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UW-Madison Department of Chemistry
1101 University Avenue
Madison, WI 53706-1396
Contact: Prof. Bassam Z. Shakhashiri
bassam@chem.wisc.edu
www.scifun.org

Development and Application of Quantitative and Qualitative Mass Spectrometry Techniques to Probe Crustacean Neuropeptides and Beyond

By

Amanda Rae Buchberger Jones

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This dissertation is approved by the following members of the Final Oral Exam Committee:

Lingjun Li, Professor, Pharmacy and Chemistry

John Wright, Professor, Chemistry

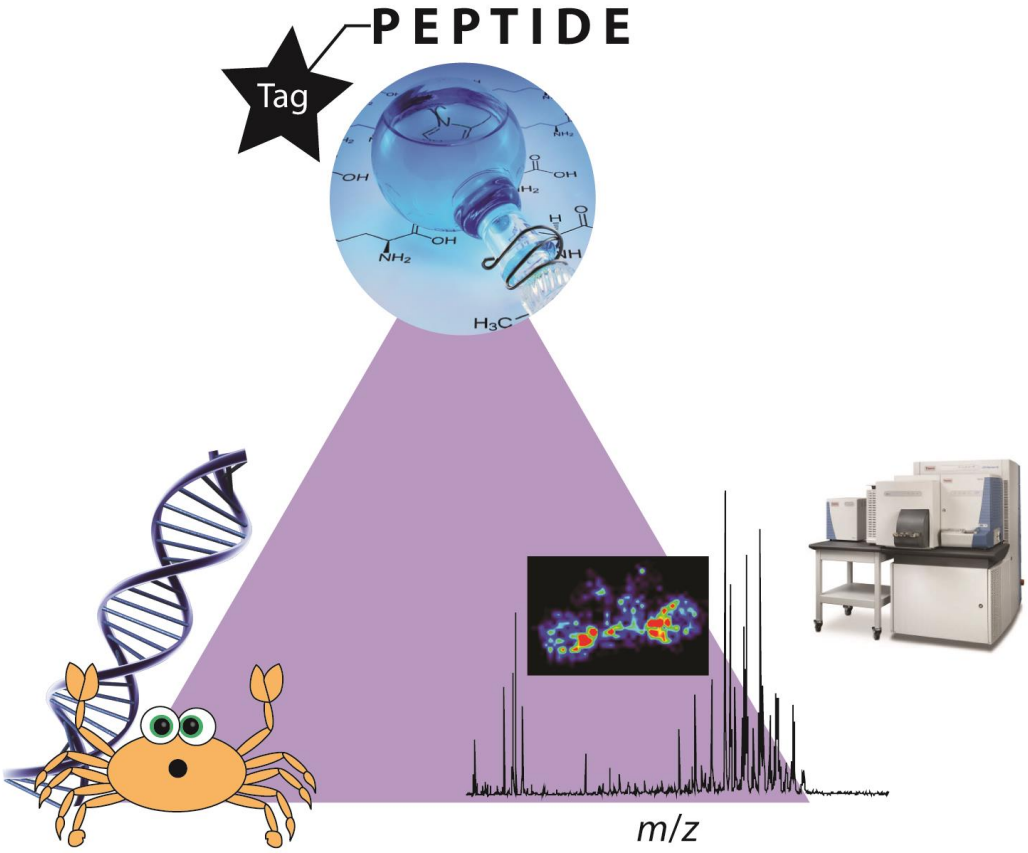
Sandro Mecozzi, Professor, Pharmacy and Chemistry

Lloyd Smith, Professor, Chemistry

Chapter 9

Seeing the Big Picture with Small Systems:

Analytical Methods to Understand Stress



Written in collaboration with the Wisconsin Initiative for Science Literacy to communicate this thesis research to non-specialists.

Key Words: Neuropeptides, Mass Spectrometry, Crustaceans, Hypoxia Stress, Mass Spectrometry Imaging, Labeling

Abstract

In order to understand how humans and animals handle stress, specifically hypoxia, also known as low oxygen levels, my thesis research has focused on utilizing mass spectrometry as an analytical technique to probe the neurological response to this stressor. Instead of humans, we use crustaceans to understand the changes through the whole body due to stress. Our results show changes due to hypoxia stress both quantitatively and how neurological molecules are located in the brain. Theoretically, future studies can apply these results, both medically and environmentally.

Why Stress?

Humans require a state of homeostasis, or the balance of everything in the body.[1] Every day external stimuli challenge our bodies. Even slight changes in homeostasis can be fatal to humans. Some key, recognizable examples of human stress are changes in blood sugar (*e.g.*, hyper- or hypo-glycemia) or body core temperature (*e.g.*, hypothermia or a fever). Our body is amazing; it has developed several different ways to handle stress. For example, when we get an infection, our immune system removes the offending virus or bacteria. Beyond the human body, ecosystems have also evolved to handle stress. During environmental fluctuations, which have increased in frequency due to human activity, stress afflicts aquatic animals. Examples of stimuli include temperature, water saltiness (*i.e.*, salinity), or even water pH changes.[2-5]

During my graduate study, my research has focused on understanding hypoxia, or low oxygen (O_2), stress. Hypoxia stress is well documented in the scientific literature to occur in aquatic estuaries, or areas where freshwater outlets meet saltwater inlets, such as the Maryland coast (*e.g.*, Chesapeake Bay).[6-8] In general, O_2 has a harder time reaching the bottom of these water reservoirs. Human activity amplifies this effect, as chemicals added into the water streams

have caused stratification, or layering. These layers make it even harder for O_2 to reach these lower water levels, creating areas with little to no O_2. Thus, animals that reside on the ocean floor developed mechanisms to either avoid or survive these stressful conditions.

Humans also suffer from hypoxia stress. Even brief periods of hypoxia can cause developmental issues within children, while long term exposure could lead to permanent damage in important regions of our brain.[9-11] In general, lack of O_2 to the organs (hypoxia) or blood (hypoxemia) prevents the cells from using glucose, causing fatigue, cell injury, and eventually death. Medical conditions, including cancer, asthma, pulmonary hypertension, and respiratory distress, tend to be associated with low O_2 levels, although there is limited knowledge about the biological and molecular changes that occur due to episodes of constant O_2 deprivation.[12-14] It is a well-known fact that losing access to O_2 is fatal, and we should investigate these effects (even in short term exposures) more thoroughly. Overall, this research will improve our understanding of the biochemical changes of hypoxia on the nervous system, both in acute and long-term exposure.

Why Neuropeptides?

Within the brain, several players are involved in regulating homeostasis. These range from very small molecules, such as metabolites and neurotransmitters, to things that are 100,000x bigger, such as proteins. The main regulators of the stress response are peptides, which are short chains of amino acids involved in signaling changes in the body. Specifically, neurological peptides (*i.e.*, neuropeptides) allow for this signaling within the brain and the nervous system. Neurons (*i.e.*, cells in the brain) synthesize and secrete these neuropeptides, as depicted in **Figure 1**.[15, 16] Secreted neuropeptides cause a chain reaction of neuron-neuron

signaling prior to affecting the final target tissue. Unlike other neurological molecules (*e.g.*, neurotransmitters), which create an instantaneous, short-term response, neuropeptides have long-lasting effects within the nervous system. Changes due to stress, especially during critical developmental times, can lead to long-term behavior and biological changes. This is also true for aquatic organisms, although they are less well characterized and understood. In general, there is a lack of understanding of the whole-body neurological changes caused by hypoxia exposure.

Why Crustaceans?

Neuropeptides are one of the most complex classes of signaling molecules in the brain. Their sizes range from just a few to several hundred amino acids.[15, 16] Neuropeptide analysis is even more complicated because several other molecules interact with, degrade, or just exist in the sample (*e.g.*, blood, *etc.*). This knowledge gap largely stems from numerous technical difficulties associated with the complete analysis of the highly complex mammalian nervous system. In complex animal models (*e.g.*, rodents), it can be challenging to confidently map the interconnected neurons and analyze the active compounds in the presence of a diverse and highly complex biological matrix. Thus, mammalian systems are much too complicated for us to use in our studies, and working with relatively simple systems is required to get an overall, more comprehensive picture of the changes occurring due to hypoxia stress. The Lingjun Li lab uses crustaceans (*e.g.*, crabs, lobsters, etc.) as model organisms for neuropeptide-based studies.[5, 17] Because crustaceans have a well-characterized and simple nervous system and structure, we can easily find the neuropeptide-rich organs of interest (shown in **Figure 2**), such as the brain.[18] Our lab also developed a very large crustacean neuropeptide database, which we use to identify several key players in environmental stressors, including hypoxia stress.

Hypoxia stress also affects crustaceans.[19-21] Estuaries are home to many crabs, including the blue crab, which has been the main focus of my graduate studies. Interestingly, crustaceans are not only ecologically relevant in terms of hypoxia stress, but they are also good model organisms for translating to higher organisms (*e.g.*, rodents). Because many of the crustacean neuropeptides have similar structures to human neuropeptides, findings using the crustacean model system are transferable to mammals. For example, RFamide-like and tachykinin peptide families are related to human peptides that are involved in how we feel pain.[22-24] These changes in the crustacean model organisms will help us identify similar signaling molecules that may play a role in adaptation to hypoxia in mammals.

Why Mass Spectrometry?

Classically, to study neuropeptides, scientists used techniques with fluorescent molecules that were thought to bind very specifically to a neuropeptide of interest.[25] As you could expect, this means we can only look at one neuropeptide at a time. When trying to look at the changes on a whole-body scale, looking at one at a time can be tedious, time consuming, and inefficient. This situation is made worse by the fact that these fluorescent molecules can actually bind non-specifically, meaning that similar neuropeptides that have distinct functional differences in stress regulation cannot be seen as different.[23] Thus, there is a need for a fast, specific, and sensitive way to look at all the neuropeptides at once.

Mass spectrometry (MS) meets all those requirements. MS is an analytical technique that works by making molecules charged (*e.g.*, become ions) by transferring energy to add or remove hydrogen, allowing them to be measured by an instrument, which is depicted in **Figure 3**. We then use software to graph the "mass-to-charge" (*m/z*) ratio along with a measured intensity onto

a mass spectrum (as shown in **Figure 4**). An *m/z* ratio is just what it sounds like: the mass of a chemical compound divided by the charge that it has. For example, if a molecule is 1000 mass units and is has one charge, then the *m/z* will be ~1000. When we have a specific *m/z* value we are interested in, we can select it to be broken up and measured separately, a process called tandem MS, in order to have its structure rebuilt using computer software.[15] This technique is extremely powerful, as we do not require any prior knowledge of the molecules (*e.g.*, neuropeptides) in order to analyze them. One major method for ionizing neuropeptides is with matrix-assisted laser/desorption ionization (MALDI)-MS. MALDI seems complicated, but, simply, we use a matrix and a laser to charge the neuropeptides. For this method, we usually put a small volume of the crushed and cleaned up brain tissue that has to be mixed with the matrix on a stainless steel plate, where we can estimate and/or quantify how much of a neuropeptide was found in the tissue.[15] While this makes getting a mass spectrum easy, we are unable to get spatial information about the neuropeptides within in the tissue. Both the quantity and the location of a neuropeptide provide important information to understand its function. Instead of crushing up the tissue, we can actually section it into thin slices (~12 μm thick), which can then be covered in matrix for MALDI analysis.[26] On this tissue section, we form a grid. On each grid point, we acquire an individual mass spectrum. The size of the grid and thus number of grid points depends on the spatial resolution, and these squares in the grid can range from 500 μm to 5 μm in length/width. For comparison of the size, blood cells have a diameter of 7-8 μm! For data processing, we can then select the mass of a neuropeptide of interest (*e.g.*, from our crustacean database), and we extract the intensity of that mass from each of the individual mass spectrum (*i.e.*, grid point), forming an image (**Figure 5**).[26] We also generate images for any peak

detected by the MS, meaning scientists can investigate unknown *m/z* values for their roles in environmental stress.[27]

With MS, we have methods we can use to directly compare samples, which is extremely important for our studies since we want to compare a hypoxia-exposed animal to a control animal. To do this, we take advantage of the existence of different, heavy versions of elements. For example, hydrogen, the most common element in the universe, has a heavy version called deuterium. Deuterium and hydrogen differ by one mass unit. This increase in mass makes it heavier in general, but it doesn't change any of its properties. By incorporating a different number of isotopes between two labels, such as adding one hydrogen to one group and one deuterium to the other group and mixing them, we see pairs of peaks in the mass spectrum that are separated by a known distance. In our case, we will be looking for a 2 or 4 *m/z* ratio difference between the peaks when they are singly charged molecules, depending on the number of different conditions we want to compare.[28] **Figure 4** shows an example where 2 samples are differentially labeled (shown in the open and closed hexagons) with 4 *m/z* mass differences in the spectrum. In this case, by comparing the intensity of the light and heavy peak pairs, we can see if neuropeptide levels increase or decrease in a hypoxia-exposed animal compared to a control, which will give us an idea about their function and/or role in hypoxia stress.

Putting it All Together

By combining MS and crustacean neuropeptide analysis, we can provide an overall idea of what is occurring when the crab is exposed to stress, specifically hypoxia. Normal levels range from 8-10 parts per million, which translates to 80-100% maximum O_2 capacity of the water. In order to consistently expose the crabs to hypoxia, we "bubbled" out the O_2 in the water

using nitrogen gas, as depicted in **Figure 6**. Once the level of O_2 reaches the level we are interested in, such as 10% O_2 (*i.e.*, severe hypoxia) compared to 100% O_2 (*i.e.*, normal), we place the crab in the water for a period of time, such as 1 hour, before sacrificing them. In addition to hypoxia-exposed crabs, we also need control crabs, which have a fully aerated tank (80-100% O_2). After sacrificing the crabs, we take out all their neuropeptide-rich organs (see **Figure 2**), isotopically label each sample, and finally analyze by MS (**Figure 7**).

Looking back at **Figure 4**, we can see that hypoxia stress does affect the neuropeptides and their concentrations. In this spectrum, the orcokinin neuropeptide family has been highlighted, and they all appear to be downregulated due to this severe hypoxia (*i.e.*, 10% O_2) stress. This is a trend across many other neuropeptide families not in the spectrum. As stated before, we can translate information from both the RFamides and tachykinin neuropeptide families of crustaceans to mammals, including humans. Several RFamides show changes ranging from 100% increase to 90% decrease. Thus, neuropeptides that are from the same family, meaning they are similar in structure, can have very different functions in the stress response.

Crustaceans are a necessary first step for overall, comprehensive studies, and the next step would be applying these results to more complex organisms, such as mice, for follow up studies. Beyond severe hypoxia (*i.e.*, 10% O_2) for 1 hour, we have also investigated moderate (*i.e.*, 20% O_2) and mild (*i.e.*, 50% O_2) hypoxia for an hour, along with a time course study (*i.e.*, 0 hour, 1 hour, 4 hours, and 8 hours) for severe (*i.e.*, 10% O_2) hypoxia. All of these conditions have shown distinct neuropeptidomic changes due to hypoxic stress.

In order to acquire spatial information of these neuropeptides, we also employed MS imaging, as seen in **Figure 5**. As you can see, some neuropeptides are located all over the tissue (*e.g.*, LNPSNFLRFamide, *m/z* 1106.611), while others are located only around the edges of the

brain (*e.g.*, LPGVNFLRFamide, *m/z* 1061.626). The most interesting neuropeptides are those that either appear or completely disappear after hypoxia stress, as it makes us believe they have a key role in how these animals handle hypoxia stress. It should be noted that when comparing control and hypoxia-exposed animals, no quantitative changes doesn't mean there is no change in the localization of neuropeptides in the tissue. For example, neuropeptides localizing to the outside or the middle can have the same overall concentration, meaning that when we crush up the tissue for labeling (see above), we would not see any change. Also, if we only find the neuropeptide on the outside of the tissue, they could be preparing for release into the crustacean blood (*i.e.*, hemolylmph). Thus, while quantitative information is important, we should consider also looking at the neuropeptide location over time to determine its role in hypoxia stress.

Conclusions and Future Directions

Using the crustacean model organism, we discovered that hypoxia stress can have a significant effect on the expression of several neuropeptides. By gaining further knowledge in basic neurobiology and neurochemistry, society will have a better understanding of the brain and how it changes in medical conditions where hypoxia occurs, including respiratory distress, cancer, asthma, *etc*. While we have found ways to probe these systems, the Lingjun Li lab will explore other areas. Another avenue I have explored is the difference between hypoxia (*i.e.*, low O_2) and hypercapnia (*i.e.*, low O_2 with increased carbon dioxide (CO_2)) stress. Several other graduate students in the lab have been inspired to look at this research from different angles, including sampling crustacean blood (*i.e.*, hemolymph) sampling while the crab is still alive or exploring other similar stressors (*e.g.*, pH).

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Figures

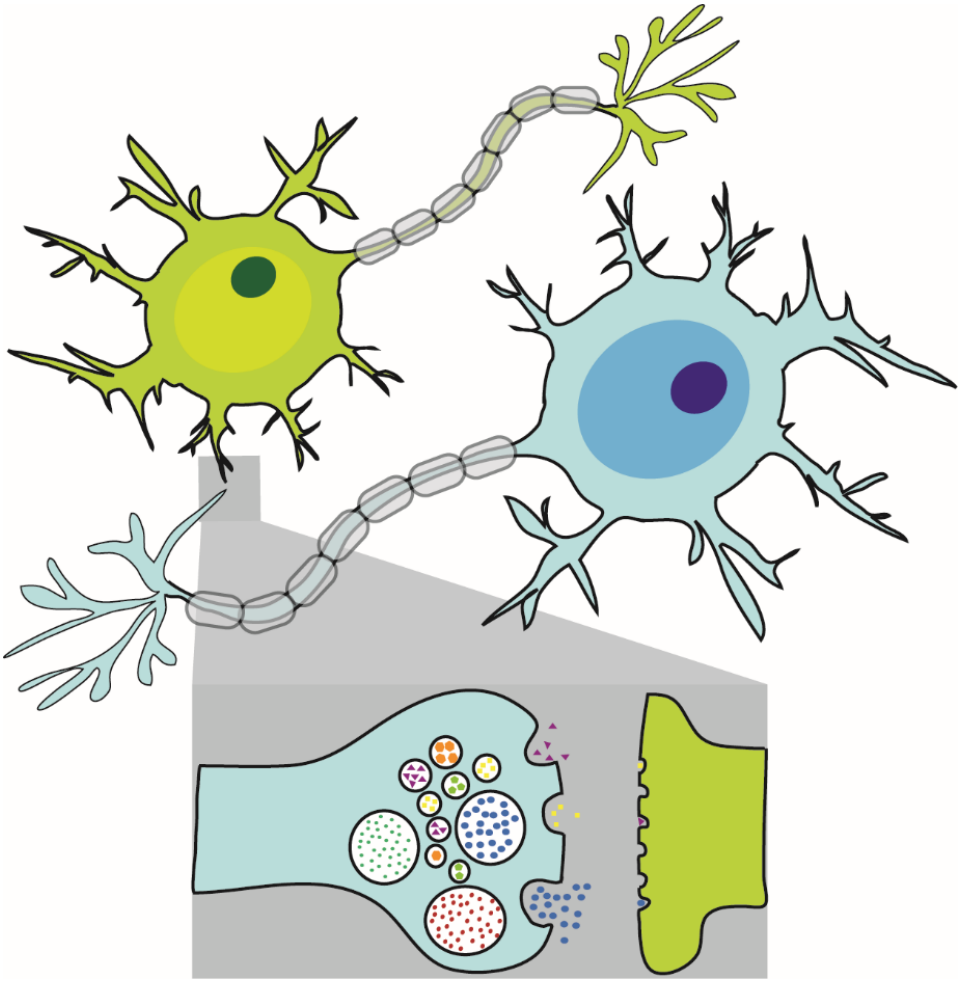

Figure 1. An artistic image of a neuron interacting with another neuron. In the zoomed in region, one can observe several molecules, including neuropeptides, being released.

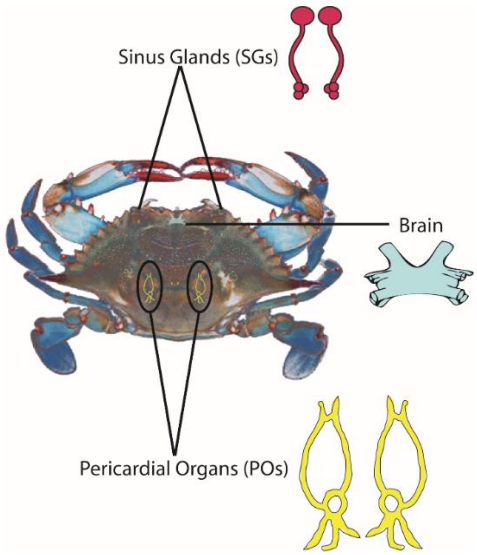

Figure 2. Locations of neuropeptide-rich organs in the crustacean. The sinus glands are located in the eyestalks. The brain is located between the eyestalks above the stomach. The pericardial organs surround the heart in the pericardial ridges.

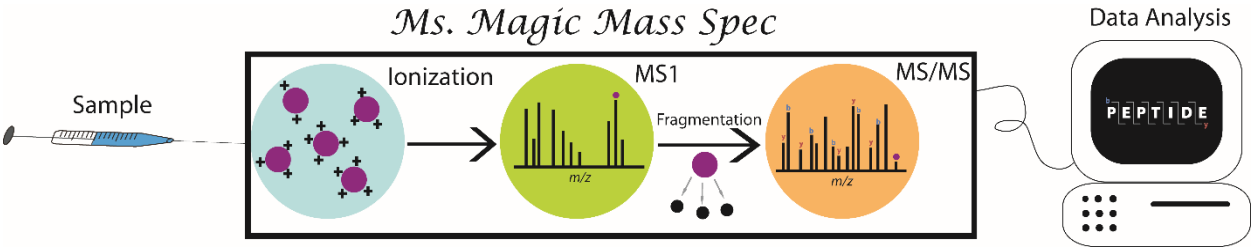

Figure 3. A schematic of a mass spectrometer. After we insert the sample into "Ms. Magic Mass Spec," the molecules become ions (*i.e.*, ionize), meaning they gain a charge. We then visualize the resulting *m/z* values in a mass spectrum (MS1). If an *m/z* value is of interest, the instrument can break it up into smaller pieces by tandem MS (MS/MS). We can then piece the fragments together into the molecule's structure using advanced computer software.

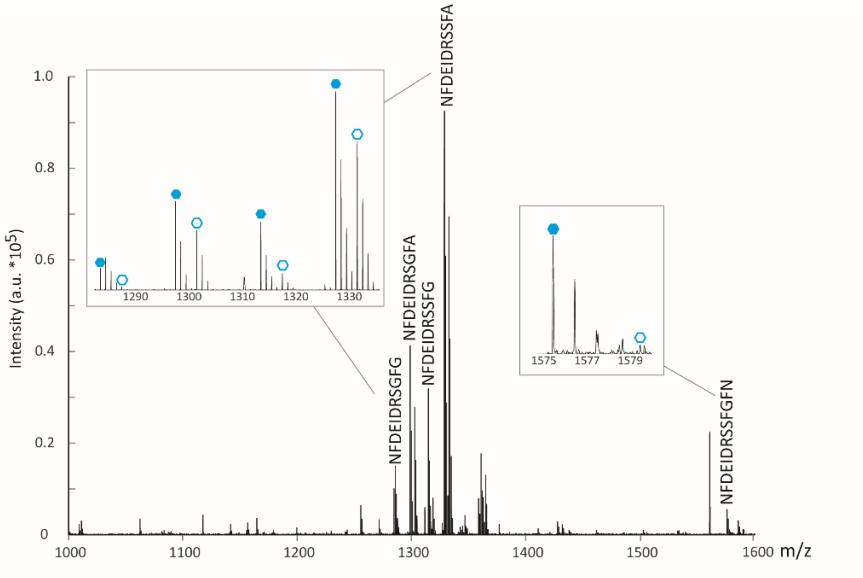

Figure 4. Example MS spectrum, highlighting the heavy and light peak pairs we see when we use duplex labeling. The closed hexagons are the neuropeptides from the control brain sample, while the open hexagons are from the severe hypoxia (*i.e.*, 10% O_2) exposed brain sample.

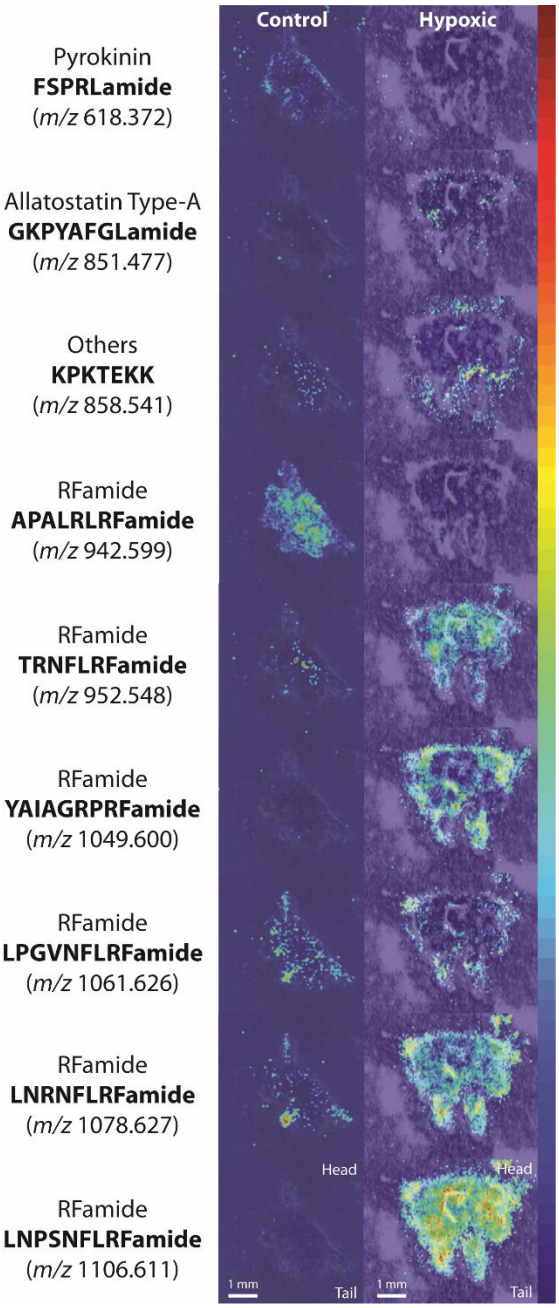

Figure 5. Nine neuropeptide brain distributions found by MS imaging comparing the control (left) and severe hypoxia (*i.e.*, 10% O_2) (right) exposed crab brains. The optical image of the brain is in the background of each of these images. A color bar shows both low (blue) and high (red) intensity.

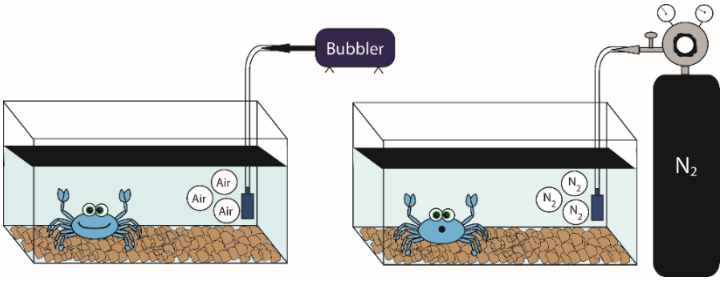

Figure 6. Tank set up for control (left) and hypoxia (right) exposure.

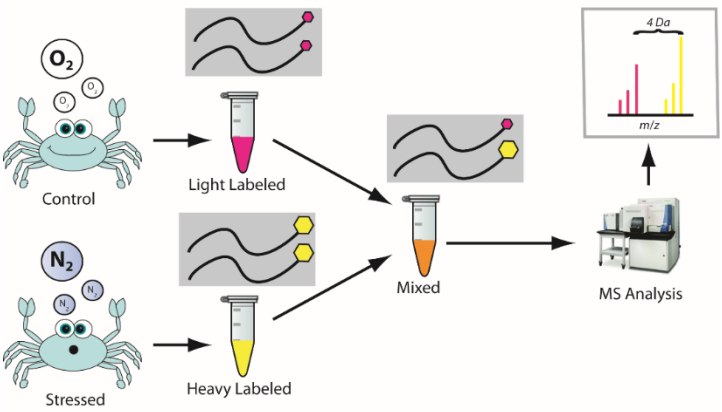

Figure 7. Schematic of the workflow for comparing a control and hypoxia stressed crab by MS. After collecting the tissues of interest, the samples are extracted and differentially labeled using different amounts of stable isotopes. We can then mix and analyze the differentially extracted and labeled peptides by MS, where we see a mass difference between these two samples.