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The Unmasking of Aggressive Chemosignaling via the Crayfish Model: an In-depth Urinalysis Using HPLC

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Abstract

It is well established in the scientific community that decapod crustaceans secrete molecules via nephropores to communicate with each other. These molecules have been shown by numerous conducted studies to affect the animal behavior, especially aggression. However, studies in decapods crustaceans have yet to elucidate the chemical nature of aggressive chemosignals during agonistic interactions. The main goal of this study was to identify the chemical profile of crayfish urine via HPLC during two conditions: when stressed and while fighting. The urine analysis of stressed crayfish showed that uric acid, guanosine, L-tryptophan, and N-acetylserotonin were present in the samples, minutes after the stressful conditions. On the other hand, the analysis of the water collected near the crayfish during the aggressive altercations revealed that xanthine, dopamine, and L-tryptophan were present in the samples. Also, an unknown molecule, which was only present in the intra-fight sample and not in any other sample, was detected during the chemical investigation. Its chemical composition and secretion source remains to be found.

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List of Abbreviations

X-Organ/Sinus Gland – XO/SG

Serotonin – 5HT

M-hydroxybenzyl-hydralazine – m-HBH

Dopamine – DA

Octopamine – OA

MHPG – 3-Methoxy-4-hydroxyphenylglycol

Uric Acid – UA

Xanthine – XA

Guanosine – GUA

L-Tryptophan – L-Trp

Homovanillic Acid – HVA

N-Acetyl-Serotonin – Nac5HT

DL-4-Hydroxy-3-methoxymandelic acid – VMA

5-hydroxyindole-3-acetic acid – 5-HIAA

5-hydroxytryptophol – 5-HTPH

Parts per million – ppm

I. Introduction

Importance in the Ecosystem

Crustaceans are considered as one of the most evolutionary diverse taxa in the animal kingdom, ranging from minute microscopic plankton to deep sea spider crabs which have meter long appendages (Duffy and Thiel, 2007). This diversity has also enabled crustaceans to adapt in a variety of Earth's habitats, ranging from hydrothermal vents to tropical coral reefs, ocean abysses, rivers and lakes, and mountain tops. Amongst crustaceans, the decapod crustaceans are the larger crustacean's species and are commonly referred by the general public as sea food: shrimp, lobsters, crabs and crayfish.

Crayfish are the only members of decapod crustaceans to live in freshwater environments (Crandall and Buhay, 2008) such as lakes, ponds, streams, rivers and other aquatic environments all over the world expect Antarctica and Africa (Taylor, 2002). They frequently reside in a variety of different habitats, ranging from sandy and muddy substrates to cobble and gravel (Taylor, 2002). To date, there are more than 640 species of crayfish classified in 3 different families: Astacidae, Cambaridae and Parastacidae (Crandall and Buhay, 2008). The Astacidae family is located mostly in Europe and Asia (31 species) and in North America (8), the Cambarid crayfish live mostly in North America (>420) and the Parastacidae habituate the Southern hemisphere.

In most ecosystems, crayfish play a critical role in maintaining the balance of the food chain. They are classified as omnivores since their dietary regime varies from detritus and macrocytes to carrion (Hill and Lodge, 1995). In streams and lakes, crayfish are also able to break up terrestrial leaf material, which will be later consumed by other aquatic species. Crayfish serve

as food for many different types of fish species ranging from bass to the northern pike and also have an impact on plant and algal communities in aquatic ecosystems.

Faxonius rusticus (known previously as *Orconectes rusticus*) is a brown to rusty-colored

Figure 1. Male rusty crayfish

freshwater crayfish that grows up to 6 inches in length and resembles a miniature lobster as seen in **(Figure 1)**. According to the Michigan Department of Natural Recourses (DNR), the *F. rusticus* ("rusty") species is usually introduced into the rivers and lakes by the fishermen, which collect and use them as fish bait during fishing (Invasive Species - Rusty Crayfish, n.d.). When introduced, it can reproduce quickly and establish significant numbers, while consuming almost every biotic item in a freshwater system (Gunderson, 1998). The juvenile crayfish diet consists mostly of benthic invertebrates, whereas the adults are opportunistic omnivores. In addition, rusty crayfish are less likely to become prey for predatorial fish or other crayfish species as they grow quickly and are relatively larger compared to other species. Another significant concern with the invasive rusty crayfish is the destruction of aquatic plant beds (Olsen *et al.,* 1991), which consequently affects other species that require these aquatic plants (Wilson *et al.,* 2004). Lastly, due to its aggressive nature, rusty crayfish have been shown to displace other crayfish species from lakes (Capelli, 1982; Lodge *et al.,* 1986; Olsen *et al.,* 1991; Garvey *et al.,* 1994; Hill

and Lodge, 1994; Olden *et al.,* 2006). They are better at occupying shelters for protection and typically outcompete other crayfish species for food sources (Garvey *et al.,* 1994; Hill and Lodge, 1994). A more comprehensive distribution of rusty crayfish is shown in **(Figure 2)**.

Functional Morphology and Anatomy

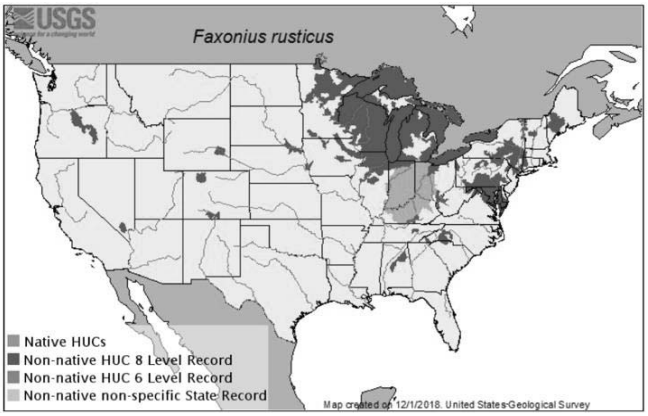


Figure 2. Faxonius rusticus distribution in the US. USGS [2019]. Nonindigenous Aquatic Species Database. Gainesville, Florida. Accessed 3/12/2019

External Anatomy

Like other arthropods, decapod crustaceans have a segmented body covered by a strong chitinous exoskeleton which protect the internal organs of the animal. Unlike an endoskeleton, the exoskeleton is shed and replaced during the molting process as the result of body growth (Duffy and Thiel, 2007). The body of most decapod crustaceans is divided into 3 regions: the head, thorax and abdomen. The head and the anterior thoracic region fuse together to form a cephalothorax, which serves as a protective carapace for the major internal organs. Furthermore, the carapace can be calcified with calcium carbonate and/or equipped with numerous spines to be a stronger armor against predators (Duffy and Thiel, 2007). The abdomen consists of different muscles and appendages that to support swimming movements.

In addition, all decapod species possess two pairs of antennae and antennules, which are mainly involved with the chemical sensation in crayfish **(Figure 3a,b)** (Duffy and Thiel, 2007; Breithaupt, 2011). In order to provide a direct access with the dissolved chemicals, the chemosensory organs have developed special small hair-like projections called sensilla (Hallberg and Skog, 2011). Sensilla are small in size and contain sensory cells called setae **(Figure 3c)**.

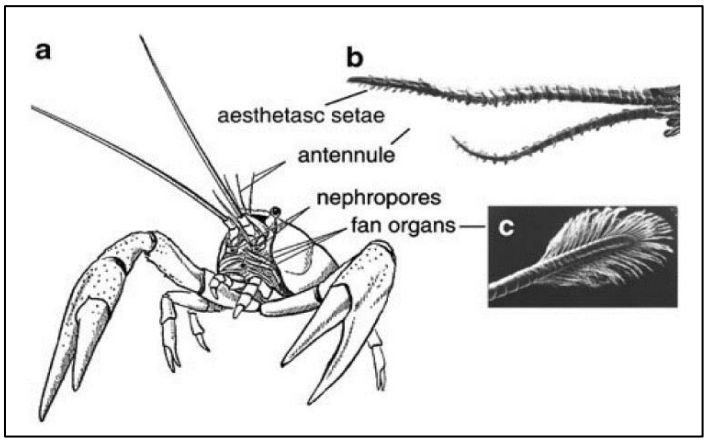


Figure 3. Crayfish's sensory organs: (a) antennae, (b) antennules and (c) setae. Adapted from (Breithaupt, 2011)

Setae assist in activities related to digging, feeding, filtering, sensing, mating and incubation of developing offspring. Since they are involved with mechano- and chemosensory functions, the setae are often concentrated anteriorly, more specifically on the antennae, near the mouth and front appendages (Breithaupt, 2011). The setae are characterized by a terminal pore through which the sensory cells protrude and contact the outside world, the waterborne molecules. One of the most important chemosensory setae are the aesthetascs, which are mostly stimulated by waterborne chemicals **(Figure 3b)**. The constant movement of the antennae reduces the thickness of the boundary layer around the aesthetasc and facilitates chemoreception of the chemicals transported via the water currents towards the crayfish (Goldman and Patek, 2002). Thus, having a very well developed chemosignaling infrastructure provides the decapod

crustaceans a good way to communicate chemically in order to transmit messages between them.

Internal Anatomy

Nervous System

The crustacean nervous system consists of cephalic ganglia and a ventral cord (Duffy and Thiel, 2007). Crustaceans obtain information about their environment through a diverse array of sensory organs, as described previously. The information from these chemoreceptors is transmitted to the "brain", where further decisions are taken (Huber *et al.,* 1997b). The primitive form of the crustacean CNS is often organized like a ladder with multiple ganglia linked longitudinally by connectives and across the midline via commissures (Sandeman, 1982). The ventral cord consists of variable number of ganglia in the thorax and abdomen that are fused across the midline but separated longitudinally in many long-bodied decapod crustaceans. The brain, which is made of fused ganglia, lies anterior and dorsal to the ventral nerve cord. It is joined with the ventral nerve by two connectives that stretch around the esophagus. Areas where communication signals are integrated, and more complex behaviors are formed lie within the brain and are referred to as the cerebral or supraesophageal ganglion. The function of many neuroanatomical structures within the brain is still unknown, and their role in processing and integrating signals, especially those used during communication is not fully understood (Herberholz, 2007).

The brain of adult decapod crustaceans is only a few millimeters in size and contains some 10000 neurons that are organized into distinct neuropils, cell clusters and neural tracts

(Sandeman, 1982). The brain **(Figure 4a)** is subdivided into three regions reflecting the three ganglia that have fused to form it:

- Protocerebrum
    - Optic ganglia – involved in visual processing (protocerebral tract).
        - Lamina ganglionaris
        - Medulla externa
        - Medulla interna
    - Lateral protocerebrum
        - This (more proximal located) and the optic ganglia are both situated in each of the two eyestalks **(Figure 4b**).
        - Made of medulla terminalis and hemiellipsoid body.
            - These integrate olfactory and other sensory inputs.
    - Median protocerebrum
        - Contains a pair of anterior and posterior lobes, the protocerebral bridge and the central body that lies between the lobes.
- Deutocerebrum
    - Includes the bilateral olfactory lobes that receive primary chemosensory input from the antennules and the lateral and median antennular neuropils, that receive information from the statocysts and from the antennular chemo- and mechanoreceptors (setae).

- Tritocerebrum
    - Contains the tegumentary neuropil, which receives afferent input through the tegumentary nerve from the dorsal carapace and the antenna II neuropils, referred also as antennal lobes, which receive mechanosensory information from the antennae.

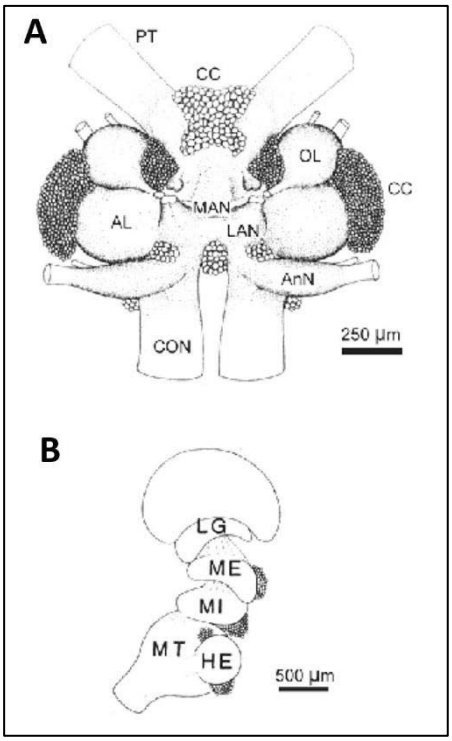


Figure 4. (A) Brain of the crayfish. Accessory lobe (AL), antenna II neuropil (AnN), cell cluster (CC), connectives (CON), lateral antennular neuropil (LAN), median antennular neuropil (MAN), olfactory lobe (OL), protocerebral tract (PT). **(B)** Eyestalk of the crayfish. Hemiellipsoid body (HE), lamina ganglionaris (LG), medulla externa (ME), medulla interna (MI) and medulla terminalis (MT). Adapted from (Herberholz, 2007).

Neuroendocrine System

Although all crustaceans are assumed to possess neuroendocrine release sites, most of the studies are done in members of decapod crustacean (Christie, 2011). Neuroendocrine sites form loosely associated clusters of cells, located along the ventral nerve cord or in peripheric

nerves. The two neuroendocrine organs in crayfish are: the X-organ – Sinus gland (XO-SG) complex and Pericardial organ (PO) shown in (**Figure 5**).

X-organ/Sinus Gland (XO-SG) Complex

In most decapods species, the XO-SG is located within the eyestalk of the animal (Cooke and Sullivan, 1982). Numerous adjacent axon terminals come togerther to form the sinus gland (SG), which is located superficially on the dorsal or dorso-lateral side of the optic ganglia **(Figure 5)**. The X-organ (XO), on the other hand, is formed by 150-200 somata of the neurosecretory cells, responsible for producing neuropeptides (Fanjul-Moles, 2006). The XO is located at the central proximal margin of the medulla terminalis and it is responsible for innervating the sinus gland (Cooke and Sullivan, 1982). The organ and the gland are connected together via the sinus gland nerve (tract), which originates from this neuropil on the dorsal or dorso-lateral side of the ganglion. The axons of this nerve project distally along the ventrolateral margin of the eyestalk until they reach the SG (Loredo-Ranjel *et al.,* 2017). Approximately 90% of the SG's terminals form the secretory terminals of the neurosecretory somata of the X-organ (Fanjul-Moles, 2006). In addition, some somata located in other regions of the eye stalk, the supraesophageal ganglion (commonly referred to as the brain) and in the thoracic ganglia are thought to project to and innervate the SG gland (Loredo-Ranjel *et al.,* 2017).

The crustacean SG is thus thought to be the major neuroendocrine center in the crayfish because it is the source of hormones that mediate many different functions (Cooke and Sullivan, 1982). These hormones are responsible for critical functions in crayfish such as molting growth, sexual maturation, and regulation of metabolism. By being superficially located on the eyestalk, the outer surface of the SG is very much accessible to the circulatory system via a

hemolymph space (Loredo-Ranjel *et al.,* 2017). Thus, the invaginating hemolymph lacunae provide circulatory access to the interior of the SG, and any released hormone will have a systemic effect.

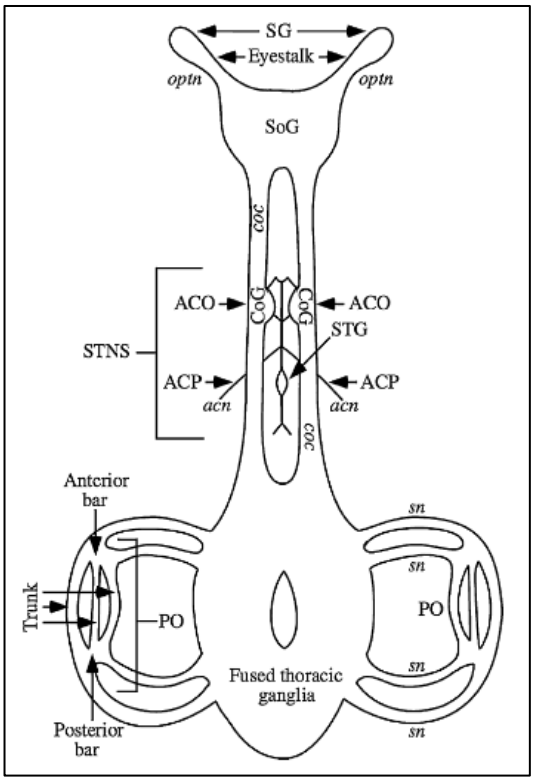


Figure 5. Neuroendocrine system in crayfish. Adapted from (Christie, 2011)

Pericardial Organ (PO)

Another important neuroendocrine center in decapod crustaceans is the pericardial organ or PO (Alexandrowicz and Carlisle, 1953). It is located along the lateral wall of the pericardial chamber that surrounds the heart **(Figure 5)** (Cooke and Sullivan, 1982; Fingerman, 1992). The proximity of the PO to the heart makes the neuromuscular system a major target for the hemolymph-borne modulators (Cooke, 2002). In members of Astacidae (clawed lobsters and crayfish) it is organized as a system of nerve trunks that divide as diffuse plexuses along the lateral walls of the pericardial chamber and on the ligaments of the heart (Christie, 2011).

Regardless of organization, the somata that innervate decapods PO include both intrinsic and extrinsic somata, thought to reside within the thoracic nervous system (Cooke and Sullivan, 1982; Fingerman, 1992).

Investigations of the decapod PO have shown that it consists of a dense collection of neurosecretory terminals located just underneath the epineurium (Cooke and Sullivan, 1982; Fingerman, 1992). Even though the XO-SG has been labeled as the main site of the neuropeptide production, the pericardial organ (PO), has been found to secrete small amount of crustacean hyperglycemic hormone (Keller *et al.,* 1985). Other studies have established that hormones secreted from the PO modulate the output of the stomatogastric neuromuscular system (Skiebe, 2001; Skiebe, 2003) and osmoregulation in air breathing decapod crustaceans (Morris, 2001).

Purine Metabolism

The metabolism of ammonia, urea and uric acid in crustaceans is believed to be similar to the other known pathways in animal phyla **(Figure 6)** (Claybrook, 1983; Weihrauch *et al.,* 2004). In this metabolic pathway, there are two main routes by which ammonia is produced: via deamination of amino acid and/or degradation of purines. Deamination of AMP, adenosine and guanine has been observed in some of the crustaceans (Roush and Betz, 1956). In addition, the three enzymes which finalize the breakdown of purine rings to ammonia, uricase, allantoinase and allantoicase, have also been detected in *F. rusticus* (Truszkowski and Chajkinówna, 1935; Sharma and Neveu, 1971). The presence of the uric acid (UA) in urine is a good indicator of the catabolism of adenine and guanine, since crustaceans lack the ability to synthesize these compounds de novo (Claybrook, 1983).

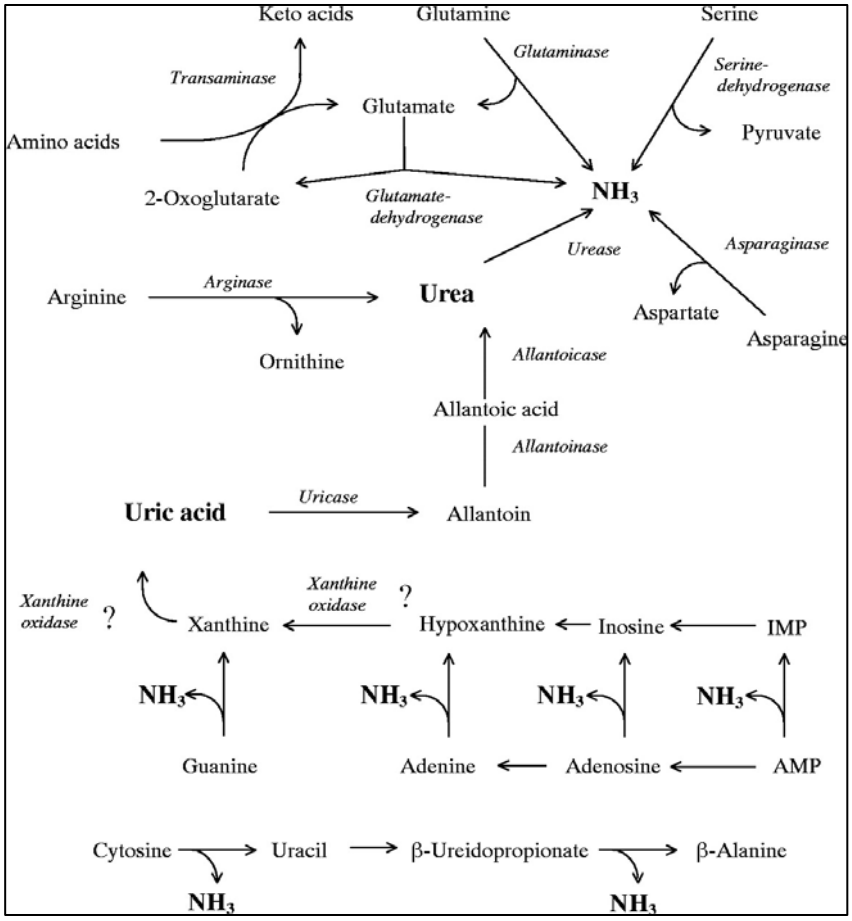


Figure 6. Purine metabolism pathway. The involvement of the enzymes in this pathway that are labeled with "?" is yet to be clarified. Adapted from (Weihrauch, 2004)

Agonistic Interactions and Social Dominance

Aggression is one of the most universal behaviors among species. Aggressive encounters between animals of the same species have been termed "agonistic behavior" in order to differentiate these behaviors from other interactions such as the aggression in predator-prey and nonsocial interactions (Moore, 2007). Agonistic interaction often follow strictly ritualized displays that usually result in nonlethal consequences. The displays may contain various forms of communication such as auditory, mechanical, visual and chemical signal, responsible for

transmitting appropriate information such as size or reproductive state of the individual (Moore, 2007). The primary effect of these interactions is the establishment of a dominance relationship that confers a hierarchy for resources such as food and shelters. Normally, dominant individuals acquire more and/or maintain longer control over resources, which can result in more mating and thereby increased evolutionary fitness of the animal (Moore, 2007).

These aggressive interactions have been shown to influence neurochemistry and can have long-lasting effects on subsequent social interactions (Huber *et al.,* 2001; Silva *et al.,* 2013). Yet, the neural and neuroendocrine mechanisms that underlie social dominance are not fully understood, largely because they include dynamic interactions within and between systems at all levels, from gene expression to complex behavioral interactions. Despite this complexity, some of the neurochemicals that appear to play significant roles in aggression and consequently influence agonistic behavior have been identified (Huber *et al.,* 2001; Audet and Anisman, 2010). Of particular importance is the fact that previous social experience of a crayfish modulates the functioning of serotonin in the nervous system (Edwards and Spitzer, 2006; Issa *et al.,* 2012). Moreover, intrinsic changes in biogenic amine neurochemistry can occur via winning or losing experiences, and these changes could be reflected in the types of chemicals released into the urine of dominant and subordinate crayfish.

General Fight Dynamics in Crayfish

Members of decapod crustaceans utilize their body posture to convey aggression/submission. An elevated body posture together with raised and spread chelipeds (meral spread) are visual signals, that usually demonstrate aggressiveness, while lowered, extended body posture and lowered claws represent visuals signals for submissiveness (Bruski and Dunham, 1987;

Herberholz, 2007). Meral spread displays have been described in many aquatic crustaceans as powerful visual signals used during intra- and interspecies encounters. This spread display is known to influence opponents to avoid engaging in more violent agonistic interactions. The fighting is initiated with one animal approaching the other and displaying the meral spread (Moore, 2007). Important factors which lead to a successful fights are body size, claw size, win/lose history, and physiological condition. In adult crayfish, the gradual decrease in the frequency and mean duration of fights has been shown to reflect the formation of a stable linear dominance hierarchy (Goessmann *et al.,* 2000). In addition, the greater the win/lose ratio of the individual is, the higher its social status/dominance will be. Finally, the maintenance of the dominance relationship was disrupted by the blockage of aesthetic hairs in P.*clarkii* (Horner *et al.,* 2008).

Factors Influencing Agonistic Behavior and Dominance

Many factors have been shown to affect aggression in agonistic interaction between crayfish. These factors include size, sex, hunger state, social experience, resource availability and shelter presence (Bergman and Moore, 2003; Davis and Huber, 2007). These same factors that influence aggression between crayfish contribute greatly to the outcome of agonistic interactions and have been shown to be accurate predictors of dominance (Davis and Huber, 2007). To fully understand the underlying mechanisms of aggression and its impact on the evolution of behavior it is necessary to better understand the extrinsic and intrinsic factors (Moore, 2007). These factors interact to influence aggression and how this leads to dominance.

Intrinsic Factors

Intrinsic factors are dependent on the individual crayfish, mainly on physical and physiological aspects of the crayfish. These factors include sex, carapace size, chelae size, social experience, neurochemistry and physiological state (Bovbjerg, 1956; Hazlett *et al.,* 1975; Rutherford *et al.,* 1995; Daws *et al.,* 2002; Bergman *et al.,* 2003).

Carapace and Chela Size

Size is one of the strongest determinant of whether an individual is likely to achieve dominance (Hazlett *et al.,* 1975; Davis and Huber, 2007). If the carapace length and chela size difference is less than 10% the outcome of the interaction is random (Pavey and Fielder, 1996; Daws *et al.,* 2002). If the difference is greater than 10%, the larger crayfish generally becomes dominant. Crayfish with larger chelae, when carapace length is similar also tend to become dominant (Rutherford *et al.,* 1995). During agonistic interactions, crayfish use their chela as a signal of aggression during meral spreads (Bruski and Dunham, 1987). During meral spread, a crayfish will spread its major chelae, displaying carapace size and chelae size. If crayfish continue to escalate their interactions after meral spread, their interaction may lead to more intense use of chelae (Schroeder and Huber, 2001). While fighting, they may continue to assess their opponent to reduce the risk of injury. Male crayfish lacking one chela have fewer aggressive displays, initiate fewer agonistic encounters and ultimately rank lower in hierarchies than do crayfish with intact chelae (Gherardi *et al.,* 1999).

Sex

Sex is another important intrinsic factor that determines aggression and dominance in crayfish. Male crayfish are typically dominant over females, but maternal females have been

shown to have an increased aggression leading to higher placement in social hierarchies when compared to nonmaternal females (Figler *et al.,* 1995; Peeke *et al.,* 1995). Generally, males will have larger carapace length and chelae size when compared to females. In social communities of crayfish, males are typically on the top of the hierarchy, even if older females are larger. This is again most likely due to overall size differences between males and females although there may be other unknown underlying factors contributing to this as well.

Motivational State

Different physiological states such as hunger also alter the level of aggression and outcomes of agonistic interactions in crayfish (Hazlett *et al.,* 1975). Starvation decreases the potential for survival leading to an increase in agonistic encounters over valuable resources (Capelli and Hamilton, 1984). It was shown in an experiment done by Hazelett *et al.,* that starved crayfish engaged in more aggressive interactions than crayfish that were fully fed (Hazlett *et al.,* 1975). The starved crayfish also had an increased rate of escalation of interactions, possibly indication their willingness to take more risks involved in agonistic interactions. Also, it is important to note the fact that crayfish are omnivorous species, and cannibalism of dead crayfish in the lab settings is not uncommon.

Previous Social Experience

Previous social experience is also a determinant of an individual's success in agonistic interactions. Crayfish lacking social interactions for seven days appear to interact as if they are socially naïve (Schneider *et al.,* 1999). Repeated previous social interactions contribute to the level of aggression and influence the outcome of future interactions (Daws *et al.,* 2002; Bergman *et al.,* 2003). Individuals that experience a win during an agonistic interaction are more

likely to win the next encounter against a familiar and naïve opponent. This increased tendency of winning is called the "winner effect". The opposite of this effect can be applied to the loser and it is more likely to lose the next encounter. The winner and loser effects influence on subsequent interactions is strong enough to overcome size difference in opponents that would otherwise accurately predict the outcome (Daws *et al.,* 2002).

These winning and losing effects can result after a single encounter that varies in duration, intensity, and repetition. Short term effects were produced from a single short encounter lasting no longer than 30 seconds (Bergman *et al.,* 2003). The effects were strengthened with repeated encounters over extended periods of time and depended on reinforcement through repeated encounters as the effect was observed to decrease after an hour. The largest influence appeared within the first 20 minutes after the first encounter. In an experiment performed by Hsu and Wolf on the winner and loser effect of *Rivulus marmoratus*, the effect lasted for at least 48 hours (Hsu and Wolf, 1999).

The mechanism of the winner and loser effect is not clear and there are a few theories in regard to an individual's change in behavior. It does not appear as if these changes are due to long-term intrinsic physiologic changes as the effect could be demonstrated after a single encounter of 30 seconds (Bergman *et al.,* 2003). The change could be related to motivation to engage in interaction, changes in the motivation and behavior could be related the neurochemistry of the individual as the effects are short-termed. Short-term neurochemical changes would be consistent with the short-term changes in behavior which could also be reinforces through repeated encounters. These effects may also alter how a crayfish perceives the fighting ability of its opponent or itself, influencing their interaction. This was not observed in the study

performed by Bergman and partners, as there was no significant change in the length of interactions or time to reach different intensity levels (Bergman *et al.,* 2003). This again seems to indicate the change is more likely related to a neurochemical or hormonal change.

Neurochemistry

It has been speculated that the behavioral difference in aggression and dominance influences nervous system chemistry and ultimately the behavior of the decapod crustaceans. Biogenic amines such as serotonin, octopamine, norepinephrine, dopamine and histamine have been shown to be the major players (Claiborne and Selverston, 1984; McClintock and Ache, 1989; Hashemzadeh-Gargari and Freschi, 1992; Yeh *et al.,* 1996; Yeh *et al.,* 1997; Edwards and Kravitz, 1997; Bergman *et al.,* 2003). It is hypothesized that individually these chemicals are engraved with specific information affecting the crayfish's overall behavior (Bergman and Moore, 2005). Intrinsic changes in neurochemistry in the crayfish "brain" could be filtered into the bladder and expressed via the urine.

Serotonin

Serotonin or 5-hydroxytryptamine or 5-HT is an indoleamine neurotransmitter and hormone (Craighead and Nemeroff, 2001). Serotonin's activity was initially described as hormonal activity in the serum from the clotted blood that caused vasoconstriction, hence the term "serum tonic factor" or serotonin. The first serotonin receptors termed "D" and "M" were characterized in guinea pig intestinal smooth muscle in 1957 (Gaddum *et al.,* 1997). The first serotonin receptors in CNS, the serotonin 1 and serotonin 2 receptor, were described in 1979, triggered an explosion of investigation and discovery in the serotonergic system (Peroutka and Snyder, 1979).

All metazoan species with organized nervous system use serotonin as a neurotransmitter (Craighead and Nemeroff, 2001). Serotonergic neurons and receptors and serotonin mediated behaviors have been described in many various species. The amount of serotonin synthesized in the CNS is controlled by the serum concentration of tryptophan (Maes *et al.,* 1990). The serum concentration determines the amount of tryptophan that crosses the blood-brain barrier, which in turn controls the amount of serotonin synthesized in the CNS (Carpenter *et al.,*

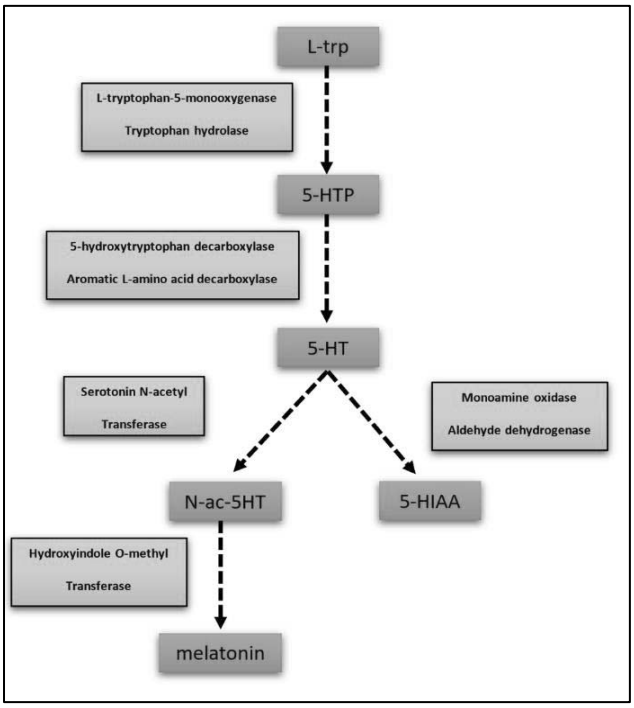


Figure 7. Biosynthesis and catabolism pathways of serotonin.
Adapted from (Craighead and Nemeroff, 2001)

1998). The biosynthesis of 5-HT in crayfish eyestalk is indicated by the presence of its immediate precursor (5-HTP) as shown in **(Figure 7)**, whereas the suppression of 5-HT production is induced by m-hydroxybenzyl-hydralazine (m-HBH), a blocker of 5-HTP decarboxylase.

The relative distribution of serotonin and its related compounds and the receptors throughout the body has been described relatively well in crayfish. Rodríguez-Sosa and partners

determined content and regional distribution of 5-HT, its precursors [L-tryptophan (L-Trp) and 5-hydroxytryptophan (5-HTP)] and of the 3 metabolites [5-hydroxytryptophol (5-HTPH), N-aceylserotonin (Nac5HT) and 5-hydroxy-indole-3-acetic acid (5-HIAA)] in the crayfish eyestalk via HPLC (Rodríguez-Sosa *et al.,* 1997). Serotonin was present in all 4 ganglia of the eyestalk. However, the distribution was not equal: medulla terminalis 40.2% had the highest concentration, whereas retina lamina ganglionaris 9.9% the lowest. As for the regional distributions of the precursors and the 3 metabolites, it was found that all of the six substances were present in the various regions of the eyestalk. The amount of the precursors was higher than that of either 5-HT and metabolites, except for the 5-HTP in the medulla terminalis. In addition, Spitzer et al. used immocytochemistry mapping and quantitative RT-PCR to localize and quantify the crustacean type 1 serotonin receptor throughout the crayfish *P.clarkii* nerve cord and in abdominal superficial flexor muscles (Spitzer *et al.,* 2005). The receptor was located in the cytoplasm and around the periphery of many somata and uniformly in neuropils throughout the nervous system such as brain and ganglia (eyestalk, circumosephageal, subesophageal and thoracic). The receptor was also expressed on nerve plexuses and on muscle tissue, implying a deep involvement in neurohormonal signaling. Take all together, these findings support the role of 5-HT as a neurotransmitter or neuromodulator in the crayfish eyestalk.

It is thought that changes in social status as a result of previous social interaction alter the function of serotonin in the nervous system of crayfish. These changes in the neurochemistry in turn affect social behavior by altering levels or aggression and dominance (Yeh *et al.,* 1997). Increased serotonin levels are closely associated with increased aggression or dominant behaviors (Edwards and Kravitz, 1997). Changes in serotonin receptor excitability have also

been observed as a consequence of achieving dominance (Yeh *et al.,* 1996). Serotonin was shown to react differently in subordinate and dominant individuals. Increased serotonergic function through injections decreased the likelihood of retreat in crayfish (Huber *et al.,* 1997b).

Serotonin has also been linked with decision making aspect of fighting crayfish. Experiments done by Huber *et al.,* showed a clear behavioral change was elicited in submissive crayfish perfused with 5-HT: both incidence and lengths of fights with dominant animals increased (Huber *et al.,* 1997b). Neurons associated with the tailflip mechanism for retreat have also been shown to exhibit reduced responsiveness due to serotonin (Edwards and Kravitz, 1997). In another study, when tested on crayfish with prior social experience, injection of serotonin or octopamine resulted in changes in aggression but did not lead to a permanent inversion of dominance as expected (Tricarico and Gherardi, 2007). Metabolites of serotonin are released with the urine and therefore may be a candidate to mediate aggressive motivation of the dominant crayfish to the subordinate receiver (Huber *et al.,* 1997a). Thus, those with increased serotonergic function in the CNS are more likely to become dominant in agonistic interactions.

Moreover, previous investigations in *P.clarkii* showed that stress induced changes in brain 5-HT concentration were responsible for inducing anxiety like behaviors (Fossat *et al.,* 2014). The researchers compare the roles of 5-HT and another biogenic amine (dopamine) in response to stress (Fossat *et al.,* 2015). By using HPLC, it was confirmed that the levels of 5-HT were significantly increased after stress are correlated to the level of anxiety like behaviors.

If serotonin and related compounds influence aggression, these same compounds also very likely influence controlled urine release as well. Aggressive behavior is effective in

intimidating opponents but only in conjunction with urine release (Breithaupt and Eger, 2002). Dominant crayfish will release urine more frequently than subordinates during encounters (Bergman *et al.,* 2005), therefore anything that changes urine release in an aggressive context likely has significant effects on the maintenance of dominance hierarchies. Studying the effects of biogenic amines requires a complex analysis of the biochemical effects on the nervous system, as well as how the subject responds behaviorally when in an altered aggressive state.

Dopamine

Dopamine (DA) or 3,4-dihydroxy-phenyl-ethyl-amine belongs to the group of chemicals known as catecholamines and it is one of the most studied major neurotransmitters in the scientific community (Craighead and Nemeroff, 2001). Dopamine synthesis starts with the conversion of the dietary amino acid tyrosine to L-DOPA by tyrosine hydrolase **(Figure 8),** which is also the rate limiting step of the pathway. Then L-DOPA gets converted into dopamine in the cytoplasm of all catecholamine neurons via dopa decarboxylase enzyme (Musacchio, 1975). Dopamine can then be metabolized into homovanillic acid (HVA), which is excreted in the urine, or be converted into the other catecholamines: norepinephrine and epinephrine. Norepinephrine and epinephrine will then be metabolized into 3-methoxy-4-hydroxyphenylglycol (MHPG) and 4-hydroxy-3-methoxymandelic acid (VMA).

There have been few studies in crustaceans to assess the role of dopamine in the aggression of the of species. In the Fossat et al. study described earlier, the levels of dopamine were also increased but were 3-5 times less abundant than 5-HT, however show no clear correlation to anxiety like behaviors (Fossat *et al.,* 2015). Furthermore, dopamine injections were not able to generate a stress or anxiety-like behaviors, compared to injections with 5-HT which did.

Treatment with chlordiazepoxide did not alter the levels of 5-HT or DA, supporting the fact that suppression of anxiety like behaviors by GABA-A receptor ligand is independent of changes in crayfish bioamine levels. On the other hand, DA has also been shown to contribute in the stress

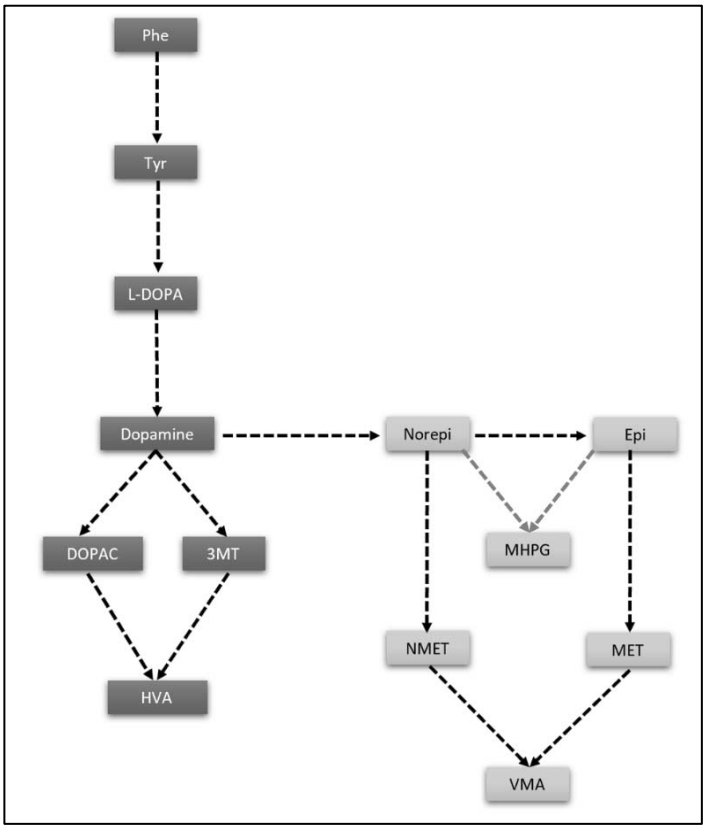


Figure 8. Biosynthesis and catabolism pathways of catecholamines. Adapted from (Musacchio, 1975).

response by accelerating the heartbeat in crayfish and crabs (Florey and Rathmayer, 1978). Dopamine has also been implicated in the release of CHH from the eyestalk ganglia of *P.clarkii* using ELISA tests (Zou *et al.,* 2003). These studies suggest that dopamine has more of support role in the stress/aggression response compared to 5-HT.

Octopamine

Octopamine (OA) has been referred to as the "fight or flight" hormone of insects (Adamo *et al.,* 1995). Octopamine levels in the cricket *Gryllus bimaculatus* increase during aggressive (agonistic) behavior, regardless of whether the cricket wins or loses the encounter. However they do not exhibit an increase in octopamine levels after performing an escape run. Therefore the neurohormonal octopamine shows some but not all of the characteristics that would be expected if it were a component of a nonspecific "arousal" system. Rather, octopamine may be released as a neurohormone to prepare the animal for a period of extended activity or to assist the animal in recovering from a period of increased energy demand (Adamo *et al.,* 1995). Octopamine as a neurohormone can induce increases in circulating levels of both lipids and sugars (Woodring *et al.,* 1989). Because octopamine levels were found to increase in the hemolymph of crickets after enforced exercise, they suggested that it may be released during "stressful" behaviors in order to mobilize energy stores.

Serotonin and octopamine have been implicated as modulators of posture and behavior in several crustaceans. Antonsen and Paul injected serotonin and octopamine in the ventral hemolymph sinus of the squat lobster *Munida quadrispina* to evaluate the potential roles of these amines in modulating agonistic behaviors (Antonsen and Paul, 1997). Injected serotonin elicits postures and behaviors in isolated individuals similar to those typical of aggressive, normally interacting animals. Injected octopamine can produce postures and behaviors typical of submissive animals and elicit behaviors which imply a modulatory role for octopamine in tailflipping. The effects of both amines are reversible and dose dependent, and the dose response curves parallel the normal progression of agonistic behaviors. However the behavioral roles and

perhaps also the mechanisms of action of 5HT and OA clearly differ among crustacean (Antonsen and Paul, 1997). In conclusion, while injections of serotonin cause anxiety like behaviors in crayfish (Fossat *et al.,* 2014) which most likely fuel the aggressive and dominance display (meral spread), octopamine injections have been shown to trigger a typical subordinate stance in other decapods such as lobsters (Livingstone *et al.,* 1980).

Hormones

There has been a lot of interest from the scientific community regarding neurosecretion and neuroendocrine modulation. It was first observed in 1944 by Abramowitz et al. that injection of crude eyestalk extract induce a hyperglycemic response (Abramowitz *et al.,* 1944). The hormone responsible for this diabetogenic effect was later characterized as the crustacean hyperglycemic hormone or CHH or HGH (Kleinholz *et al.,* 1967; Keller and Andrew, 1973; Kleinholz, 1976). CHH is a neuropeptide that is synthesized, stored and released by neuroendocrine cells in the medulla terminalis of X-organ in the crustacean eye stalk (Loredo-Ranjel *et al.,* 2017). CHH has also been detected using immunocytochemistry in the second roots of subesophagic ganglion of the lobster *A. Homarus* (Chang *et al.,* 1999) and in the retina of the *P. clarkii* crayfish (Escamilla-Chimal *et al.,* 2001). Loredo-Ranjel was able to observe CHH mRNA in cell clusters located in both the eyestalk and the brain of *P.clarkii* (Loredo-Ranjel *et al.,* 2017). Both the cytoplasm and the nuclei of these cells expressed differential CHH positivity that was associated with the time of day, indicating that CHH transcription is likely controlled by the circadian clock in decapods. Thus, CHH may provide metabolic support and increase glucose levels to support of these regions.

As for the physiological secretion of CHH, it has also been reported that the circadian changes in CHH secretion are related to circadian changes in glucose levels in hemolymph (Hamann, 1974) in addition to both eye stalk and retina (Fanjul-Moles *et al.,* 2010). Serotonergic inputs to the medulla terminalis-X-organ are implicated to invoke the release of CHH into the circulation via the ophthalmic artery (Santos *et al.,* 2001; Escamilla-Chimal *et al.,* 2002), causing a 5-HT induced hyperglycemia (Lee *et al.,* 2001). CHH activity varies during the life cycle of the decapod crustacean and contributes to mechanism the underlying adaptation to stressful conditions through a dual feedback control system (Santos and Keller, 1993b) and that the synthesis and secretion is homeostatically controlled (Santos and Keller, 1993a; Santos *et al.,* 2001; Fanjul-Moles, 2006).

CHH also has been shown to participate in other physiological processes such as molting, reproduction and osmoregulatory responses to stress (Fanjul-Moles, 2006; Webster *et al.,* 2012). It induces hyperglycemia and hyperlipemia in the hemolymph, playing a crucial role providing glucose and lipids to the internal organs and tissues of crayfish, particularly the midgut and musculature (Kummer and Keller, 1993). CHH has been also been implicated as a modulator of aggression in crayfish *P.clarkii* (Aquiloni *et al.,* 2012). This study found that independently of the crayfish's prior social experience, CHH injections induced the expression of dominance behavior, higher glycemic levels and lower time spent motionless. For the first time their data show that similarly to serotonin, CHH enhances individual aggression (Aquiloni *et al.,* 2012).

Extrinsic Factors

Extrinsic factors are dependent on the environment and include visual signaling, chemical signaling, mechanical signaling and resources such as food, shelter and mates (Capelli and Hamilton, 1984; Bruski and Dunham, 1987; Bergman *et al.,* 2003; Bergman *et al.,* 2005). Communication is used by crayfish during agonistic interactions supposedly to provide information about an individual's social status and fighting ability. The information is transferred during interactions using various methods involving visual, chemical and mechanical signal to communicate.

Visual Communications

Visual signals contribute to crayfish aggression, particularly during initial stages of fighting (Bruski and Dunham, 1987). During encounters, crayfish will exhibit signals such as meral spread, heightened and lowered body posture, and approach and retreat behaviors. These signals communicate information about an individual to influence another conspecific they have encountered. This relayed information will allow individuals to adjust their behaviors for further interactions and can provide benefit to both crayfish. It appears that visual signals are important in agonistic interaction as crayfish exhibited changes in their fight dynamics under different light conditions (Bruski and Dunham, 1987). Behaviors such as tailflipping and retreat were performed by subordinate crayfish when dominant crayfish approached in well-lit conditions. In darker conditions, these behaviors were observed less frequently, suggesting visualization of the dominant crayfish is an important factor for subordinate crayfish (Bruski and Dunham, 1987).

Mechanical Communications

Mechanical signals such as antennal whipping and chelae contact are observed during agonistic interactions and are thought to convey tactile information between opponents (Bruski and Dunham, 1987; Bergman *et al.,* 2005). The use of information currents during agonistic interactions can also be considered mechanical communication. Although these mechanical signals have been observed, it is unclear what information is exchanged during antennal whops and chelae grasps.

Resources

Resources also have a large role influencing aggression and social behaviors in decapod crustaceans. The ability to acquire and protect resources, known as resource holding potential (RHP) can be fined by an individual's likelihood to win a fight (Parker, 1974). The ultimate consequence of attaining dominance is access to resources such as mates, shelter and food (Wilson, 1975; Fero *et al.,* 2007). In agonistic interactions, resources may be required through dominance establishment or through allocation with respect to relative dominance rank within a hierarchy.

The presence of shelter and food has been shown to increase aggression in crayfish (Capelli and Hamilton, 1984). Ownership of a resource is more likely to increase aggression to defend the resource against other conspecific (Peeke *et al.,* 1995). Crayfish have been observed to occupy and defend shelters (Capelli and Hamilton, 1984; Martin and Moore, 2007). In general, the dominant crayfish inhabit bigger shelter and are less likely to get evicted compared to less dominant conspecifics (Martin and Moore, 2008). The presence of shelters resulted in longer and more intense interactions than those involving available food resources (Bergman and

Moore, 2003). Fights on detritus patches exhibited higher overall intensities and ended with more tailflips from an opponent than when in macrophyte beds. It was concluded that fight intensity and duration correlated with resource availability. In summary, fighting intensity and levels of aggression are increased when fights occur over valuable resources.

Chemical Communications

Decapods, such as crayfish, rely heavily on olfactory signals during social interaction. Olfaction is important for recognition and determination of dominance in crayfish (Schneider *et al.,* 1999; Schneider *et al.,* 2001; Bergman *et al.,* 2003). Crayfish create and control water currents during social interactions to actively send urine or to sample urine from opponents (Breithaupt and Eger, 2002; Bergman *et al.*, 2005). Urine is released through nephropores and is almost exclusively released during social interactions. Crayfish's urine likely contains social pheromones (Schneider *et al.,* 2001; Bergman *et al.,* 2005). Antennules are one of the most important chemosensory organs in crayfish. They are involved in sending and receiving chemical signals during interactions, sex recognition and dominance status (Bruski and Dunham, 1987; Pavey and Fielder, 1996; Schneider *et al.,* 1999; Schneider *et al.,* 2001; Gherardi and Daniels, 2003). Recognition of social status in crayfish is perceived through their antennae and antennules via chemical signals (Tierney *et al.,* 1984).

Information is received through the antennules may alter crayfish's behavior during an interaction. If chemical information is blocked, agonistic interactions are longer in duration and take longer to escalate to higher levels of intensities (Schneider *et al.,* 2001). When crayfish with a winning experience fight against chemoreceptor blocked individuals, the winner effect is eliminated, indicating that chemicals are necessary in the detection of previous social

interaction (Bergman *et al.,* 2003). Crayfish will create currents, called information currents, using maxillipeds, pleopods and gills, along with nephropores propulsion to communicate past social experience. They will use currents to project or draw an opponent's urine towards their antennules (Breithaupt, 2001; Bergman *et al.,* 2005). Urine released during these interactions shows differences in the number of times urine was released and duration of release between dominant and subordinate crayfish(Bergman *et al.,* 2005).

The presentation of chemical signals alone is able to bring about a threat display (Schneider *et al.*, 1999; Breithaupt and Eger, 2002). Hence, chemical signals appear to play a role in the outcomes of social interactions as well as fighting dynamics. Crayfish exposed to dominant and subordinate odors adapted a social status that is contrary to the odors to which they were exposed, whereas crayfish exposed to odors from naïve crayfish do not alter behavior (Bergman and Moore, 2005). Based on these studies, it appears previous odor exposure through urinary signals alter subsequent interactions. Communication using urine demonstrates that chemical signaling plays a significant role in agonistic interaction between crayfish.

In conclusion, numerous studies have shown that the agonistic behavior in crayfish is a complex behavior and depends on various factors, chemical signaling being one of them. Yet, there is still a gap of knowledge in the chemical identity of the molecules (aggressive chemosignals) used to transmit signals between two opponents. In this experiment, we will aim to identify the chemical nature of the aggressive chemosignals and their implication in altering the aggressive behavior in crayfish.

II. Material and Methods

Crayfish Collection and Maintenance

Male crayfish, *F. propinquus* will be collected using nets from Muskegon river near Newaygo, Michigan between May and August. Once collected, the crayfish will be put in a bucket with aerator and transported to the crayfish lab at Grand Valley State University. The aquatic laboratory is composed of different tanks filled with dechlorinated water. The room temperature will be between 22-25^{o}C. The lights will be on a 14:10 – day:night cycle to replicate natural conditions. The tanks will be fed twice a week with Meijer brand pet rabbit food currently used as feed in the lab, usually 2-3 pellets per crayfish in the tank. While invertebrate research is not overseen by IACUC, our lab is well established with rules and proper training of new members to ensure that every animal is cared for properly. There is a weekly cleaning schedule, where each tank is cleaned from debris and filled with dechlorinated freshwater.

Preparation Work

In order to eliminate any previous established dominance, the crayfish was isolated socially and physically in 1.5-liter boxes for at least 5 days prior to any experiment. The sample size for the direct injection protocol was n= 3, for the behavioral significance study n= 17, whereas for the aggression trials 15 groups, or n= 30 crayfish. Each crayfish was removed from the tank, blot dried with a paper towel and weighed on an Ohaus balance. Next, its carapace length was measured using a caliper (Bergman *et al.,* 2003) and the data was recorded in Microsoft Excel for subsequent statistical analysis.

Behavioral Significance of the Urine

Before running the aggression protocol, we were interested to investigate the behavior significance of the urine sample and whether or not the crayfish could recognize their own urine and/or the urine from a bigger crayfish, which it had never interacted in any way. Crayfish were isolated and sized as in the previous protocol. To be more consistent with the food stimulus placement in the tank, three concentric circles were drawn in the fighting tank as shown in **(Figure 9)**. The smaller crayfish was acclimated for 10 minutes before the behavioral study was to begin. Right before the trial started, a 0.40 – 0.50 g piece of fish filet was placed at the center of the concentric circles **(Figure 9).** Then, 4.0 mL of either crayfish's own urine, a larger crayfish's urine or water was pipetted into the tank above the marked location. A blue-green food dye was added to the 4.0 mL sample in order to make it visible in the water. The behavior of the smaller crayfish in these various conditions was recorded for 5 min and the time it took to reach the food, time interacting with food and the success rate were calculated.

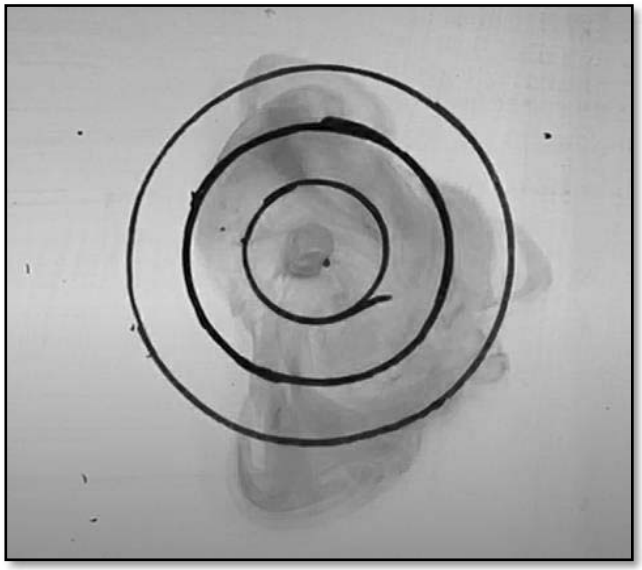


Figure 9. Food dye trials setup. Circles radii were 16 mm, 30 mm, and 45 mm respectively.

Urine Collection Protocol for HPLC

Urine Collection During Aggressive Fights

The next goal of the study was to identify the chemical profile of the urine released during agonistic interactions. To do so, urine samples were collected at various behavioral phases of crayfish such as not-fighting, fighting and post-fighting. The crayfish were size-matched, +20 % body size/weight difference, to establish a clear a winner and loser after a 15-minute interaction in the fight arena. Three mL of urine was collected from the isolation tank of each individual crayfish prior to fighting, placed in an Eppendorf tube and frozen right away in a -20 ^{o}C General Electric fridge. Then the crayfish were transferred into the fighting arena, making sure that the net was rinsed after each transfer in order to eliminate the introduction of the urine of the resting crayfish. The fight arena was made of opaque Plexiglas (40 × 40 × 14 cm), divided into four quadrants and separated by opaque retractable walls. Five liters of dechlorinated water was placed into the fighting arena and the crayfish were allowed to acclimate for at least 5 minutes. The divider was then lifted, and the two crayfish were allowed to interact for 15 minutes. The fighting was recorded using an iPhone Xs max. During the fighting time, 5 x 1mL urine samples were collected near the two crayfish. The 5 samples were pooled together and labeled as the intra-fighting samples. Next, the respective isolation tank was wiped out using a paper towel to remove any food particles and waste and each crayfish was bathed in 1.00 L of dechlorinated water. The crayfish remained there for at least 30 minutes post-fighting to allow enough time for the release of the compounds via urine. Then, 3.00 mL of the water from the isolation tank was extracted using a pipette and put into an Eppendorf tube as described above. The vial was labeled as post-fighting and stored with the other samples in the fridge. The

fighting arena was rinsed thoroughly with enough water after each fight to ensure that no odors were left behind. A summary of this study is illustrated in **(Figure 10)**.

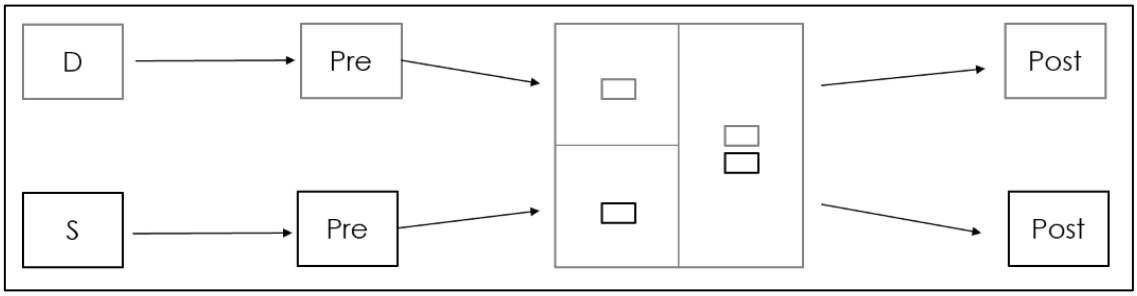


Figure 10. Aggressive trial layout. D = dominant, S = subordinate

Before each urine sample was run through HPLC, the behavioral significance of 9 of the collected samples and thereby, its contents was also determined in the following behavioral study. The loser/subordinate from the fight on day 6, was put alone in the fighting arena as shown in **(Figure 11)**. Once it was acclimated for 10 minutes in the fighting tank, 0.20 – 0.30 gr of Meijer brand farm raised tilapia fillets piece was placed on the opposite corner from him. As the crayfish was approaching the fish piece, multiple drops of urine sample (2.00 ml total) was provided on top of the food stimulus. On day 7, the subordinate was exposed to his own non-fighting urine and water (control), on day 8 with the non-fighting dominant urine and on day 9 with the urine collected during the fighting period. The behavioral response such as coming towards or running away from the corner was evaluated and the total time spend with the food was calculated. The remaining of the samples were chemically investigated via HPLC to elucidate the differences.

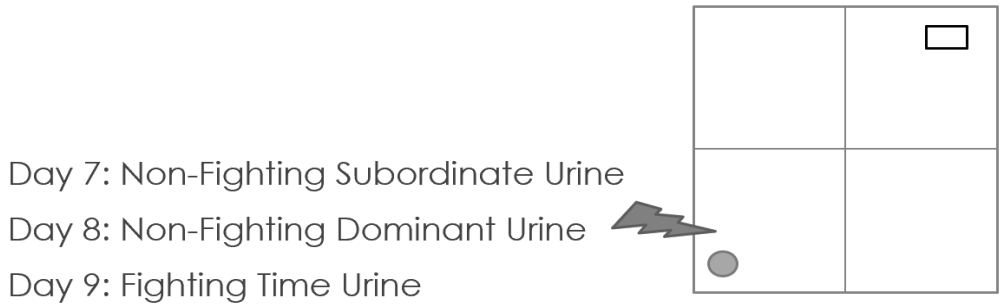


Figure 11. The behavioral significance of urine samples. Black rectangle symbolizes the subordinate crayfish from day 6 and the yellow circle, the food pellet.

Direct Urine Collection via Direct Injection

Besides investigating the urine samples during aggressive interactions, we were also very intrigued to know the chemical profile of a stressed crayfish. To do so, 3 crayfish with the following masses, subject 1 = 16.27 g, subject 2 = 10.47 g and subject 3 = 4.25 g, were used. The crayfish were secured into the Y board using rubber bands to restrict their movement **(Figure 12)**. A 25G 1 ½ syringe was gently inserted into the nephropore and the urine was extracted directly from the bladder. The samples were put into an Eppendorf tube and frozen in -20 ^{o}C.

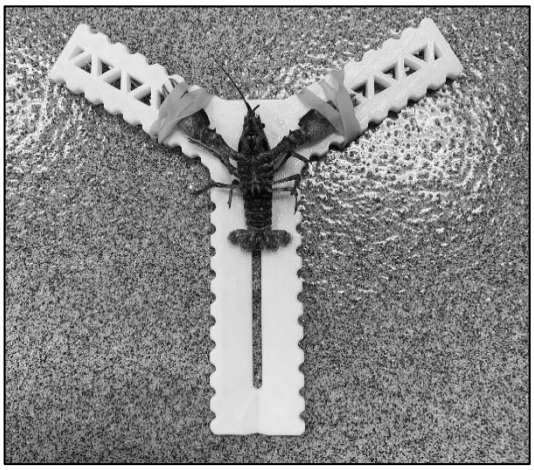


Figure 12. Urine extraction via direct injection. A 25G 1 ½ syringe was used to extract urine directly from the crayfish's bladder.

Samples

The samples were stored in -20 ^{o}C and thawed at room temperature right before the trials. The urine samples collected during the aggressive fights were blown continuously with nitrogen in the dark to concentrate the samples. Then enough E-pure water was added to a known volume (1.0 mL) and the samples were filtered via 13 mm/ 0.45 µm ALLPure syringe filters. For the injection samples, the concentration step was bypassed since they were taken directly from the subjects. They were filtered via 0.22 micron, 4 mm Millex-GV$_4$ filter unit.

Chemical Investigations

Reagents

HPLC grade methanol (≥ 99.9) and L-tryptophan (L-Trp) (≥ 98%) were obtained from Sigma-Aldrich, guanosine hydrate (98%), xanthine and dopamine-HCL from Aldrich, serotonin hydrochloride (5-HT) (98%) was obtained from Alfa Aesar, and DL-4-Hydroxy-3-methoxymandelic acid (VMA), 5-hydroxyindole-3-acetic acid (5-HIAA), Homovanillic acid (HVA) (98%), Uric acid (UA) (99%) and N-Acetyo-5-HT (Nac5HT) from Fisher Scientific company, LLC. Analytical sodium acetate trihydrate, 6M sodium hydroxide, 5M hydrochloric acid and 3M acetic acid were obtained from the stock room of the Chemistry department at Grand Valley State University. All aqueous solutions were prepared with ultra-pure water (18 MOhm) purified with Thermo Scientific Barnstead water purification system. The acetate buffer (50 mM) was adjusted to pH 4.42 with 5M hydrochloric acid.

Preparations of Standards and Calibration Curves

There were ten stock solutions prepared for this experiment with a concentration approximately 50 ppm solutions. Guanosine hydrate stock was prepared by also adding approximately 10 drops of 3M acetic acid and sonified for 5 minutes, to increase the solubility of guanosine in water. Xanthine and UA stocks were prepared by adding drops of strong base (6M NaOH) to achieve the same result. All the standards were prepared freshly every week and were stored in the dark, at room temperature.

The stock solutions were then diluted in series 1:25 (v/v), 1:100 (v/v), 1:400 (v/v), 1:800 (v/v) and 1:1600 (v/v) to create standards with the following concentrations: 2 ppm (dilution 0), 0.5 ppm (dilution 1), 0.125 ppm (dilution 2), 0.0625 ppm (dilution 3) and 0.0313 ppm (dilution 4). Dilution 1 was prepared by pipetting xanthine and UA first in the flask and then adding 3-4 drops of 3M acetic acid to neutralize them; the rest of the solutions were added in a random manner. Each of the dilutions was run in triplicates via the HPLC procedure and the average peak areas vs concentration (in ppm) were graphed.

Instrumentation and Chromatographic Conditions

For this experiment, the HPLC system (UltiMate 3000, Thermo Scientific) was equipped with an auto injector (ACC-3000), a system controller (Cromeleon Console), a pump (SD), a column oven (TCC-3000SD), a 15 µL injection loop, a sampler (WPS-3000SL) and a UV detector (VWD-3100). The HPLC analysis was performed using YMC-Pack ODS-AM column, 150 x 4.6 mmI.D. and with a particle size S-3 µm, 12nm. The mobile phase consisted of a degassed mixture of methanol (B) and 50 mM sodium acetate buffer (A) filtered through a 0.45 µm membrane. The analyte separation was performed using the following elution gradient: from 0 to 5

minutes the mobile phase consisted of 94% A and 9% B, from 5 to 17 minutes the % of B was gradually increased up to 38%, while the % of A was decreased by the same amount, from 17 to 19 minutes B was kept at 38 % and from 19 to 20 minutes the conditions were reversed back to the initial ones (94% A and 6% B) to equilibrate the column before the next trial. The flow rate was kept at 1.100 mL/min and the column temperature at 28 ^{o}C. The UV based detection was monitored at 273 nm. These parameters were determined based on the other experiments (Umeda *et al.*, 2005; Sánchez-Machado *et al.*, 2008; Giuliani *et al.*, 2016; Changenet-Barret *et al.*, 2016; Qu *et al.*, 2017). For the direct injection samples, 0.3 mL vial inserts were used due to small volume of the samples.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel. One-way ANOVA test as well as t-Test: Two-Sample Assuming Equal Variances were performed to determine the statistical significance of the data in the behavioral significance samples. The alpha factor for both tests was $\alpha = 0.05$. The statistical results are shown in Appendix A.

III. Results and Discussion

Behavioral Significance of the Urine

Before starting the chemical investigations of the urine samples, we sought to examine the behavioral significance of the urine in crayfish. For this reason, we devised a procedure which exposed the behavior of a hungry crayfish towards a food stimulus "bathed" with various samples: water, own urine and a bigger crayfish urine, which the subject had never interacted before. We also implemented a food dye in the samples, to better visualize the location of the urine cloud. At the beginning of the study, we were concerned whether the presence of the green food dye would interfere with eagerness of the crayfish to reach the food stimulus. However, during the 17 control trials 13 crayfish successfully interacted with the food stimulus, indicating that the presence of the food dye had little to no effect in the behavior of hungry crayfish to interact with food.

The behavior significance of the urine samples study had the following results: 13 crayfish were successful to interact with the food during water exposure, 10 during their own exposure and 7 during bigger exposure. These translated into the following success rates for water, own and bigger: 76.5 %, 58.8 % and 41.2 %, respectively **(Figure 13)**. The one-way ANOVA test for the success rates yielded a p value = 0.12 and F_{crit} > F value, indicating that the success rates were not significantly different **(Table 1)**. However, when comparing the success rates as a group of 2 via the t-T test **(Table 2)**, there was a significance difference between the water and bigger exposure p= 0.019. In addition, there was not a statistical difference between the own and bigger exposure success rates. These results indicate that crayfish were more successful to

reach the food stimulus during the water and dye exposure compared to the other exposures.

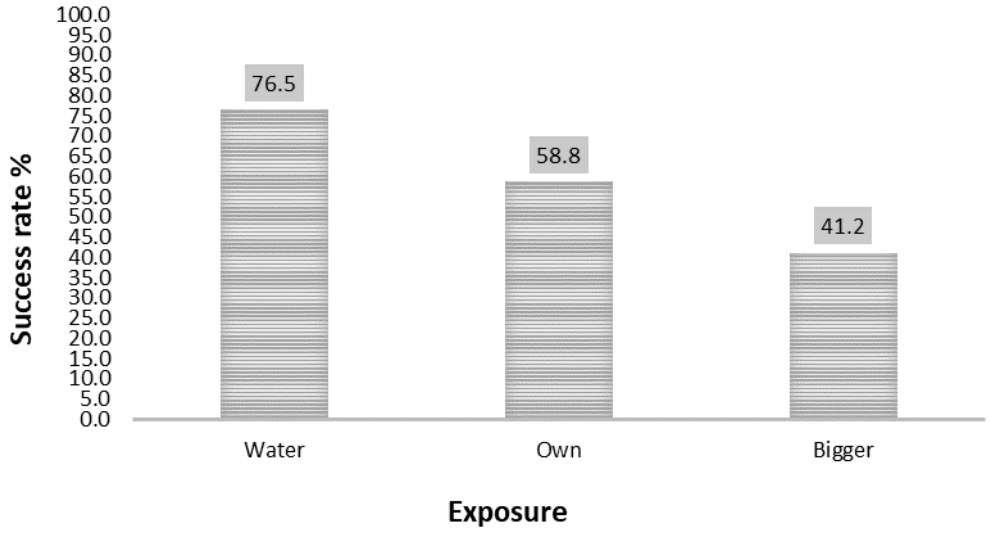


Figure 13. Success rate of the different exposures in the food dye trials. Out 17 trials, only 13 crayfish were successful to interact with the food stimulus during water exposure, 10 during their own exposure and 7 during bigger exposure.

Next, we were interested to know how much time it took for each subject to reach the food stimulus (**Figure 14**) and how much time did each one of them spent with the food during each exposure (**Figure 15**). As seen in **(Figure 14)**, the crayfish took significantly less time to reach the food stimulus during the water exposure compared to the other ones. The average time to reach the food during the water, own and bigger exposures were 88.9 s, 168.4 s and 183.7 s, respectively. In addition, the crayfish spent significantly more time with the food stimulus during the water exposure than the other exposures **(Figure 15)**. The average time spent with the food was as the following: 140.8 s for the water exposure, 24.5 s for their own exposure and 23.6 s for the bigger exposure. Both statistical test indicated significant results between the different exposure **(Table 1 and Table 2).**

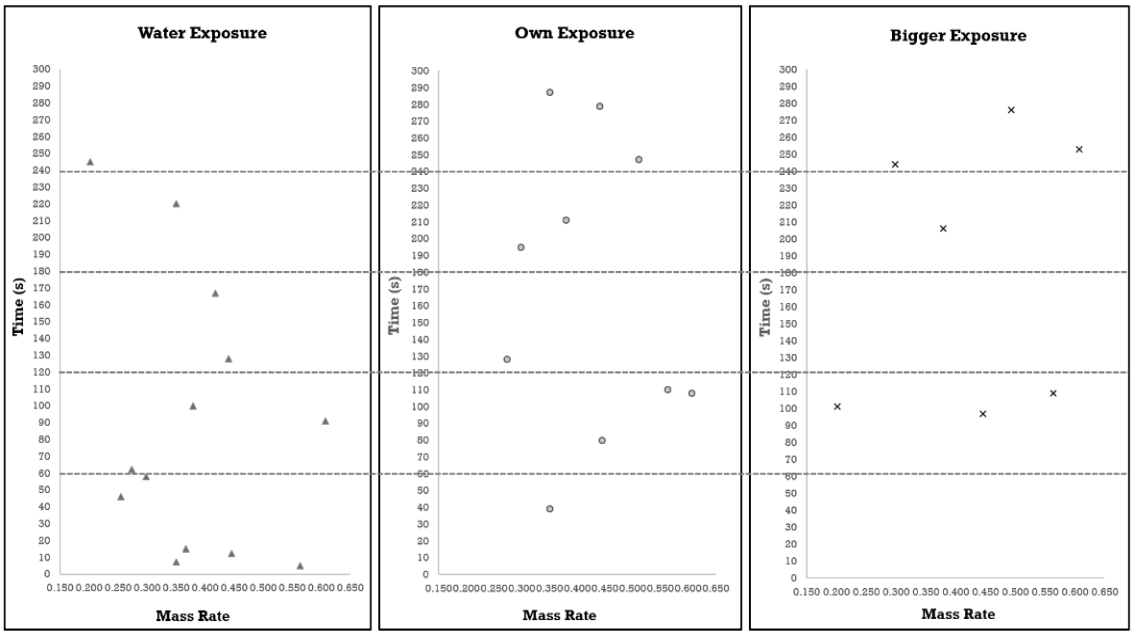


Figure 14. Time it took to interact with the food stimulus. On average, it took 88.9 s for the crayfish to interact with the food stimulus in the water exposure, 168.4 s in the own exposure and 183.7 s for the bigger exposure.

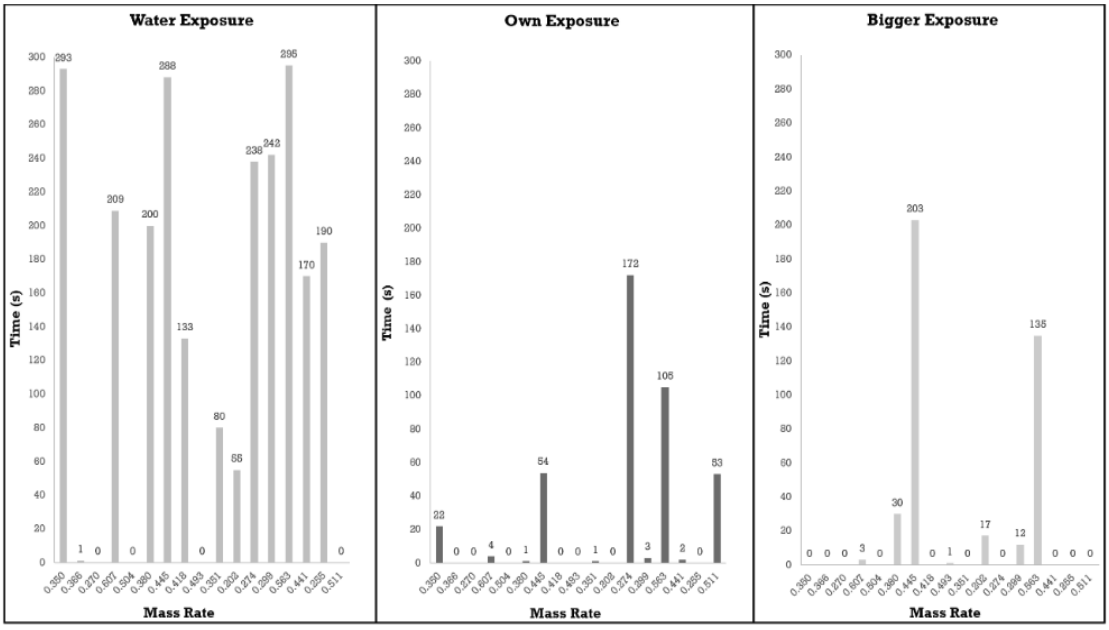


Figure 15. Time spent with the food stimulus. On average, crayfish spent 140.8 s interacting with the food stimulus during water exposure, 24.5 s during their own exposure and 23.6 s during bigger exposure.

Based on these results, we can safely conclude that there are chemicals present in the urine of the crayfish which conveys specific messages. In addition, data show that crayfish cannot recognize their own urine, since the time needed to reach and interact with food were similar to the bigger crayfish urine exposure.

HPLC Results

Standards

The goal of this study was to generate a HPLC method to successfully separate the 10 standards of interest. The separation method was a success in the separation of the 10 standards since there were no overlaying peaks displayed in the chromatogram**.** The reagents were identified by making different combinations with or without the presence of a certain chemical. The runs were compared, and the missing peaks were assigned to the particular reagents. Based on the UV detection the standards eluted in the following order: VMA, UA, DA, XA, GUA, 5-HT, L-Trp, 5-HIAA, HVA and Nac5HT and with the following average timing: 2.46, 2.90, 3.46, 3.86, 7.48. 8.68, 11.40, 12.73, 14.22, 14.22 and 15.13 min **(Figure 16)**. For more information, check calibration tables and curves in Appendices. Important to note is that the neutralization of XA and UA with acetic acid was a success since the dopamine produced only 1 peak. This was not the case when these compounds were not neutralized (data not shown).

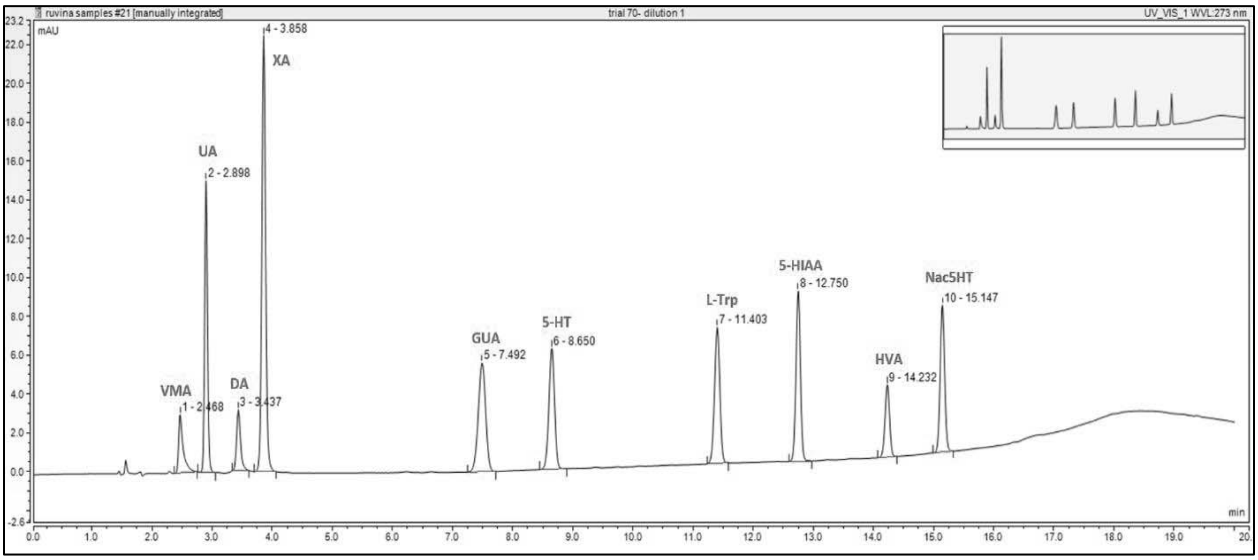


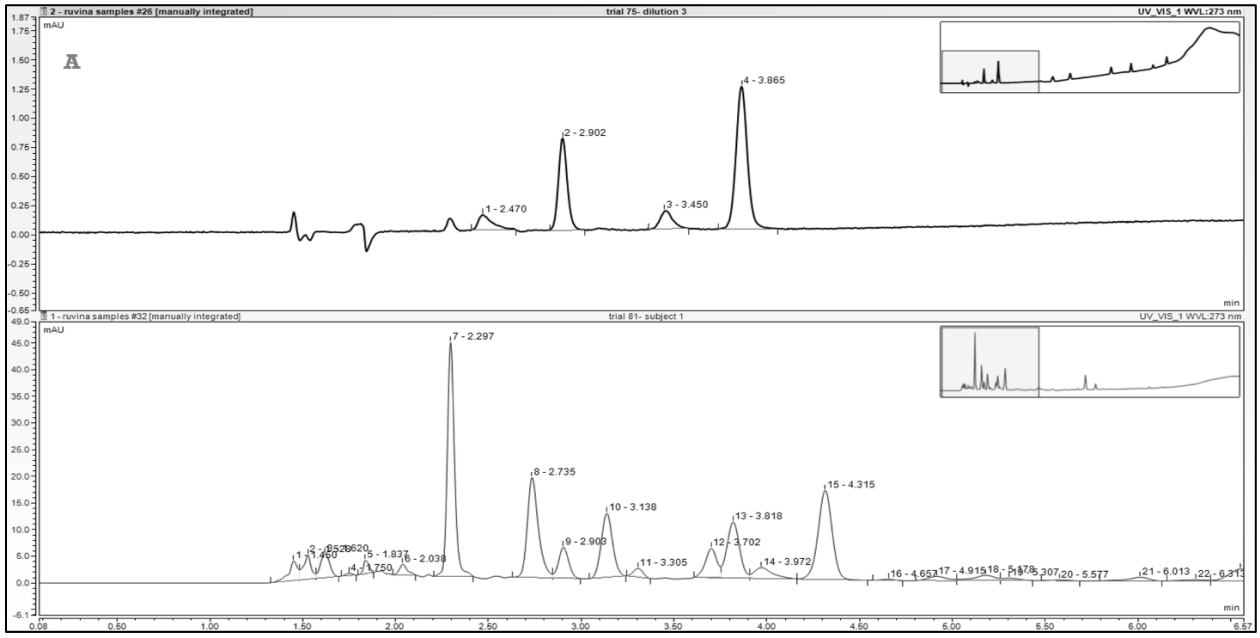
Figure 16. Separation of the 10 standards via UV detection. The absorption wavelength was set at 273 nm since most of the compounds are related to the catecholamines and contain aromatic rings. As seen in this figure, the 10 standards were separated successfully.

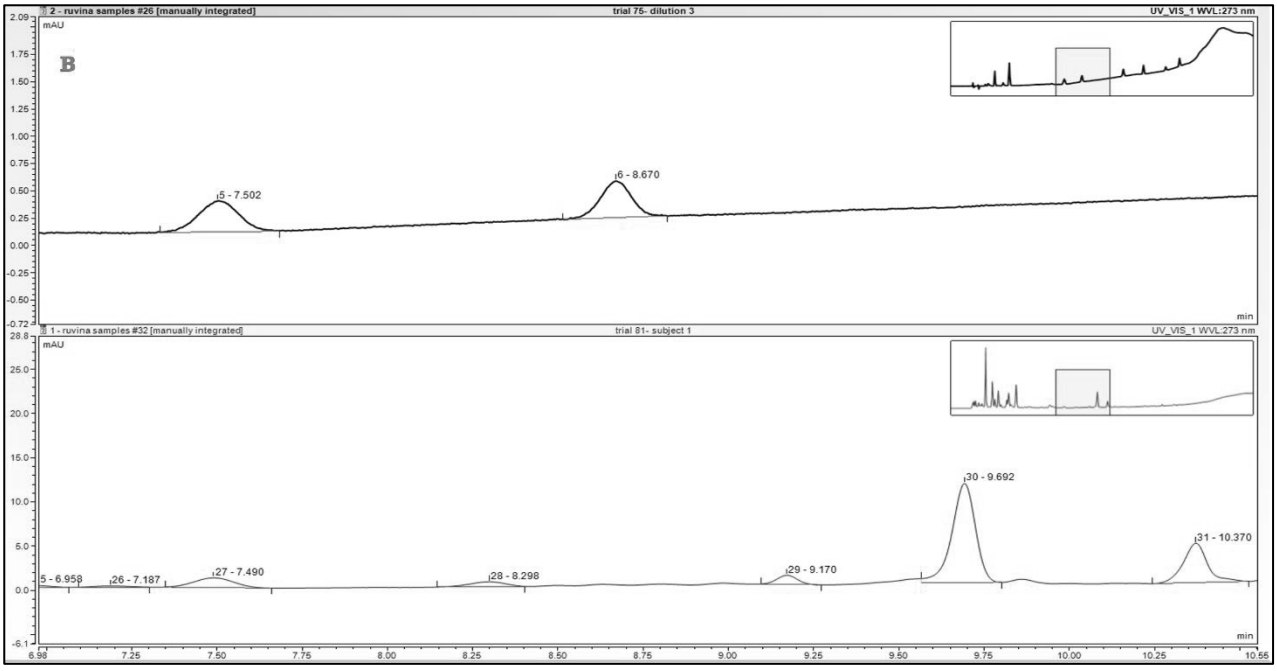
Direct Injections Samples

For the direct injection samples, we chose to extract the urine directly from the nephropore in only 3 subjects. Since the crayfish were tied up in the Y board, there were stressed. Consequently, the urine would contain chemical messengers related to stress and or potentially aggression. During this experiment, the dilutions were run in triplicates before the samples. The data for the calibration curves for subject 1-3 are shown in **(Table 3)** and **(Figure 17-19)** .

The chromatogram for subject 1 showed 43 peaks in total as seen in **(Figure 17)**. The complete details are shown on **(Table 4)**. Based on the retention times of the all 43 peaks, we were able to identify peaks 9, 27, 33 and 40. These peaks correspond to uric acid, guanosine, L-Trp and N-acetyl-serotonin, respectively and imply that these compounds were present in the bladder of the subject 1 right before the time of extraction. Based on the calibration curves

(Figure 26), the concentration of these substances were 0.713, 0.310, 0.045 and 0.082 ppm, respectively.





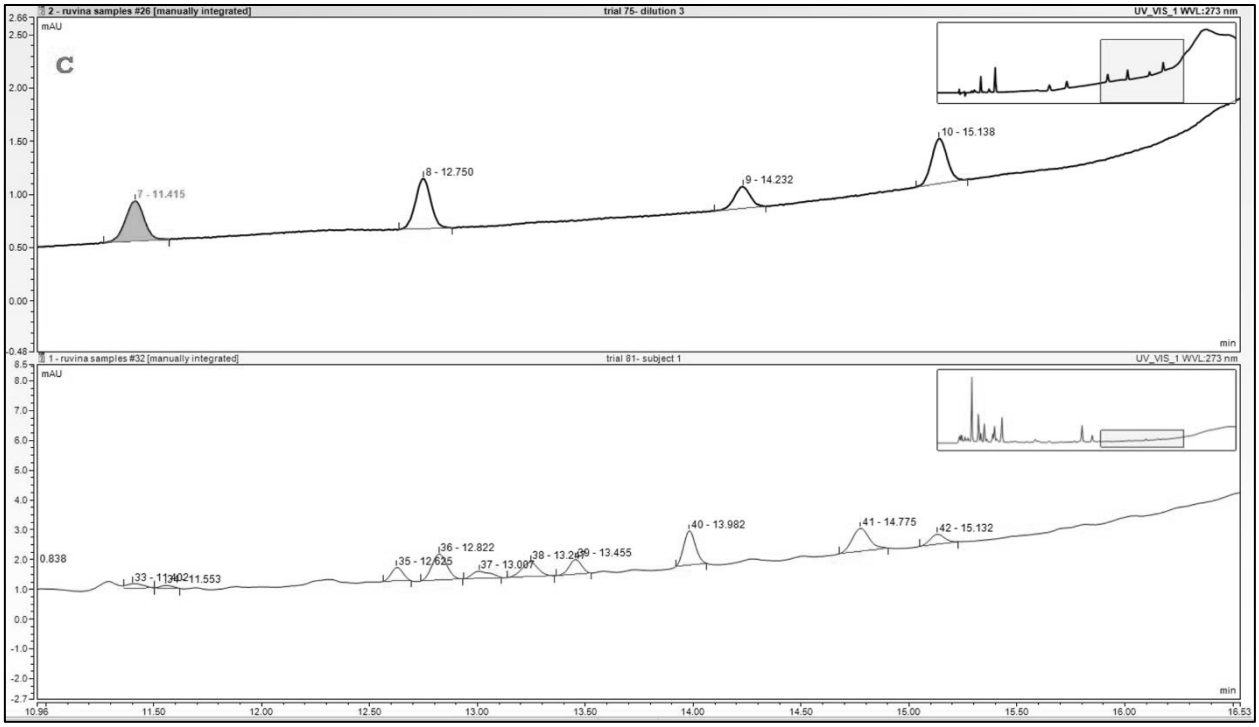
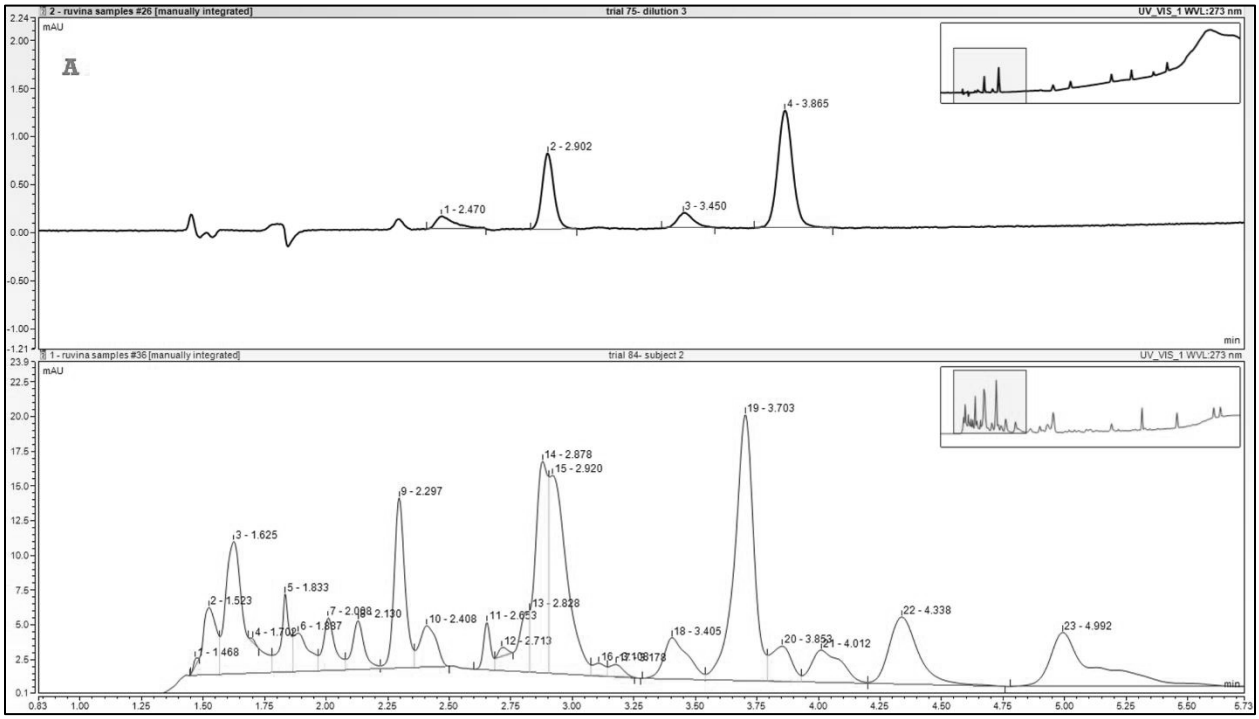
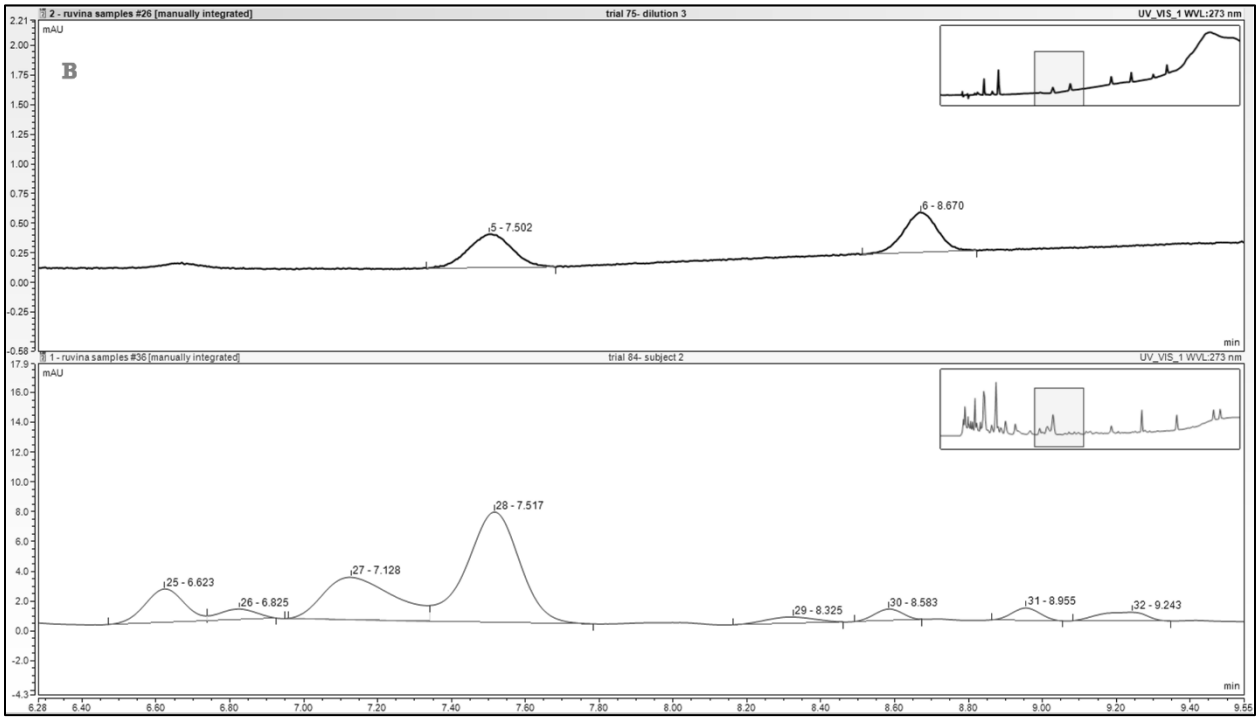


Figure 17. Urine analysis for subject 1. This urine sample resulted in 43 peaks. Out of these 43 peaks, we were able to identify only 4 peaks. Peak 9 correspond to uric acid, peak 27 to guanosine, peak 33 to L-Trp and peak 40 to N-acetyl-serotonin, a byproduct of 5HT. The colored peak in figure C denotes the peak area measured for each of the samples, and it is the value graphed vs the concentration of the standard.

The chromatogram for subject 2, showed 56 peaks in total, **(Figure 18)**. The complete details are shown on **(Table 5)**. Based on the retention times of the all 57 peaks, we were able to identify peaks 15, 28 and 38. These peaks correspond to uric acid, guanosine and L-trp, respectively and imply that these compounds were present in the bladder of the subject 2 right before the time of extraction. The concentration of these substances were 0.054, 3.127, and 0.916 ppm and were measured via the calibration curves in **(Figure 26).** In addition, we suspect that peaks 18 and 30 belongs to the DA and 5-HT, since the retention time of the unknown peaks are very similar to the standards. The shift in the retention times might be explained due

to intermolecular forces between various chemicals in the samples, which can either push the reagents faster or retain them longer in the HPLC column.





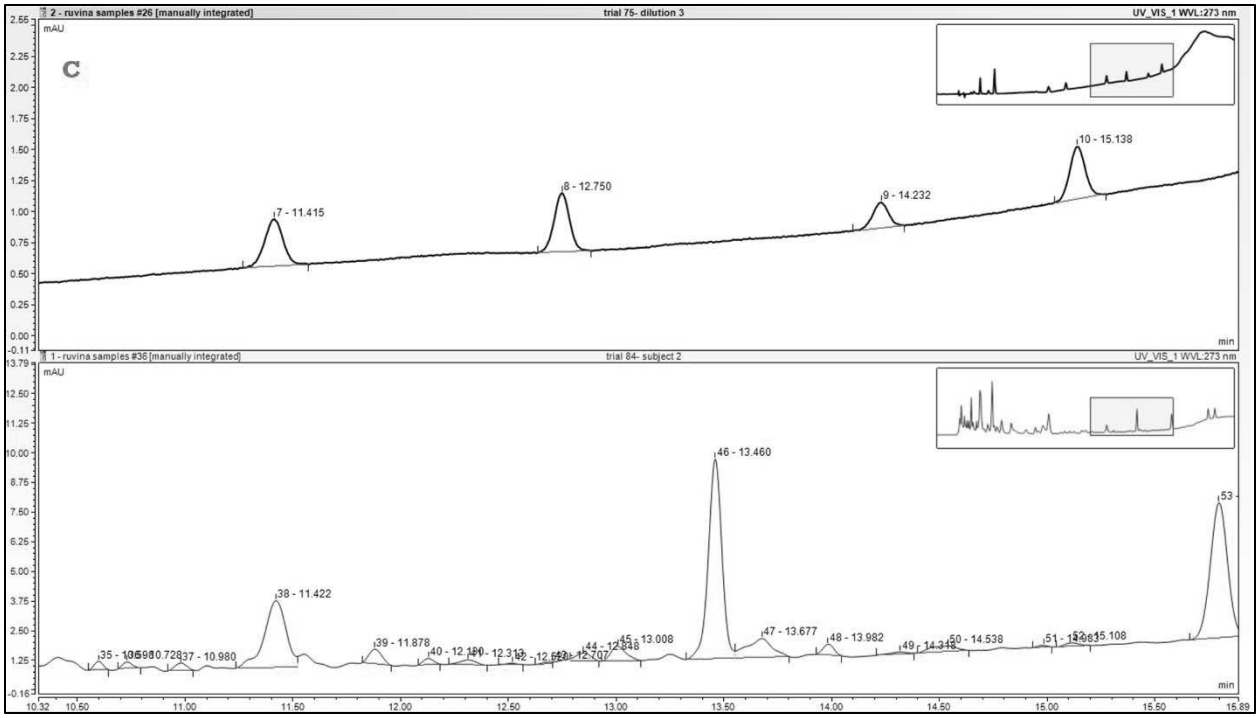
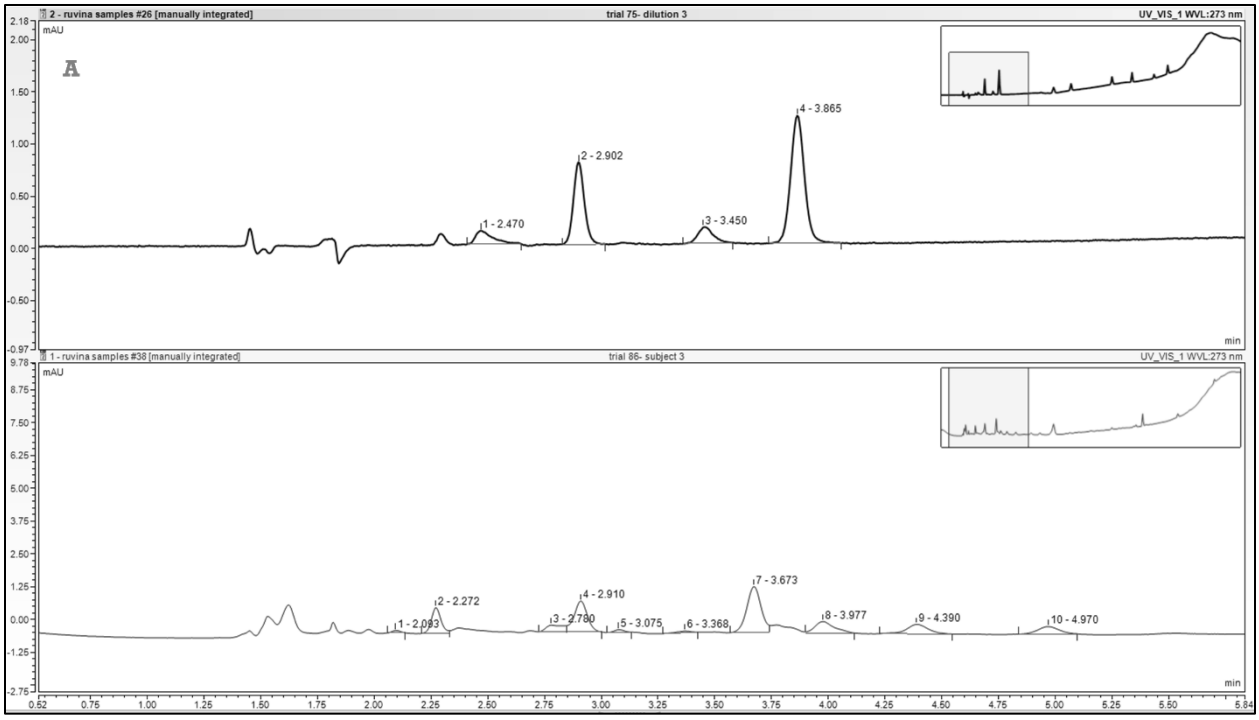
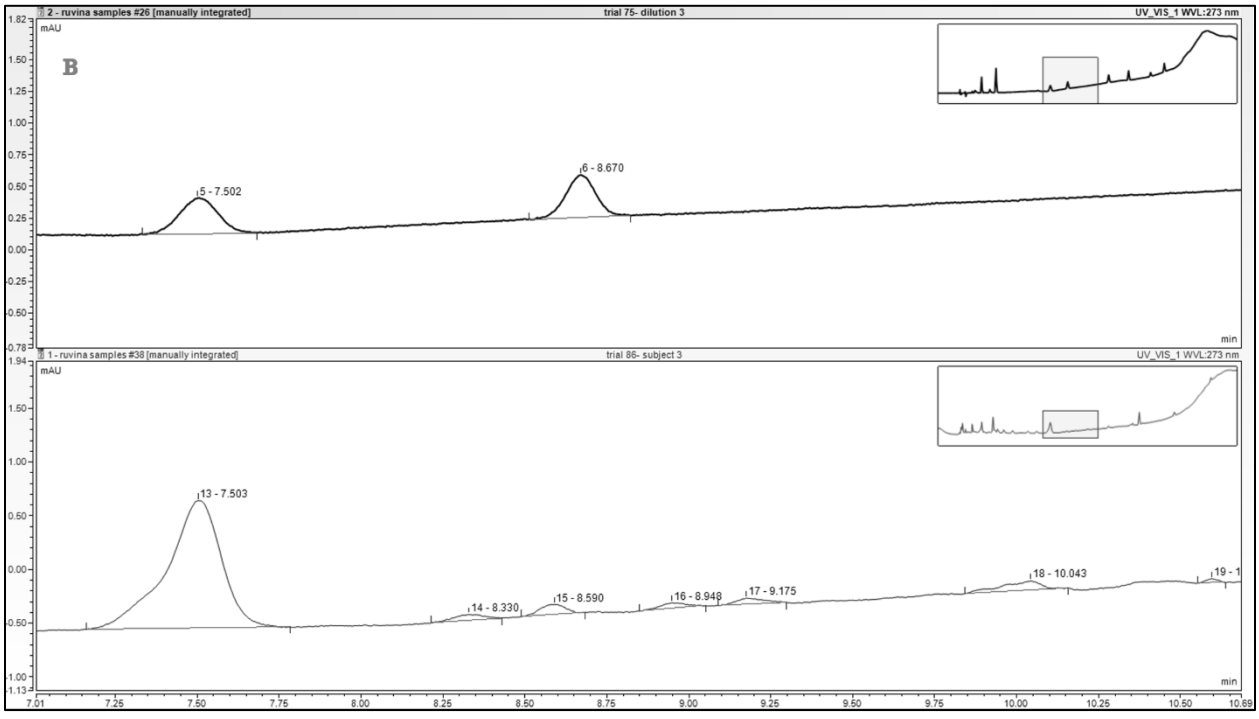


Figure 18. Urine analysis for subject 2. This urine sample resulted in 57 peaks. Out of these 47 peaks, we were able to identify only 3 peaks. Peak 15 correspond to uric acid, peak 28 to guanosine and peak 38 to L-Trp.

The chromatogram for subject 3, showed 31 peaks in total, **(Figure 19)**. The complete details are shown on **(Table 6)**. Based on the retention times of the all 31 peaks, we were able to identify peaks 4, 13 and 21. These peaks correspond to uric acid, guanosine and L-Trp, respectively and imply that these compounds were present in the bladder of the subject 1 during the time of extraction. The concentration of these substances were 0.018, 0.070, and 0.066 ppm, respectively and were measured via the calibration curves in **(Figure 26).** We suspect that peak 15 belongs to the 5-HT since the retention times are very similar between the two peaks.


2 - ruvina samples #26 [manually integrated]
trial 75- dilution 3
UV_VIS_1 WVL:273 nm
mAU
A
1 - 2.470
2 - 2.902
3 - 3.450
4 - 3.865
min
1 - ruvina samples #38 [manually integrated]
trial 86- subject 3
UV_VIS_1 WVL:273 nm
mAU
1 - 2.093
2 - 2.272
3 - 2.780
4 - 2.910
5 - 3.075
6 - 3.368
7 - 3.673
8 - 3.977
9 - 4.390
10 - 4.970
min


2 - ruvina samples #26 [manually integrated]
trial 75- dilution 3
UV_VIS_1 WVL:273 nm
mAU
B
5 - 7.502
6 - 8.670
min
1 - ruvina samples #38 [manually integrated]
trial 86- subject 3
UV_VIS_1 WVL:273 nm
mAU
13 - 7.503
14 - 8.330
15 - 8.590
16 - 8.948
17 - 9.175
18 - 10.043
min

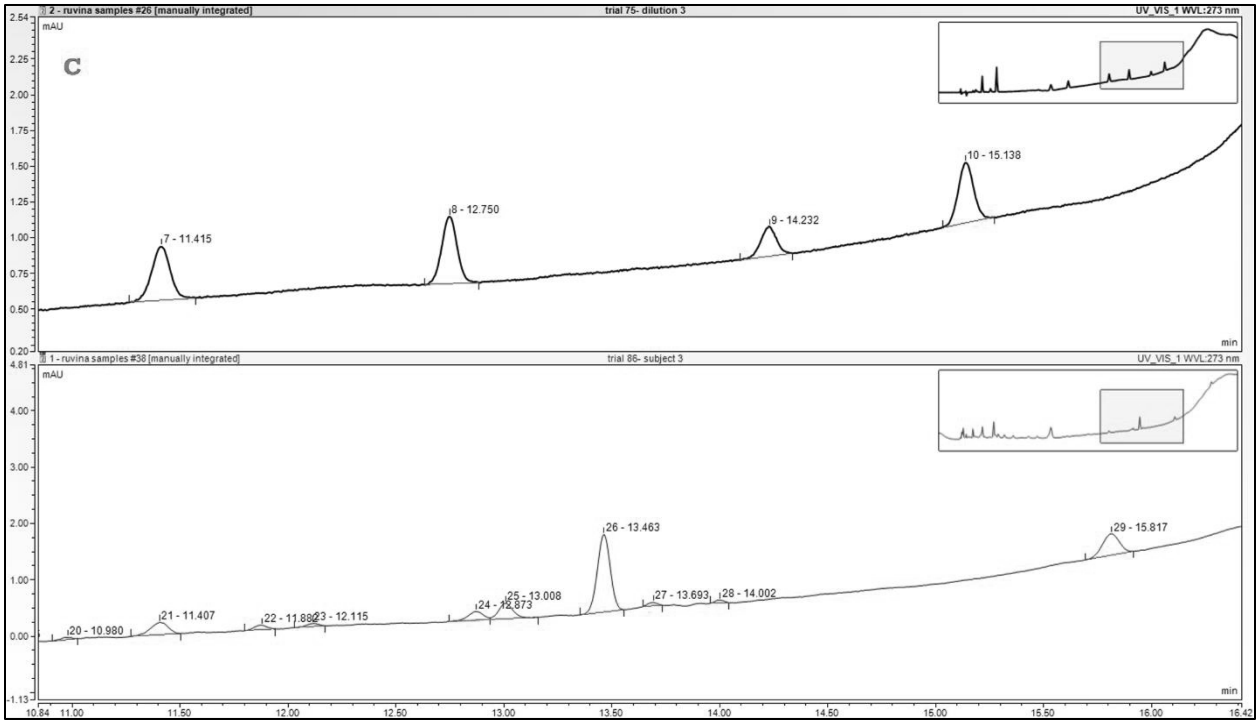


Figure 19. Urine analysis for subject 3. This urine sample resulted in 31 peaks. Out of these 41 peaks, we were able to identify only 3 peaks. Peak 4 correspond to uric acid, peak 13 to guanosine and peak 21 to L-Trp.

Taking these together, we conclude that UA, L-Trp, GUA and Nac5HT were present in the bladder of a stressed crayfish at various amounts. We found that UA, GUA and L-Trp were present consistently in all of the three samples, while Nac5HT in only one of the samples. We also suspect that DA and 5-HT might be present samples, since the retention times between the samples and standards were very close. However, a LC-MS study must be conducted in the future to further clarify the presence of serotonin in the urine of the crayfish.

Aggressive Fights Samples

Behavior Significance of the Samples

Before we ran the HPLC study for the aggressive fight samples, we were interested to investigate the behavioral significance of the samples using the protocol depicted in **(Figure 11)**.

Based on the results **(Figure 20 A-D)**, we concluded that this procedure was not ideal, since the crayfish might have detected the hand movement and/or the water ripples created by the pipetting and perceived it as a danger situation. This is indicated in the **(Figure 20 A)** since the majority of the crayfish did not even approach the water treatment. Another possibility might have been that the samples were not concentrated enough or that not the right chemicals were caught during the agonistic interactions. However, based on the video analysis we conclude that the crayfish were actually scared due to their decreased level of activity during the trial. They were positioned in the corner and not willing to engage with the food stimulus. Thus, we modified the protocol for the food dye trials slightly; the sample was pipetted before the gate was lifted and the experimenter stayed away from the tank while the trials were being recorded.

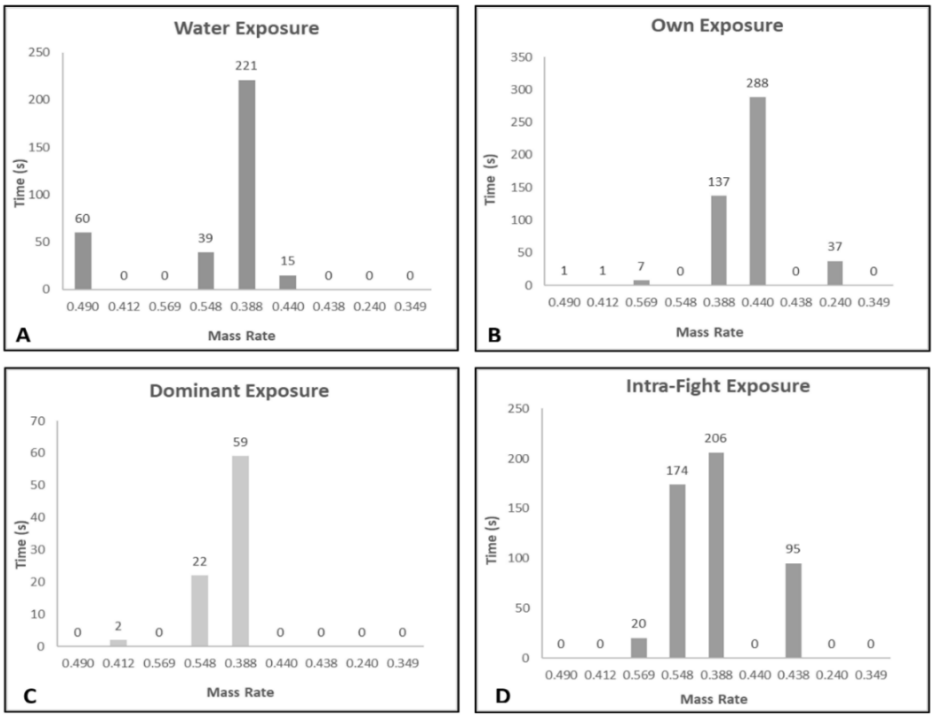


Figure 20. Behavioral significance of the agonistic interactions' trials. We concluded that this procedure was not ideal, since the crayfish may interpret the movement of the investigator's hand and/or the water ripples as danger, and not try to reach for the food stimulus.

HPLC Results of Agonistic Fights

The population for this study was n = 30 crayfish, which equaled 15 fight trials. The trials were numbered from 1-15. Each fight trial consisted of 5 samples: subordinate pre-fight, subordinate post-fight, intra fight, dominant pre-fight and dominant post-fight. The subordinate was denoted with number 1, whereas the dominant with number 2; the pre-fight was marked with letter A, whereas the post-fight with letter B. For example, the subordinate post-fight sample for trial 8 would be labeled 8.1B. The intra-fight sample was labeled with number 3 (for ex: 8.3).

Once the samples were thawed, nitrogen was blown on top of the samples in order to concentrate them. They were concentrated until the volume of the samples was lower than 0.5 mL and then ultrapure water was added to bring the volume back to 0.5 mL. This way, the 1A, 1B, 2A and 2B samples were concentrated by a factor of 6, whereas the 3 samples by a factor of 10. The samples were filtered via 13 mm/ 0.45 µm ALLPure syringe filters and were run in the HPLC. For the HPLC experiment, we decided to investigate fight number 12, 13 and 14. The calibration data **(Table 7-8)** and curves **(Figure 27-28)** are shown in Appendix D, whereas the complete HPLC chromatograms for the fights 12, 13 and 14 are shown in **(Figure 29-31)** in Appendix E. Based on the HPLC results we conclude the following:

Fight 12. The HPLC chromatograms for the 12.2A, 12.2B, 12.3, 12.1A and 12.1B showed 3, 8, 10, 20 and 19 peaks, respectively **(Figure 21)**. Peak 1 on the 12.2A was present on only this sample and none other, whereas peaks 2 and 3 were consistent across the other samples. In the 12.2B chromatogram, we could only identify peak 6, which corresponds to xanthine (9.02 ppb). Xanthine was also present in the intra-fight, prefight subordinate and post-fight

subordinate samples as well with the following concentrations: 6.30 ppb, 58.2 ppb, and 31.9 ppb, respectively. In the subordinate samples (12.1A and 12.1B) we concluded that peaks 8 and 9 belong to dopamine, since the retention times in these samples were consistent with the retention time of the dopamine standard, 3.45 min with a concentration of 60.9 ppb and 18.3 ppb, respectively. We also suspect that peak 12 on 12.1B corresponds to guanosine, whereas, peak 3 on 12.1A and peak 4 in 12.1B belong to VMA, since their retention time is very similar to

Fight 12				
12.2A	12.2B	12.3	12.1A	12.1B
-	-	-	1.815	-
-	-	1.883	-	1.883
-	2.128	2.138	2.142	2.093
-	2.270	2.267	-	2.268
-	2.367	-	-	-
-	-	-	2.415	2.412
-	2.608	-	2.605	-
-	-	-	-	2.765
-	-	-	-	2.862
-	-	2.963	2.950	-
-	3.077	3.072	-	3.070
-	-	-	3.150	-
-	-	-	3.355	3.365
-	-	-	3.450	3.452
-	-	-	3.643	3.650
-	3.823	3.823	3.813	3.818
-	-	4.150	-	-
-	-	-	4.797	-
5.037	-	-	-	-
-	-	-	5.955	-
-	-	-	6.595	-
-	-	-	-	7.410
-	-	-	8.277	-
-	-	-	8.867	8.895
-	-	-	9.433	-
-	-	-	9.763	9.757
-	-	-	-	11.347
-	-	-	-	11.567
-	-	-	12.122	12.153
16.773	16.833	16.812	16.238	16.810
17.918	18.173	17.942	18.052	18.023
-	-	18.877	-	-

Figure 21. Fight 12 HPLC results. Peak 1 on 12.2A was present only present in this sample. Xanthine was present in the dominant post-fight, intra-fight, prefight subordinate and post-fight subordinate samples as well. Dopamine was found in both pre and post-fight samples for the subordinate crayfish only. 5-HT was found only in 12.1A. UA and L-Trp were found only in 12.1B. The molecule with retention time 4.150 min was unique to only intra-fight sample.

the standards as well. Peaks 1, 4, 6, 11, 12, 13, 14 and 16 were only present on the 12.1A sample and nowhere else. Peak 15 in 12.1A had similar elution time to 5-HT standard and its concentration was found to be 37.1 ppb. Peaks 6 in 12.1B corresponds to uric acid, with a concentration of 47.5 ppb, whereas peak 15 to L-Trp with a concentration of 41.4 ppb. As for the intra-fight sample (12.3), the molecule with retention time 4.150 min (peak 7) was unique to this sample. Its identity remains to be unknown since its retention time was not similar to any of the standards. What we can speculate is that in terms of size and polarity, this molecule is between xanthine (ret. time = 3.84 min) and guanosine (ret. time = 7.46 min).

Fight 13. The HPLC chromatograms for the 13.2A, 13.2B, 13.3, 13.1A and 13.1B showed 4, 8, 4, 0 and 17 peaks, respectively **(Figure 22).** Peak 2 in 13.2A was unique to only this sample (dominant pre-fighting sample). We do not refute the idea that it might be a food metabolite since the crayfish had consumed food before the fight. Xanthine was found in the dominant

Fight 13				
13.2A	13.2B	13.3	13.1A	13.1B
1.887	1.883	-	-	1.877
-	2.142	-	-	-
-	2.270	-	-	2.258
-	-	-	-	2.410
-	2.607	2.618	-	-
-	-	-	-	2.763
-	-	-	-	2.862
-	3.078	3.085	-	3.047
-	-	-	-	3.342
-	-	-	-	3.443
-	-	-	-	3.642
-	3.827	-	-	3.805
-	-	4.193	-	-
-	-	-	-	4.802
4.977	-	-	-	-
-	-	-	-	7.347
-	-	-	-	8.863
-	-	-	-	9.733
-	-	-	-	11.540
17.043	16.823	-	-	16.775
18.21	18.147	18.038	-	17.982

Figure 22. Fight 13 HPLC results. Uric acid, dopamine and 5-HT were only detected in 1B sample, whereas xanthine was present in the 1B an 2B.

(5.19 ppb) and subordinate (35.4 ppb) post-fight samples. Dopamine (48.7 ppb) and uric acid (31.2 ppb) were found in the subordinate post-fight sample, which is consistent with the previous results. Likewise fight 12, we found 5-HT (31.9 ppb) was present in the subordinate sample. Serotonin was not detected in the other samples of this trial. As for the intra-fight sample, peak 3 is consistent with the unknown molecule from trial 12 and it is present only in the intra-fight sample. It is unclear why the 13.1A produced no peaks.

Fight 14. The HPLC chromatograms for the 14.2A, 14.2B, 14.3, 14.1A and 14.1B showed 9, 15, 26, 0 and 0 peaks, respectively **(Figure 23).** Xanthine (74.1, 27.4 and 30.3 ppb) and uric acid (44.8 and 29.2 ppb) were consistently found again in the samples. Dopamine was found

Fight 14				
14.2A	14.2B	14.3	14.1A	14.1B
-	-	1.820	-	-
-	1.900	-	-	-
2.130	-	2.123	-	-
2.257	2.273	2.263	-	-
2.408	2.425	2.408	-	-
-	-	2.617	-	-
-	2.782	2.767	-	-
2.857	-	2.857	-	-
-	-	2.955	-	-
-	3.080	3.062	-	-
3.340	3.357	3.343	-	-
-	3.472	3.452	-	-
3.635	3.655	3.640	-	-
3.803	3.828	3.807	-	-
4.785	-	4.785	-	-
-	-	6.515	-	-
-	7.560	7.417	-	-
7.943	-	-	-	-
-	9.050	8.882	-	-
-	9.832	9.760	-	-
-	-	10.332	-	-
-	-	11.367	-	-
-	-	11.553	-	-
	11.647	-	-	-
-	-	12.125	-	-
-	-	12.842	-	-
-	-	13.338	-	-
-	16.952	16.757	-	-
17.959	18.057	18.035	-	-

Figure 23. Fight 14 HPLC results. Uric acid and dopamine were detected only in the intra-fight sample, whereas xanthine in both 2A and 3 samples. L-trp was detected in the intra-fight sample.

during the dominant pre-fight (53.0 ppb), intra-fight conditions (7.83 ppb) and dominant post-fight sample (22.6 ppb). We also detected L-tryptophan in the intra-fight sample (32.3 ppb). Furthermore, we suspect that peak 16 in 14.3 sample corresponds to guanosine since its retention time is very close to the standard time. As for the samples that produced no peaks, it is still unclear why the pre-fighting sample (14.1A) produced no peaks. We were not surprised with the post-fight sample (14.1B) producing no peaks since the crayfish tank was refilled with clean water and the samples could have simply contained just pure water.

Combined results. Based on the results from fight 12-14, we lined up the peaks **(Table 9)** and combined the results to have a better understanding of the chemical profile of the gathered samples **(Figure 24)**. Based on these results, we conclude the following regarding the urine profiles of the samples: First, L-tryptophan (av. ret. time = 11.37 min) was found consistently in the subordinate and intra-fight samples, but not in the dominant samples. Secondly, dopamine (av. ret. time = 3.45 min) and xanthine (av.ret. time = 3.84 min) were detected consistently across the samples. Uric acid was detected in the dominant pre-fight, intra-fight and subordinate post-fight samples. One explanation to why we were unable to detect it in the rest of the samples, might be that UA was very diluted in those samples and exceed the detection range of the instrument. As for serotonin, it was eluted from the column in a range of 8.61-8.71 min, with an average retention time of 8.66 min **(Table 8)**. Peak 15 in one of the subordinate pre-fight samples is within the elution time range. As for the rest of the samples, we obtained peaks with elution times similar to 5-HT's elution time, but further investigations need to be conducted to validate the presence of 5-HT in those samples. As for the VMA (av. ret. time = 2.45 min) and guanosine (av. ret. time = 7.46 min), we were able to obtain similar retention time

results in the samples. We suspect that the molecule with retention time = 2.41 min obtained in the samples is either VMA or a very similar compound. Similarly, we conclude the same for the molecule with the retention time = 7.41 min, which could possibly be guanosine or related to guanosine, such as guanine. The most novel finding from this chromatograms is the molecule with retention time 4.150 min. This molecule was detected in only the intra-fight samples and not the rest. We refute the idea that it might be a food metabolite, because if it were so, it would have showed up in the pre-fight samples in a much higher concentration; also, the fighting tank was filled simply with clean water.

Combined Peaks				
Combined 2A	Combined 2B	Combined 3	Combined 1A	Combined 1B
-	-	1.820	1.815	-
1.887	1.883	1.883	-	1.883
2.130	2.128	2.123	2.142	2.093
2.257	2.270	2.263	-	2.268
-	2.367	-	-	-
2.408	2.425	2.408	2.415	2.412
-	2.608	2.617	2.605	-
-	-	2.767	-	2.765
2.857	-	2.857	-	2.862
-	-	2.955	2.950	-
-	3.077	3.062	-	3.070
-	-	-	3.150	-
3.340	3.357	3.343	3.355	3.365
-	3.472	3.452	3.450	3.452
3.635	3.655	3.640	3.643	3.650
3.803	3.828	3.807	3.813	3.818
-	-	4.150	-	-
4.785	-	4.785	4.797	-
5.037	-	-	-	-
-	-	-	5.955	-
-	-	6.515	6.595	-
-	7.560	7.417	-	7.410
7.943	-	-	-	-
-	-	-	8.277	-
-	9.050	8.882	8.867	8.895
-	-	-	9.433	-
-	9.832	9.760	9.763	9.757
-	-	10.332	-	-
-	-	11.367	-	11.347
-	-	11.553	-	11.567
-	11.647	-	-	-
-	-	12.125	12.122	12.153
-	-	12.842	-	-
	-	13.338	-	-
16.773	16.833	16.757	16.238	16.810
17.959	18.173	18.035	18.052	18.023
-	-	18.877	-	-

Figure 24. Combined HPLC results for fight 12, 13 and 14. The molecule with ret. time = 4.150 is unique to only the intra-fight sample. Dopamine, xanthine, and uric acid was found consistently throughout all the samples. Serotonin was detected in only one of the subordinate samples (1A) and intra-fight sample.

Stress vs aggression. Next, we wanted to compare these results to the stress study. We compiled and lined up the data again as seen in **(Figure 25)**. There are some significant changes between the aggression and stress urine profiles. To be more concise, there are 2 compounds with retention time 4.15 min and 4.79 min present in the intra-fight samples but not in the direct injection samples. From these two, the molecule with the retention time 4.79 min was also detected in the prefight samples, which does not eliminate the possibility that it can be a food metabolite. On the other hand, the molecule with the retention time 4.15 was only detected in the intra-fight sample and not in any other aggression or stress samples. Thus, this molecule cannot be a food metabolite. Furthermore, since it was not detected in the urine samples extracted directly from the bladder, and detected only in the intra-fight samples, this molecule can potentially be an aggression chemosignal. We also suspect it is being secreted from another gland via a different route as compared to the normal urine which is secreted from the nephropore. The chemical composition of this molecule and the location and identity of the gland secreting it remains to be founded for future studies.

Summary. Crayfish cannot recognize their own urine since own and bigger exposures yielded similar results. Significant behavioral difference between water vs own and bigger validating the presence of chemo-messaging via urine. We were able to identify only four of ten chemicals of interest in a stressed crayfish: uric acid, guanosine, L-tryptophan and N-acetyl-serotonin. We were able to identify only four of ten chemicals of interest: uric acid, xanthine, dopamine and L-tryptophan. A unique signal which can potentially be an aggression chemosignal was detected in the intra-fight sample. Future studies need to be conducted to elucidate its chemical profile.

Combined Peaks					
Combined 2A	Combined 2B	Combined 3	Combined 1A	Combined 1B	Combined Stress
-	-	1.820	1.815	-	1.833
1.887	1.883	1.883	-	1.883	1.887
-	-	-	-	-	2.008
2.130	2.128	2.123	2.142	2.093	2.093
-	-	-	-	-	2.130
2.257	2.270	2.263	-	2.268	2.272
-	-	-	-	-	2.297
-	2.367	-	-	-	-
2.408	2.425	2.408	2.415	2.412	2.408
-	2.608	2.617	2.605	-	2.653
-	-	2.767	-	2.765	2.713
-	-	-	-	-	2.828
2.857	-	2.857	-	2.862	2.878
-	-	-	-	-	2.920
-	-	2.955	2.950	-	-
-	3.077	3.062	-	3.070	3.108
-	-	-	3.150	-	3.178
3.340	3.357	3.343	3.355	3.365	3.305
-	3.472	3.452	3.450	3.452	3.405
3.635	3.655	3.640	3.643	3.650	3.703
3.803	3.828	3.807	3.813	3.818	3.860
-	-	-	-	-	4.012
-	-	4.150	-	-	-
-	-	-	-	-	4.338
-	-	-	-	-	4.657
4.785	-	4.785	4.797	-	-
5.037	-	-	-	-	4.992
-	-	-	-	-	5.178
-	-	-	-	-	5.307
-	-	-	-	-	5.577
-	-	-	5.955	-	5.990
-	-	-	-	-	6.313
-	-	6.515	6.595	-	6.623
-	-	-	-	-	6.825
-	-	-	-	-	6.958

Combined Peaks					
Combined 2A	Combined 2B	Combined 3	Combined 1A	Combined 1B	Combined Stress
-	-	-	-	-	7.128
-	7.560	7.417	-	7.410	7.517
7.943	-	-	-	-	-
-	-	-	8.277	-	8.325
-	-	-	-	-	8.583
-	9.050	8.882	8.867	8.895	8.955
-	-	-	-	-	9.243
-	-	-	9.433	-	-
-	9.832	9.760	9.763	9.757	9.737
-	-	-	-	-	10.040
-	-	10.332	-	-	10.370
-	-	-	-	-	10.598
-	-	-	-	-	10.728
-	-	-	-	-	10.980
-	-	11.367	-	11.347	11.422
-	-	11.553	-	11.567	11.553
-	11.647	-	-	-	-
-	-	-	-	-	11.878
-	-	12.125	12.122	12.153	12.130
-	-	-	-	-	12.313
-	-	-	-	-	12.520
-	-	-	-	-	12.707
-	-	12.842	-	-	12.848
-	-	-	-	-	13.008
-	-	13.338	-	-	13.247
-	-	-	-	-	13.460
-	-	-	-	-	13.677
-	-	-	-	-	13.982
-	-	-	-	-	14.318
-	-	-	-	-	14.538
-	-	-	-	-	15.108
-	-	-	-	-	15.797
-	-	-	-	-	16.547
16.773	16.833	16.757	16.238	16.810	16.858
17.959	18.173	18.035	18.052	18.023	18.258
-	-	18.877	-	-	18.698

Figure 25. Stress vs Aggression. The molecule with retention time 4.150 min is only present in the intra-fight sample and none of the rest. This molecule can potentially be one of the aggression chemosignal.

IV. Appendices

Appendix A. Statistical Analysis of the Behavioral Samples

Table 1. One-way ANOVA test for the behavioral significance samples

One-way ANOVA: Time interacting with food						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	154526.1569	2	77263.08	12.46058	4.38E-05	3.190727
Within Groups	297628.8235	48	6200.6			
Total	452154.9804	50				

One-way ANOVA: Time it took to reach the food						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	55174.71502	2	27587.36	4.057169	0.028799	3.354131
Within Groups	183590.7516	27	6799.657			
Total	238765.4667	29				

One-way ANOVA: Success rates						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.058823529	2	0.529412	2.25	0.116404	3.190727
Within Groups	11.29411765	48	0.235294			
Total	12.35294118	50				

Table 2. T-test for the behavioral significance samples

t-Test: Time interacting with food						
Condition	*Water*	*Own*	*Water*	*Bigger*	*Own*	*Bigger*
Mean	140.8235	24.52941176	140.8235	23.58824	24.52941	23.58824
Variance	13105.65	2288.764706	13105.65	3207.382	2288.765	3207.382
Observations	17	17	17	17	17	17
Pooled Variance	7697.21		8156.518		2748.074	
Hypothesized Mean Difference	0		0		0	
df	32		32		32	
t Stat	3.864564		3.78456		0.052344	
P(T<=t) one-tail	0.000256		0.000319		0.47929	
t Critical one-tail	1.693889		1.693889		1.693889	
P(T<=t) two-tail	0.000512		0.000639		0.95858	
t Critical two-tail	2.036933		2.036933		2.036933	

t-Test: Time it took to reach food						
Condition	*Water*	*Own*	*Water*	*Bigger*	*Own*	*Bigger*
Mean	88.92308	168.4	88.92308	183.7143	168.4	183.7143
Variance	6495.91	7583.155556	6495.91	6231.905	7583.156	6231.905
Observations	13	10	13	7	10	7
Pooled Variance	6961.873		6407.908		7042.655	
Hypothesized Mean Difference	0		0		0	
df	21		18		15	
t Stat	-2.26457		-2.5259		-0.3703	
P(T<=t) one-tail	0.017122		0.010569		0.358168	
t Critical one-tail	1.720743		1.734064		1.75305	
P(T<=t) two-tail	0.034244		0.021137		0.716336	
t Critical two-tail	2.079614		2.100922		2.13145	

t-Test: Succes rate						
Condition	*Water*	*Own*	*Water*	*Bigger*	*Own*	*Bigger*
Mean	0.764706	0.588235294	0.764706	0.411765	0.588235	0.411765
Variance	0.191176	0.257352941	0.191176	0.257353	0.257353	0.257353
Observations	17	17	17	17	17	17
Pooled Variance	0.224265		0.224265		0.257353	
Hypothesized Mean Difference	0		0		0	
df	32		32		32	
t Stat	1.086429		2.172858		1.014185	
P(T<=t) one-tail	0.142703		0.018653		0.159051	
t Critical one-tail	1.693889		1.693889		1.693889	
P(T<=t) two-tail	0.285406		0.037306		0.318102	
t Critical two-tail	2.036933		2.036933		2.036933	

Appendix B. Calibrations Data for Subjects 1-3

Table 3. Calibration data for subjects 1-3. The average retention time for the VMA, UA, DA, XA, GUA, 5HT, L-Trp, 5-HIAA, HVA and Nac5HT were 2.47, 2.90, 3.45, 3.86, 7.51, 8.67, 11.41, 12.75, 14.23, 15.14 min, respectively.

VMA									
		UV							
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.8								
Dilution 1	2.112	0.2614	0.2669	0.2630	0.2638	2.463	2.473	2.468	2.468
Dilution 2	0.528	0.0638	0.0659	0.0632	0.0643	2.470	2.468	2.467	2.468
Dilution 3	0.132	0.0116	0.0115	0.0133	0.0121	2.465	2.470	2.478	2.471

Uric Acid									
		UV							
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 1	2.064	0.8171	0.8233	0.8237	0.8214	2.892	2.910	2.898	2.900
Dilution 2	0.516	0.2136	0.2132	0.2130	0.2133	2.905	2.903	2.902	2.903
Dilution 3	0.129	0.0439	0.0443	0.0431	0.0438	2.905	2.902	2.910	2.906
Dilution 4	0.0323	0.0101	0.0101	0.0106	0.0103	2.902	2.902	2.902	2.902

Dopamine									
		UV							
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 1	2.064	0.2399	0.2398	0.2399	0.2399	3.425	3.448	3.437	3.437
Dilution 2	0.516	0.0624	0.0642	0.0641	0.0636	3.448	3.452	3.450	3.450
Dilution 3	0.129	0.0126	0.0122	0.0125	0.0124	3.457	3.450	3.465	3.457

Xanthine									
		UV							
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	53.2								
Dilution 1	2.128	1.6265	1.641	1.6313	1.6329	3.852	3.877	3.858	3.862
Dilution 2	0.532	0.4258	0.4252	0.4242	0.4251	3.867	3.868	3.862	3.866
Dilution 3	0.133	0.0882	0.0875	0.0876	0.0878	3.863	3.865	3.875	3.868
Dilution 4	0.0333	0.0181	0.0199	0.0184	0.0188	3.862	3.867	3.862	3.864

Guanosine									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.2								
Dilution 1	2.048	0.8027	0.8026	0.8030	0.8028	7.465	7.523	7.492	7.493
Dilution 2	0.512	0.2042	0.2043	0.2053	0.2046	7.513	7.517	7.498	7.509
Dilution 3	0.128	0.0380	0.0394	0.0389	0.0388	7.502	7.502	7.547	7.517
Dilution 4	0.0320	0.0099	0.0090	0.0090	0.0093	7.515	7.512	7.522	7.516

5-HT									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 1	2.064	0.6784	0.6786	0.6801	0.6790	8.623	8.667	8.65	8.647
Dilution 2	0.516	0.1773	0.1745	0.1744	0.1754	8.670	8.672	8.667	8.670
Dilution 3	0.129	0.0358	0.0360	0.0358	0.0359	8.672	8.670	8.700	8.681
Dilution 4	0.0323	0.0098	0.0076	0.0071	0.0082	8.693	8.675	8.705	8.691

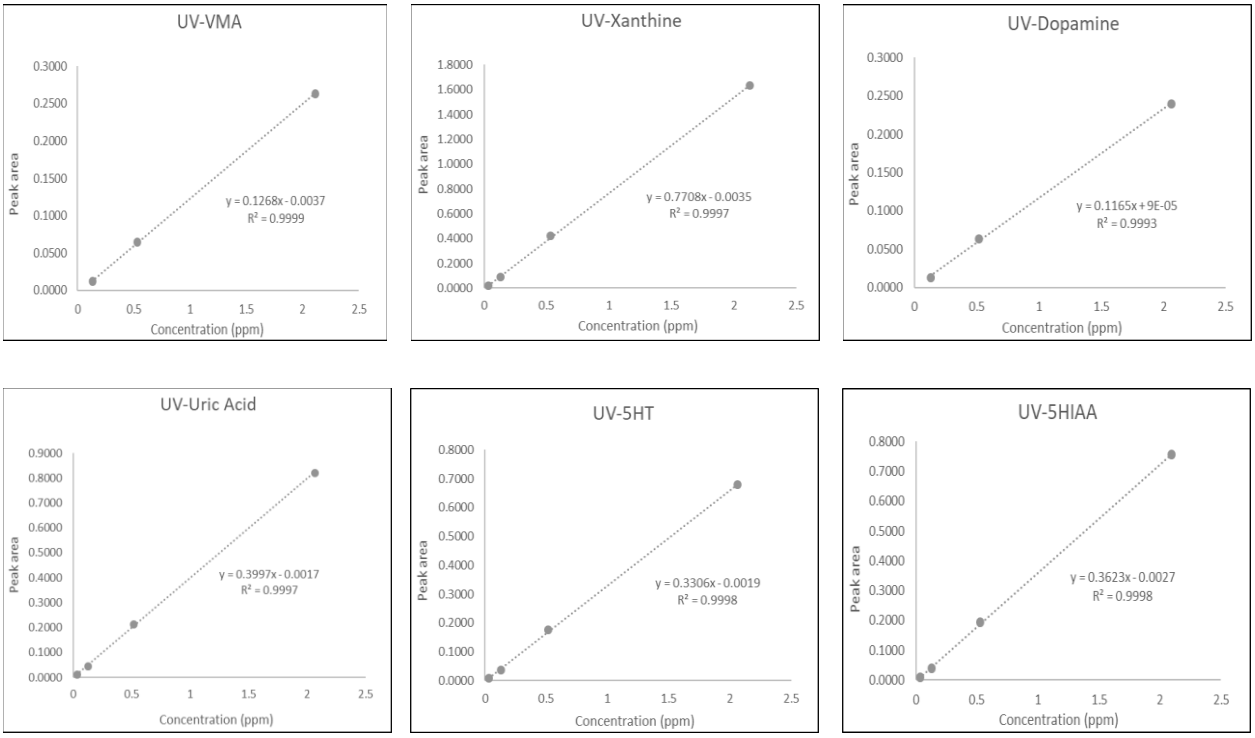
L-Trp									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52								
Dilution 1	2.08	0.7186	0.7183	0.7148	0.7172	11.390	11.418	11.403	11.404
Dilution 2	0.52	0.1867	0.1876	0.1875	0.1873	11.417	11.413	11.407	11.412
Dilution 3	0.13	0.0376	0.0381	0.0377	0.0378	11.408	11.415	11.425	11.416
Dilution 4	0.0325	0.0079	0.0083	0.0081	0.0081	11.408	11.408	11.420	11.412

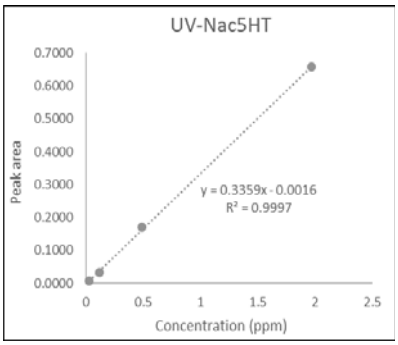
5-HIAA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.4								
Dilution 1	2.096	0.7521	0.7581	0.7559	0.7554	12.738	12.763	12.750	12.750
Dilution 2	0.524	0.1949	0.1948	0.1930	0.1942	12.760	12.750	12.745	12.752
Dilution 3	0.131	0.0394	0.0393	0.0393	0.0393	12.750	12.750	12.757	12.752
Dilution 4	0.0328	0.0086	0.0098	0.0086	0.0090	12.737	12.738	12.757	12.744

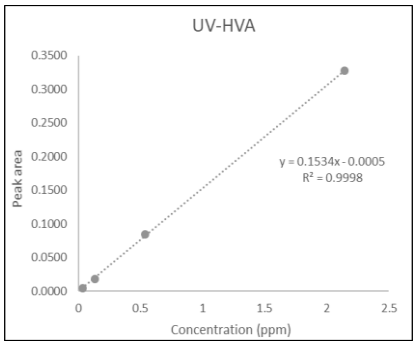
HVA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	53.6								
Dilution 1	2.144	0.3275	0.3278	0.3277	0.3277	14.215	14.238	14.232	14.228
Dilution 2	0.536	0.0851	0.0847	0.0846	0.0848	14.233	14.228	14.227	14.229
Dilution 3	0.134	0.0174	0.0179	0.0185	0.0179	14.240	14.232	14.235	14.236
Dilution 4	0.0335	0.0042	0.0044	0.0044	0.0043	14.230	14.232	14.232	14.231

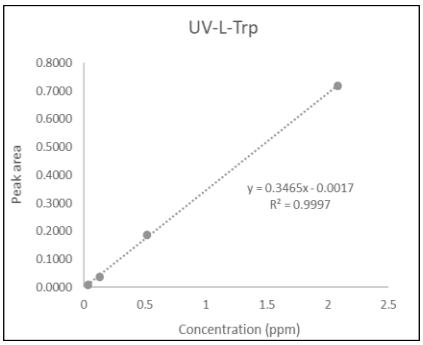
Nac5HT									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	49.2								
Dilution 1	1.968	0.6592	0.6616	0.6531	0.6580	15.132	15.153	15.147	15.144
Dilution 2	0.492	0.1713	0.1701	0.171	0.1708	15.140	15.142	15.138	15.140
Dilution 3	0.123	0.0338	0.0354	0.0337	0.0343	15.157	15.138	15.150	15.148
Dilution 4	0.0308	0.0085	0.0089	0.0079	0.0084	15.148	15.143	15.138	15.143

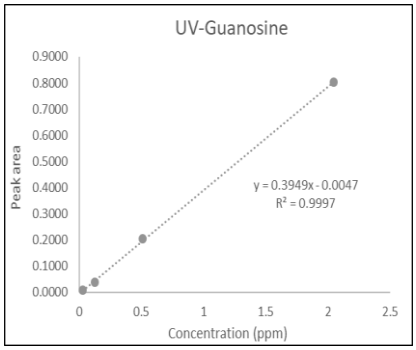
Figure 26. Calibration curves for subjects 1-3.




UV-Nac5HT
Peak area
Concentration (ppm)
y = 0.3359x - 0.0016
R² = 0.9997


UV-HVA
Peak area
Concentration (ppm)
y = 0.1534x - 0.0005
R² = 0.9998


UV-L-Trp
Peak area
Concentration (ppm)
y = 0.3465x - 0.0017
R² = 0.9997


UV-Guanosine
Peak area
Concentration (ppm)
y = 0.3949x - 0.0047
R² = 0.9997

Appendix C. The chromatogram data for subjects 1-3.

Table 4. Subject 1 HPLC results. Peak 9, 17, 33 and 40 correspond to UA, GUA, L-Trp and Nac5HT respectively.

Subject 1		
Peak Number	Retetion Time (min)	Peak Area (mAU*min)
1	1.450	0.2036
2	1.528	0.2223
3	1.620	0.2637
4	1.750	0.0176
5	1.837	0.0862
6	2.038	0.0955
7	2.297	1.9683
8	2.735	1.2988
9	2.903	0.3653
10	3.138	0.8545
11	3.305	0.0994
12	3.702	0.3889
13	3.818	0.8393
14	3.972	0.2371
15	4.315	1.4783
16	4.657	0.0174
17	4.915	0.0949
18	5.178	0.1373
19	5.307	0.0461
20	5.577	0.0145
21	6.013	0.0890

Subject 1		
Peak Number	Retetion Time (min)	Peak Area (mAU*min)
22	6.313	0.0343
23	6.553	0.3546
24	6.755	0.1601
25	6.958	0.0233
26	7.187	0.0150
27	7.490	0.1487
28	8.298	0.0662
29	9.170	0.0716
30	9.692	0.9047
31	10.370	0.3667
32	10.838	0.0471
33	11.402	0.0133
34	11.553	0.0057
35	12.625	0.0284
36	12.822	0.0703
37	12.007	0.0232
38	13.247	0.0427
39	13.455	0.0331
40	13.982	0.0730
41	14.775	0.0712
42	15.132	0.0261
43	16.743	0.0186

Table 5. Subject 2 HPLC results. Peak 15, 28 and 38 correspond to UA, GUA and L-Trp, respectively.

Subject 2			Subject 2		
Peak Number	Retetion Time (min)	Peak Area (mAU*min)	Peak Number	Retetion Time (min)	Peak Area (mAU*min)
1	1.468	0.0093	**29**	8.325	0.0634
2	1.523	0.3198	**30**	8.583	0.0664
3	1.625	0.8732	**31**	8.955	0.0729
4	1.702	0.0054	**32**	9.243	0.0883
5	1.833	0.2261	**33**	9.737	0.1287
6	1.887	0.2047	**34**	10.040	0.1242
7	2.008	0.2120	**35**	10.598	0.0175
8	2.130	0.1991	**36**	10.728	0.0129
9	2.297	0.6314	**37**	10.980	0.3314
10	2.408	0.2340	**38**	11.422	0.3314
11	2.653	0.1136	**39**	11.878	0.0425
12	2.713	0.0247	**40**	12.130	0.0147
13	2.828	0.2978	**41**	12.313	0.0166
14	2.878	0.8646	**42**	12.520	0.0030
15	2.920	1.1.994	**43**	12.707	0.0018
16	3.108	0.0555	**44**	12.848	0.0318
17	3.178	0.0569	**45**	13.008	0.0503
18	3.405	0.3376	**46**	13.460	0.6137
19	3.703	1.8840	**47**	13.677	0.1087
20	3.860	0.0914	**48**	13.982	0.0271
21	4.012	0.3259	**49**	14.318	0.0074
22	4.338	0.6702	**50**	14.538	0.0338
23	4.992	0.8380	**51**	15.108	0.0104
24	5.990	0.2223	**52**	15.797	0.5298
25	6.623	0.2766	**53**	16.547	0.0090
26	6.825	0.8250	**54**	16.858	0.0250
27	7.128	0.6382	**55**	18.258	0.3163
28	7.517	1.2303	**56**	18.698	0.2886

Table 6. Subject 3 HPLC results. Peak 4, 13, 21 correspond to UA, GUA and L-Trp, respectively

Subject 3		
Peak Number	**Retetion Time (min)**	**Peak Area (mAU*min)**
1	2.093	0.0035
2	2.272	0.0473
3	2.780	0.0220
4	2.910	0.0790
5	3.075	0.0050
6	3.368	0.0045
7	3.673	0.1283
8	3.977	0.0436
9	4.390	0.0397
10	4.970	0.0301
11	6.005	0.0273
12	6.600	0.0250
13	7.503	0.2301
14	8.330	0.0059
15	8.590	0.0083
16	8.948	0.0042
17	9.175	0.0053
18	10.043	0.0119
19	10.595	0.0026
20	10.980	0.0026
21	11.407	0.0211
22	11.882	0.0053
23	12.115	0.0036
24	12.873	0.0127
25	13.008	0.0215
26	13.463	0.0961
27	13.693	0.0033
28	14.002	0.0025
29	15.817	0.0346
30	18.267	0.0284
31	18.705	0.0072

Appendix D. Calibration Data for aggression samples

Table 7. Calibration data for samples 12.2A,3,2B – 13.2A,3,2B. The average retention time for the VMA, UA, DA, XA, GUA, 5HT, L-Trp, 5-HIAA, HVA and Nac5HT were 2.44, 2.88, 3.45, 3.83, 7.45, 8.65, 11.39, 12.68, 14.15 and 15.10 min, respectively. We decided to add an additional dilution (1:800) to have a better relationship at the lower concentrations.

VMA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.8								
Dilution 0	2.112								
Dilution1	0.528	0.0639	0.0634	0.0638	0.0637	2.448	2.443	2.447	2.446
Dilution 2	0.132	0.0158	0.0156	0.0158	0.0157	2.442	2.445	2.445	2.444
Dilution 3	0.066	0.0091	0.0091	0.0090	0.0091	2.438	2.440	2.430	2.436
Dilution 4	0.0330	0.0049	0.0043	0.0050	0.0047	2.445	2.438	2.435	2.439

Uric Acid									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 0	2.064								
Dilution 1	0.516	0.0696	0.0697	0.0697	0.0697	2.888	2.878	2.877	2.881
Dilution 2	0.129	0.0184	0.0175	0.0177	0.0179	2.875	2.888	2.883	2.882
Dilution 3	0.0645	0.0089	0.0093	0.0092	0.0091	2.877	2.875	2.867	2.873
Dilution 4	0.0323	0.0037	0.0040	0.0039	0.0039	2.875	2.872	2.872	2.873

Dopamine									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 0	2.064								
Dilution 1	0.516	0.0621	0.0617	0.0612	0.0617	3.427	3.422	3.425	3.4247
Dilution 2	0.129	0.0193	0.0188	0.0184	0.0188	3.422	3.442	3.433	3.4323
Dilution 3	0.0645	0.0162	0.0149	0.0164	0.0158	3.475	3.472	3.462	3.4697
Dilution 4	0.0323	0.0097	0.0089	0.0099	0.0095	3.472	3.465	3.465	3.4673

Xanthine									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	53.2								
Dilution 0	2.128								
Dilution 1	0.532	0.4261	0.4264	0.4257	0.4261	3.845	3.833	3.828	3.835
Dilution 2	0.133	0.1081	0.1077	0.1077	0.1078	3.827	3.840	3.838	3.835
Dilution 3	0.0665	0.0543	0.0542	0.0535	0.0540	3.830	3.828	3.817	3.825
Dilution 4	0.0333	0.0167	0.0169	0.0165	0.0167	3.823	3.815	3.822	3.820

Guanosine									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.8								
Dilution 0	2.112								
Dilution 1	0.528	0.3982	0.3987	0.3986	0.3985	7.500	7.467	7.44	7.469
Dilution 2	0.132	0.0997	0.0995	0.0998	0.0997	7.447	7.467	7.475	7.463
Dilution 3	0.066	0.0390	0.0383	0.0383	0.0385	7.453	7.453	7.410	7.439
Dilution 4	0.0330	0.0064	0.0062	0.0054	0.0060	7.405	7.398	7.435	7.413

5-HT									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 0	2.064								
Dilution 1	0.516	0.1681	0.1678	0.1681	0.1680	8.667	8.657	8.640	8.6547
Dilution 2	0.129	0.0416	0.0412	0.0416	0.0415	8.638	8.657	8.663	8.6527
Dilution 3	0.0645	0.0208	0.0205	0.0207	0.0207	8.653	8.648	8.623	8.6413
Dilution 4	0.0323	0.0087	0.0096	0.0088	0.0090	8.633	8.623	8.645	8.6337

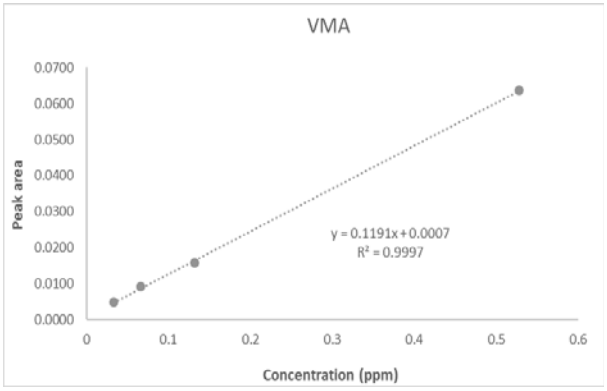
L-Trp									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52								
Dilution 0	2.08								
Dilution 1	0.520	0.1220	0.1226	0.1221	0.1222	11.398	11.385	11.375	11.3860
Dilution 2	0.130	0.0298	0.0304	0.0302	0.0301	11.375	11.380	11.382	11.3790
Dilution 3	0.065	0.0150	0.0154	0.0154	0.0153	11.372	11.362	11.358	11.3640
Dilution 4	0.0325	0.0039	0.0039	0.0037	0.0038	11.530	11.355	11.370	11.4183

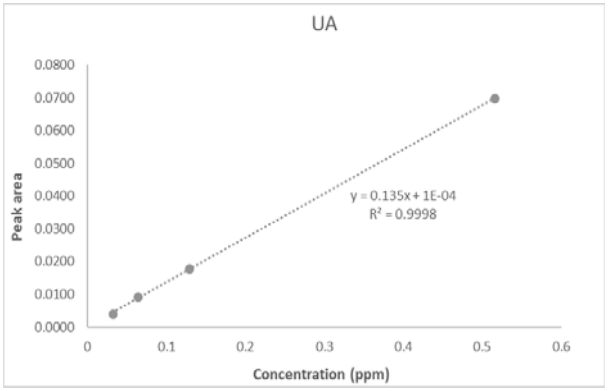
5-HIAA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.4								
Dilution 0	2.096								
Dilution 1	0.524	0.1848	0.1839	0.1842	0.1843	12.717	12.700	12.6880	12.7017
Dilution 2	0.131	0.0471	0.0467	0.0470	0.0469	12.690	12.690	12.693	12.6910
Dilution 3	0.0655	0.0234	0.0236	0.0234	0.0235	12.687	12.677	12.667	12.6770
Dilution 4	0.0328	0.0101	0.0099	0.0099	0.0100	12.672	12.660	12.655	12.6623

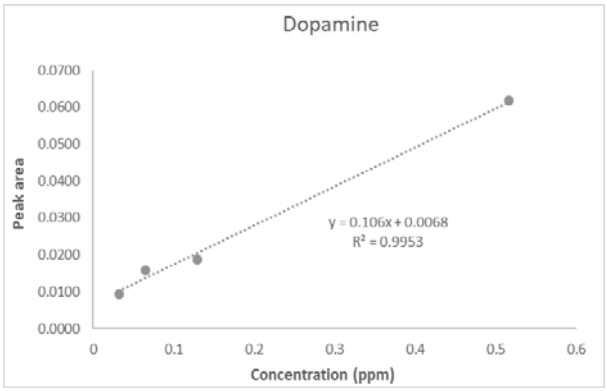
HVA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	53.6								
Dilution 0	2.144								
Dilution 1	0.536	0.0799	0.0812	0.0802	0.0804	14.173	14.160	14.152	14.1617
Dilution 2	0.134	0.0205	0.0206	0.0209	0.0207	14.153	14.160	14.157	14.1567
Dilution 3	0.067	0.0114	0.0114	0.0114	0.0114	14.162	14.145	14.140	14.1490
Dilution 4	0.0335	0.0043	0.0053	0.0050	0.0049	14.142	14.145	14.145	14.1440

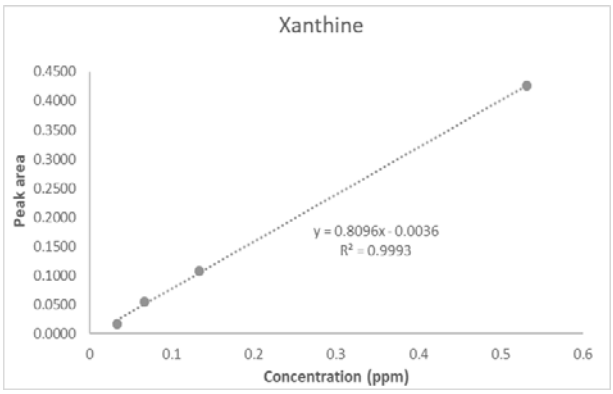
Nac5HT									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	49.2								
Dilution 0	1.968								
Dilution 1	0.492	0.1547	0.155	0.1547	0.1548	15.120	15.103	15.095	15.106
Dilution 2	0.123	0.0392	0.0391	0.0393	0.0392	15.097	15.102	15.098	15.099
Dilution 3	0.0615	0.0191	0.0191	0.0191	0.0191	15.092	15.090	15.085	15.089
Dilution 4	0.0308	0.0080	0.0094	0.0080	0.0085	15.107	15.087	15.083	15.092

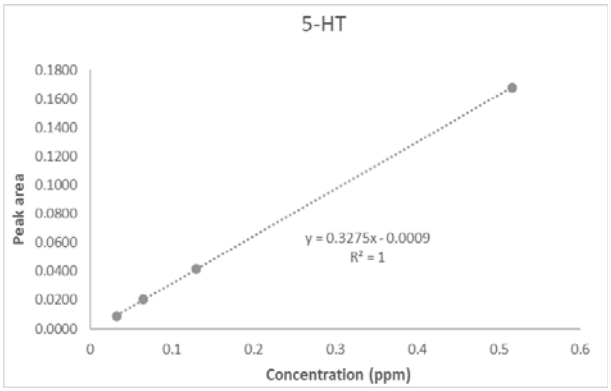
Figure 27. Calibration curves for samples 12.2A,3,2B – 13.2A,3,2B.

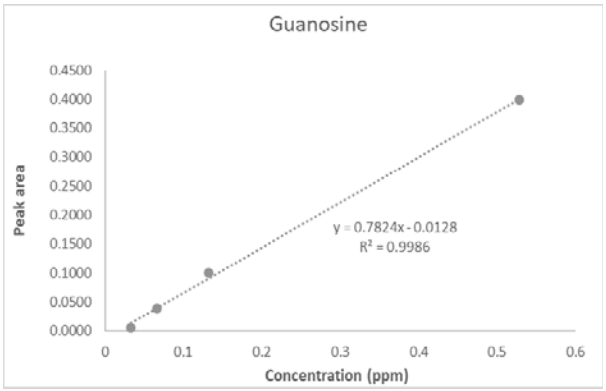


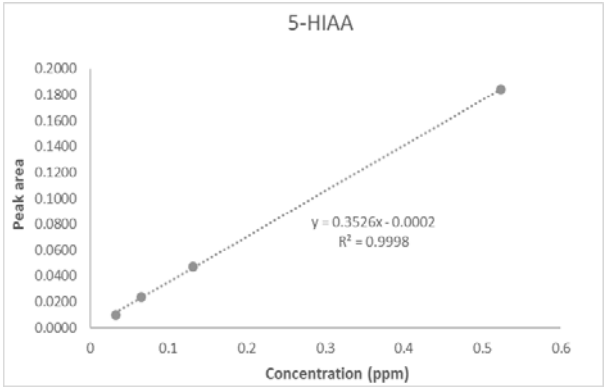


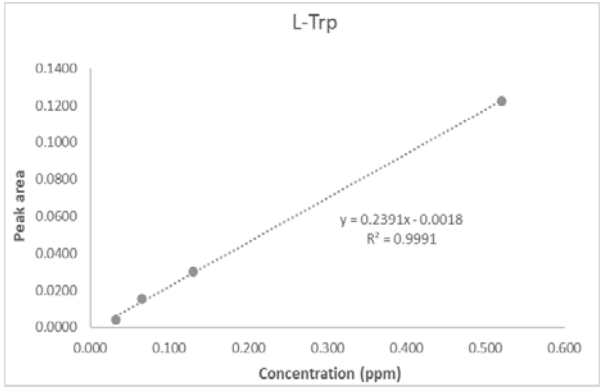


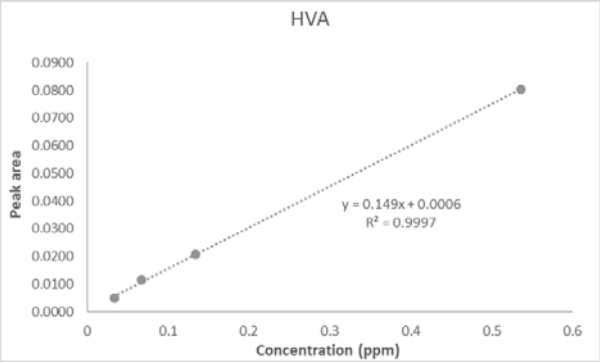












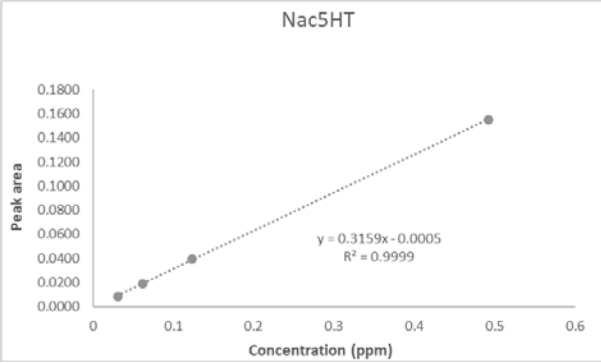


Table 8. Calibration data for samples 12.1A,1B, 13.1A,1B and 14. The average retention time for the VMA, UA, DA, XA, GUA, 5HT, L-Trp, 5-HIAA, HVA and Nac5HT were 2.45, 2.88, 3.45, 3.84, 7.46, 8.66, 11.37, 12.68, 14.16 and 15.10 min, respectively.

VMA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.8								
Dilution 0	2.112								
Dilution1	0.528	0.0637	0.0634	0.0635	0.0635	2.453	2.455	2.447	2.452
Dilution 2	0.132	0.0132	0.0133	0.0132	0.0132	2.452	2.455	2.448	2.452
Dilution 3	0.066	0.0056	0.0056	0.0060	0.0057	2.442	2.447	2.447	2.445

Uric Acid									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 0	2.064								
Dilution 1	0.516	0.0731	0.0732	0.0731	0.0731	2.880	2.873	2.870	2.874
Dilution 2	0.129	0.0134	0.0130	0.0133	0.0132	2.882	2.890	2.875	2.882
Dilution 3	0.0645	0.0068	0.0067	0.0068	0.0068	2.860	2.867	2.867	2.865
Dilution 4	0.0323	0.0026	0.0025	0.0027	0.0026	2.895	2.878	2.870	2.881

Dopamine									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 0	2.064								
Dilution 1	0.516	0.0601	0.0605	0.0609	0.0605	3.433	3.427	3.422	3.4273
Dilution 2	0.129	0.0128	0.0132	0.0135	0.0132	3.442	3.445	3.455	3.4473
Dilution 3	0.0645	0.0095	0.0096	0.0096	0.0096	3.433	3.448	3.440	3.4403
Dilution 4	0.0323	0.0048	0.0056	0.0049	0.0051	3.513	3.447	3.448	3.4693

Xanthine									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	53.2								
Dilution 0	2.128								
Dilution 1	0.532	0.4427	0.4424	0.4252	0.4368	3.833	3.828	3.822	3.828
Dilution 2	0.133	0.0782	0.0776	0.0779	0.0779	3.833	3.838	3.845	3.839
Dilution 3	0.0665	0.0382	0.0381	0.0390	0.0384	3.807	3.812	3.813	3.811
Dilution 4	0.0333	0.0166	0.0169	0.0168	0.0168	3.922	3.813	3.922	3.886

Guanosine									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.8								
Dilution 0	2.112								
Dilution 1	0.528	0.4264	0.4264	0.4269	0.4266	7.452	7.457	7.400	7.436
Dilution 2	0.132	0.0712	0.0717	0.0715	0.0715	7.428	7.452	7.575	7.485
Dilution 3	0.066	0.0319	0.0317	0.0326	0.0321	7.388	7.390	7.392	7.390
Dilution 4	0.0330	0.0132	0.0118	0.0117	0.0122	7.843	7.397	7.403	7.548

5-HT									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	51.6								
Dilution 0	2.064								
Dilution 1	0.516	0.1804	0.1806	0.1805	0.1805	8.655	8.655	8.613	8.6410
Dilution 2	0.129	0.0302	0.0305	0.0302	0.0303	8.628	8.700	8.705	8.6777
Dilution 3	0.0645	0.0150	0.0153	0.0153	0.0152	8.612	8.613	8.612	8.6123
Dilution 4	0.0323	0.0055	0.0061	0.0067	0.0061	8.860	8.622	8.620	8.7007

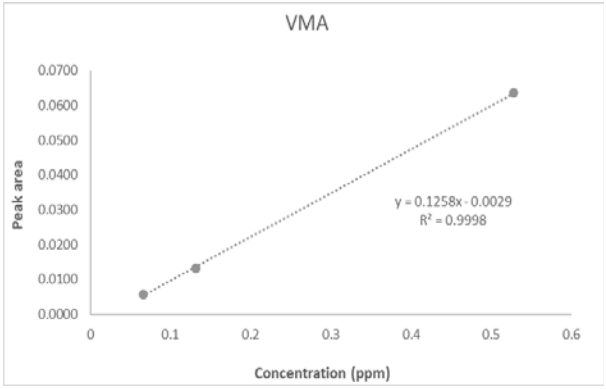
L-Trp									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52								
Dilution 0	2.08								
Dilution 1	0.520	0.1305	0.1308	0.1300	0.1304	11.372	11.383	11.345	11.3667
Dilution 2	0.130	0.0234	0.0225	0.0226	0.0228	11.350	11.418	11.383	11.3837
Dilution 3	0.065	0.0113	0.0110	0.0112	0.0112	11.350	11.345	11.340	11.3450
Dilution 4	0.0325	0.0036	0.0036	0.0042	0.0038	11.462	11.335	11.358	11.3850

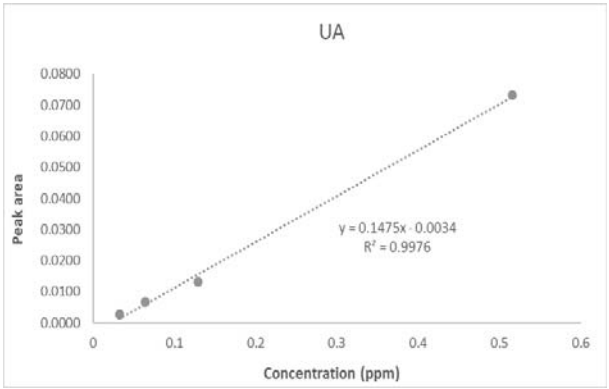
5-HIAA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	52.4								
Dilution 0	2.096								
Dilution 1	0.524	0.1938	0.1931	0.1930	0.1933	12.692	12.698	12.665	12.6850
Dilution 2	0.131	0.0343	0.0343	0.0337	0.0341	12.655	12.720	12.693	12.6893
Dilution 3	0.0655	0.0166	0.0167	0.0166	0.0166	12.667	12.657	12.663	12.6623
Dilution 4	0.0328	0.0070	0.0073	0.0068	0.0070	12.740	12.667	12.672	12.6930

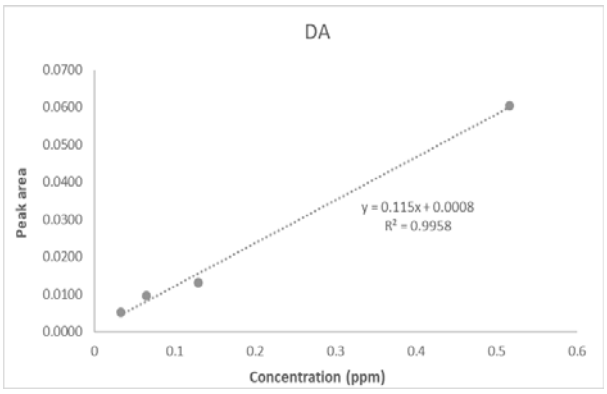
HVA									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	53.6								
Dilution 0	2.144								
Dilution 1	0.536	0.0849	0.0843	0.0841	0.0844	14.167	14.165	14.133	14.1550
Dilution 2	0.134	0.0148	0.0143	0.0139	0.0143	14.143	14.180	14.158	14.1603
Dilution 3	0.067	0.0064	0.0062	0.0066	0.0064	14.148	14.145	14.145	14.1460
Dilution 4	0.0335	0.0027	0.0026	0.0021	0.0025	14.195	14.153	14.162	14.1700

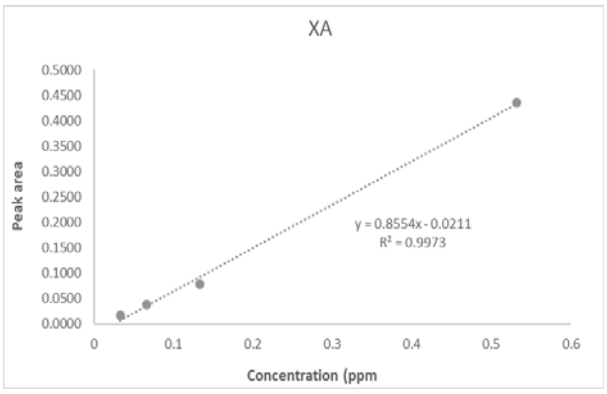
Nac5HT									
UV									
Dilutions	Concentration (ppm)	Peak area	Peak area	Peak area	Average peak area	Ret time	Ret time	Ret time	Average ret time
Stock	49.2								
Dilution 0	1.968								
Dilution 1	0.492	0.1630	0.1625	0.1628	0.1628	15.093	15.095	15.067	15.085
Dilution 2	0.123	0.0289	0.0288	0.028	0.0286	15.072	15.100	15.083	15.085
Dilution 3	0.0615	0.0140	0.0141	0.0141	0.0141	15.075	15.065	15.072	15.071
Dilution 4	0.0308	0.0055	0.0057	0.0056	0.0056	15.115	15.083	15.075	15.091

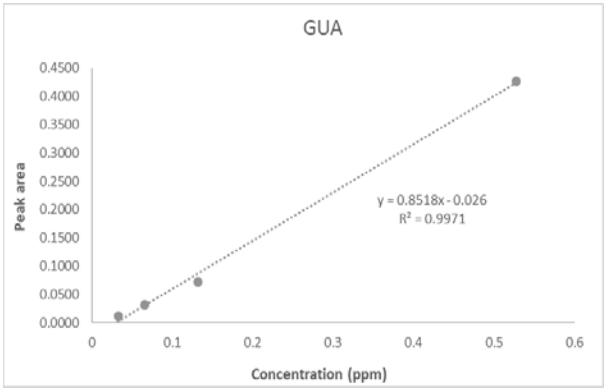
Figure 28. Calibration curves for samples 12.1A,1B, 13.1A,1B and 14.

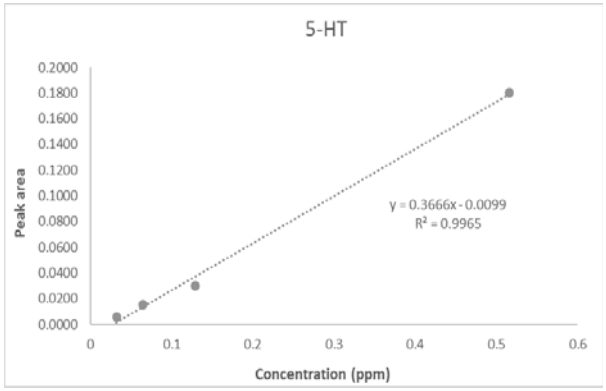


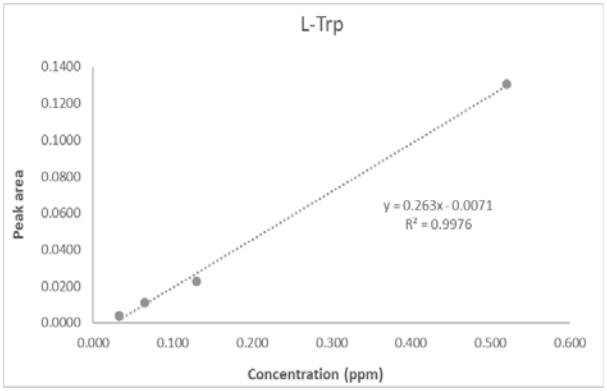


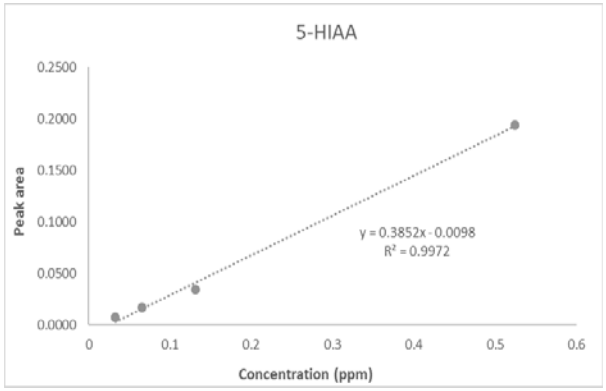

DA
Peak area
Concentration (ppm)
y = 0.115x + 0.0008
R² = 0.9958

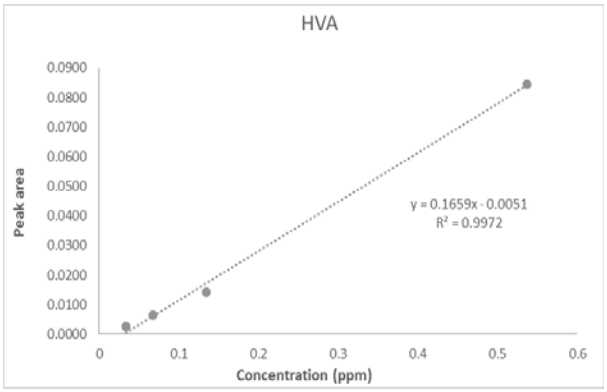

XA
Peak area
Concentration (ppm
y = 0.8554x - 0.0211
R² = 0.9973

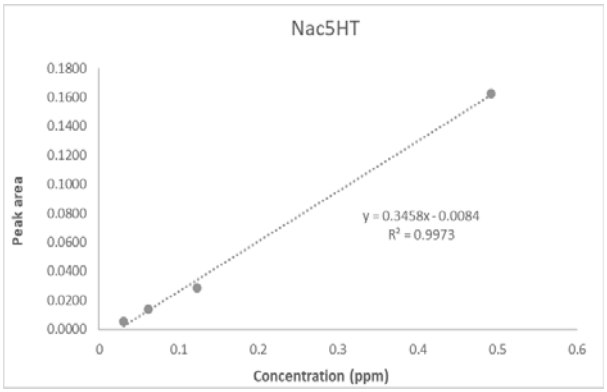

GUA
Peak area
Concentration (ppm)
y = 0.8518x - 0.026
R² = 0.9971


5-HT
Peak area
Concentration (ppm)
y = 0.3666x - 0.0099
R² = 0.9965


L-Trp
Peak area
Concentration (ppm)
y = 0.263x - 0.0071
R² = 0.9976

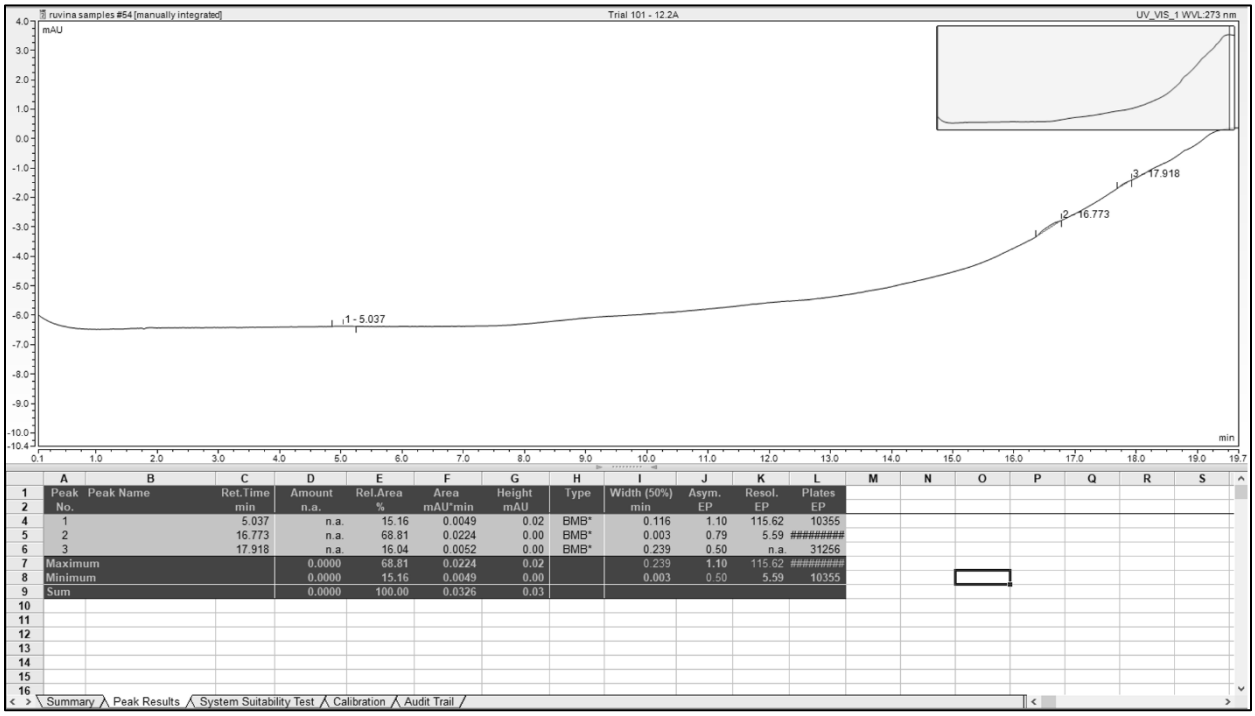

5-HIAA
Peak area
Concentration (ppm)
y = 0.3852x - 0.0098
R² = 0.9972


HVA
Peak area
Concentration (ppm)
y = 0.1659x - 0.0051
R² = 0.9972


Nac5HT
Peak area
Concentration (ppm)
y = 0.3458x - 0.0084
R² = 0.9973

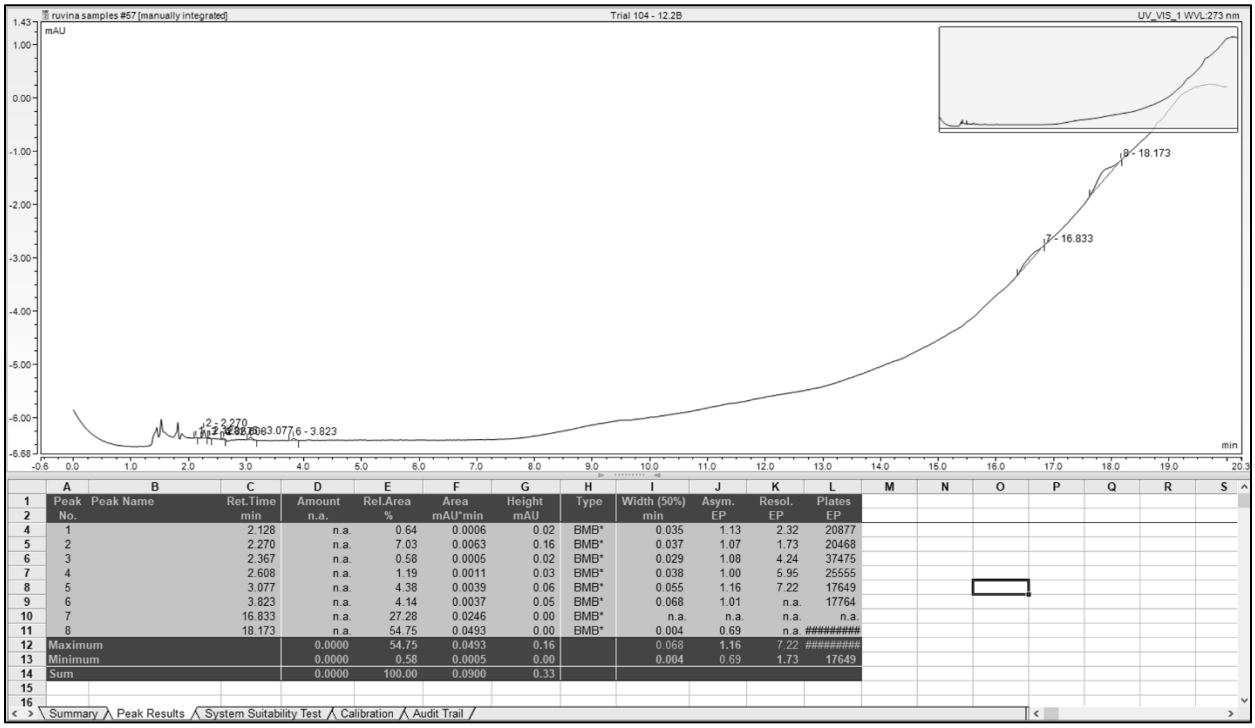
Appendix E. Complete chromatograms for the fights 12, 13 and 14.

Figure 29. Fight 12 complete HPLC chromatograms. The number 1 denotes the small crayfish (subordinate), 2 the bigger crayfish (dominant) and 3 the sample collected during the fight. The letter A symbolizes the prefight samples, whereas B the post-fight sample. The number 12 is the fight number.



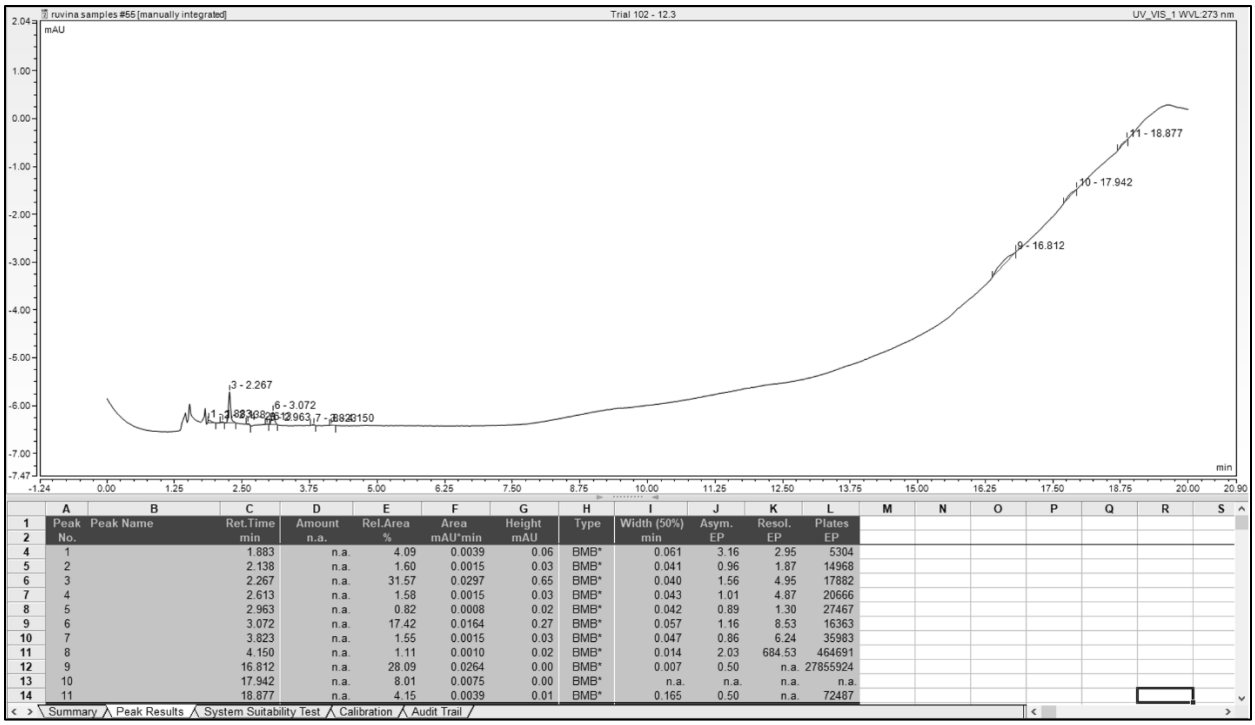
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		5.037	n.a.	15.16	0.0049	0.02	BMB*	0.116	1.10	115.62	10355
2		16.773	n.a.	68.81	0.0224	0.00	BMB*	0.003	0.79	5.59	#########
3		17.918	n.a.	16.04	0.0052	0.00	BMB*	0.239	0.50	n.a.	31256
Maximum			0.0000	68.81	0.0224	0.02		0.239	1.10	115.62	#########
Minimum			0.0000	15.16	0.0049	0.00		0.003	0.50	5.59	10355
Sum			0.0000	100.00	0.0326	0.03					

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



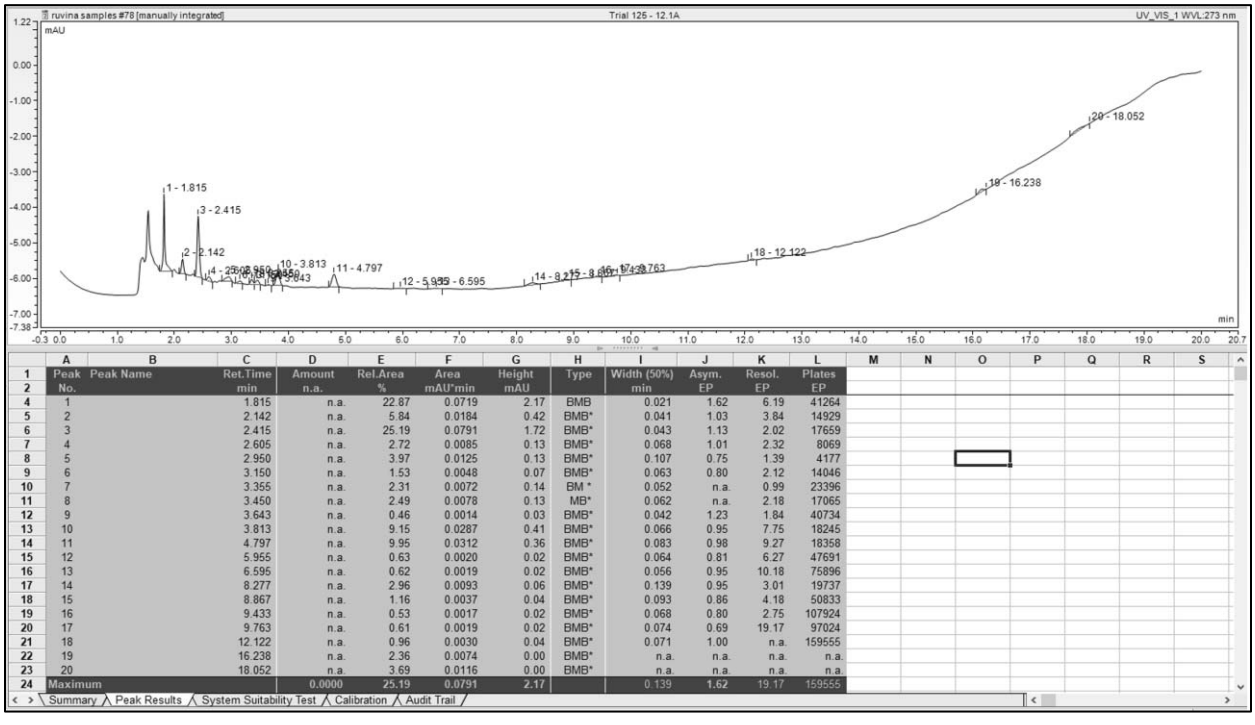
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		2.128	n.a.	0.64	0.0006	0.02	BMB*	0.035	1.13	2.32	20877
2		2.270	n.a.	7.03	0.0063	0.16	BMB*	0.037	1.07	1.73	20468
3		2.367	n.a.	0.58	0.0005	0.02	BMB*	0.029	1.08	4.24	37475
4		2.608	n.a.	1.19	0.0011	0.03	BMB*	0.038	1.00	5.95	25555
5		3.077	n.a.	4.38	0.0039	0.06	BMB*	0.055	1.16	7.22	17649
6		3.823	n.a.	4.14	0.0037	0.05	BMB*	0.068	1.01	n.a.	17764
7		16.833	n.a.	27.28	0.0246	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
8		18.173	n.a.	54.75	0.0493	0.00	BMB*	0.004	0.69	n.a.	#########
Maximum			0.0000	54.75	0.0493	0.16		0.068	1.16	7.22	#########
Minimum			0.0000	0.58	0.0005	0.00		0.004	0.69	1.73	17649
Sum			0.0000	100.00	0.0900	0.33					

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



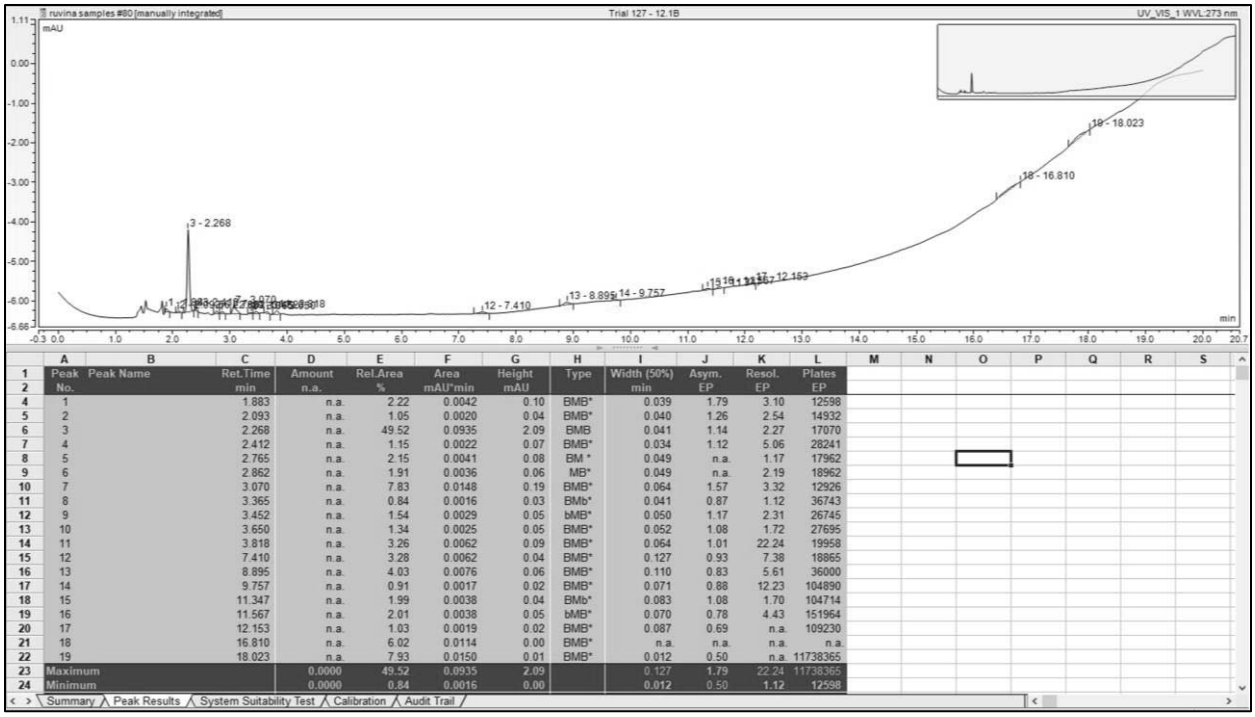
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		1.883	n.a.	4.09	0.0039	0.06	BMB*	0.061	3.16	2.95	5304
2		2.138	n.a.	1.60	0.0015	0.03	BMB*	0.041	0.96	1.87	14968
3		2.267	n.a.	31.57	0.0297	0.65	BMB*	0.040	1.56	4.95	17882
4		2.613	n.a.	1.58	0.0015	0.03	BMB*	0.043	1.01	4.87	20666
5		2.963	n.a.	0.82	0.0008	0.02	BMB*	0.042	0.89	1.30	27467
6		3.072	n.a.	17.42	0.0164	0.27	BMB*	0.057	1.16	8.53	16363
7		3.823	n.a.	1.55	0.0015	0.03	BMB*	0.047	0.86	6.24	35983
8		4.150	n.a.	1.11	0.0010	0.02	BMB*	0.014	2.03	684.53	464691
9		16.812	n.a.	28.09	0.0264	0.00	BMB*	0.007	0.50	n.a.	27855924
10		17.942	n.a.	8.01	0.0075	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
11		18.877	n.a.	4.15	0.0039	0.01	BMB*	0.165	0.50	n.a.	72487

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		1.815	n.a.	22.87	0.0719	2.17	BMB	0.021	1.62	6.19	41264
2		2.142	n.a.	5.84	0.0184	0.42	BMB*	0.041	1.03	3.84	14929
3		2.415	n.a.	25.19	0.0791	1.72	BMB*	0.043	1.13	2.02	17659
4		2.605	n.a.	2.72	0.0085	0.13	BMB*	0.068	1.01	2.32	8069
5		2.950	n.a.	3.97	0.0125	0.13	BMB*	0.107	0.75	1.39	4177
6		3.150	n.a.	1.53	0.0048	0.07	BMB*	0.063	0.80	2.12	14046
7		3.355	n.a.	2.31	0.0072	0.14	BM *	0.052	n.a.	0.99	23396
8		3.450	n.a.	2.49	0.0078	0.13	MB*	0.062	n.a.	2.18	17065
9		3.643	n.a.	0.46	0.0014	0.03	BMB*	0.042	1.23	1.84	40734
10		3.813	n.a.	9.15	0.0287	0.41	BMB*	0.066	0.95	7.75	18245
11		4.797	n.a.	9.95	0.0312	0.36	BMB*	0.083	0.98	9.27	18358
12		5.955	n.a.	0.63	0.0020	0.02	BMB*	0.064	0.81	6.27	47691
13		6.595	n.a.	0.62	0.0019	0.02	BMB*	0.056	0.95	10.18	75896
14		8.277	n.a.	2.96	0.0093	0.06	BMB*	0.139	0.95	3.01	19737
15		8.867	n.a.	1.16	0.0037	0.04	BMB*	0.093	0.86	4.18	50833
16		9.433	n.a.	0.53	0.0017	0.02	BMB*	0.068	0.80	2.75	107924
17		9.763	n.a.	0.61	0.0019	0.02	BMB*	0.074	0.69	19.17	97024
18		12.122	n.a.	0.96	0.0030	0.04	BMB*	0.071	1.00	n.a.	159555
19		16.238	n.a.	2.36	0.0074	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
20		18.052	n.a.	3.69	0.0116	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
Maximum			0.0000	25.19	0.0791	2.17		0.139	1.62	19.17	159555

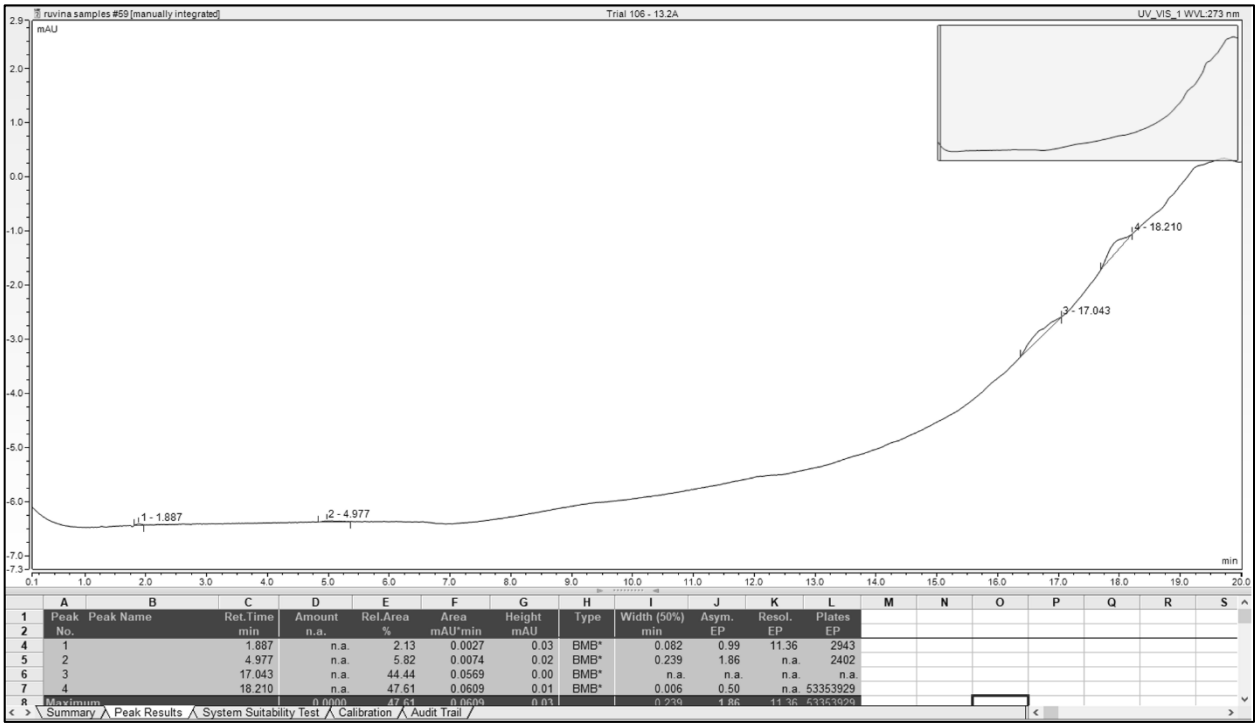
Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



	A	B	C	D	E	F	G	H	I	J	K	L
1	Peak	Peak Name	Ret.Time	Amount	Rel.Area	Area	Height	Type	Width (50%)	Asym.	Resol.	Plates
2	No.		min	n.a.	%	mAU*min	mAU		min	EP	EP	EP
4	1		1.883	n.a.	2.22	0.0042	0.10	BMB*	0.039	1.79	3.10	12598
5	2		2.093	n.a.	1.05	0.0020	0.04	BMB*	0.040	1.26	2.54	14932
6	3		2.268	n.a.	49.52	0.0935	2.09	BMB	0.041	1.14	2.27	17070
7	4		2.412	n.a.	1.15	0.0022	0.07	BMB*	0.034	1.12	5.06	28241
8	5		2.765	n.a.	2.15	0.0041	0.08	BM *	0.049	n.a.	1.17	17962
9	6		2.862	n.a.	1.91	0.0036	0.06	MB*	0.049	n.a.	2.19	18962
10	7		3.070	n.a.	7.83	0.0148	0.19	BMB*	0.064	1.57	3.32	12926
11	8		3.365	n.a.	0.84	0.0016	0.03	BMb*	0.041	0.87	1.12	36743
12	9		3.452	n.a.	1.54	0.0029	0.05	bMB*	0.050	1.17	2.31	26745
13	10		3.650	n.a.	1.34	0.0025	0.05	BMB*	0.052	1.08	1.72	27695
14	11		3.818	n.a.	3.26	0.0062	0.09	BMB*	0.064	1.01	22.24	19958
15	12		7.410	n.a.	3.28	0.0062	0.04	BMB*	0.127	0.93	7.38	18865
16	13		8.895	n.a.	4.03	0.0076	0.06	BMB*	0.110	0.83	5.61	36000
17	14		9.757	n.a.	0.91	0.0017	0.02	BMB*	0.071	0.88	12.23	104890
18	15		11.347	n.a.	1.99	0.0038	0.04	BMb*	0.083	1.08	1.70	104714
19	16		11.567	n.a.	2.01	0.0038	0.05	bMB*	0.070	0.78	4.43	151964
20	17		12.153	n.a.	1.03	0.0019	0.02	BMB*	0.087	0.69	n.a.	109230
21	18		16.810	n.a.	6.02	0.0114	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
22	19		18.023	n.a.	7.93	0.0150	0.01	BMB*	0.012	0.50	n.a.	11738365
23	Maximum			0.0000	49.52	0.0935	2.09		0.127	1.79	22.24	11738365
24	Minimum			0.0000	0.84	0.0016	0.00		0.012	0.50	1.12	12598

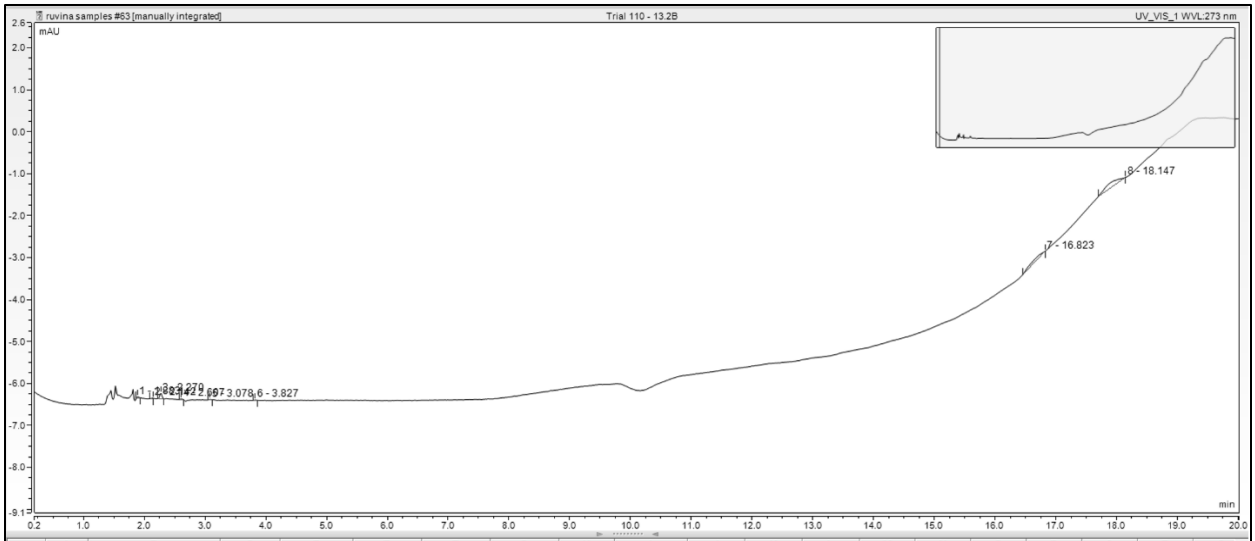
Summary | Peak Results | System Suitability Test | Calibration | Audit Trail

Figure 30. Fight 13 complete HPLC chromatograms. The number 1 denotes the small crayfish (subordinate), 2 the bigger crayfish (dominant) and 3 the sample collected during the fight. The letter A symbolizes the prefight samples, whereas B the post-fight sample. The number 13 is the fight number.



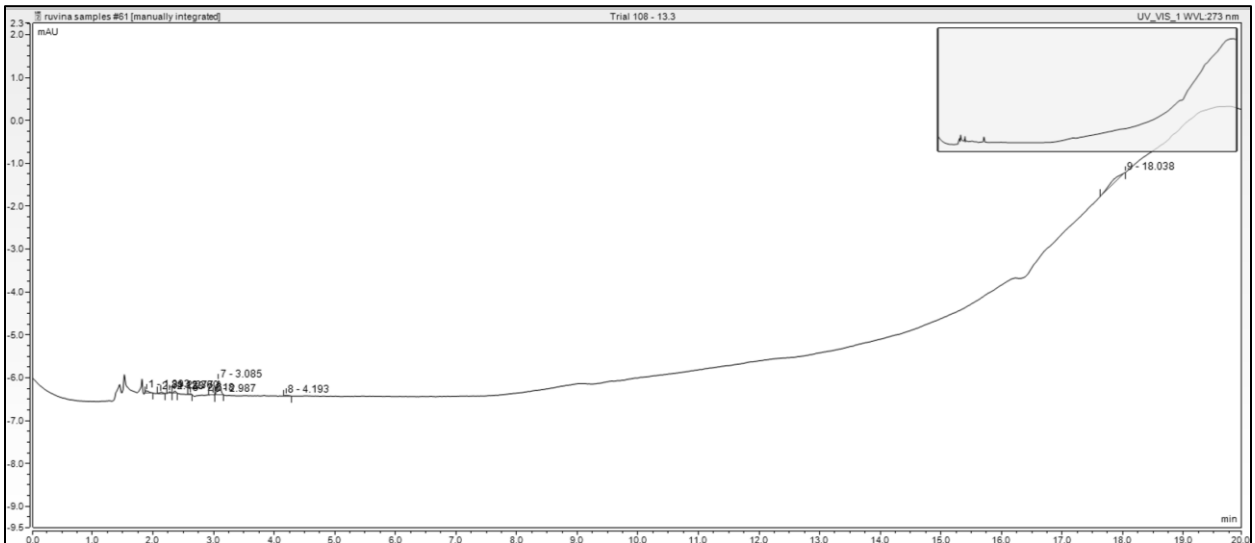
	A	B	C	D	E	F	G	H	I	J	K	L
1	Peak	Peak Name	Ret.Time	Amount	Rel.Area	Area	Height	Type	Width (50%)	Asym.	Resol.	Plates
2	No.		min	n.a.	%	mAU*min	mAU		min	EP	EP	EP
4	1		1.887	n.a.	2.13	0.0027	0.03	BMB*	0.082	0.99	11.36	2943
5	2		4.977	n.a.	5.82	0.0074	0.02	BMB*	0.239	1.86	n.a.	2402
6	3		17.043	n.a.	44.44	0.0569	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
7	4		18.210	n.a.	47.61	0.0609	0.01	BMB*	0.006	0.50	n.a.	53353929
8	Maximum			0.0000	47.61	0.0609	0.03		0.239	1.86	11.36	53353929

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



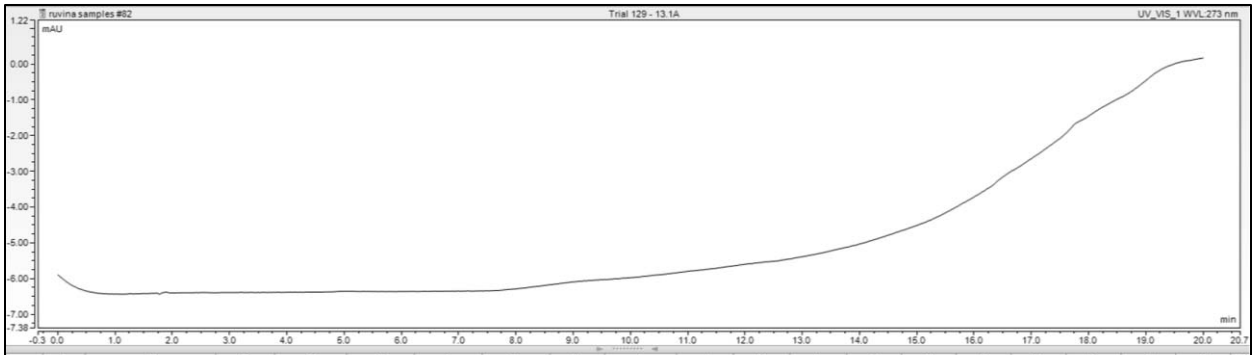
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		1.883	n.a.	2.46	0.0015	0.04	BMB*	0.044	2.26	4.10	9962
2		2.142	n.a.	0.66	0.0004	0.01	BMB*	0.030	0.65	2.14	28413
3		2.270	n.a.	8.78	0.0053	0.13	BMB*	0.041	1.12	5.57	17033
4		2.607	n.a.	1.02	0.0006	0.02	BMB*	0.030	1.23	9.43	40599
5		3.078	n.a.	0.98	0.0006	0.01	BMB*	0.029	1.03	17.33	64435
6		3.827	n.a.	0.92	0.0006	0.01	BMB*	0.022	1.09	n.a.	161423
7		16.823	n.a.	29.44	0.0177	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
8		18.147	n.a.	55.75	0.0335	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
Maximum			0.0000	55.75	0.0335	0.13		0.044	2.26	17.33	161423

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



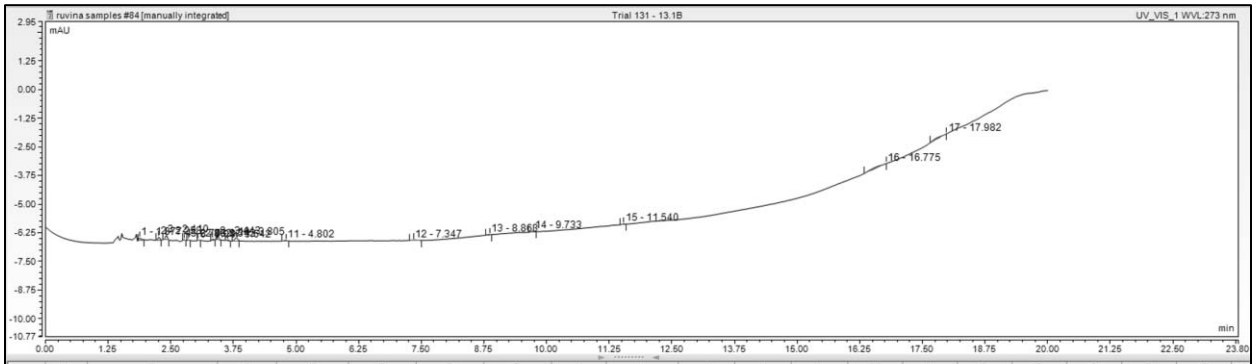
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
4		2.360	n.a.	4.66	0.0025	0.06	bMB*	0.042	0.97	3.72	17292
5		2.618	n.a.	1.35	0.0007	0.02	BMB*	0.040	0.77	5.46	24129
6		2.987	n.a.	1.40	0.0008	0.02	BMB*	0.040	0.73	1.21	30911
7		3.085	n.a.	37.60	0.0201	0.34	BMB*	0.056	1.11	10.48	16862
8		4.193	n.a.	3.15	0.0017	0.02	BMB*	0.069	1.56	n.a.	20562
9		18.038	n.a.	37.23	0.0199	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
Maximum			0.0000	37.60	0.0201	0.34		0.069	2.19	10.48	30911
Minimum			0.0000	1.35	0.0007	0.00		0.035	0.73	1.21	4896

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Peak	Peak Name	Ret.Time	Amount	Rel.Area	Area	Height	Type	Width (50%)	Asym.	Resol.	Plates							
2	No.		min	n.a.	%	mAU*min	mAU		min	EP	EP	EP							
6	Maximum			0.0000	0.00	0.0000	0.00		0.000	0.00	0.00	0							
7	Minimum			0.0000	0.00	0.0000	0.00		0.000	0.00	0.00	0							
8	Sum			0.0000	0.00	0.0000	0.00												

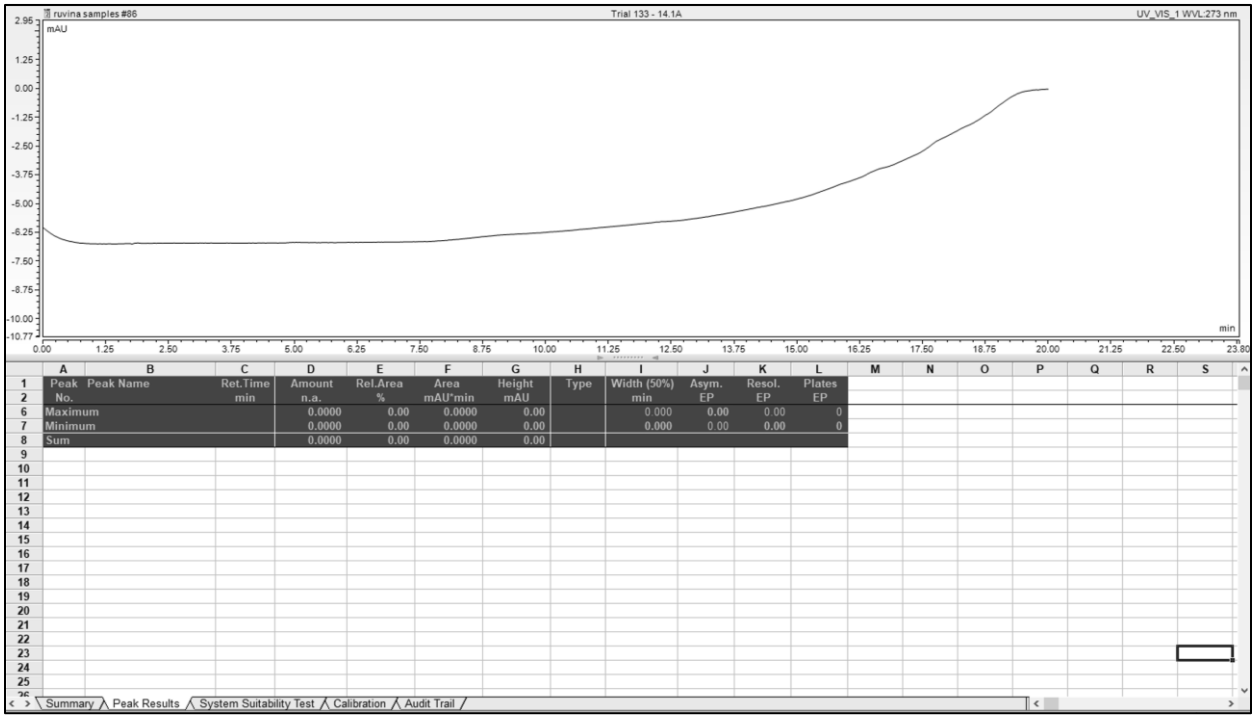
Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



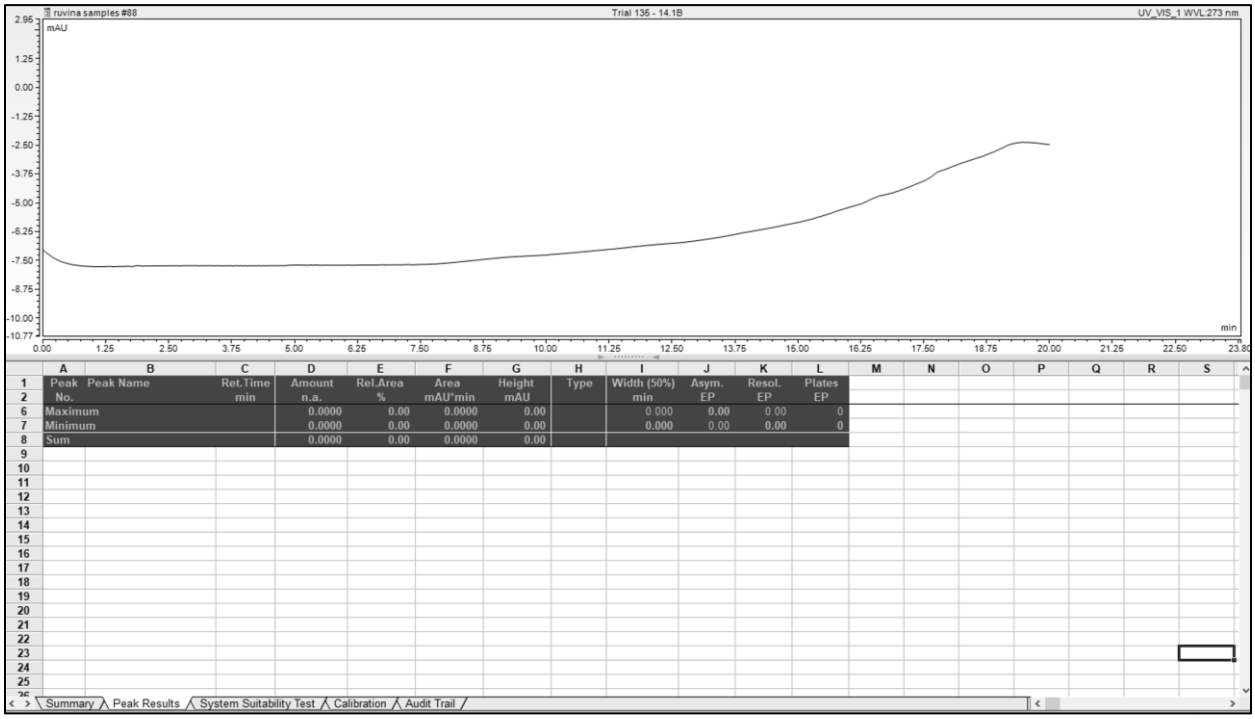
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Peak	Peak Name	Ret.Time	Amount	Rel.Area	Area	Height	Type	Width (50%)	Asym.	Resol.	Plates							
2	No.		min	n.a.	%	mAU*min	mAU		min	EP	EP	EP							
4	1		1.877	n.a.	5.89	0.0047	0.10	BMB*	0.038	2.20	5.83	13700							
5	2		2.258	n.a.	6.91	0.0055	0.13	BMB*	0.040	1.18	2.23	18095							
6	3		2.410	n.a.	11.40	0.0091	0.21	BMB*	0.041	0.91	5.30	19505							
7	4		2.763	n.a.	2.10	0.0017	0.05	BMB*	0.038	1.01	1.54	29284							
8	5		2.862	n.a.	1.57	0.0012	0.03	BMB*	0.037	0.85	2.88	32353							
9	6		3.047	n.a.	1.37	0.0011	0.03	BMB*	0.038	1.85	4.08	34737							
10	7		3.342	n.a.	3.60	0.0029	0.06	BMB*	0.047	0.94	1.18	28221							
11	8		3.443	n.a.	8.01	0.0064	0.11	BMB*	0.055	1.12	2.12	21628							
12	9		3.642	n.a.	2.55	0.0020	0.04	BMB*	0.056	0.82	1.63	23835							
13	10		3.805	n.a.	11.57	0.0092	0.14	BMB*	0.063	0.94	8.81	20202							
14	11		4.802	n.a.	2.63	0.0021	0.03	BMB*	0.070	0.78	15.45	25744							
15	12		7.347	n.a.	4.77	0.0038	0.03	BMB*	0.124	1.40	9.17	19463							
16	13		8.863	n.a.	2.23	0.0018	0.02	BMB*	0.071	0.73	6.50	85677							
17	14		9.733	n.a.	2.68	0.0021	0.02	BMB*	0.087	0.85	13.99	69852							
18	15		11.540	n.a.	2.61	0.0021	0.03	BMB*	0.066	0.89	83.28	170877							
19	16		16.775	n.a.	17.48	0.0139	0.01	BMB*	0.008	0.50	n.a.	21723508							
20	17		17.982	n.a.	12.63	0.0100	0.00	BMB*	n.a.	n.a.	n.a.	n.a.							
21	Maximum			0.0000	17.48	0.0139	0.21		0.124	2.20	83.28	21723508							
22	Minimum			0.0000	1.37	0.0011	0.00		0.008	0.50	1.18	13700							
23	Sum			0.0000	100.00	0.0794	1.04												

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail

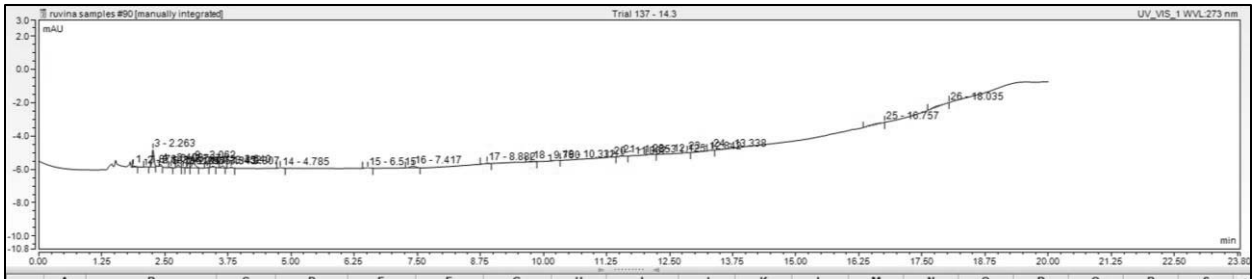
Figure 31. Fight 14 complete HPLC chromatograms. The number 1 denotes the small crayfish (subordinate), 2 the bigger crayfish (dominant) and 3 the sample collected during the fight. The letter A symbolizes the prefight samples, whereas B the post-fight sample. The number 14 is the fight number.



Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
Maximum			0.0000	0.00	0.0000	0.00		0.000	0.00	0.00	0
Minimum			0.0000	0.00	0.0000	0.00		0.000	0.00	0.00	0
Sum			0.0000	0.00	0.0000	0.00					

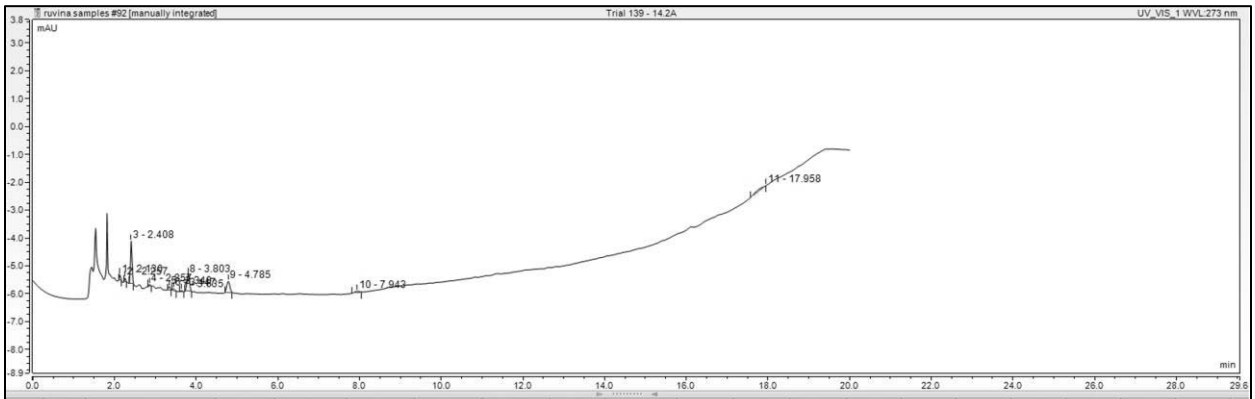


Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
Maximum			0.0000	0.00	0.0000	0.00		0.000	0.00	0.00	0
Minimum			0.0000	0.00	0.0000	0.00		0.000	0.00	0.00	0
Sum			0.0000	0.00	0.0000	0.00					



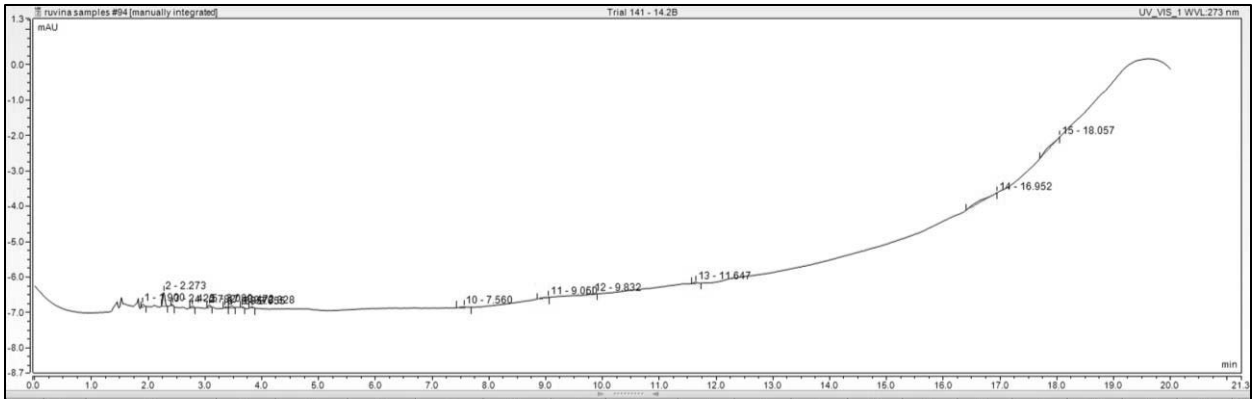
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		1.878	n.a.	1.82	0.0033	0.06	BMB*	0.052	2.28	3.39	7220
2		2.123	n.a.	0.51	0.0009	0.02	BMB*	0.033	1.33	2.22	22579
3		2.263	n.a.	25.61	0.0461	1.03	BM *	0.041	n.a.	2.16	16653
4		2.408	n.a.	2.93	0.0053	0.14	BMB*	0.038	1.19	2.76	22435
5		2.617	n.a.	0.75	0.0013	0.03	BMB*	0.051	1.02	1.83	14490
6		2.767	n.a.	4.73	0.0085	0.18	BMB*	0.046	1.00	1.26	20420
7		2.857	n.a.	0.51	0.0009	0.02	BMB*	0.039	1.34	1.78	29925
8		2.955	n.a.	0.36	0.0006	0.02	BMB*	0.026	1.15	1.47	69191
9		3.062	n.a.	14.91	0.0268	0.40	BMB*	0.059	1.41	3.12	14809
10		3.343	n.a.	0.96	0.0017	0.04	BMB*	0.047	0.88	1.23	27589
11		3.452	n.a.	3.24	0.0058	0.10	BMB*	0.056	1.01	1.94	20899
12		3.640	n.a.	4.36	0.0078	0.13	BMB*	0.058	1.00	1.55	21527
13		3.807	n.a.	2.65	0.0048	0.07	BMB*	0.068	1.09	8.38	17229
14		4.785	n.a.	1.65	0.0030	0.04	BMB*	0.069	1.13	11.25	26271
15		6.515	n.a.	1.72	0.0031	0.03	BMB*	0.112	1.06	4.44	18776
16		7.417	n.a.	7.04	0.0127	0.09	BMB*	0.128	0.99	8.36	18739
17		8.882	n.a.	2.18	0.0039	0.04	BMB*	0.079	0.80	6.27	69363
18		9.760	n.a.	2.23	0.0040	0.04	BMB*	0.086	0.79	7.72	71579
19		10.332	n.a.	0.43	0.0008	0.00	BMB*	0.001	0.63	14.40	#########
20		11.367	n.a.	0.80	0.0014	0.02	BMB*	0.083	0.77	1.45	103007
21		11.553	n.a.	3.28	0.0059	0.08	BMB*	0.069	1.04	4.68	157025
22		12.125	n.a.	1.53	0.0027	0.03	BMB*	0.076	0.96	6.08	142550
23		12.842	n.a.	1.10	0.0020	0.03	BMB*	0.063	0.82	4.71	227132
24		13.338	n.a.	1.11	0.0020	0.03	BMB*	0.061	0.69	n.a.	265181
25		16.757	n.a.	6.51	0.0117	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
26		18.035	n.a.	7.08	0.0127	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
Maximum			0.0000	25.61	0.0461	1.03		0.128	2.28	14.40	#########

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		2.130	n.a.	3.52	0.0069	0.19	BMB*	0.037	1.09	2.18	18821
2		2.257	n.a.	2.28	0.0044	0.14	BMB*	0.032	1.06	2.39	27781
3		2.408	n.a.	35.45	0.0691	1.53	BMB*	0.043	1.07	6.14	17406
4		2.857	n.a.	1.64	0.0032	0.08	BMB*	0.043	0.97	6.01	24176
5		3.340	n.a.	3.56	0.0069	0.13	BMB*	0.052	1.04	1.18	23146
6		3.447	n.a.	1.97	0.0038	0.07	BMB*	0.055	1.14	1.97	21919
7		3.635	n.a.	1.28	0.0025	0.04	BMB*	0.058	1.05	1.58	21897
8		3.803	n.a.	21.71	0.0423	0.60	BMB*	0.068	1.02	7.68	17384
9		4.785	n.a.	17.64	0.0344	0.40	BMB*	0.083	1.00	18.38	18430
10		7.943	n.a.	3.37	0.0066	0.05	BMB*	0.120	0.96	96.15	24351
11		17.958	n.a.	7.59	0.0148	0.01	BMB*	0.003	0.71	n.a.	#########
Maximum			0.0000	35.45	0.0691	1.53		0.120	1.14	96.15	#########
Minimum			0.0000	1.28	0.0025	0.01		0.003	0.71	1.18	17384
Sum			0.0000	100.00	0.1949	3.24					

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail



Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		1.900	n.a.	5.30	0.0040	0.08	BMB*	0.047	1.21	5.09	9133
2		2.273	n.a.	21.61	0.0163	0.39	BMB*	0.040	1.17	2.53	18154
3		2.425	n.a.	2.25	0.0017	0.05	BMB*	0.031	1.23	5.52	33747
4		2.782	n.a.	3.85	0.0029	0.06	BMB*	0.045	0.99	3.63	21002
5		3.080	n.a.	4.98	0.0037	0.07	BMB*	0.052	0.98	3.51	19569
6		3.357	n.a.	1.83	0.0014	0.03	BMB*	0.041	1.41	1.43	37013
7		3.472	n.a.	4.53	0.0034	0.06	BMB*	0.054	0.97	2.19	23284
8		3.655	n.a.	1.41	0.0011	0.03	BMB*	0.045	1.16	2.13	36299
9		3.828	n.a.	3.08	0.0023	0.05	BMB*	0.051	1.03	29.00	31493
10		7.560	n.a.	4.59	0.0035	0.03	BMB*	0.101	1.16	17.08	31003
11		9.050	n.a.	3.72	0.0028	0.01	BMB*	0.002	1.34	11.58	#########
12		9.832	n.a.	2.28	0.0017	0.02	BMB*	0.078	0.76	14.92	88524
13		11.647	n.a.	3.36	0.0025	0.03	BMB*	0.066	0.76	n.a.	173575
14		16.952	n.a.	20.79	0.0156	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
15		18.057	n.a.	16.41	0.0123	0.00	BMB*	n.a.	n.a.	n.a.	n.a.
Maximum			0.0000	21.61	0.0163	0.39		0.101	1.41	29.00	#########
Minimum			0.0000	1.41	0.0011	0.00		0.002	0.76	1.43	9133
Sum			0.0000	100.00	0.0752	0.91					

Summary | Peak Results | System Suitability Test | Calibration | Audit Trail

Table 9. Peak comparison for fights 12,13 and 14.

Fight 12					Fight 13					Fight 14				
12.2A	12.2B	12.3	12.1A	12.1B	13.2A	13.2B	13.3	13.1A	13.1B	14.2A	14.2B	14.3	14.1A	14.1B
-	-	-	1.815	-	-	-	-	-	-	-	-	1.820	-	-
-	-	1.883	-	1.883	1.887	1.883	-	-	1.877	-	1.900	-	-	-
-	2.128	2.138	2.142	2.093	-	2.142	-	-	-	2.130	-	2.123	-	-
-	2.270	2.267	-	2.268	-	2.270	-	-	2.258	2.257	2.273	2.263	-	-
-	2.367	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	2.415	2.412	-	-	-	-	2.410	2.408	2.425	2.408	-	-
-	2.608	-	2.605	-	-	2.607	2.618	-	-	-	-	2.617	-	-
-	-	-	-	2.765	-	-	-	-	2.763	-	2.782	2.767	-	-
-	-	-	-	2.862	-	-	-	-	2.862	2.857	-	2.857	-	-
-	-	2.963	2.950	-	-	-	-	-	-	-	-	2.955	-	-
-	3.077	3.072	-	3.070	-	3.078	3.085	-	3.047	-	3.080	3.062	-	-
-	-	-	3.150	-	-	-	-	-	-	-	-	-	-	-
-	-	-	3.355	3.365	-	-	-	-	3.342	3.340	3.357	3.343	-	-
-	-	-	3.450	3.452	-	-	-	-	3.443	-	3.472	3.452	-	-
-	-	-	3.643	3.650	-	-	-	-	3.642	3.635	3.655	3.640	-	-
-	3.823	3.823	3.813	3.818	-	3.827	-	-	3.805	3.803	3.828	3.807	-	-
-	-	4.150	-	-	-	-	4.193	-	-	-	-	-	-	-
-	-	-	4.797	-	-	-	-	-	4.802	4.785	-	4.785	-	-
5.037	-	-	-	-	4.977	-	-	-	-	-	-	-	-	-
-	-	-	5.955	-	-	-	-	-	-	-	-	-	-	-
-	-	-	6.595	-	-	-	-	-	-	-	-	6.515	-	-
-	-	-	-	7.410	-	-	-	-	7.347	-	7.560	7.417	-	-
-	-	-	-	-	-	-	-	-	-	7.943	-	-	-	-
-	-	-	8.277	-	-	-	-	-	-	-	-	-	-	-
-	-	-	8.867	8.895	-	-	-	-	8.863	-	9.050	8.882	-	-
-	-	-	9.433	-	-	-	-	-	-	-	-	-	-	-
-	-	-	9.763	9.757	-	-	-	-	9.733	-	9.832	9.760	-	-
-	-	-	-	-	-	-	-	-	-	-	-	10.332	-	-
-	-	-	-	11.347	-	-	-	-	-	-	-	11.367	-	-
-	-	-	-	11.567	-	-	-	-	11.540	-	-	11.553	-	-
-	-	-	-	-	-	-	-	-	-	-	11.647	-	-	-
-	-	-	12.122	12.153	-	-	-	-	-	-	-	12.125	-	-
-	-	-	-	-	-	-	-	-	-	-	-	12.842	-	-
-	-	-	-	-	-	-	-	-	-	-	-	13.338	-	-
16.773	16.833	16.812	16.238	16.810	17.043	16.823	-	-	16.775	-	16.952	16.757	-	-
17.918	18.173	17.942	18.052	18.023	18.21	18.147	18.038	-	17.982	17.959	18.057	18.035	-	-
-	-	18.877	-	-	-	-	-	-	-	-	-	-	-	-

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