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Expression and distribution of neuropeptides in the nervous system of the crab *Carcinus maenas* and their roles in environmental stress

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Abstract

Environmental fluctuations, such as salinity, impose serious challenges to marine animal survival. Neuropeptides, signaling molecules involved in the regulation process, and the dynamic changes of their full complement in the stress response have yet to be investigated. Here, a MALDI-MS-based stable isotope labeling quantitation strategy was used to investigate the relationship between neuropeptide expression and adaptability of *Carcinus maenas* to various salinity levels, including high (60 p.p.t.) and low (0 p.p.t.) salinity, in both the crustacean pericardial organ (PO) and brain. Moreover, a high salinity stress time course study was conducted. MS imaging (MSI) of neuropeptide localization in *Carcinus maenas* PO was also performed. As a result of salinity stress, multiple neuropeptide families exhibited changes in their relative abundances, including RFamides (e.g. APQGNFLRFamide), RYamides (e.g. SSFRVGGSRYamide), B type-allatostatins (AST-B) (e.g. VPNDWAHFRGSWamide), and orcokinin (e.g. NFDEIDRSSFGFV). The MSI data revealed distribution differences in several neuropeptides (e.g. SGFYANRYamide) between color morphs, but salinity stress appeared to not have a major effect on the localization of the neuropeptides.

Keywords

crustacean; mass spectrometry imaging; neuropeptides; isotopic labeling; salinity stress

Introduction

Significant environmental fluctuations, such as in salinity levels, pose great physiological challenges to a variety of organisms. Acclimation to different conditions is crucial to an animal's survival, however the precise mechanism of how this process occurs remains elusive. Increasing evidence suggests that neuropeptides play an important role in stress

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regulation [1–6]. Unfortunately, a comprehensive view of the neuropeptides involved in the stress response is lacking and required to understand their functions inside the nervous system. As one of the most important and complex class of signaling molecules, neuropeptides are bioactive peptides that occur in the nervous system but can also be expressed in non-neuronal tissue. Due to neuropeptides' natural diversity and chemical complexity, a nervous system that is relatively simple would be beneficial to investigate a neuropeptide's modulatory effect (*i.e.* function) at the system and circuit level. Crustaceans (*e.g.* crabs and lobsters) provide a simple, well-characterized neurological platform for the study of behaviors, including feeding, molting, and reproduction [7–16]. The crustacean nervous system is comprised of the neuroendocrine nervous system (*e.g.*, pericardial organs (PO)), stomatogastric nervous system (STNS), and the CNS (*e.g.* brain) [17]. In addition, this model organism has previously served as an important system for the research of environmental stress related responses [3, 4, 6, 18–25], providing valuable information on neuropeptides' roles in the regulation process.

In this study, the green crab *Carcinus maenas* is employed as an experimental model. Originating from Europe, green crabs are one of the most invasive marine species well known for their adaptability to a wide range of environmental conditions [26]. Therefore, *C. maenas* serves as a model organism to explore the roles of neuropeptides in acclimation to salinity changes [21, 24, 26, 27]. Largely determined by the local environment, the shell colors of green crabs are variable, ranging from a dark green to a reddish brown [28]. It should be noted that crabs with red shells are stronger, more aggressive, but appear to avoid areas with undesirable environmental conditions, such as low salinity [29]. Given the negative biological and economic impact of *C. maenas* [30], great effort should be devoted to exploring their nervous system to develop molecular tools to prevent further ecological damage. A recent study of the green crab identified 122 neuropeptides, including 49 peptides first-time described in *C. maenas* and 42 novel peptides in any crustaceans [31]. Furthermore, crustacean hyperglycemic hormones (CHHs), a neuropeptide superfamily, were identified and found to be involved in the stress response in the green crab using a radioimmunoassay (RIA) [15, 25].

MS has gained a lot of attention for neuropeptidomic analysis studies [32]. With its tolerance to salts, simple sample preparation, and fast acquisition time, MALDI-based methods have been extensively used to investigate neuropeptide changes, including several studies involving stressors [6, 8, 21]. Recently, MALDI-based MS imaging (MSI) has gained increased attention as a powerful tool to obtain molecular maps of biomolecules [32–36]. This technique can provide spatial distribution of compounds of interest in a similar manner to traditional techniques such as chemical staining, immunohistochemistry, and RIAs [37–39] without requiring prior knowledge of the target analytes. Moreover, MSI can maintain an analyte's molecular distribution while avoiding the disadvantages of sample extraction, purification, and separation [40, 41].

Here, isotopic dimethyl labeling coupled to MALDI-MS was utilized to investigate the quantitative changes of several neuropeptide families in the crustacean brain and PO. The results showed that green crabs with different shell colors (*i.e.* red or green) responded differently towards stress, with red shell individuals being less tolerant. Comparing low and

high salinity stress, all crabs showed a more significant response to high salinity conditions. A time course study of high salinity stress was also performed to determine the dynamics of neuropeptide expression upon exposure to salinity changes in the green shell green crabs. MSI was also employed to illustrate the distribution changes of neuropeptides of interest in the PO due to salinity stress, enabling detailed mapping of individual isoforms of various neuropeptides.

Materials and Methods

All methods are described briefly below. Please see the supplementary information for more details.

1. Animals and Salinity Stress Experiments

Both red and green shell green crabs *Carcinus maenas* were exposed to normal (30 parts per thousand (p.p.t.)), high (60 p.p.t.), or low (0 p.p.t.) salinity for a predetermined amount of time ranging from 5 hours (h) to 48h. Crabs were cold anesthetized, and the neuronal organs were dissected out [42]. All the dissection and sample processing steps were exactly the same between the two shell types.

2. Sample Preparation

Neuropeptides were extracted from the sample tissue by manual homogenization with acidified methanol and desalted with C18 Ziptips. To allow for relative quantitation via dimethylation, extracts were either labeled with formaldehyde (CH₂O) (control) or deuterium formaldehyde (CD₂O) (stressed) and mixed in equal amounts. The resulting mixture was spotted with CHCA and analyzed by MALDI-TOF/TOF. For imaging experiments, the dissected PO was mounted onto the MALDI-TOF/TOF sample plate and coated with DHB.

3. MS Analysis and Imaging

A model 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser was used for extract quantitation and tissue imaging. Image acquisition was performed with the 4800 Imaging application (Novartis, Basel, Switzerland).

4. Quantitative Data Analysis

The same approaches were used for both high salinity and low salinity stress experiments. Neuropeptide extracts from salinity stressed and control animals were differentially labeled and mixed with 1:1 ratio. The abundance ratio for each neuropeptide in stressed versus control crabs was determined by dividing the heavy labeled peak intensity (stressed) with the respective light labeled peak intensity (control).

Results and Discussion

1. Neuropeptide Content Changes between High Salinity Stress and Control in the PO

As a major neuroendocrine organ in crustaceans, the PO can release peptide hormones to modulate the motor patterns of neuronal circuits in the STNS, which are located very near the crustacean's heart. Thus, the involvement of this neuroendocrine organ with stress regulation by releasing neuropeptides can be expected. Here, to investigate how neuropeptides in the PO are involved in the stress regulation, a duplex dimethyl labeling strategy with formaldehyde in conjunction with MALDI-MS is utilized to measure quantitative changes in neuropeptides between control and stressed animals.

Fourteen neuropeptides from 4 families were examined for both green (n=6) and red (n=6) shell individuals' POs. Significant changes were observed for members from several neuropeptide families ($p<0.05$) after high salinity stress, indicated with asterisks in Figure 1A. All of the important values are included in Table S1. In general, the results were variable depending on the color morph studied. For green shell individuals, only a few neuropeptides exhibited significant changes in relative content levels. For example, B-type allatostatin (AST-B) VPNDWAHFRGSWamide (m/z 1470.7) showed great increase after stress ($p<0.005$), which could be indicative of no release from the tissue due to a silent neuron or a delayed release. Other neuropeptides, such as the RYamide SSRFVGGSRYamide (m/z 1114.5), decreased significantly ($p<0.05$). This observation suggests that these peptides, once the crab has been exposed to high salinity stress, could have been released from the PO or degraded.

Compared to the green color morphs, red individuals after high salinity stress showed a more extreme response, as almost all neuropeptide content levels changed upon exposure to the high salinity stress. AST-B VPNDWAHFRGSWamide (m/z 1470.7) levels were significantly elevated ($p<0.005$), whereas the RYamide SSRFVGGSRYamide (m/z 1114.5) showed a decreasing trend ($p<0.005$). Both of these content changes were consistent with the green color morph crabs. However, all RFamide peptide levels in red individuals rose significantly after high salinity stress ($p<0.05$).

It was found that only 5 neuropeptides in green shell crabs showed significant content changes, whereas 10 neuropeptides in red shell crabs exhibited significant content changes. Furthermore, the neuropeptide content changes seen in the red morph were much more intense than those observed in the green morph. These findings suggest that green morph crabs show not only better tolerance to low salinity stress [29] but also to high salinity stress. From this observation, greater peptidome stability, which is a potential molecular clue for better adaptability, is implied for green individuals compared to red shell variants. It should be noted that a neuropeptide may or may not carry the same weight in terms of stabilizing the peptidome, and further studies will need to be performed to validate our conclusions. As discussed, this color morph difference is not only genetically related but also largely due to local environment factors which affect the crab molting [28]. A red shell color indicates that the crab took a longer time to molt, particularly inter molt, which helps them to gain greater size, heavier weight, thicker carapaces, and thus an advantage in mating [43]. However, a tradeoff is that the red morph crabs are physiologically less tolerant of harsh environments

[29]. These observations lead us to believe that there is a molecular, specifically neuropeptidomic, connection among reproductive success, morphological evolution, and environmental adaptability. More in-depth investigations will be required to fully understand the underlying mechanism and establish the correlation.

2. Neuropeptide Content Changes in the Brain Tissue of Animals Subjected to High Salinity Stress and Controls

The brain is an important neural organ in the CNS, which plays a crucial role in behavior and physiological condition regulation in both mammals [44, 45] and crustaceans [46–48]. Figure 1B illustrates the neuropeptide changes in green and red morph brain tissue in response to high salinity stress, with all valuable numbers included in Table S2. In green individuals (n=4), all 8 neuropeptides in 5 families kept the same levels after stress, while in red individuals (n=4), all orckinins and most RFamide peptides exhibited elevated content levels after stress ($p<0.05$).

RFamides are a conserved peptide family throughout different animal nervous systems, and they have been shown to be involved in cardiovascular function, modulation of muscle contraction, control of locomotor activity, and water balance [49–51]. Orckinin peptides are the most abundant neuropeptide family in crab brain, which have been reported to exert neuromodulatory effects on the lobster pyloric neural circuits [52]. It should be noted that, while the STNS contains many intrinsic orckinin neurons, it is not clear how the brain can exert direct effects on the pyloric circuits. Overall, the increase of RFamide and orckinin isoforms after high salinity exposure suggests their possible involvement in the stress response. In contrast, SIFamide, tachykinin, and HIGSLYRamide (m/z 844.5) peptides remained unchanged in both color morphs, suggesting they have no role in the acute stress response. While not detected here, previous research has shown that other peptide families (*e.g.* angiotensin-like peptides) would exhibit elevated levels during chronic stress [3].

These different neuropeptide changes in *Carcinus maenas* with diverse shell colors in response to high salinity stress in both the PO and the brain indicate that different growth environments may determine the tolerance to environmental stress for the crustacean, which is consistent with previous ecological research [29]. Thus, the relationship between environments and evolutionary benefits (*e.g.* shell differences) would be an area for future investigation. Also, special considerations should be made when executing physiological studies, as the origin of animals should be always an important control factor taken into account.

3. Neuropeptide Content Changes in the PO and Brain of Animals Subjected to Low Salinity Stress and Control Animals

In addition to high salinity stress, neuropeptide changes in response to low salinity stress were also investigated. Figure 2A compares the neuropeptide content change between green (n=5) and red (n=6) shell crab POs, with all the important values included in Table S3. All RYamides (*e.g.* FVGGSRYamide (m/z 784.4)) in red individuals experienced a significant decrease ($p<0.05$), reduced by almost 25% of the original levels. A smaller degree of decrease was observed for RFamides (*e.g.* NRNFLRFamide (m/z 965.5)), exhibiting only a

~10% decrease after low salinity stress ($p<0.05$). In contrast, in the green color morphs, RFamide DGNRNFLRFamide (m/z 1137.6) and all AST-B family members (e.g. VPNDWAHFRGSWamide (m/z 1470.7)) were significantly increased after low salinity stress ($p<0.05$).

Figure 3B shows the neuropeptide content changes in the brains between crabs with red ($n=5$) and green ($n=5$) shell colors, and Table S4 details all of these observations. Interestingly, red and green individuals almost showed no significant response to low salinity stress. All neuropeptide expression besides the RFamide DGNRNFLRFamide in red shell crabs stayed at the same level, while only 4 neuropeptides showed a significant increase of less than 5% in green shell crabs.

4. Neuropeptide Content Differences in Response to Different Salinity Stresses

When comparing high and low salinity stress in the two shell colors, there is a distinct difference in expression of the measured peptides. In the high salinity stress study, 10 neuropeptides in the red shell crab PO showed significant changes. 9 of these neuropeptides showed significant increase, most of which were larger than 20% ($p<0.05$). In contrast, in the red shell crab PO low salinity stress study, only 7 neuropeptides showed significant decrease, and only 2 showed more than a 20% level change ($p<0.05$). For green shell crabs, four neuropeptides showed significant content level changes in high salinity stress PO experiments. Only five neuropeptides were involved in low salinity stress regulation of the green morph PO exhibited elevation in their content levels ($p<0.05$). We can thus conclude that high salinity stress is a more severe stress for *Carcinus maenas* than low salinity stress. This is inferred from not only the number and amount of neuropeptides having content changes due to the particular salinity stress but also the severity of the responses observed.

Animals have different adaptability to low or high salinity, which can be related to genetic factors. Many crustacean species were reported to have a better tolerance to low salinity stress than high salinity stress, like *Moina affinis* [53] and *Homarus americanus* [54]. As mentioned above, our research results show that *Carcinus maenas* crustaceans also have a better tolerance to low salinity stress, which can be explained by green crabs living habitat. *C. maenas* is a non-native invasive predator that frequently migrates across oceans and experiences fresh water regions, thus it has developed a better tolerance to low salinity environment. When comparing between color morphs, we found even though the green shell crab showed a better stress tolerance to high salinity stress, but the neuropeptide families involved and trends in the regulation were still similar between the red and green shell crabs. However, in low salinity stress, most neuropeptides in the red morph experienced a reduction in their expression levels, while those in green morph exhibited an increase in their content levels. This phenomenon is interesting because it raises the question whether different color morphs in the same species can have different neuropeptide regulation mechanisms under certain physiological conditions. Further investigation with electrophysiology experiments, such as extracellular recording of the STNS and PO, will need to be performed to provide robust evidence.

When comparing the neuropeptide content changes in response to acute stress in the PO and brain, it is interesting to observe that the extent of regulatory responses in the brain is

smaller than that of PO regardless of the color morphs and different salinity stress conditions. This observation triggers an interest in exploring the relationship between neuroendocrine organs and the CNS. In previous research, signaling molecules in neuroendocrine organs were shown to play a role in regulating the mammalian nervous system [55–57]. Combining research on mammalian model systems and our own study, it is believed that neuroendocrine organs in the crustacean can also play a role in the regulation of CNS responses. Our data show that the PO exhibits a more intense response compared to brain in acute stress, although it is likely they work together to overcome the stress caused by salinity changes. However, a more in-depth investigation of chronic stress and adaptability will need to be performed to fully understand the underlying mechanism.

5. Time Course Study

In order to better understand the dynamics of neuropeptide changes in response to stress, we also conducted a time course study of the green color morph of *C. maenas* in response to high salinity stress. Measurements were taken at 5h, 12h, 24h, and 48h after exposure, and all results are shown in Figure 3, Table S5, and Table S6. In a previous experimental study, researchers performed more acute stress studies ranging from 1h to 2h [4–58]. We are more interested in a longer time adaptability; thus we selected 24h and 48h time periods, setting our time points according to a similar experimental design [59]. For each time point, at least 6 replicates of experiment were performed. In Figure 3A, a majority of neuropeptide levels stayed at the same level after 5h stress in the crustacean PO. Two exceptions were AST-B neuropeptide VPNDWAHFRGSWamide, which increased by ~20%, and orcokinin NFDEIDRSSFGFV, which decreased by ~25%. After 12h of stress, only the AST-B peptide VPNDWAHFRGSWamide showed a statistical change, increasing from the 5h increase. It should be noted that we did see two out of ten groups of experimental animals exhibit an unusual high levels of NFDEIDRSSFGN (m/z 1547.7), producing a clearly higher average relative ratio (~1.4). After 24h elapsed, four neuropeptide levels dropped compared to a control. Finally, only three neuropeptides, two AST-B neuropeptides and one orcokinin, showed a significant difference after 48h exposure.

The brain tissue results are shown in Figure 3B. None of the neuropeptide content levels showed significant changes after 5h of stress, as discussed before, but several neuropeptides exhibited extreme changes in their neuropeptide content over time. For example, tachykinin and NRNFLRFamide showed significant increase after 12h of high salinity stress. After 24h stress, a majority of neuropeptide content levels experienced a drop, including HIGSLYRamide, tachykinin, and SIFamide. These 10% drops were also maintained after 48h. All other neuropeptides had no major changes after 48h when compared to a control. Overall, the differential time course response of neuropeptide expressions observed in the PO and brain after high salinity stress could be related to the release, degradation, and/or accumulation of these peptides, accentuating the diverse physiological roles played by different types of neuropeptides in the decapod crustaceans. More information on these mechanisms could be determined by the study of the crustacean hemolymph during salinity stress, including the use of microdialysis [32].

6. Neuropeptide Localization in the PO

Previous studies have shown that a rich repertoire of neuropeptides exist in the crustacean PO [7, 60, 61]. To investigate correlation of neuropeptide function and localization, MALDI-MSI was used to map the distribution of several neuropeptides of interest in the PO. Intact POs were collected from both green (n=3) and red (n=3) shell *C. maenas*, and images of numerous neuropeptides were obtained.

Figure 4 shows the localization of 3 most abundant neuropeptide families of green and red shell green crab PO. AST-Bs (e.g. STNWSSLRSAWamide (m/z 1293.6)) showed very similar spatial localization within the family. They were prevalent all over the PO tissue with highest concentration in the anterior bar and posterior bar region of both green and red shell crustacean PO. The RFamide family peptides (e.g. DGNRNFLRFamide (m/z 1137.6)) were not as abundant as the AST-Bs in the *C. maenas* PO. They also existed throughout the whole tissue in both morphs, but they were more concentrated in posterior part of green shell crabs. The small difference in localization of the RFamide family between these two morphs during high and low salinity can be related to the quantitative study.

The most abundant neuropeptides in the *C. maenas* PO belong to the RYamide family (e.g. SSRFVGGSRYamide (m/z 1114.6)). The localization pattern among the family members is quite similar, as all RYamide peptides were highly concentrated in dorsal trunk, anterior, and posterior region in crabs of both shell colors. They were most abundant in posterior region especially for red shell green crabs. Other peptides like proctolin RYLPT (m/z 649.4), CCAP PFCNAFTCamide (m/z 956.4), and HIGSLYRamide (m/z 844.5) shared similar localization patterns to the AST-B family.

Neuropeptide localization patterns between the two morphs were quite similar in RYamide and AST-B families. For RFamide, their patterns exhibited slight differences not only between morphs but also among peptide families, indicating the distinct physiological functions of each member of the respective family, which also can be related to the quantitation study. Intensity variation between two morph images could also be due to the error induced by sample preparation and matrix application. It should be noted though that all of the above localizations are consistent with previously collected immunochemistry data in both *C. maenas* [62, 63] and other crustacean species [64, 65]. Our study presents the first survey of neuropeptide localization in the *C. maenas* PO using MALDI-MSI, which suggests that the peptide content changes due to stress may correlate to the peptide's distribution pattern.

Concluding Remarks

In summary, we employed isotopic dimethyl labeling and MALDI-MS based methodology to quantitatively study neuropeptide regulation of environmental stress in both the brain and the PO. Green color morphs of *C. maenas* demonstrated a better tolerance to salinity stress, with fewer, less intense changes in relative abundances of neuropeptides compared to the red morphs. Different neuropeptides were observed to be involved in stress regulation between the two morphs, associating environmental factors with possible genetic involvement. Time course studies were also carried out to investigate the dynamics of neuropeptide expression

changes upon exposure to environmental stress. Finally, MALDI-MS imaging was utilized to characterize the special distribution of a multitude of neuropeptides within various peptide families in their respective neuronal terminals in the PO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MSI	mass spectrometry imaging
PO	pericardial organ
AST-B	B type-allatostatin
CHH	crustacean hyperglycemic hormone
CNS	central nervous system
STNS	stomatogastric nervous system
CH₂O	formaldehyde
CD₂O	deuterium formaldehyde
p.p.t.	parts per thousand
h	hour
v	volume

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Statement of Significance

This study provides a comprehensive understanding of the neuropeptide content changes in both the red and green color morphs of the green crab *Carcinus maenas* upon both high and low salinity stress. This work could provide an initial glimpse at the neuropeptidome involved in environmental adaptability. Various neuropeptides within the same families showed distinct content changes, providing evidence of familial isoforms having different functions within the crustacean nervous system. This research also provided the first-ever survey of neuropeptide localization in the pericardial organ (PO) of *C. maenas* using MS imaging (MSI). Given that *C. maenas* is considered an invasive species capable of adapting to wide-range of environments, the simple, well-characterized crustacean provides an excellent model for the understanding of neuromodulation due to stress. Since many of the features are conserved between neurochemical systems, the research findings presented in this study will facilitate our understanding of molecular mechanisms that govern more complex organisms under environmentally stressful conditions.

Figure 1.

Content changes of neuropeptides of interest in *Carcinus maenas* (A) PO (n=6) and (B) brain (n=4) between a control and 5h of high salinity stress. For each pair, the bars on the left refer to changes in the green morph, while the bars on the right correspond to the red morph. Each group contained two POs or one brain. A ratio equal to one denotes no change between stress and control conditions, marked by a dotted line. The error bars depict the SEM of the measured ratio. Asterisks indicate significance levels (student's t test): *, $p<0.05$, **, $p<0.005$.

Figure 2.

Content changes of neuropeptides of interest in *Carcinus maenas* (A) PO (n=5) and (B) brain (n=5) between a control and 5h of low salinity stress. For each pair, the bars on the left refer to changes in the green morph, while the bars on the right correspond to the red morph. Each group contained two POs or one brain. A ratio equal to one denotes no change between stress and control conditions, marked by a dotted line. The error bars depict the SEM of the measured ratio. Asterisks indicate significance levels (student's t test): *, $p < 0.05$, **, $p < 0.005$.

Figure 3.

Time course study of *Carcinus maenas* neuropeptide content changes due to high salinity stress for the (A) PO and (B) brain for the green color morph. 6 groups for 5h, 10 groups for 12 h, 6 groups for 24h, 6 groups for 48h (each group has two POs or one brain). A ratio equal to one denotes no change between stress and control conditions, marked by a dotted line. The error bars depict the SEM of the measured ratio. Asterisks indicated significance levels (student's t test): *, $p < 0.05$, **, $p < 0.005$. ANOVA tests where the peptide changes were found to be significant are indicated by a pound sign (#) by the peptide name.

Figure 4.

MALDI-MSI analysis of several neuropeptides in the *C. maenas* PO. (A) Optical image of a PO on a MALDI plate. The main body of the decapod crustacean PO consists of anterior region, posterior region, dorsal trunk, and ventral trunk. Neuropeptide localization in (B) green shell and (C) red shell *C. maenas* PO for both control (left) and stressed (right) animal. Visualized neuropeptides pertain to three families, including RYamides ((i) FVGGSRYa (m/z 784.5), (ii) SGFYANRYa (m/z 976.5), and (iii) pEGFYYSQRYa (m/z 1030.5)), RFamides ((iv) NRNFLRFa (m/z 965.5), (v) GNRNFLRFa (m/z 1022.6), and (vi) DGNRNFLRFa (m/z 1137.6)), and AST-Bs ((vii) QWSSMRGAWa (m/z 1107.6), (viii) STNWSSLRSAWa (m/z 1293.6), and (ix) VPNDWAHFRGSWa (m/z 1470.7)).