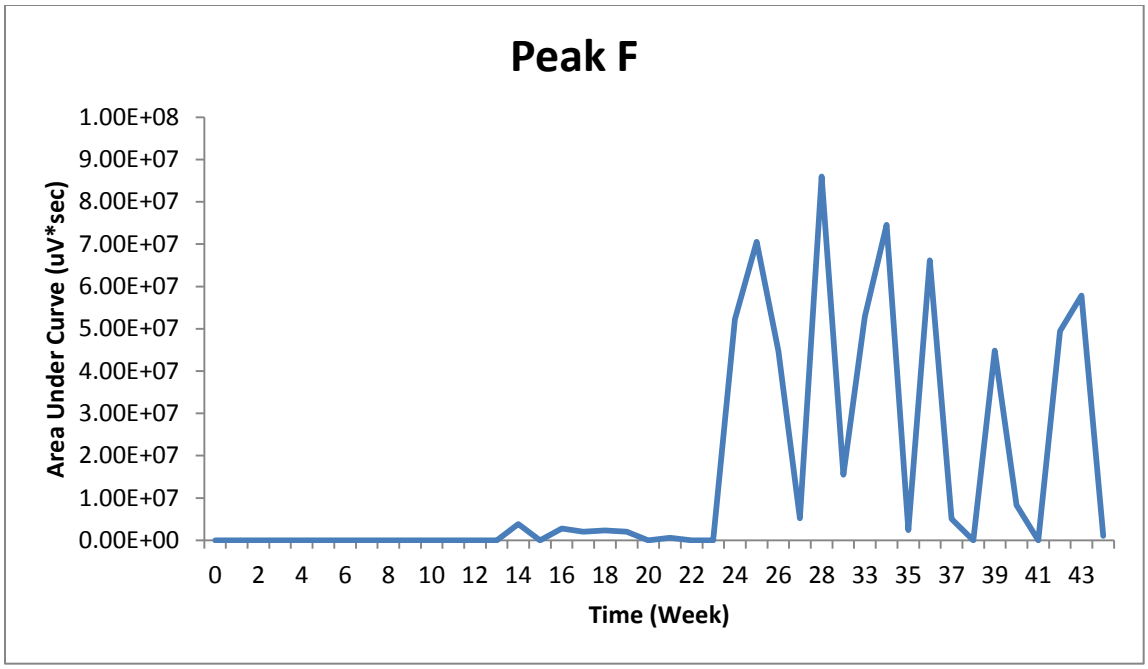

Figure 86. Illustrating area under curve for isolated Peak F.

Snail 8.1 produced all isolated peaks as well. We also observe the same spike in AUC across all peaks at approximately week 24. All three isolated peak profiles for this snail appear to mirror each in cycling.

Table 47. Snail 8.1 (Fish Food + Vitamin Pack + Bromine) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	811051	0	0	0	1553033	0	Pill
1	0	0	0	0	0	0	Fish
2	0	0	0	0	0	0	Pill
3	1153648	14572217	0	46316328	2377711	0	Fish
4	0	0	0	0	0	0	Pill
5	397441	937787	0	3177448	6488232	0	Fish
6	0	0	0	0	0	0	Pill
7	0	3208623	0	14232071	7723329	0	Fish
8	0	1290075	0	6702835	4176761	0	Pill
9	0	0	0	0	0	0	Fish
10	0	0	0	0	0	0	Pill

Table 48. Snail 8.1 (Fish Food + Vitamin Pack + Bromine) Area Under Curve and schedule of diet

11	0	11239812		45381568	22198675	0	Fish
12	0	0	0	1604674	6541074	0	Pill
13	0	0	0	844750	1983861	0	Fish
14	0	7510762	23815578	0	9750398	3812282	Pill
15	0	3729982	11681914	1616966	7501302	0	Fish
16	0	6260889	19326595	0	0	2787364	Pill
17	0	3329560	11602328	932364	5499063	2045336	Fish
18	0	4456749	15164658	2973073	7643722	2329270	Pill
19	0	4614432	0	0	8229291	2038020	Fish
20	0	840256	0	0	425682	0	Pill
21	0	2713997	0	0	772140	625297	Fish
22	0	0	0	0	0	0	Pill
23	31464332	26413272	94831152	7261420	43701864	0	Fish
24	83287984	69027909	92537397	22085212	27787839	52282032	Pill
25	1.12E+08	92569099	2.04E+08	22246151	24701891	70532501	Fish
26	63747114	52757667	1.43E+08	14659211	16572167	44711181	Pill
27	5417754	5923641	12949699	6381811	9414381	5191111	Fish
28	1.39E+08	1.02E+08	2.2E+08	18478311	17689674	85953541	Pill
29	22865160	24011853	66567388	5543181	2726266	15478820	Pill
30	68253026	50891806	1.43E+08	8707483	10046715	52889345	Fish
31	1.21E+08	89934246	1.96E+08	26331985	35083307	74544517	Fish
32	0	421469	1495656	3345003	3103910	2430873	Pill
33	90262284	68495638	1.59E+08	15366771	18457529	66190185	Fish
34	7601361	3071723	18508710	7063910	13909752	5076660	Pill
35	0	0	0	0	0	0	Fish
36	67819624	49943211	1.35E+08	14477314	16734473	44836182	Pill
37	10976582	4157203	27551199	6571033	10144784	8318263	Fish
38	0	0	0	2162050	3129018	0	Pill
39	63312708	48407067	1.44E+08	18702519	21397504	49459447	Fish
40	70822646	55645465	1.57E+08	14109563	17637630	57854549	Pill
41	0	0	0	26301452	12077292	1084417	Fish
42	63312708	48407067	1.44E+08	18702519	21397504	49459447	Pill
43	70822646	55645465	1.57E+08	14109563	17637630	57854549	Fish
44	0	0	0	26301452	12077292	1084417	Pill
45	0	0	0	0	0	0	Fish
46	0	0	0	0	0	0	Pill

Appendix N : Snail 9.1 – Predominant Isolated Peaks

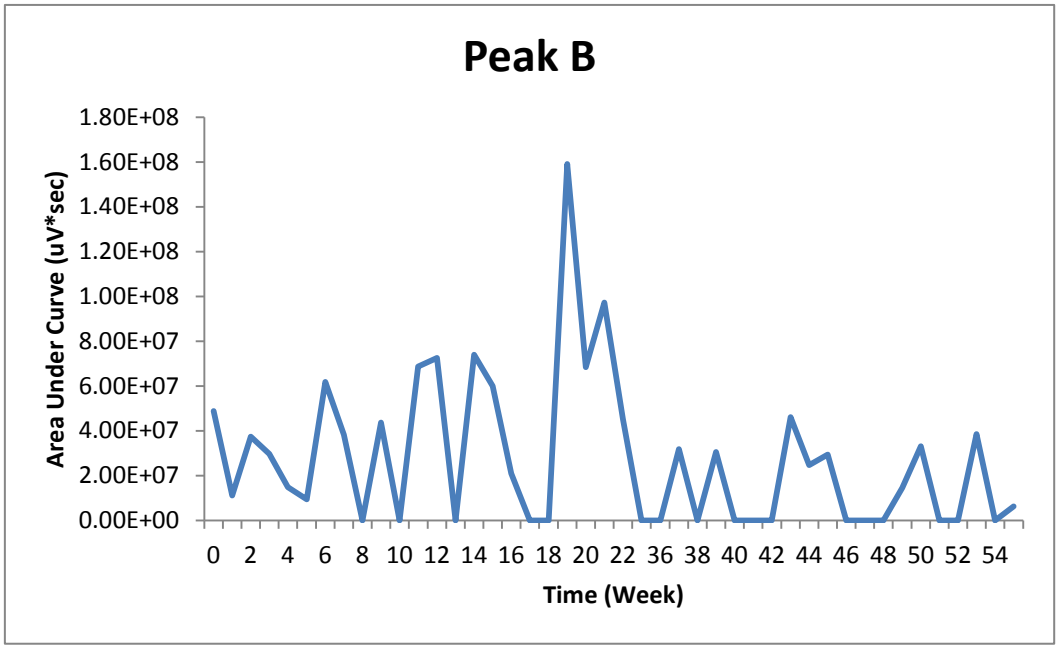

Figure 87. Illustrating area under curve for isolated Peak B.

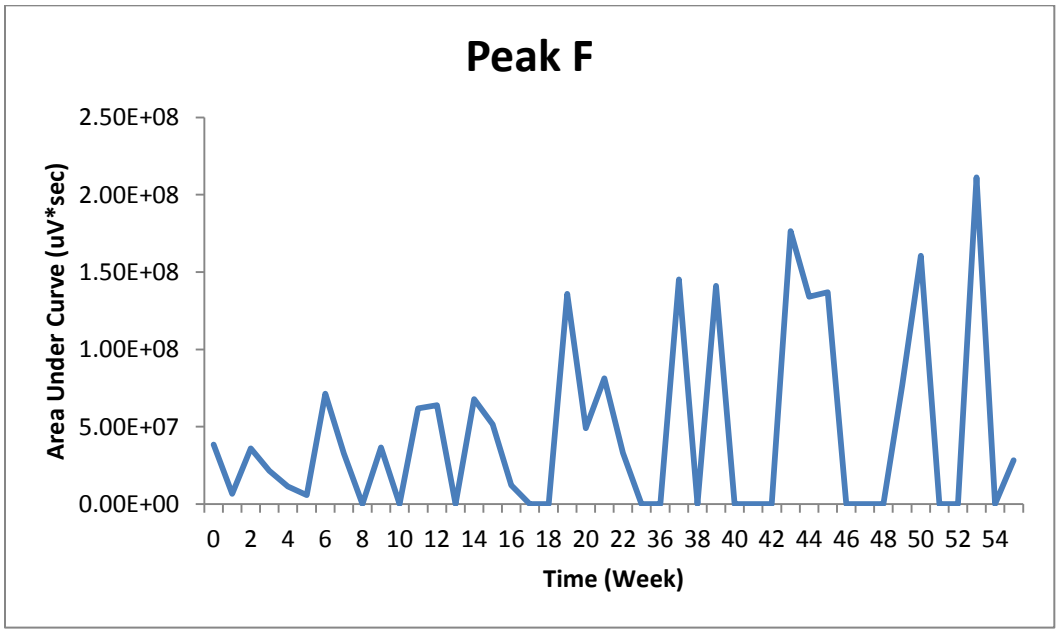

Figure 88. Illustrating area under curve for isolated Peak F.

Snail 9.1 predominantly produced peaks B and F. It looks as though at approximately week 36 as AUC dropped for Peak B, Peak F AUC increased in response.

Table 49. Snail 9.1 (Fish Food + Vitamin Pack + Bromine) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	48797819	0	0	0	38581907	Pill
1	0	11068194	0	0	0	6665552	Fish
2	0	37446371	0	0	0	35940067	Pill
3	0	29716113	0	0	0	21587720	Fish
4	0	14852330	0	0	0	11302885	Pill
5	0	9430774	0	0	0	5764101	Fish
6	0	61761508	0	0	0	71366925	Pill
7	0	38314364	0	0	0	32811502	Fish
8	0	0	0	0	0	0	Pill
9	0	43655973	0	0	0	36708679	Fish
10	0	0	0	0	0	0	Pill
11	0	68620990	0	0	0	61837005	Fish
12	0	72563216	0	0	0	63985504	Pill
13	0	0	0	0	0	0	Fish
14	0	74004153	0	0	0	67860484	Pill
15	0	59933329	0	6627388	0	51441978	Fish
16	0	20920351	0	0	0	12145609	Pill
17	0	0	0	0	0	0	Fish
18	0	0	0	0	0	0	Pill
19	0	1.59E+08	0	0	0	1.36E+08	Fish
20	0	68352274	0	0	0	48952255	Pill
21	0	97199788	0	0	0	81331501	Fish
22	0	45202722	0	0	0	33208495	Pill
23	0	0	0	0	0	0	Fish
36	0	0	0	0	0	0	Fish
37	2.38E+08	31863012	1.74E+08	9757891	0	1.45E+08	Pill
38	0	0	0	0	0	0	Fish
39	2.29E+08	30582590	1.7E+08	4984866	0	1.41E+08	Pill
40	0	0	0	0	0	0	Fish
41	0	0	0	0	0	0	Pill
42	0	0	0	0	0	0	Fish
43	2.85E+08	46128479	1.87E+08	20590432	0	1.77E+08	Pill
44	2.05E+08	24650521	1.38E+08	6975734	0	1.34E+08	Fish
45	2.12E+08	29345505	1.39E+08	6530899	0	1.37E+08	Pill
46	0	0	0	0	0	0	Fish
47	0	0	0	0	0	0	Pill

Table 50. Snail 9.1 (Fish Food + Vitamin Pack + Bromine) Area Under Curve Data and schedule of diet

48	0	0	0	0	0	0	Fish
49	1.22E+08	14527367	96446648	39933438	0	77052032	Pill
50	2.26E+08	33055491	1.58E+08	32260086	0	1.61E+08	Fish
51	0	0	0	0	0	0	Pill
52	0	0	0	0	0	0	Fish
53	2.5E+08	38520736	1.81E+08	43431758	0	2.11E+08	Pill
54	0	0	0	0	0	0	Fish
55	45219891	6276284	40026861	2638619	0	28377541	Pill

Appendix O : Snail 9.2 – Predominant Isolated Peaks

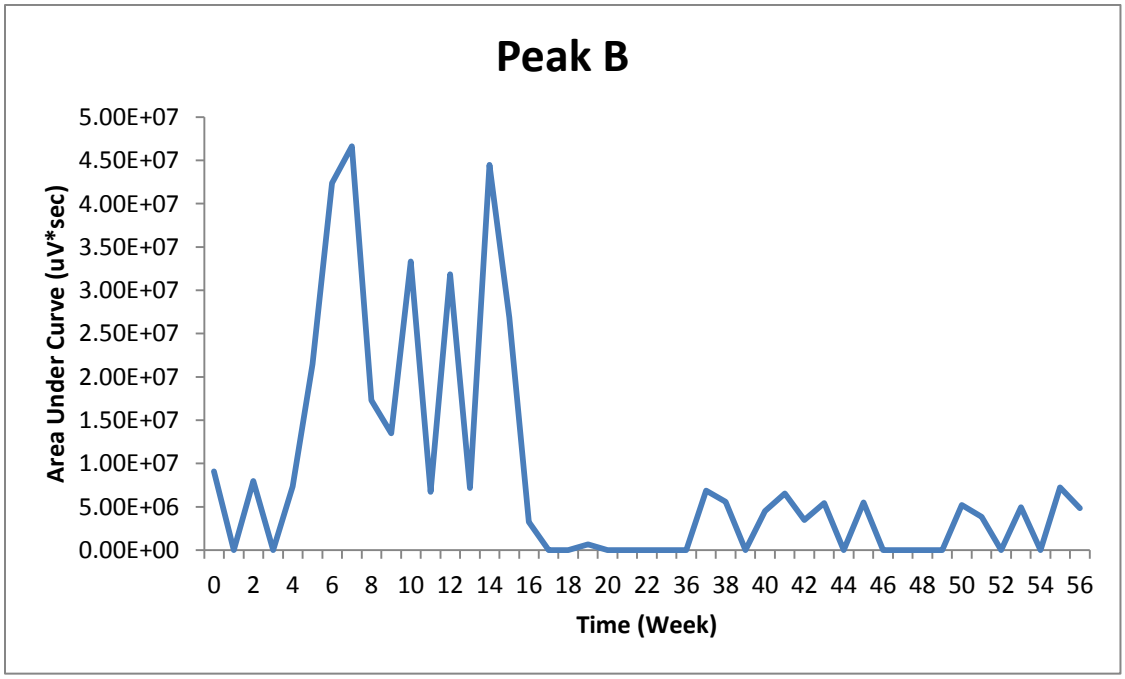

Figure 89. Illustrating area under curve for isolated Peak B.

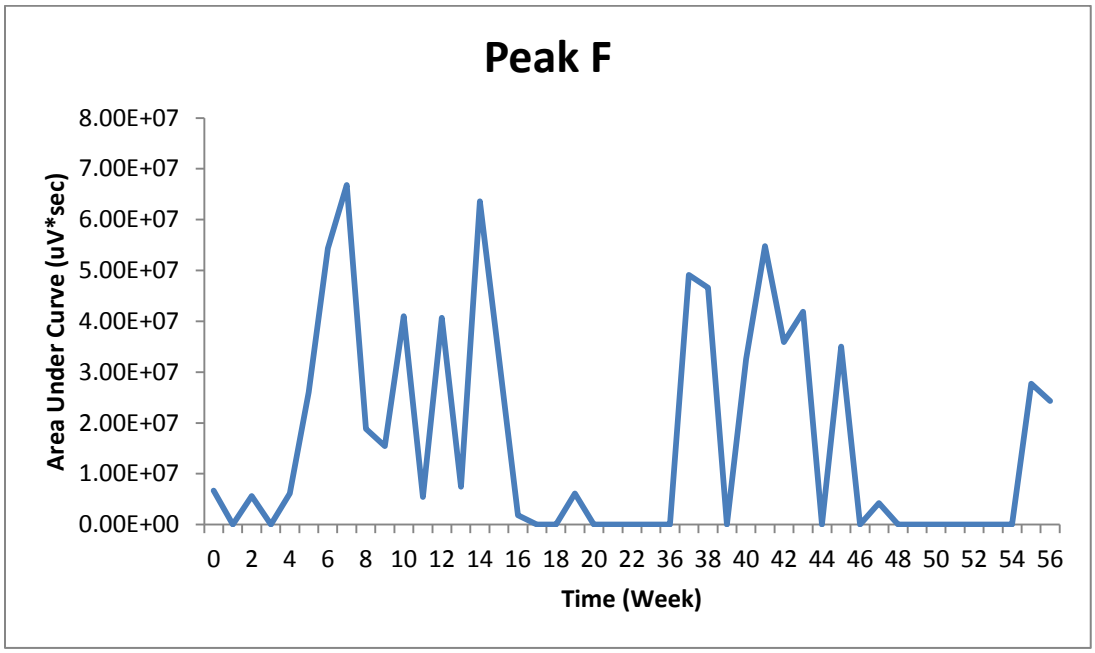

Figure 89. Illustrating area under curve for isolated Peak F.

Snail 9.2 also predominantly produced peaks B and F. It looks as though at approximately week 36 as AUC dropped for Peak B, Peak F AUC increased in response.

Table 51. Snail 9.2 (Fish Food + Vitamin Pack + Bromine) Area Under Curve Data and schedule of diet

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Diet
0	0	9097105	0	0	0	6694204	Pill
1	0	0	0	0	0	0	Fish
2	0	7984035	0	0	0	5620462	Pill
3	0	0	0	0	0	0	Fish
4	0	7338836	0	0	0	6134521	Pill
5	0	21458792	0	0	0	26028042	Fish
6	1194094	42364903	0	0	0	54341051	Pill
7	1123392	46633168	0	0	0	66832730	Fish
8	0	17290347	0	0	0	18819628	Pill
9	0	13477329	0	0	0	15439459	Fish
10	0	33341325	0	0	0	41023121	Pill

Table 52. Snail 9.2 (Fish Food + Vitamin Pack + Bromine) Area Under Curve Data and schedule of diet

11	0	6719320	0	0	0	5428202	Fish
12	0	31840227	0	0	0	40674851	Pill
13	0	7170919	0	0	0	7439546	Fish
14	0	44494226	0	0	0	63595605	Pill
15	0	26872556	0	0	0	33128079	Fish
16	0	3251106	0	0	0	1830067	Pill
17	0	0	0	0	0	0	Fish
18	0	0	0	0	0	0	Pill
19	0	667934	0	0	0	6148070	Fish
20	0	0	0	0	0	0	Pill
21	0	0	0	0	0	0	Fish
22	0	0	0	0	0	0	Pill
35	0	0	0	0	0	0	Pill
36	0	0	0	0	0	0	Fish
37	55279644	6877720	88405148	0	71250913	49148767	Pill
38	47124270	5588906	92507124	0	61828053	46612717	Fish
39	0	0	0	0	0	0	Pill
40	34056708	4512750	72474772	0	44045248	32594667	Fish
41	52879043	6553903	1.01E+08	0	67138677	54777041	Pill
42	29070350	3494304	63214464	0	44130436	35923258	Fish
43	37918880	5422917	75754667	0	49358406	41857956	Pill
44	0	0	0	0	0	0	Fish
45	26694053	5504514	5.12E+08	0	37481128	35016878	Pill
46	6773304	0	12115062	0	8161342	0	Fish
47	9323522	0	14545599	0	19479072	4225238	Pill
48	0	0	0	0	0	0	Fish
49	0	0	0	0	0	0	Pill
50	0	5225384	7639415	0	5596314	0	Fish
51	0	3864407	5372162	0	3885141	0	Pill
52	0	0	0	0	0	0	Fish
53	0	4946159	10619531	0	8287677	0	Pill
54	0	0	0	0	0	0	Fish
55	28695405	7246201	53918094	0	43076068	27723223	Pill
56	25901762	4833166	43938764	0	35171073	24305953	Fish

Appendix P : Snail 2.1 – Predominant Isolated Peaks

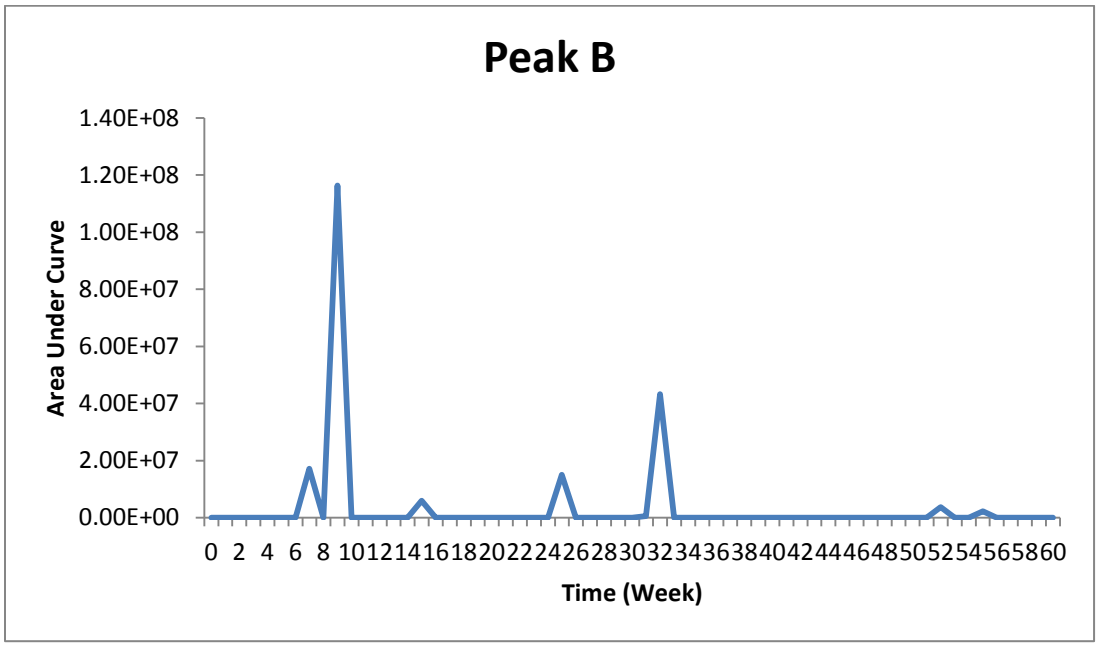

Figure 90. Illustrating area under curve for isolated Peak B.

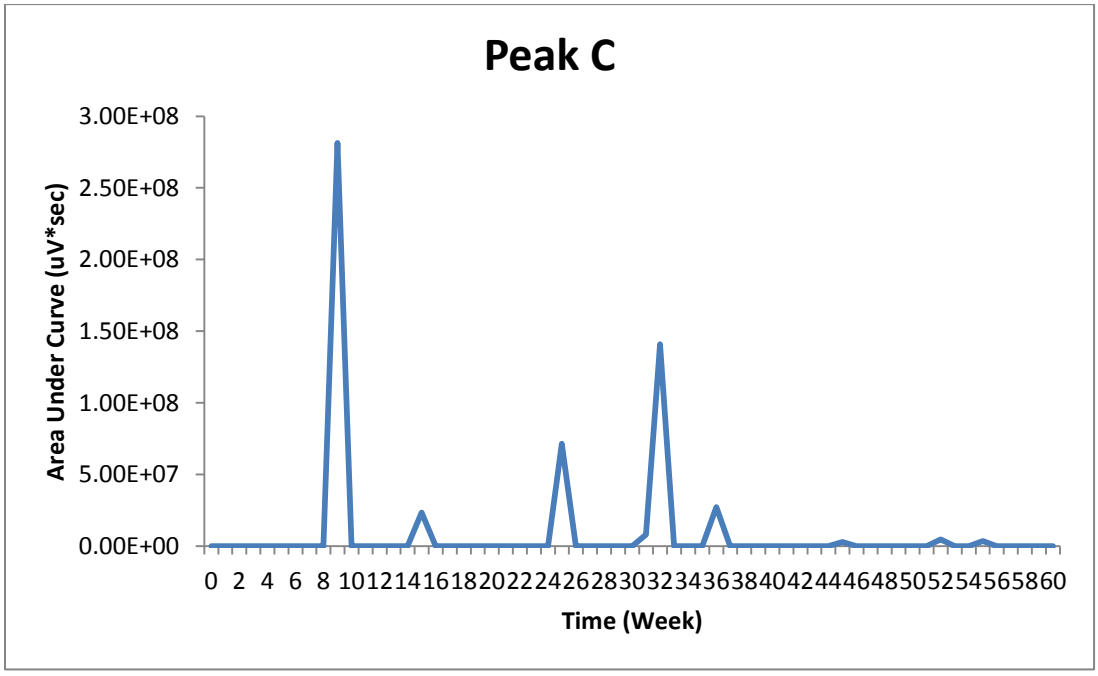

Figure 91. Illustrating area under curve for isolated Peak C.

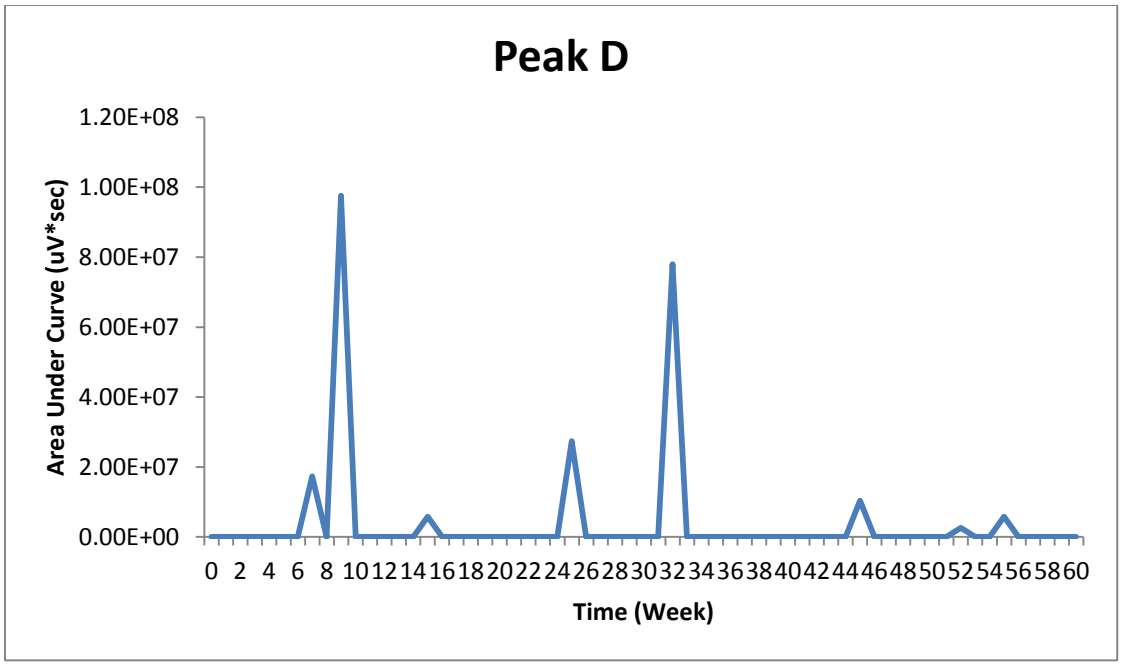

Figure 92. Illustrating area under curve for isolated Peak D.

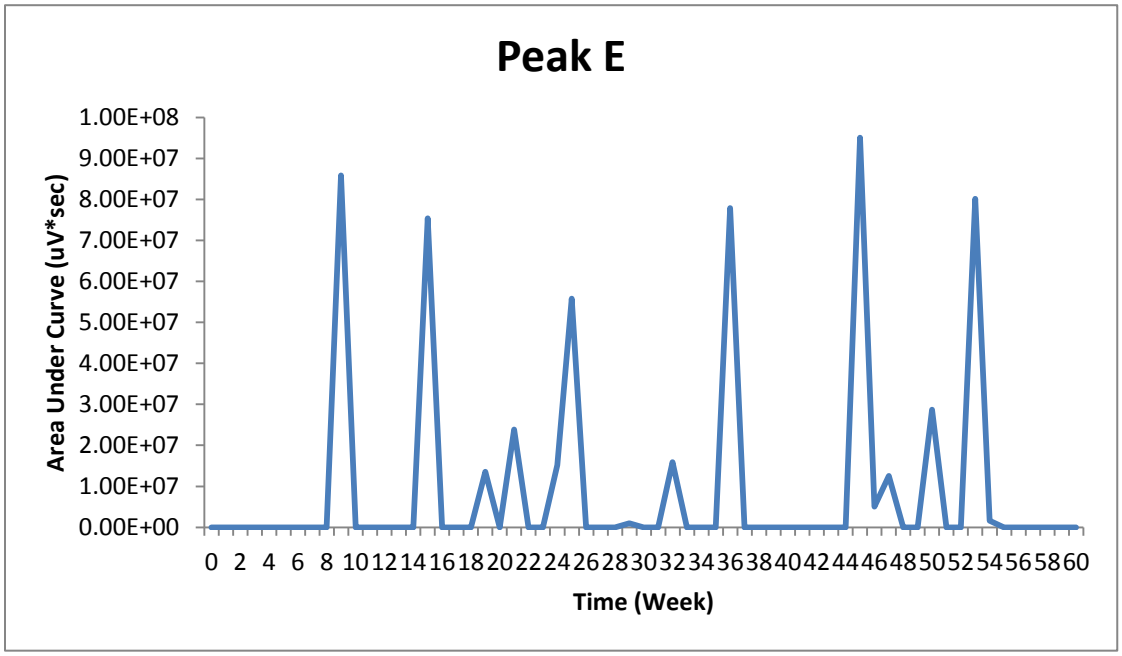

Figure 93. Illustrating area under curve for isolated Peak E.

Control snail 2.1 predominantly produced Peaks B, C, D, and E. Profiles for Peaks B, C, D, and E mirror each other. Peak E has more consistent spikes in AUC.

Table 53. Snail 2.1 (Control - Fish) Area Under Curve Data

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F
6	0	0	0	0	0	0
7	7388625	17201323	0	17363142	0	0
8	0	0	0	0	0	0
9	1.64E+08	1.16E+08	2.81E+08	97539920	85843210	1.43E+08
10	0	0	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	0	5974486	23415153	5784961	75399403	7948058
16	0	0	0	0	0	0
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	13514345	0
20	0	0	0	0	0	0
21	0	0	0	0	23854260	0
22	0	0	0	0	0	0
23	0	0	0	0	0	0
24	0	0	0	0	15202930	0
25	0	15072920	71461465	27425541	55822968	6931953
26	0	0	0	0	0	0
27	0	0	0	0	0	0
28	0	0	0	0	0	0
29	0	0	0	0	965828	0
30	0	0	0	0	0	0
31	8610720	619202	7948102	0	0	1117082
32	47523014	43309227	1.41E+08	77936905	15925435	59603898
33	0	0	0	0	0	0
34	0	0	0	0	0	0
35	0	0	0	0	0	0
36	0	0	27127919	0	77886159	7088760
37	0	0	0	0	0	0
38	0	0	0	0	0	0
39	0	0	0	0	0	0
40	0	0	0	0	0	0
41	0	0	0	0	0	0
42	0	0	0	0	0	0

Table 54. Snail 2.1 (Control - Fish) Area Under Curve Data

43	0	0	0	0	0	0
44	0	0	0	0	0	0
45	0	0	2794395	10385764	95087261	0
46	0	0	0	0	5025270	0
47	0	0	0	0	12529700	0
48	0	0	0	0	0	0
49	0	0	0	0	0	0
50	0	0	0	0	28660752	0
51	0	0	0	0	0	0
52	0	3756302	4723760	2605660	0	0
53	0	0	0	0	80180113	0
54	0	0	0	0	1644674	0
55	0	2231387	3483066	5846002	0	0
56	0	0	0	0	0	0
57	0	0	0	0	0	0
58	0	0	0	0	0	0
59	0	0	0	0	0	0
60	0	0	0	0	0	0

Appendix Q : Snail 4.2 – Predominant Isolated Peaks

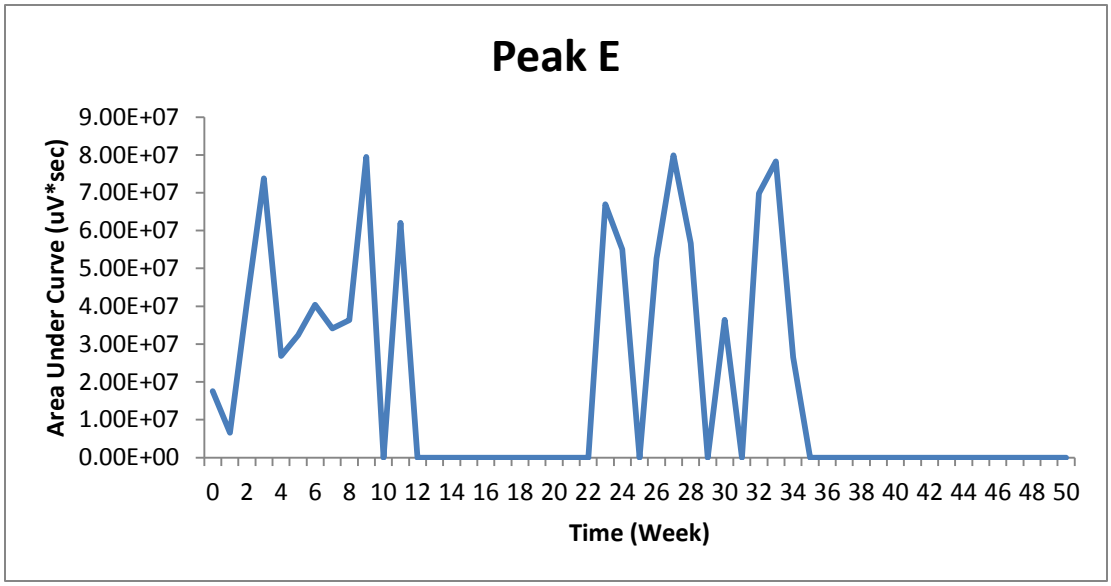

Figure 94. Illustrating area under curve for isolated Peak E.

Control snail 4.2 predominantly produced Peaks E.

Table 55. Snail 4.2 (Control - Fish) Area Under Curve Data

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F
0	0	0	0	0	17543953	0
1	0	0	0	0	6577686	0
2	0	0	0	0	41022791	0
3	0	0	0	0	73817516	0
4	0	0	0	0	26883124	0
5	0	0	0	0	32323049	0
6	0	0	0	0	40374389	0
7	0	0	5975745	0	34150289	0
8	0	0	0	0	36307513	0
9	0	0	0	0	79493815	0
10	0	0	0	0	0	0
11	0	0	0	0	62069904	0
12	0	0	0	0	0	0

Table 56. Snail 4.2 (Control - Fish) Area Under Curve Data

13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0
16	0	0	0	0	0	0
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	0	0	0	0	0	0
21	0	0	0	0	0	0
22	0	0	0	0	0	0
23	0	0	0	0	66950785	0
24	0	0	0	0	55032791	0
25	0	0	0	0	0	0
26	0	0	0	0	52672796	0
27	0	0	0	0	79894143	0
28	0	0	0	0	56616716	0
29	0	0	4285161	0	0	0
30	0	0	0	0	36446805	0
31	0	0	0	0	0	0
32	0	0	0	0	69807283	0
33	0	0	0	0	78255468	0
34	0	0	0	0	26559861	0
35	0	0	0	0	0	0
36	0	0	0	0	0	0
37	0	0	0	0	0	0
38	0	0	0	0	0	0
39	0	0	0	0	0	0
40	0	0	0	0	0	0
41	0	0	0	0	0	0
42	0	0	0	0	0	0
43	0	0	0	0	0	0
44	0	0	0	0	0	0
45	0	0	0	0	0	0
46	0	0	0	0	0	0
47	0	0	0	0	0	0
48	0	0	0	0	0	0
49	0	0	0	0	0	0
50	0	0	0	0	0	0

Appendix R : Snail 7.1 – Predominant Isolated Peaks

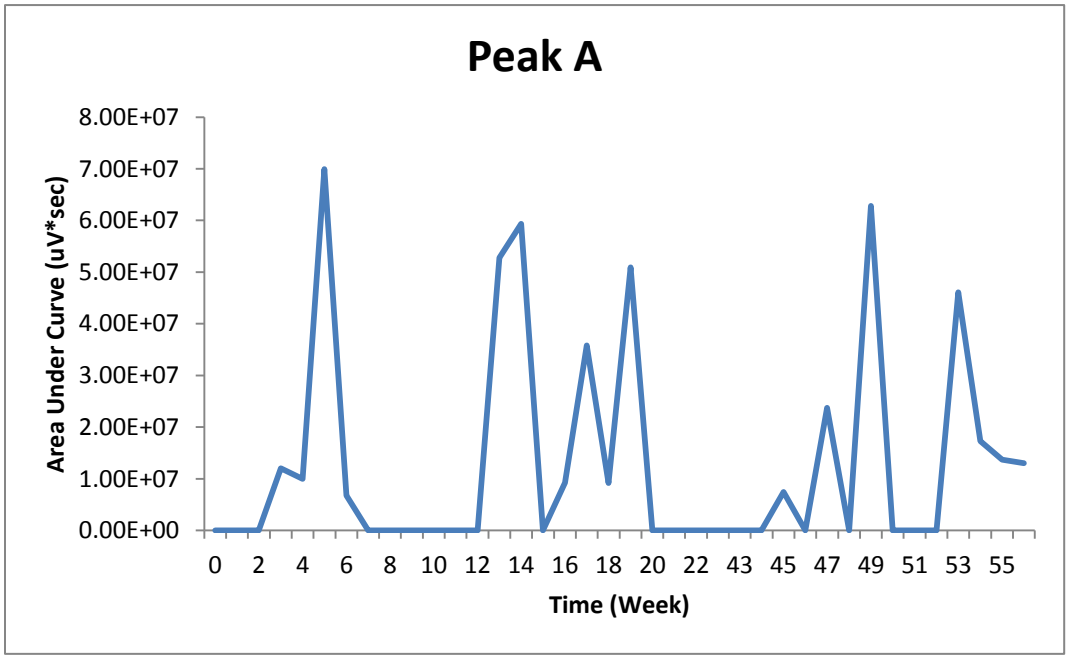

Figure 95. Illustrating area under curve for isolated Peak A.

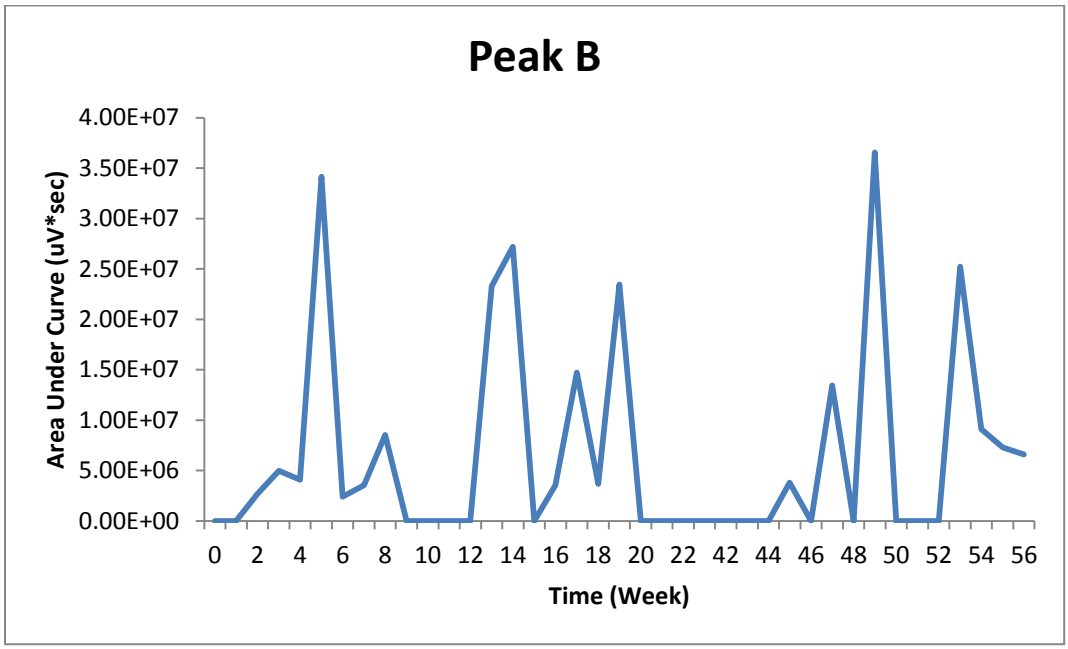

Figure 96. Illustrating area under curve for isolated Peak B.

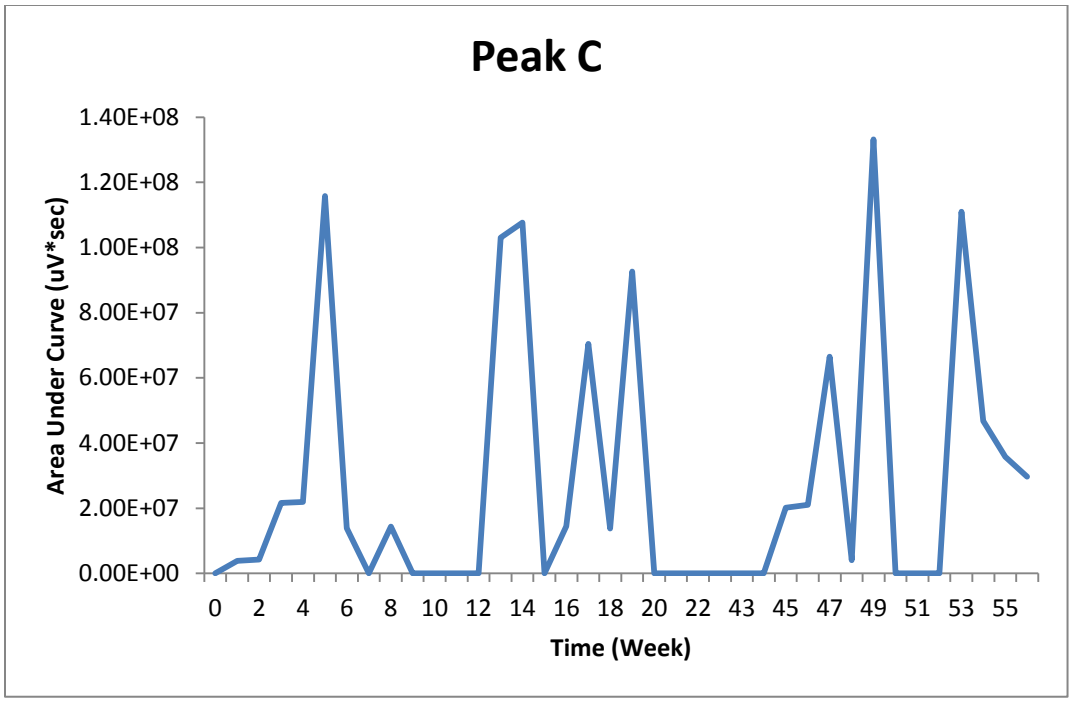

Figure 97. Illustrating area under curve for isolated Peak C.

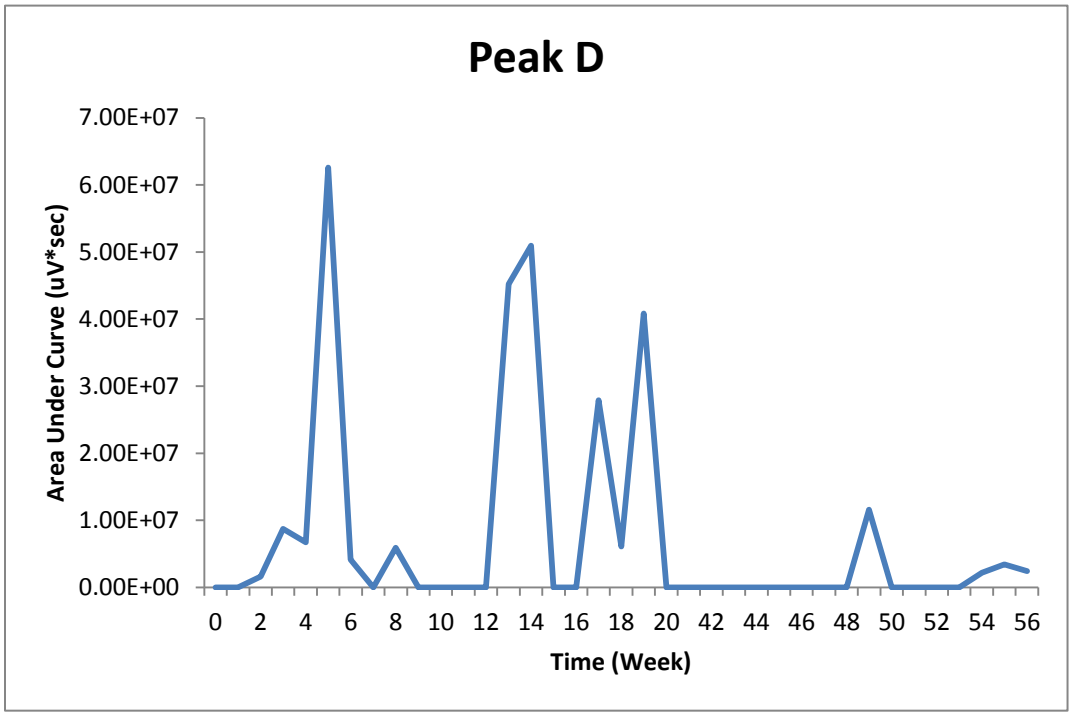

Figure 98. Illustrating area under curve for isolated Peak D.

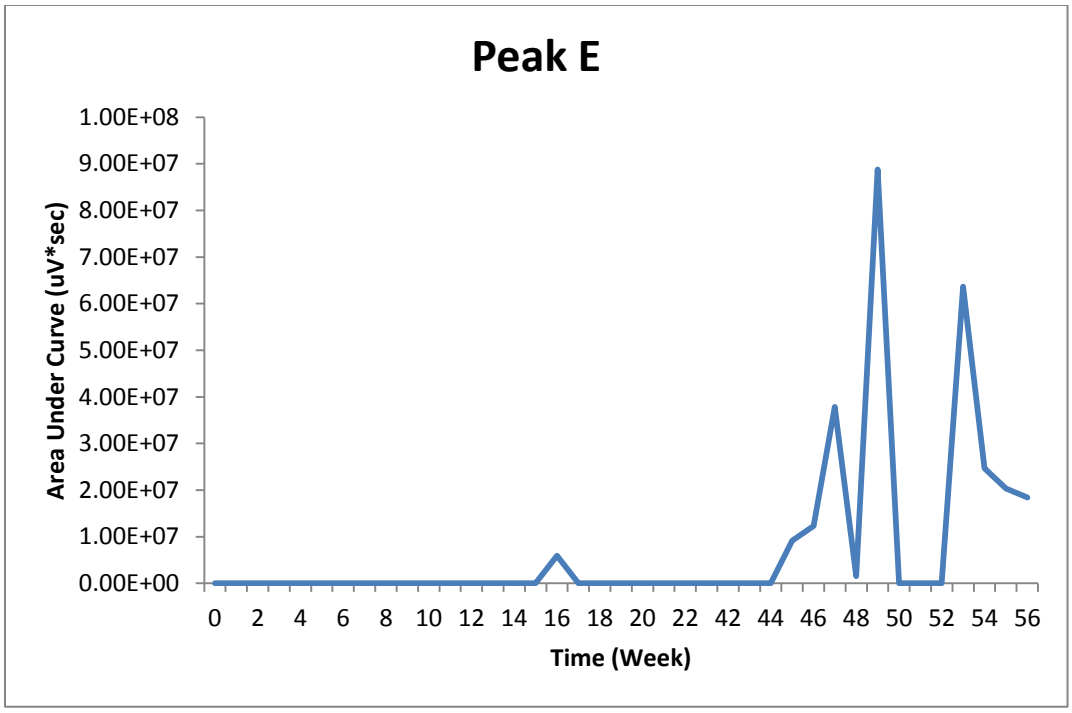

Figure 99. Illustrating area under curve for isolated Peak E.

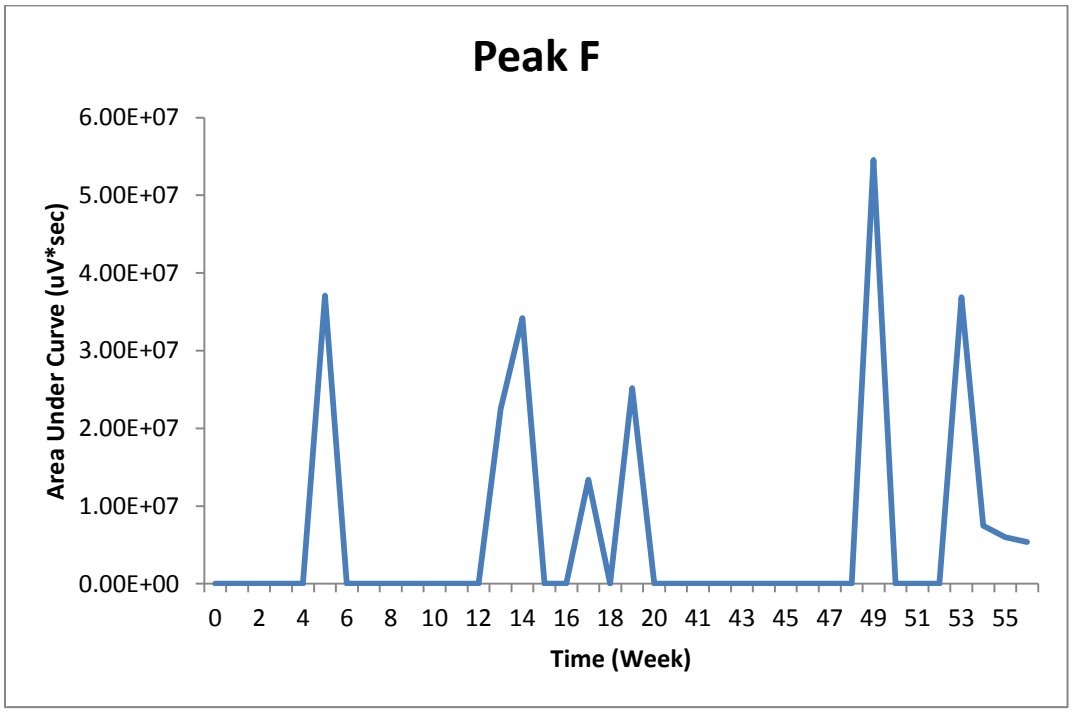

Figure 100. Illustrating area under curve for isolated Peak F.

Snail 7.1 predominantly produced Peaks A – F. With varying AUCs, the individual peak profiles almost mirror each other.

Table 57. Snail 7.1 (Control - Fish) Area Under Curve Data

Week	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F
0	0	0	0	0	0	0
1	0	0	3882498	0	0	0
2	0	2696787	4245505	1604561	0	0
3	12008773	4975531	21649361	8728697	0	0
4	9992847	4092908	21935097	6729167	0	0
5	69920967	34144828	1.16E+08	62606705	0	37087938
6	6758799	2416298	13866686	4122221	0	0
7	0	3541686	0	0	0	0
8	0	8539507	14328035	5893465	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	52805303	23302015	1.03E+08	45224146	0	22509947
14	59351026	27197502	1.08E+08	50944357	0	34193561
15	0	0	0	0	0	0
16	9215208	3555178	14493838	0	5967311	0
17	35782416	14727108	70430124	27894966	0	13392676
18	9178090	3674726	13800118	6118736	0	0
19	50953791	23436020	92636869	40819265	0	25180447
20	0	0	0	0	0	0
21	0	0	0	0	0	0
22	0	0	0	0	0	0
41	0	0	0	0	0	0
42	0	0	0	0	0	0
43	0	0	0	0	0	0
44	0	0	0	0	0	0
45	7431961	3799252	20148929	0	9130311	0
46	0	0	20997763	0	12301672	0
47	23739436	13467687	66517177	0	37887329	0
48	0	0	4038961	0	1529339	0
49	62818474	36575127	1.33E+08	11565200	88766209	54567832
50	0	0	0	0	0	0
51	0	0	0	0	0	0
52	0	0	0	0	0	0
53	46071403	25238655	1.11E+08	0	63674432	36860461
54	17305264	9108172	46720390	2180116	24690924	7471477
55	13668381	7281576	35791647	3406414	20354584	5992665
56	12996476	6598435	29694763	2428144	18433108	5361362

Appendix S: Snail 8.2 – Predominant Isolated Peaks

Snail 8.2 did not produce any of the isolated peaks.