The molecular mechanism for the pain caused by *Pterois volitans* venom

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Abstract

The wealth of biodiversity in the world's library of venoms and their toxins represents an enormous untapped resource that could contain the scaffolds for novel therapeutic drugs. The red lionfish (*Pterois volitans*) is a venomous species of fish originating from the Indo-Pacific but now invasive in many regions, where it poses a significant stress on marine ecosystems and produces one of the most painful stings in the ocean. In a study I completed prior to this thesis, we demonstrated the qualities of the pain elicited by the venom (in mice) as well as its high specificity for its cellular target – nonpeptidergic nociceptors. These cells are responsible for detecting pain in the peripheral nervous system and the venom somehow specifically activates them over other sensory neurons responsible for touch or proprioception. How can the venom target these cells specifically? This was the broad question that led to this doctoral project. The objective of this thesis was to gain insights into the human pain experience of lionfish stings, the proteinaceous toxin components of the venom and its molecular mechanism of action.

In chapter 2, I used a detailed pain questionnaire, completed by over 500 lionfish sting victims, to understand the pain they experienced and its impact on their daily lives. This was the first broad-scale study of lionfish stings ever performed and provided key insights into the average duration of pain caused by lionfish stings, causes of stings, locations of stings and other important variables.

In chapter 3, I examined the toxin composition of the venom using a combinatorial transcriptomic and proteomic approach. We performed *de novo* RNA sequencing of the lionfish's venomous spines and assembled a transcriptome which we used in mass spectrometry experiments to identify the proteins expressed in the lionfish venom. From there, I characterized the most abundant proteins and transcripts, screened venom fractions for their ability to activate nociceptors,

synthesized toxins and tested them individually for their ability to activate nociceptors. From this, I identified a highly abundant pain-causing toxin, whose sequence is a close match to Apolipoprotein-E, a molecule that has been intrinsically linked to pain sensation but whose function and mechanism of action remains poorly understood.

In chapter 4, I examined the mechanism through which the venom activates sensory neurons to understand what receptor is responsible for the venom's activation of nonpeptidergic nociceptors. I showed that the venom activates nociceptors through activation of P2X3, and following this activation, triggers ATP release from these cells to further activate neighbouring cells. Furthermore, blocking the P2X3 receptor significantly reduces the pain caused by lionfish venom in mice, suggesting that the pain-causing mechanism of action of the venom relies heavily on direct or indirect activation of P2X3, through a mechanism that remains unknown.

The findings revealed in my thesis form the basis of a new understanding of the components of lionfish venom, their mechanism of action and the human experience of lionfish stings. It has brought to light potential molecules that may be used in the study of the P2X3 receptor channel and sheds light on how the lionfish's venom has evolved to specifically cause pain in its victims.

Résumé

La richesse de la biodiversité dans les venins et de leurs toxines représente une énorme ressource inexploitée qui pourrait contenir les structures de base de nouveaux médicaments thérapeutiques. Le poisson-lion rouge (*Pterois volitans*) est une espèce de poisson venimeux originaire de l'Indo-Pacifique, et est maintenant invasive dans de nombreuses régions, où elle exerce une pression significative sur les écosystèmes marins et inflige l'une des piqûres les plus douloureuses de l'océan. Dans une étude que j'ai réalisée avant cette thèse, nous avons démontré les caractéristiques de la douleur provoquée par le venin (chez les souris) ainsi que sa spécificité pour sa cible cellulaire - les nocicepteurs non peptidergiques. Ces cellules sont responsables de la détection de la douleur dans le système nerveux périphérique et le venin les active de manière spécifique par rapport aux autres neurones sensoriels responsables du toucher ou de la proprioception. Comment le venin peut-il cibler spécifiquement ces cellules? C'était la question générale qui a conduit à ce projet de thèse. L'objectif de ma thèse était de mieux comprendre l'expérience de la douleur chez l'Homme causée par les piqûres du poisson-lion, la composition en toxines protéiques du venin et son mécanisme moléculaire d'action causant la douleur.

Dans le chapitre 2, j'ai codéveloppé un questionnaire détaillé sur la douleur qui a été complété par plus de 500 personnes piquées par un poisson-lion afin de comprendre la douleur qu'ils ont ressenti, et l'impact de cette douleur sur leurs activités quotidiennes. Il s'agissait de la première étude à grande échelle sur les piqûres de poisson-lion et elle a révélé des informations clés sur la durée moyenne de la douleur causée par les piqûres de poisson-lion, les causes des piqûres, leurs emplacements, et autres variables importantes.

Dans le chapitre 3, j'ai examiné la composition en toxine du venin en utilisant une approche combinatoire transcriptomique et protéomique. Nous avons réalisé un séquençage d'ARN *de novo* des épines venimeuses du poisson-lion et avons assemblé un transcriptome, que nous avons utilisé dans des expériences de spectrométrie de masse pour identifier les protéines exprimées dans le venin du poisson-lion. J'ai ensuite caractérisé les protéines et les transcrits les plus abondants du venin, identifié les fractions d'après leur capacité à activer les nocicepteurs, synthétisé des toxines candidates et les ai testées individuellement pour leur capacité à activer les nocicepteurs. Ces étapes m'ont permis d'identifier une toxine fortement présente qui cause de la douleur et dont la séquence est très proche de celle de l'apolipoprotéine E, une molécule intrinsèquement liée à la sensation de douleur, mais dont la fonction et le mécanisme d'action restent peu connus. Cette étude a identifié une toxine présente dans le venin du poisson-lion ayant un fort potentiel à provoquer la douleur.

Dans le chapitre 4, j'ai examiné le mécanisme par lequel le venin active les neurones sensoriels afin de comprendre quelle cible moléculaire sous-tend la spécificité du venin pour les nocicepteurs non peptidergiques. J'ai démontré que le venin active les nocicepteurs en activant le récepteur P2X3, et après cette activation, déclenche la libération d'ATP à partir de ces cellules afin d'activer davantage les neurones voisins et les cellules non neuronales. De plus, l'antagonisme du récepteur P2X3 réduit significativement la douleur causée par le venin du poisson-lion chez les souris, indiquant que le mécanisme algogénique du venin repose sur l'activation directe du P2X3, ou indirecte par le biais d'un mécanisme encore inconnu. Les résultats révélés dans ma thèse constituent la base d'une nouvelle compréhension des composants du venin du poisson-lion, de leur mécanisme d'action et de l'expérience des piqûres du poisson-lion chez l'Homme. Ils ont mis en lumière des molécules potentielles qui pourraient être utilisées dans l'étude du canal récepteur

P2X3, et éclairent la manière dont le venin du poisson-lion a évolué pour causer spécifiquement de la douleur chez ses victimes.

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If you know me, you know that one of my mottos in life is FIDLAR and it's (of course) how I convinced myself it was a good idea to embark on a PhD in the first place. As I write this, I don't quite know exactly what my future looks like, but I feel a lot of confidence knowing that I have come this far thanks to the support of all the people mentioned in this section. So many told me that doing a PhD would be the hardest thing I have ever done, and I never understood or believed any of them until this year. Thank you to everyone for helping me get across this finish line. It has been the hardest and most rewarding journey I have ever been on.

Contribution to original knowledge

The results of this thesis provide the first insights into the human pain experience of a lionfish sting, the venom's protein components and the venom's pain-causing mechanism of action. I show that lionfish venom is a composed of a variety of proteinaceous toxins, one of which is particularly similar to ApoE, a known component of the pain pathway. Furthermore, I show that lionfish venom takes advantage of a unique P2X3-dependent pathway to cause pain, and that blockade of this channel alone can significantly reduce the pain behaviours shown by mice after envenomation. Furthermore, we performed the first sequencing of the lionfish venom gland transcriptome as well as its proteome, both of which will be made available online in the Uniprot Venoms and Toxins Database upon publication of the manuscript. These datasets will certainly prove valuable to other groups in the future performing studies of aquatic venoms and their components. The findings of this thesis are shown in 3 original manuscripts:

Mouchbahani-Constance S., Choinière M.; Sharif-Naeini R. Understanding the pain experience of lionfish envenomation. PAIN Reports 8(5):p e1090, September 2023. | DOI: 10.1097/PR9.0000000000001090

Mouchbahani-Constance S., Pidgeon R., Taylor L., Castagner B., Trempe J-F., Sharif-Naeini R. Detailed transcriptomic and proteomic analysis of *Pterois volitans* venom identifies a highly abundant and pain-causing Apolipoprotein-E like toxin, 2023, *In preparation*

Mouchbahani-Constance S., Sharif-Naeini R. Lionfish venom elicits pain through a P2X3-dependent mechanism, 2023, *In preparation*

Aside from the three manuscripts in my thesis, I have contributed to 6 other manuscripts over the course of my PhD, 3 of which I was first author of. In my first first-authored manuscript, discussed in the introduction of this thesis, I provided the first comprehensive study of the pain caused by lionfish venom in mice and we characterized the cellular target of lionfish venom. This work laid the foundation for my PhD. In my second first-authored manuscript, I wrote a review of proteomic and transcriptomic techniques in the study of venoms. This allowed me to become

deeply acquainted with the techniques I would use in the third chapter of this thesis. In my third first-authored manuscript, I evaluated the ability of a novel nociceptor-specific viral vector tool to infect human nociceptors and express functional cargo in these cells. For this approach, I used a technique that I adapted for our lab to culture primary human DRG neurons from organ donor tissue. Once cultured, I infected the cells with the viruses and evaluated their responses in calcium imaging and electrophysiology experiments.

First-authored manuscripts:

Mouchbahani-Constance S., Lesperance L. S., Petitjean H., Davidova A., MacPherson A., Prescott S. A., Sharif-Naeini R. (2018) Lionfish venom elicits pain predominantly through the activation of non-peptidergic nociceptors. PAIN. 159(11): 2255-2266. PMID: 29965829. doi: 10.1097/j.pain.0000000000001326. **(cover of the November 2018 issue)**

Mouchbahani-Constance S., Sharif-Naeini R. (2021) Proteomic and Transcriptomic Techniques to Decipher the Molecular Evolution of Venoms. Toxins. 154(13). doi:10.3390/toxins13020154

Mouchbahani-Constance S, Lagard C, Schweizer J, Labonté I, Georgiopoulos M, Otis C, St-Louis M, Troncy E, Sarret P, Ribeiro-Da-Silva A, Ouellet JA, Séguéla P, Paquet ME, Sharif-Naeini R. Modulating the activity of human nociceptors with a SCN10A promoter-specific viral vector tool. Neurobiol Pain. 2023 Jan 30;13:100120. doi: 10.1016/j.ynpai.2023.100120. PMID: 36816616; PMCID: PMC9932673.

Other manuscripts:

He B. H.*, Christin M.*, Mouchbahani-Constance S., Davidova A., Sharif-Naeini R. (2017) Mechanosensitive ion channels in articular nociceptors drive mechanical allodynia in osteoarthritis. Osteoarthritis & Cartilage. 25(12): 2091-2099. doi: 10.1016/j.joca.2017.08.012

Beaulieu-Laroche L.*, Christin M.*, Donoghue A., Agosti F., Yousefpour N., Petitjean H., Davidova A., Stanton C., Khan U., Dietz C., Faure E., Fatima T., MacPherson A., Mouchbahani-Constance S., Bisson D.G., Haglund L., Ouellet J.A., Stone L.S., Samson K., Smith M. J., Ask K., Ribeiro-da-Silva A., Blunck R., Poole K., Bourinet E., Sharif-Naeini R. (2020) TACAN is an ion channel involved in sensing mechanical pain. Cell. 180(5): 956-967. PMID: 32084332. doi: 10.1016/j.cell.2020.01.033.

Petitjean H., Fatima T., Mouchbahani-Constance S., Davidova A., Ferland CE., Orlowski J., Sharif-Naeini R. (2020) Loss of SLC9A6/NHE6 impairs nociception in a mouse model of Christianson Syndrome. PAIN. 161(11):2619-2628. doi: 10.1097/j.pain.0000000000001961.

Contribution of authors

The following statements describe the responsibilities of all the authors in the co-authored manuscripts in this thesis:

Dr. Reza Sharif-Naeini: Principal investigator of all projects, intellectual influence on all projects, experimental design, writing/editing manuscripts, funding all projects

Dr. Gergely Lukacs: Co-supervisor, intellectual influence on projects

Stephanie Mouchbahani-Constance: Main intellectual influence on all projects, designed and planned all experiments, collected and analyzed data, writing/editing manuscripts

Dr. Manon Choinière: Assisted in writing the lionfish pain questionnaire, intellectual influence and writing/editing manuscript

Dr. Lois Miraucourt: Electrophysiological expertise, assisted in editing manuscripts

Reilly Pidgeon: HPLC expertise, designed and performed HPLC experiments

Lorne Taylor: Mass spectrometry expertise, designed and performed MS/MS experiments

Dr. Jean-François Trempe: Proteomics expertise, intellectual influence, design of HPLC and MS/MS experiments

Dr. Bastien Castagner: Proteomics expertise, HPLC access

Chapter 1 – General Introduction

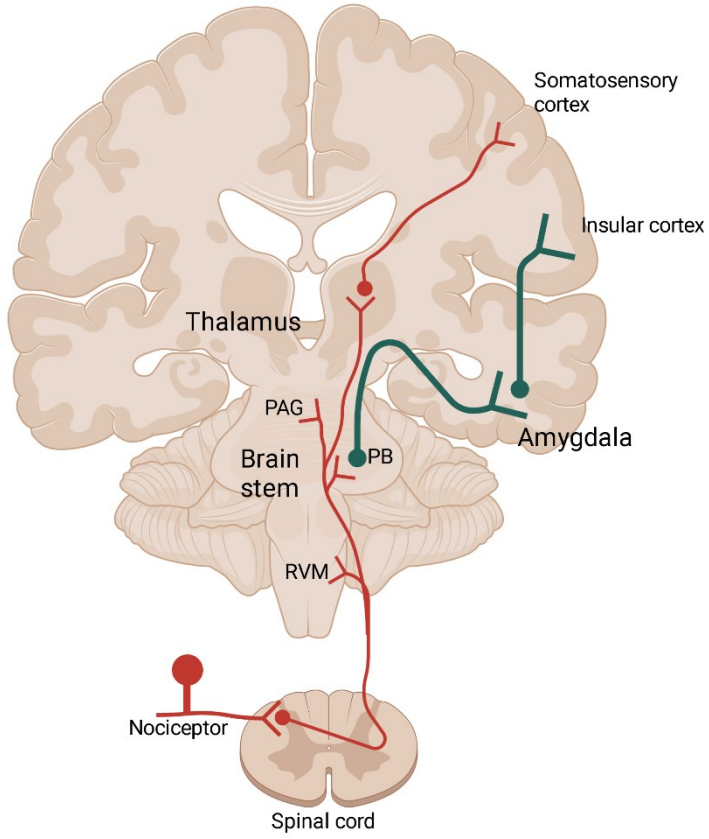
1.1 Pain

Nociception, the capacity to detect high intensity thermal, mechanical, and/or chemical stimuli, is fundamental to the survival and continuity of a species. The subjective experience of this nociception, leading to protection of an injured limb or avoidance of a hazardous situation, is referred to as pain (Raja et al. 2020). The ability to feel pain is essential to survival: a hyposensitivity to pain may lead an animal to being unaware of injuries and potentially land itself in a life threatening situation, while a hypersensitivity to pain may become chronic and debilitating for its victims (Basbaum et al. 2009; Fry et al. 2009). Acute pain is short-lived and, in many cases, is a symptom of a physiological healing process whereby it protects healing tissue from overuse and re-injury. This may manifest itself as allodynia, during which innocuous stimuli such as light touch are perceived as painful, or hyperalgesia, during which normally painful stimuli evoke exaggerated pain responses (Basbaum et al. 2009; Fry et al. 2009). Chronic pain takes place when pain becomes exaggerated in its time course; this typically occurs when pain persists after an acute injury has healed and typically originates in the peripheral or central nervous systems (Basbaum et al. 2009; Ronald Melzack and Wall 1965; R. Melzack and Wall 1962). Unfortunately, chronic pain is an epidemic that affects 1 in 5 Canadians, with an economic burden estimated at $7 billion per year, and occurring mostly in female, Indigenous, veteran and older populations (Schopflocher, Taenzer, and Jovey 2011).

1.2 Neurobiology of pain processing

Pain processing begins in the periphery and involves multiple cell types across both the peripheral and central nervous system before an individual is conscious of their pain from a physical and emotional point of view. First, primary afferent nociceptors detect noxious stimuli and convey this information to projection neurons in the dorsal horn of the spinal cord via

interneuron intermediates (see Introduction Section 1 Figure 1). These projection neurons then transmit this information to the thalamus, which connects to the somatosensory cortex where the intensity and location of the painful stimulus is assessed. In parallel to this, other projection neurons from the spinal cord connect to the parabrachial nucleus in the brain stem. These neurons send connections into the amygdala and insular cortex, one of the sites where the emotional component of the pain experience is processed (Basbaum et al. 2009).



Introduction Section 1.2 Figure 1: Anatomy of the Pain Pathway, adapted from (Basbaum et al. 2009). Red lines indicate ascending pain information, and blue-green line indicate circuits involved in the processing of the emotional component of pain.

1.2.1 Primary afferents and dorsal root ganglia

Nociceptors are peripheral pain-sensing fibers innervating the skin, muscle, and other visceral organs. They are pseudounipolar neurons with their cell body in the dorsal root (DRG) or trigeminal ganglia (TG), a peripheral axon that terminates in tissue with free nerve endings located in the epidermis and dermis, and a central axon that terminates in the upper laminae of the dorsal horn of the spinal cord (Dubin and Patapoutian 2010). On their peripheral terminals, they express ion channels and receptors which transduce painful mechanical, thermal and/or chemical stimuli into electrical signals, making them as critical to nociception as photoreceptors are for our sense of sight (Dubin and Patapoutian 2010). The activation of these channels results in a local depolarization at the site of transduction, called a receptor potential. This depolarization will then recruit voltage-gated ion channels to generate an all-or-none action potential which can then travel along the length of the peripheral and central axon to the spinal cord (Sharif-Naeini 2020, 2015). From their central terminals in the spinal cord, nociceptors release neurotransmitters to activate their postsynaptic targets to recruit the ascending pain circuits mentioned above (Basbaum et al. 2009; Dubin and Patapoutian 2010).

Overall, there exist over eleven different specialized types of peripheral sensory neurons for the different modalities of somatosensation, with each cell type expressing a different combination of ion channels and receptors to specifically tune them to their modality (Usoskin et al. 2014; Kupari et al. 2021; Braz et al. 2005). There are two subtypes of low-threshold mechanoreceptive neurons (myelinated A-LTMRs and unmyelinated C-LTMRs), two subtypes of proprioceptive neurons (A-α and A-β fibers) and two major classes of nociceptors, which are thinly myelinated A-δ fibers (which tend to mediate acute and “fast” pain) and unmyelinated C fibers (which tend to mediate longer or “slow” pain) (Usoskin et al. 2014; Braz et al. 2005;

Basbaum et al. 2009). Within the A-δ class, there are two clear groups of electrophysiologically distinct nociceptors: Type I A-δ nociceptors, which have a high activation threshold for thermal (>50 ºC), mechanical and chemical stimuli. Type II A-δ nociceptors have a lower heat threshold but a high mechanical threshold (Basbaum et al. 2009). Most C fibres are considered to be polymodal, meaning they are both mechanically and heat sensitive, and some (termed silent nociceptors) are heat-responsive and only become mechanically responsive after injury (Perl 2007). C fibers can also be divided into those expressing substance P (SP) and calcitonin gene-related peptide (CGRP) – called peptidergic nociceptors and those without neuropeptides, but that bind to isolectin B4 and express MrgprD receptors (Mas-related G protein-coupled receptor), called nonpeptidergic nociceptors (Le Pichon and Chesler 2014). Recent single cell RNA-sequencing studies have added additional subtypes into this mix, including NP1, NP2, NP3 and PEP1, PEP2 and PEP3 subpopulations, each slightly different due to their expression patterns of channels and receptors and thus each transmitting slightly different information (Usoskin et al. 2014; Kupari et al. 2021). All of these DRG neurons are excitatory and release glutamate, SP, somatostatin or other excitatory neurotransmitters/neuropeptides onto their post-synaptic spinal cord targets (Abraira and Ginty 2013).

1.2.2 Spinal Cord and Brain

The central terminals of sensory fibers terminate in the dorsal horn of the spinal cord in a unique dorso-ventral pattern that is determined by their function. Peptidergic nociceptors terminate in the most superficial layers of the dorsal horn [(laminae I and outer laminae II (IIo)] while nonpeptidergic nociceptors terminate lower [(the inner laminae II (IIi)] and touch-sensitive mechanoreceptors terminating even lower (laminae III-V) (Kupari et al. 2021; Usoskin et al. 2014). In addition to the stratification of their terminals, nociceptors engage distinct circuits

involving different patterns of excitatory and inhibitory interneurons, responsible for complex processing and gating of nociceptive information before the pain signals reach projection neurons (Braz et al. 2005; Snider and McMahon 1998; Qiu et al. 2022). The majority of the dorsal horn's output is carried by projection neurons originating in laminae I and V; they project to the thalamus and parabrachial nucleus, respectively (Basbaum et al. 2009). From there, signals reach cortical structures where the sensory-discriminative properties of the pain as well as its emotional aspects are processed (Apkarian et al. 2005).

1.3 Ion channels and receptors for pain transduction

The molecules responsible for the different functionalities of sensory neurons are the receptors and channels they express on their membranes. These are ion channels, gated pores that allow ions to flow across the membrane, or G protein-coupled receptors which upon activation trigger second messenger cascades that ultimately excite the neuron.

1.3.1 Ion channels

Ion channels are specialized protein structures that span the membranes of living organisms. They function to selectively allow ions across the cell membrane and, depending on the ion (sodium, potassium, calcium or chloride ions), underlie neuronal signaling and contribute to a wide range of physiological processes (Hille 2001). In order for a thermal, mechanical or chemical stimulus to activate a nociceptor and result in pain, the stimulus must activate an ion channel and result in a receptor potential strong enough to activate voltage-gated sodium channels and trigger an action potential (Hille 2001). In this section, I will describe the major temperature-sensing, mechanically gated and ligand-gated ion channels responsible for transducing these stimuli into electrical signals.

1.3.1.1 Temperature-sensing channels

Many different classes of nociceptors display an increase in activity in response to heat or cold stimuli at a variety of intensities. The most studied heat-activated channel is the transient receptor potential vanilloid 1 (TRPV1) channel, which is also activated by low pH and capsaicin, the painful component of chili peppers (K. Zhang, Julius, and Cheng 2021; Caterina et al. 1997; Tominaga et al. 1998). The S1-S4 membrane domains of TRPV1 are thought to contain its temperature sensing domain which, upon exposure to high (>43 ºC) heat, undergoes a conformational change causing the pore to open and allow sodium and calcium ions to flow into the cell, resulting in a depolarization (Kim et al. 2020). However, the details of how this process works are still relatively unknown. A related channel, TRPV2, has been shown to be activated by temperatures greater than 52 ºC, as well as TRPV3 (>50 ºC for initial activation and ~33 ºC for subsequent activation) and TRPV4 (> 27 ºC) (on keratinocytes) (Zimmermann et al. 2005; Patapoutian, Tate, and Woolf 2009; Caterina et al. 1999; Singh et al. 2019).

Cold sensing has not been studied in the same depth as heat sensing, likely due to the fact that the mammalian threshold for painful cold varies dramatically since the rate of the cooling plays a large role in cold-evoked fiber activity (Allchorne, Broom, and Woolf 2005; Davis and Pope 2002; Foulkes and Wood 2007). The menthol-activated channel TRPM8 is the most studied cold-sensitive channel and is responsible for the detection of innocuous cooling and some sensing in the noxious range (overall, the pain threshold is roughly 15 ºC and the noxious range is between 10 and 15 ºC) (Patapoutian, Tate, and Woolf 2009; Gentry et al. 2010). TRPA1 has also been shown in both *in vitro* and *in vivo* mouse studies to be required for cold sensing, and it is activated near 17 ºC (Foulkes and Wood 2007; Gentry et al. 2010). It is thought that this expression of both

channels in these cells may underlie the burning sensation felt in response to very cold stimuli (Schepers and Ringkamp 2010).

1.3.1.2 Mechanosensitive channels

Mechanosensitive channels are the gatekeepers to our ability to sense touch, pressure, a hard smack and even a pinprick. The variety of these channels as well as their pattern of expression on the surface of sensory neurons allows us to sense a variety of different pressures as pleasant or painful. Touch sensation is mostly mediated by the Piezo2 channel, which is activated at low pressure thresholds and is broadly expressed across touch-sensitive neurons and proprioceptors (Coste et al. 2010). TACAN, a channel identified by our group, has been shown to be involved in sensing high intensity mechanical pain, but not the pain mediated by a pinprick (Beaulieu-Laroche et al. 2020). There are also members of the two-pore potassium channel family that are hyperpolarizing and are mechanosensitive: TRAAK, TREK1, TREK2 and TRESK are expressed on sensory neurons and are activated at a wide range of membrane tension levels (Brohawn 2015). Upon activation by pressure, mechanosensitive channels undergo a conformational change resulting in the opening of their pore and allowing ions to flow across the membrane. The specific mechanisms for activation of mechanosensitive channels are still unclear, but likely differ between different channels due to major differences in their structures (Chen et al. 2022).

1.3.1.3 Ligand-gated channels

Ligand-gated ion channels play a major role across the entire nervous system and are responsible for binding their ligands, present in the extracellular space, causing a conformational change and allowing ions to flow across the cell membrane to depolarize (or in some cases, hyperpolarize) neurons. These ligands can be neurotransmitters, hormones, or in the case of pain sensation,

noxious chemical irritants (Dubin and Patapoutian 2010). Chemo-nociception is the process by which nociceptors detect irritants present in the environment to deter the victim from potential harm, or the detection of endogenous irritant molecules produced in response to tissue damage and physiological stress. Chemical irritants include (but are not limited to) capsaicin (binds to TRPV1), menthol (binds to TRPM8), mustard oil (binds to TRPA1) and ATP (binds to P2X3) (Basbaum et al. 2009). The mechanism for activation of each channel by their agonist differs from channel to channel, but the purpose is the same: to detect any irritant molecules and trigger activation of nociceptors to transmit the pain signal to the victim.

1.3.1.3.1 TRP and ASIC channels

The TRPA1 channel is a unique member of the TRP channel family due to its ability to bind and be activated by multiple, structurally diverse compounds, the only unifying characteristic of its major agonists are their thiol groups (Bautista et al. 2006). This property has made it a key target for irritants including toxins, molecules from mosquito saliva, wasabi, acrolein (irritant in tear gas) and has been implicated as a key molecule in the development of chronic itch in addition to its roles in temperature sensing discussed above (Lin King et al. 2019; Chung et al. 2021; Derouiche et al. 2021; Melo et al. 2021; Wilson et al. 2013).

TRPV1 was first discovered due to its affinity for capsaicin, the painful ingredient in chili peppers, and its thermal sensitivity. Since, the channel has been shown to be able to bind to and be modulated by protons, lipids and is the target of a variety of different animal toxins, all of which can act as positive allosteric modulators of the channel's intrinsic thermal sensitivity (Siemens et al. 2006; Y. Wang et al. 2021; Basbaum et al. 2009). TRPV1 has also been implicated in sensing the inflammatory stage of tissue injury-evoked pain such as sunburn, infections, osteoarthritis, rheumatoid arthritis and inflammatory bowel disease (Basbaum et al. 2009).

ASIC channels are activated by acidification and are also a common target of toxins or components of the inflammatory reaction (Baron et al. 2013; Stephan et al. 2018). There are 4 different ASIC genes which can form 6 homomeric and heteromeric channels (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4) that each have distinct proton sensitivities and expression patterns (Basbaum et al. 2009; Baron et al. 2013). ASIC3 is the most prominently expressed in nociceptors and is highly sensitive to changes in acidity, mostly activated by moderate acidification (drop from pH 7.4 to 7.0), which corresponds to the range in which lactic acid buildup would acidify muscle tissues (Yagi et al. 2006). Some specific toxins targeting ASIC channels have been identified in venoms from spiders, anemone, and snakes (Baron et al. 2013; Bohlen et al. 2011).

1.3.1.3.2 P2X3 and other purinergic channels and receptors

P2X3 is another important ligand-gated ion channel expressed on the surface of nociceptors. Like other members of the purinergic receptor family, it is activated by ATP and has the highest affinity for the ATP analog αβMe-ATP (Evans et al. 1995). ATP has been widely known as an important chemical energy currency in eukaryotic cells, but is also now known to be an important signaling molecule between cells (North 2016). The first evidence that ATP could depolarize neurons and open an ion channel came from the 1980s, and later the molecule was shown to be able to mediate fast synaptic transmission in both the PNS and CNS neurons, but the channels responsible had yet to be cloned and studied in detail (Jahr and Jessell 1983; Krishtal, Marchenko, and Pidoplichko 1983; Edwards, Gibb, and Colquhoun 1992; Evans, Derkach, and Surprenant 1992; Silinsky, Gerzanich, and Vanner 1992). In 1994, the first P2X genes were cloned and the rest were identified in the years that followed as well as the metabotropic purinergic receptors, the P2Y class (Brake, Wagenbach, and Julius 1994; Buell, Collo, and Rassendren 1996;

Chen et al. 1995; Collo et al. 1996; Soto et al. 1996; Surprenant 1996; Valera et al. 1994; Seguela et al. 1996).

In all, there are 7 mammalian P2X subunits that have 2 hydrophobic membrane-spanning segments that are separated by a highly folded extracellular domain which is held together by its 10 conserved cysteine residues that form disulphide bonds and have a pore that is non-selectively permeable to cations (Khakh and North 2012; Kasuya et al. 2017). The complex folding of the extracellular domain is essential for proper ATP binding and for antagonist binding. The channels are trimeric and have intracellular N- and C- termini that are the targets of intracellular modulators (Kasuya et al. 2017; Mansoor et al. 2016). After the monomers assemble into trimers, the channel's extracellular folds interact to create multiple bindings sites for ATP (Mansoor et al. 2016). Many P2X channels are expressed in DRG neurons: P2X1 is very lowly expressed in peptidergic nociceptors, P2X3 is highly expressed in nonpeptidergic nociceptors and lowly expressed in some peptidergic, P2X4 is lowly expressed across all DRG neuron subtypes but highly expressed in microglia, P2X5 and P2X6 are highly expressed in myelinated neurons that are positive for the marker Neurofilament 200 (NF200), important for touch with some moderate expression in nonpeptidergic nociceptors and P2X7 is highly expressed on immune cells and microglia (Zeisel et al. 2018). Interestingly, P2X2, 4 and 7 have the ability to dilate their pores and allow for influx or efflux of larger molecules and have even been shown to allow for the efflux of ATP following prolonged channel activation (Chaumont and Khakh 2008; Khadra et al. 2012; Yan et al. 2011, 2008).

By far, P2X3 has been the P2X channel most implicated with pain and is a high value target for the development of pain-reducing therapeutics (Ballini et al. 2011; Bleehen, Hobbiger, and Keele 1976; Chen et al. 1995; Cockayne et al. 2000; Guo et al. 1999; Jarvis et al. 2002; M. C. G.

Oliveira et al. 2009; Vulchanova et al. 1998; Bernier, Ase, and Séguéla 2018). It is thought that post-translational modifications of the N- and C- termini of P2X3 contribute to the sensitization of neurons in chronic pain conditions (Bernier, Ase, and Séguéla 2018). Prostaglandin-E2, a major signaling molecule produced during inflammation, has been shown to indirectly enhance P2X3 currents through a second messenger cascade mediated by its receptor, EP3. Upon activation by PGE2, EP3 receptors activate a PKA-dependent pathway that enhances both expression and activation of P2X3 receptors and the currents they mediate (C. Wang, Li, and Huang 2007). Activated metabotropic P2Y receptors have also been shown to modulate P2X3 activity in nociceptors through a G_q-coupled PLC-dependent pathway (Mo et al. 2009). This may be a homeostatic mechanism to control excessive P2X3 activation. Blocking P2X3 receptors has been shown to reduce pain in a variety of preclinical models of inflammatory, neuropathic and cancer-induced pain and is a focus of some clinical trials in these spaces as well as chronic cough (Chawla et al. 2023; Gever et al. 2010; Y. Wang et al. 2021).

1.4 Venom

Many organisms that do not possess the physical size or capacity to protect themselves from predators or to hunt their prey have specifically evolved their own personalized chemical weapons – potent venoms. Venoms are secretions produced by animals or invertebrates that are composed of cocktails of molecules, referred to as toxins, which have each been evolutionarily honed to execute their function and work in concert with one another to execute the venom's purpose (Bohlen and Julius 2012; Fry et al. 2009; Wong and Belov 2012; Morgenstern and King 2013; Casewell et al. 2013). Venoms are often confused with poisons, but the key difference is the mode of administration in the victim. In the case of poisons, a victim must ingest the poison, whereas a

venom is directly injected into the victim through an envenomating apparatus evolved by the venomous organism (Bordon et al. 2020).

1.4.1 Venom evolution

Some venoms only cause pain in victims while others seek to paralyze and some even seek to kill; all of these arise due to different evolutionary pressures. Painful venoms typically serve as protection for animals to save themselves from predators. By causing excruciating pain, painful venoms produce a lasting memory of the experience in their victims to serve as a reminder to avoid them in the future. Some groups even suggest that because sting victims learn to avoid such venomous prey in the future and potentially teach their offspring to do so as well, producing pain may be a stronger deterrent than the killing of the predator (Harris et al. 2021). On the other hand, some venoms have evolved as an aid for predators hunting their prey; these venoms tend to paralyze or kill victims in order to facilitate the capturing of a meal (Dutertre et al. 2014; Bricelj et al. 2005; Asakawa et al. 2019; Tan et al. 2015).

Toxins arise through "toxin recruitment events" whereby ordinary (non-toxin) genes that are involved in key regulatory processes are duplicated. The duplicated gene then becomes selectively expressed in the venom gland and undergoes one of three fates – the gene is either deleted, mutated into a non-functional gene, or adopts a new function (neofunctionalization) as a toxin. Typically, a neofunctionalized gene will maintain the same molecular scaffold as the parent gene but will perform some sort of toxin function instead of its original fate (Fry et al. 2009; Wong and Belov 2012).

1.4.2 Venom compositions

Venoms are complex mixtures of bioactive molecules that serve as effective tools for predation and defense among various animal species. From snakes and spiders to scorpions and cone snails, these creatures have evolved sophisticated venomous systems to immobilize and control their prey or foes (Morgenstern and King 2013). Close phylogenetic relatives often exhibit similar venom compositions, implying a conserved and adaptive pattern in venom evolution (Morgenstern and King 2013). In this section, I will describe general venom compositions from some highly studied groups of species as well as the venom compositions of fish closely related to the lionfish.

1.4.2.1 Snakes, spiders, and scorpions

Nearly all snake venoms possess at least 4 families of toxins in their venoms: phospholipase A-2 toxins, snake venom metalloproteases, snake venom serine proteases and 3-finger toxins (Tan et al. 2015; Martill, Tischlinger, and Longrich 2015; Fry et al. 2006; Sanz and Calvete 2016; Vonk et al. 2013; Leonardi et al. 2019). Some families of snakes have venoms with toxins that are biased to some of these families, for example elapids tend to have more 3-finger toxins and phospholipase A2 toxins, while vipers tend to have more metalloproteases, serine proteases and phospholipase A2 toxins than 3-finger toxins (Tasoulis and Isbister 2017).

Spiders tend to use their venoms to subdue their prey, typically insects or small vertebrates, so their toxin composition differs from that of snakes and larger venomous species that exclusively hunt small vertebrates. Their most abundant toxins are disulfide-rich peptides and serine proteases. Disulfide-rich peptides can range from neurotoxins (that immobilize prey) to cytotoxins or enzymes which aim to break down the prey's tissues and partially pre-digest it for the spider. Serine proteases tend to aid in digestion or modulation of the prey's immune response (Haney et al. 2016; Huang et al. 2018; Li et al. 2017). These toxins act in concert to facilitate the capture and consumption of food for spiders.

Scorpions have somewhat similar prey to spiders (small vertebrates and insects), and therefore a related venom composition. Their venoms are mostly composed of toxins that can immobilize and pre-digest prey, but their approach to this immobilization is slightly different. They also have neurotoxins, but theirs are uniquely able to target a variety of potassium and sodium channels with high efficiency, as well as cytotoxins and enzymes to help pre-digest prey (Fry et al. 2009; de Oliveira et al. 2018; Ward, Ellsworth, and Rokyta 2018). However, they also possess 2 toxin families that are not frequently found elsewhere: natriuretic peptides and antimicrobial peptides. Natriuretic peptides serve to trigger blood pressure changes in victims and affect the prey's cardiovascular system, while antimicrobial peptides are protective for the scorpion to help defend them from potential infections coming from the prey's gut flora or from environmental microbes (Fry et al. 2009; D. Oliveira et al. 2018; Ward, Ellsworth, and Rokyta 2018). Together, these toxins serve to aid the scorpion in hunting prey and protect it from potential infections.

1.4.2.2 Cone snails, fish and other marine species

While the venoms of many land-dwelling species have been highly studied, venoms from aquatic species have been studied in far less depth (Asakawa et al. 2019). An exception to this rule are the cone snails (family *Conidae*), whose venoms have been studied in great depth thanks to the pioneering work of Dr. Baldomero Olivera (Dutertre et al. 2014; Kohn 1956; Safavi-Hemami et al. 2015; Terlau et al. 1996; Olivera et al. 1985; Sousa et al. 2018; Cordeiro et al. 2019; Cruz, Gray, and Olivera 1978; Cruz and Olivera 1986). Cone snails are fascinating for many reasons, but a favourite of mine is their ability to swap their venoms between defense and predatory venoms depending on the situation. It has been shown that their defensive venom contains mostly paralytic toxins, while their predatory venom contains more prey-specific toxins that can only incapacitate their prey, leaving other non-prey species unaffected (Dutertre et al. 2014). Toxins from cone snail

venoms are called conotoxins and major families of conotoxins that have been identified – α-conotoxins (target nicotinic acetylcholine receptors), δ-conotoxins (cause fast inactivation of voltage-gated sodium channels), κ-conotoxins (block potassium channels), μ-conotoxins (block muscle-specific voltage-gated sodium channels) and ω-conotoxins (block N-type voltage-gated calcium channels) (Dutertre et al. 2014; Safavi-Hemami et al. 2015; Terlau et al. 1996; Olivera et al. 1985; French et al. 2010; Sousa et al. 2018; Cordeiro et al. 2019; Cruz and Olivera 1986).

The blue-ringed octopus, comprised of 4 species of the genus *Hapalochlaena*, are some of the only venomous species of octopus and are found in the Pacific and Indian oceans. The toxin in their venom which makes them lethal to humans is tetrodotoxin, a non-proteinaceous toxin which they sequester from bacteria located in the salivary gland. Other toxin families found in their venom include hyaluronidases, metalloproteases, phospholipases and serine proteases (Asakawa et al. 2019; Geffeney et al. 2019; Fingerhut et al. 2018; Whitelaw et al. 2016). Cnidaria, also known as jellyfish, are particularly dependent on their venoms for protection since their soft and squishy bodies provide them with none. The major toxin families identified from their venoms are enzymes, cytolysins and a variety of neurotoxins targeting ion channels including voltage-gated sodium channels, voltage-gated potassium channels, ASIC channels and TRPV1 (Jouiaei et al. 2015; Orts et al. 2013; Brinkman et al. 2015; Lewis Ames et al. 2016). Stingrays have venomous tail spines, termed dermal denticles, and their venom is mostly composed of hyaluronidases and cytotoxins, but their venoms have not been studied very broadly and little is known about other key venom components (Júnior et al. 2016; F. Silva et al. 2018).

The *Scorpaenidae* family contains numerous species of venomous fish, in particular the scorpionfish (*Scorpaenopsis cirrose, S. neglecta* and *S. possi*), the stonefish (*Synanceia verrucosa*) and the lionfish (*Pterois volitans* and *Pterois miles*). These venoms have not been studied in as

much depth as spider and scorpion venoms due to a variety of reasons, including the difficulty of accessing these fish, the difficulty of extracting venom from the fish and the fact that the glandular structures of these fish are still unknown. It is thought that venom is produced in these species by clusters of secretory cells that line the grooves in the cartilage of their venomous spines, although this has never been verified experimentally (Xie et al. 2019). Some key toxins have been identified in these venoms, as well as some common ones between them, these include hyaluronidases, phospholipases, and some large proteinaceous toxins which are thought to act as cytolysins (Harris et al. 2021; Kiriake and Shiomi 2011; Gwee et al. 1994; Ghadessy et al. 1995; Xie et al. 2019; Rensch and Murphy-Lavoie 2021). The venom from the lionfish will be described in a later section of the introduction and in depth in chapter 3 of this thesis.

1.4.3 Pharmacological interest in venoms

By studying venoms and their overall molecular mechanism of action, we can gain valuable insights into the molecular basis of pain sensation and take advantage of their structures and function to design more efficient pharmaceutical therapies. There are currently over 16 FDA-approved drugs or therapies derived from animal venom toxins, and many more in clinical trial pipelines around the world (Bordon et al. 2020; Harvey 2014; Peigneur, de Lima, and Tytgat 2018; McDermott 2020; King 2011; C. R. Wang et al. 2020). These include popular and highly prescribed medications such as Ziconotide, derived from toxins found in cone snails and treats very severe chronic pain, and Enalapril, derived from the venom of the Jamaica pit viper that treats hypertension (Bordon et al. 2020; Lewis et al. 2012; Sajevic, Leonardi, and Križaj 2011).

In addition to helping develop new therapies, the study of toxins and their molecular mechanisms of action has also led to numerous important discoveries in basic science. By simple virtue of the fact that toxins have evolved to bind to their target with high efficiency and specificity,

they make for perfect tools in the identification and manipulation of molecules involved in normal physiological processes including haemostasis, synaptic transmission, receptor activation and action potential propagation (Sajevic, Leonardi, and Križaj 2011; French et al. 2010; Caleo and Schiavo 2009; Brodie 1999; Schmidtko et al. 2010; Dutton and Craik 2001). Many toxins exploit features exclusive to certain receptors, for example unique gating cycles, ligand binding sites and voltage sensing domains, while others can even penetrate cells to bind to intracellular channel residues to modulate activity from within the cell (Lin King et al. 2019; Gross and Mackinnon 1996; Mackinnon, Reinhart, and White+ 1988; Doyle et al. 1998; Swartz and MacKinnon 1997; Lee and MacKinnon 2004; Alabi et al. 2007; Tsetlin, Utkin, and Kasheverov 2009). For example, charybdotoxin (from scorpion *Leiurus quinquestriatus hebraeus*), agitoxin (from scorpion *Leiurus quinquestriatus hebraeus*) and hanatoxin (from tarantula *Grammostola spatulata*), have highly refined specificities for voltage-gated potassium channels. Their use was instrumental in experiments geared at elucidating the channels' gating cycles, structural features and ion conduction properties and led in part to the 2003 Nobel Prize in Chemistry, awarded to Roderick MacKinnon and others for the study of ion channels (Gross and Mackinnon 1996; Mackinnon, Reinhart, and White+ 1988; Doyle et al. 1998; Swartz and MacKinnon 1997; Lee and MacKinnon 2004). All these properties and more make toxins great tools for studying the molecular underpinnings of pain, neuroscience, and other physiological pathways.

1.4.4 Resistance mechanisms to venoms

Since venoms are so powerful, interacting species that share the same habitat often evolve resistance mechanisms to toxins to continue co-existing and/or competing with the venomous species. This parallel evolution of toxins and resistance mechanisms is often referred to as an evolutionary arms race, where toxins and their targets are evolving in parallel to adapt to changes

evolved in the opponent (Nei and Rooney 2005; Calvete 2017; Dawkins and Krebs 1979; Lynch and Conery 2000). The basis for these adaptations is survival – either a venomous species will become a resistant species' dinner and perish, or a resistant species will evade a venomous predator's venom with its resistance mechanism, leaving the venomous predator to die from hunger. Survival is central in driving the phenotypic evolution of venoms, and the genetic variants underlying these developments can provide insights into the molecular basis for different evolutionary processes (Calvete 2017; Daltry, Wüster, and Thorpe 1996; Holding et al. 2021; D. Silva and Aird 2001).

1.5 Lionfish

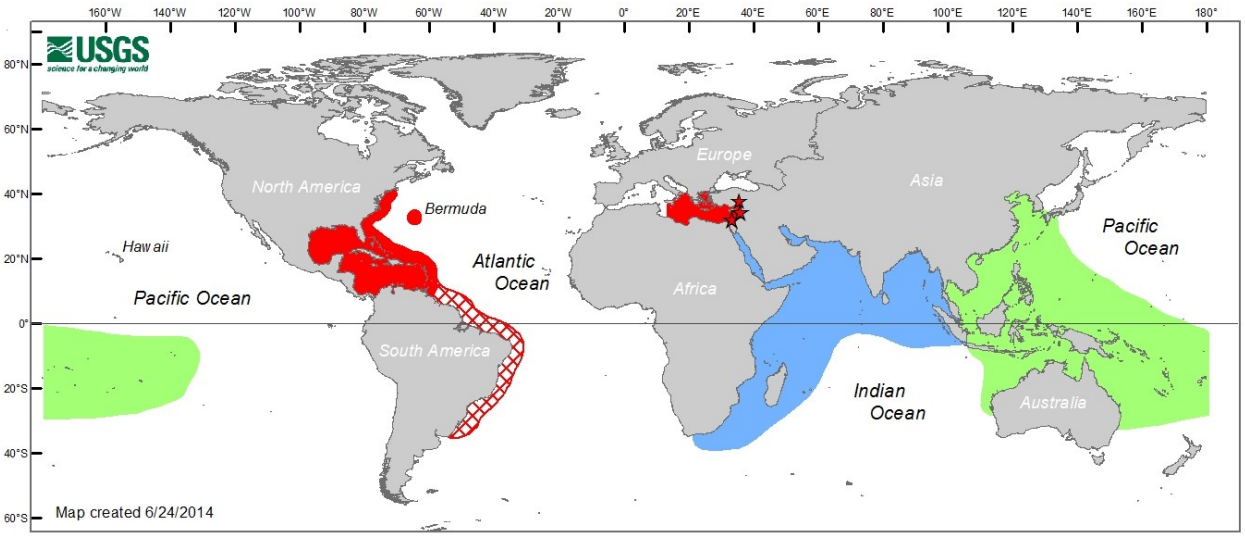
Lionfish (*Pterois volitans*) are a venomous species of fish that are native to the Indo-Pacific, but in the past 30 years have invaded the Gulf of Mexico, Caribbean Sea, Atlantic coasts of North and South America as well as the Mediterranean Sea (Johnson and Swenarton 2016). First reports of the fish outside of its native habitat were along the east coast of southern Florida in 1985, with sporadic sightings until 2000 when the fish became increasingly documented in the region (Schofield 2009; Morris et al. 2008). They have a characteristic coloration with vertical red, brown or black stripes across their bodies and wide fans of projecting fins from their side and spines from their backs (see Introduction Section 1.5 Figure 1) (Karleskint, Turner, and Small 2009). The lionfish is a member of the Scorpaenidae family which includes many of the worlds venomous species of fish, all have spines on their dorsal area which are covered in a skin sheath and contain their venom (see Introduction Section 1.5 Figure 1, inset) (Nelson, Grande, and Wilson 2016; Betancur-R et al. 2017). Lionfish tend to live five to fifteen years; males grow to an average of 100 mm in length and females grow to an average of 180 mm in length, but can grow up to 470 mm in length in some cases (James A. Morris and Akins 2009; Ruiz-Carus et al. 2006). With a

very high annual fecundity of over two million eggs and spawning roughly every four days, they reproduce very quickly, adding fuel to the fire of their invasion (James A. Morris and Akins 2009).

Introduction Section 1.5 Figure 1: Picture of a lionfish (*Pterois volitans*), inset shows retracted sheath on venomous spine, revealing sharp cartilaginous structure underneath. (Adapted (with permissions) from an image taken by the Cape Eleuthera Institute)

Lionfish are native to the Indian Ocean and Western Pacific Ocean, in particular the subtropical and tropical regions around southern Japan and Korea, the east coast of Australia, and between Sumatra and Sri Lanka (see Introduction Section 1.5 Figure 2) (Andradi-Brown 2019). They are typically found on the seaward edge of coral reefs and rocks at depths varying from very shallow waters to 300m depth (Dahl and Patterson 2020; Gress et al. 2017). Their invasion of the Caribbean, Gulf of Mexico and Atlantic coasts of North and South America at large has been

incredibly successful and they are now considered to be the most abundant fish predators in most reef systems in these areas (Albins and Hixon 2008; Côté, Green, and Hixon 2013), reaching record densities that even exceed those in their native habitat of the Indo-Pacific (Green and Côté 2009). This success is due to a variety of factors, including highly efficient hunting strategies, an ability to tolerate a variety of different salinity levels, depths and water temperatures, high fecundity, venomous defenses and an absence of natural predators in their newly colonized environments (James A. Morris and Freshwater 2008; Norton and Norton 2021; Morris et al. 2008; Mouchlianitis et al. 2022). Lionfish feed on a broad diversity of crustaceans and reef fishes, and due to their hunting strategy, these feeding habits can result in a devastating 90% reduction of species richness and abundance on some small patches of reef (Albins and Hixon 2008; Santamaria, Locascio, and Greenan 2020; Valdez-Moreno et al. 2012). In studies examining the contents of lionfish stomachs, small lionfish have even been found in the stomachs of larger ones, suggesting that they are capable of cannibalizing their own kind in low food source situations (Santamaria, Locascio, and Greenan 2020; Valdez-Moreno et al. 2012).



Introduction Section 1.5 Figure 2: Map demonstrating the native range of *Pterois volitans* (in green) and *Pterois miles* (in blue), another species of lionfish. Red areas indicate the invasive range of the lionfish, and hatched red areas indicate predicted future distribution of the fish. (Image source, with permissions: (Schofield, P.J., J.A. Morris, Jr, J.N. Langston, and P.L. Fuller 2020))

The lionfish's hunting strategy is puzzling – they disorient their prey by partially closing their mouth and blowing a strong and directed water jet at them prior to their capture. This is a highly unusual predatory strategy in the regions it has invaded, so prey yield easily to the lionfish since they have yet to evolve effective defense strategies (Albins and Lyons 2012). Interestingly, several other teleost fish display this behaviour to catch marine invertebrate prey, but lionfish are so far the only recorded marine predator to use this technique to capture other fish (Wainwright and Turingan 1997; Frazer, Lindberg, and Stanton 1991). This hunting behaviour seems to be an evolutionary adaptation of an existing coughing mechanism that teleost fish use to expel indigestible or unpalatable items from their mouths (Wainwright and Turingan 1997).

1.5.1 Lionfish stings

As a defensive measure, lionfish have evolved a highly painful venom to deter predators from hunting them. Envenomation by a lionfish occurs following the injection of the fish's venom through a puncture wound created by one or many of the lionfish's spines (Resiere, Cerland, et al. 2016; Robertson et al. 2014; Vetrano, Lebowitz, and Marcus 2002). There are 13 dorsal, 2 pelvic and 3 anal cartilaginous spines that contain venom, each of which is covered by an integumentary sheath (Resiere, Cerland, et al. 2016; Robertson et al. 2014; Vetrano, Lebowitz, and Marcus 2002). Each spine is structured with three lateral grooves, which are thought to contain the lionfish's venom and venom gland tissue, making each spine its own venom factory, storage site and delivery system (Saenz et al. 2017).

There are three grades of lionfish envenomations: grade I occurs most frequently and is characterized by local redness and swelling, grade II involves blister or vesicle formation and grade III results in local tissue necrosis (Patel and Wells 1993; Auerbach et al. 1987). However, it is likely that the necrosis observed in grade III envenomations is not directly caused by a toxin in the venom, but rather by a local infection that may go untreated and subsequently result in necrosis (Schult et al. 2017). There have been several case studies of instances of lionfish stings on tourists and divers who characterize the pain as excruciating, with immediately intense pain extending from the wound and reaching its full potency about 1-2 hours after envenomation and maintaining this peak level for anywhere from days to weeks. Extreme cases result in hospitalization due to shock, excessive pain, necrosis or allergic reactions (Resiere, Cerland, et al. 2016; Vetrano, Lebowitz, and Marcus 2002; Saenz et al. 2017; Hobday et al. 2016; Kizer, McKinney, and Auerbach 1985; Henn et al. 2016; Haddad et al. 2015; Patel and Wells 1993). However, no study has characterized the *average* experience of a person stung by a lionfish, a question I seek to answer in the study I completed in chapter 2 of this thesis.

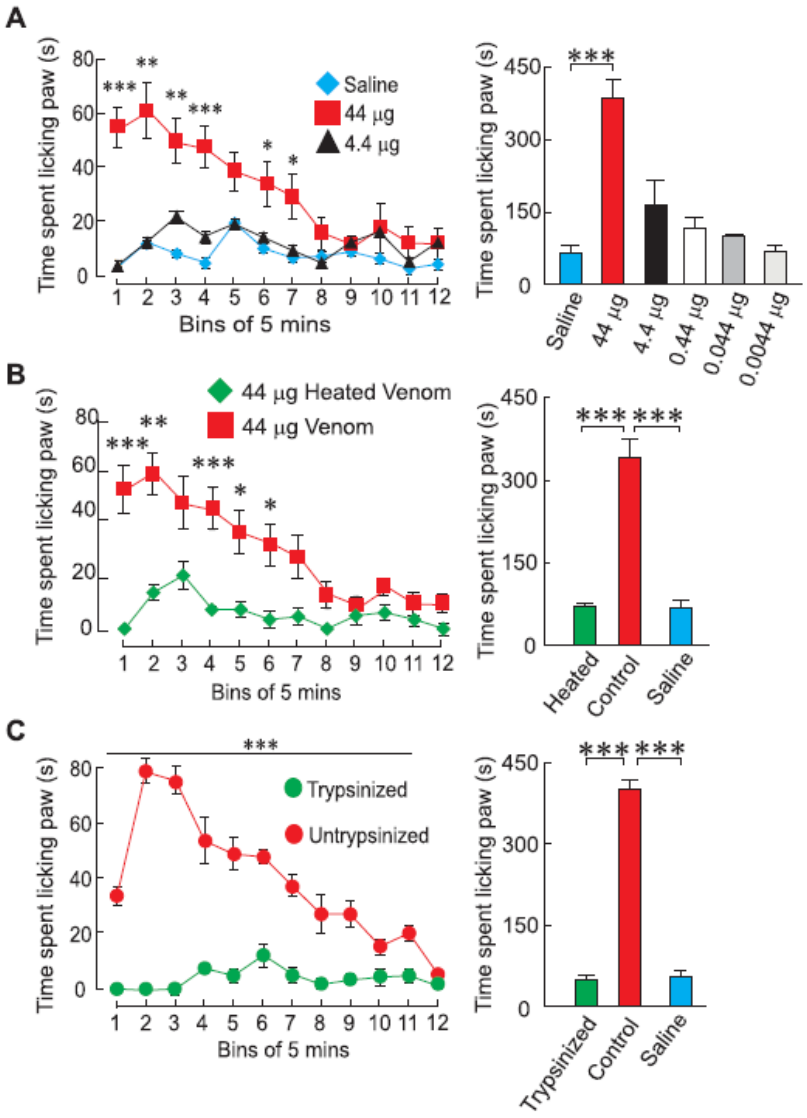
1.5.2 Lionfish venom

Prior studies of lionfish venom have shown that it contains some small proteases, a 59 kDa hyaluronidase and a 150 kDa porin-type toxin similar to one found in stonefish and scorpionfish venom (Kiriake and Shiomi 2011; Memar et al. 2016; Balasubashini et al. 2006; Kiriake, Shiomi, and Madokoro 2014; Campos et al. 2021). In biochemical analyses of the venom, it has been shown to possess low myotoxic activity and no hemorrhagic activity, but does cause an increase in vascular permeability and some thrombotic activity in mice (Saenz et al. 2017). In one particular study focused on the cardiovascular effects of lionfish venom, application of venom directly onto dissected rat hearts elicited some pumping activity which could be abolished using acetylcholine

and noradrenaline blockers, suggesting that the venom may trigger hypotension in its victims (Jarrod E. Church 2002). Other than these studies, there exists no knowledge of the toxin components of lionfish venom, and I seek to fill this large knowledge gap with the study I completed in chapter 3.

1.5.2.1 Cellular target of lionfish venom

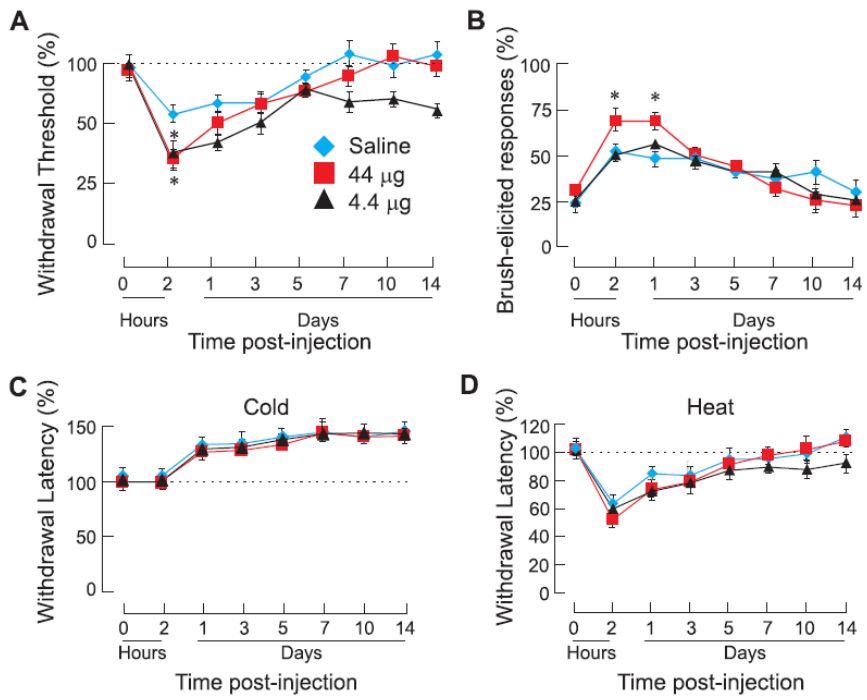
Before I began this PhD, our group completed the first study of the cellular target of lionfish venom in mice to gain insights into the algogenic (pain-causing) mechanism of action of lionfish venom. In that study, I performed a variety of pain experiments to characterize the pain and inflammation caused by lionfish venom, which I will describe in this section of my thesis introduction (Mouchbahani-Constance et al. 2018). First, I determined that 44 μg of venom protein was a dose sufficient to cause pain in mice and that both heating the venom and treating it with trypsin were effective at eliminating pain behaviours, suggesting that the main algogenic component of the venom is a (or are) heat-labile proteinaceous toxin(s) (Introduction Section 1.5 Figure 3A – C).



Introduction Section 1.5 Figure 3: Lionfish venom injection elicits sustained spontaneous pain behaviour. **(A)** Mean (± s.e.m.) duration of spontaneous pain behavior assessed for 1 hour after the intraplantar injection of 44 µg (n = 12; red symbols), 4.4 µg (n = 6; black symbols), or 0 µg (saline; n = 5; blue symbols) of venom. **Left:** Spontaneous pain behavior 1 hour immediately after injection, divided into 5-minute bins. **Right:** Sum of the time spent displaying spontaneous pain behavior in the 1 hour immediately following the injection. **(B)** Mean (± s.e.m.) duration of spontaneous pain behavior after injection with 44 µg of boiled (n = 4; red symbols) or unboiled (n = 12; blue symbols) venom. **Left:** Spontaneous pain behavior 1 hour immediately after injection, divided into 5-minute bins. **Right:** Sum of the time spent displaying spontaneous pain behavior in the 1 hour immediately following the injection. **(C)** Spontaneous pain behavior after injection

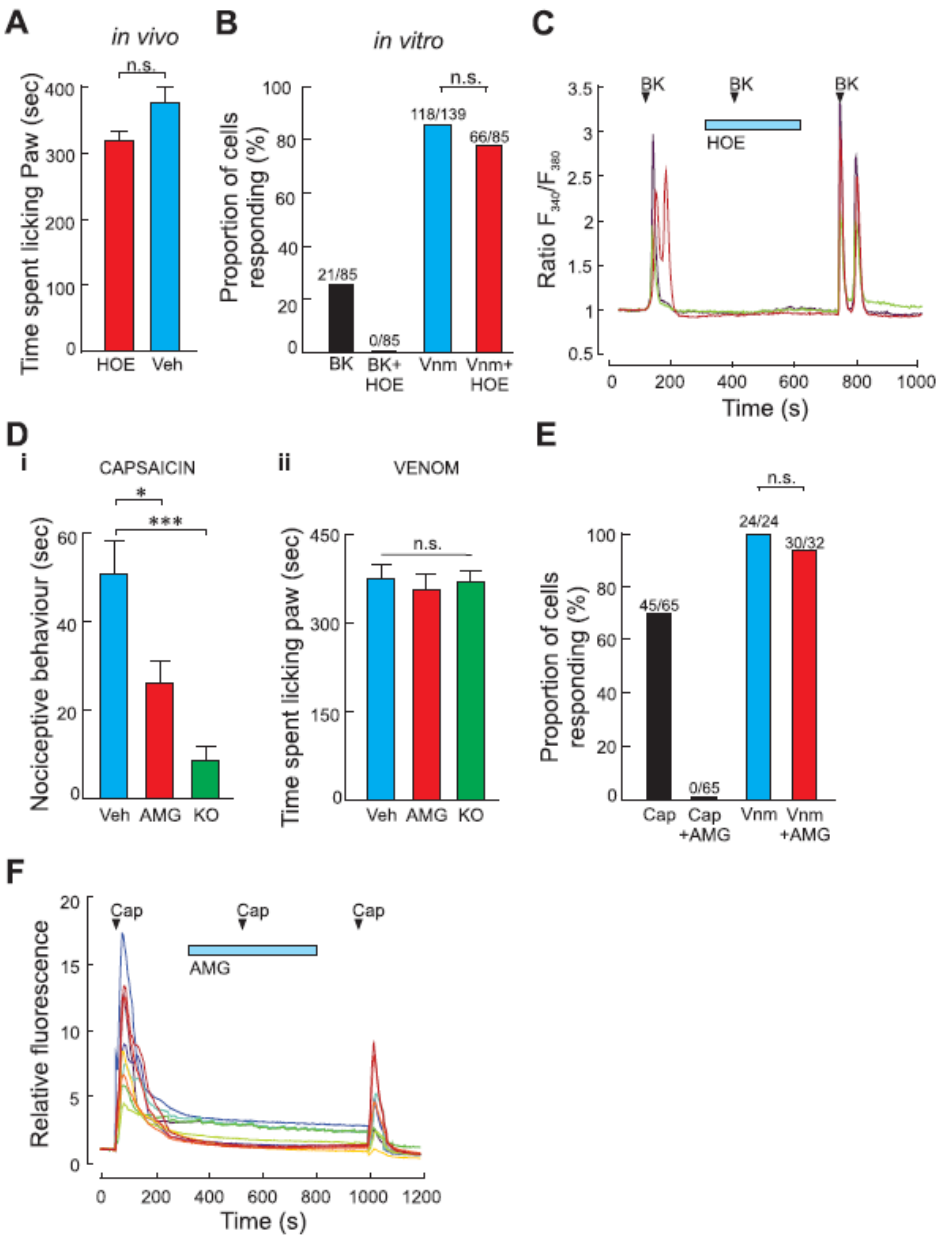
with 44 μg of trypsinized (n = 5; red symbols) or untrypsinized (n = 5; blue symbols) venom. **Left:** Spontaneous pain behavior 1 hour immediately after injection, divided into 5-minute bins. **Right:** Sum of the time spent displaying spontaneous pain behavior in the 1 hour immediately following the injection. * = significantly different from control group ($p < 0.05$), ** = ($p < 0.01$), *** = ($p < 0.001$) (two-way ANOVA followed by Tukey's post hoc test)

The next series of experiments I performed focused on the qualities of the pain elicited by lionfish venom in mice and its duration. After injecting mice with venom, I performed experiments using von Frey filaments to characterize whether the venom elicited mechanical or thermal hypersensitivities in mice. Overall, the mice developed a mechanical hypersensitivity to both static and dynamic stimuli – the hypersensitivity to static stimuli resolved itself within 1 day and the hypersensitivity to dynamic stimuli took more than 1 day but less than 3 days to resolve itself (Introduction Section 1.5 Figure 4A and B). However, I did not observe any hypersensitivity to cold or heat (Introduction Section 1.5 Figure 4C and D).



Introduction Section 1.5 Figure 4: Lionfish venom injection elicits lasting mechanical and thermal hypersensitivity (A) Static mechanical hypersensitivity, quantified in the von Frey test on saline (n = 5; blue symbols), 44 µg dose (n = 6; red symbols) or 4.4 µg dose (n = 6; black symbols) groups over a 14-day period. Values are normalized and expressed as a percentage (mean ± s.e.m.) of baseline values. (B) Mean (± s.e.m.) dynamic mechanical hypersensitivity, quantified with the dynamic touch test on saline (n = 5; blue symbols), 44 µg dose (n = 6; red symbols) or 4.4 µg dose (n = 6; black symbols) injected groups over a 14-day period. (C) Cold hypersensitivity, quantified with the cold syringe test on saline (n = 5; blue symbols), 44 µg dose (n = 6; red symbols) or 4.4 µg dose (n = 6; black symbols) injected groups over a 14-day period. Values were normalized and expressed as a percent (mean ± s.e.m.) of baseline values. (D) Heat hypersensitivity, quantified with the Hargreaves' Test on Saline (n = 5; blue symbols), 44 µg dose (n = 6; red symbols) and 4.4 µg dose (n = 6; black symbols) injected groups. Values were normalized and expressed as a percent (mean ± s.e.m.) of baseline values. * = significantly different from saline group ($p < 0.05$) (two way ANOVA followed by multiple comparisons using Holm-Sidak Method).

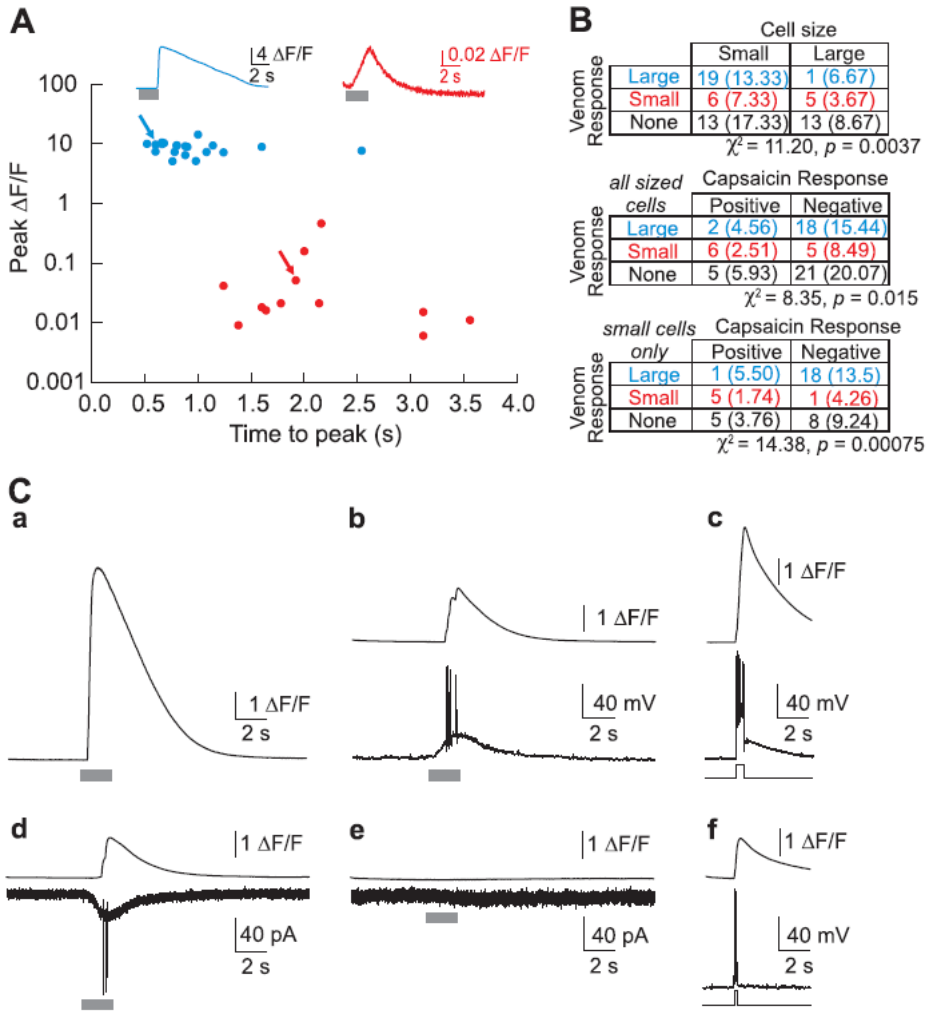
In the literature, it has been reported that lionfish venom may potentially contain bradykinin, or might activate cardiovascular cells via the BK2 receptor, and that this would likely be the mechanism through which the venom causes pain since sensory neurons express this receptor. I sought to verify this using the BK receptor antagonist HOE-140 and showed that in both *in vivo* and *in vitro* experiments, it was ineffective at blocking the pain behaviour and calcium influx elicited by lionfish venom (Introduction Section 1.5 Figure 5A-C). Since TRPV1 is a common target of pain-causing venoms (Bohlen and Julius 2012), I used the TRPV1 antagonist AMG-9810 in experiments with lionfish venom to see if it had anti-nociceptive properties, as well as TRPV1 knockout mouse (provided by the Bourque lab). Antagonism of TRPV1 was also ineffective at blocking venom-induced pain behaviour and calcium influx (Introduction Section 1.5 Figure 5D-F), showing that TRPV1 is not the target of lionfish venom in sensory neurons.



Introduction Section 1.5 Figure 5: Venom-elicited behavioral and cellular responses are independent of the TRPV1 or bradykinin receptors (A) Mean (± s.e.m.) spontaneous pain behavior quantified for a 1-hour period after intraplantar injection of 44 µg of venom in mice pretreated with a systemic (i.p.) injection of saline (blue bar, n = 5) or 0.1 µmol/kg of HOE-140 (red bar, n = 5) (**B**) Proportion of cells responding to BK alone and in the presence of HOE-140 (black bars, n = 21/85 and 0/85 respectively) and proportion of cells responding to the venom alone and in the presence of HOE-140 (blue and red bars, n = 118/139 and 66/85 respectively; Fisher's Exact Test, p = 0.939). (**C**) Sample traces of HEK293T cells (n = 3 cells) showing a rise in intracellular calcium

in response to a 1 sec puff of bradykinin (BK, 100 μM), a blocked response to a second puff of BK after bathing cells in HOE-140 (1 mM) and a response to a third puff of BK after washing the antagonist out of the dish. **(D)** Mean (± s.e.m.) spontaneous pain behavior quantified for a 1-hour period after intraplantar injection of 44 μg of venom in mice pretreated with a systemic (i.p.) injection of DMSO (blue bar, n = 5) or 30 mg/kg of AMG-9810 (red bar, n = 5; Kruskal-Wallis one way ANOVA on ranks p = 0.067 for the HOE-140 group and p = 0.841 for the AMG-9810 group) (**E**) Proportion of cells responding to capsaicin (Cap) alone and in the presence of AMG-9810 (black bars, n = 45/65 and 0/65 respectively) and proportion of cells responding to the venom alone and in the presence of AMG-9810 (blue and red bars, n = 24/24 and 30/32 respectively; Fisher's Exact Test, p = 0.322). (**F**) Sample traces of DRG neurons (n = 10 cells) showing a rise in intracellular calcium in response to a 1 sec puff of capsaicin (Cap., 1 μM), a blocked response to a second puff of capsaicin after bathing cells in AMG-9810 (10 μM) and a response to a third puff of capsaicin after washing the antagonist out of the dish.

Finally, through a collaboration with the laboratory of Dr. Steven Prescott at the University of Toronto, we determined the cellular target of the venom. Using an Advillin-Cre x GCaMP6f mouse line, whereby all sensory neurons express the genetically encoded calcium sensor GCaMP6f, lionfish venom and capsaicin were applied to primary cultures of dorsal root ganglia in calcium imaging experiments and the responses were categorized based on cell diameter and responsiveness to capsaicin. Cells that displayed a large response to lionfish venom were overwhelmingly small-diameter, capsaicin-insensitive neurons, suggesting that the venom specifically activates nonpeptidergic nociceptors (which are small-diameter and TRPV1-negative) (Introduction Section 1.5 Figure 6A-B). In electrophysiology experiments, currents coincided with spiking activity in cells, and were only observed when patching cells in a perforated patch configuration and were lost when, in the same cell, the patch was converted to whole-cell mode (Introduction Section 1.5 Figure 6C).



Introduction Section 1.5 Figure 6: Lionfish venom predominantly excites nonpeptidergic nociceptors. (A) Inset: Sample GCaMP fluorescence responses to venom application (gray bar). Peak ΔF/F and latency to peak from response onset was measured for each cell based on first venom application. Venom responses fell into three groups: large, abrupt-onset response (blue); small, gradual-onset response (red); no response (not shown). Note difference between vertical scale bars for blue and red sample responses; data point for each response is highlighted by arrow. (B) Tables show *Observed (Expected)* number of cells showing each type of venom response, subdivided according to cell size (top) and capsaicin sensitivity (middle and bottom). As expected, large cells did not respond to capsaicin, hence bottom table shows relationship between venom response and capsaicin response based only on small cells. To summarize, the cells exhibiting large venom responses were disproportionately small and TRPV1-negative. (C) Sample GCaMP response to venom before patching (a) and during current clamp recording with perforated patch

(b). Calcium signal reflects spiking. (c) Response to injected current (40 pA) to show spike-triggered calcium signal. (d) Voltage-clamp response to venom in same cell; escape spikes occurred because of incomplete voltage clamp when using perforated patch. Venom response was abruptly lost upon conversion to whole cell configuration (e) although cell continued to exhibit a large calcium response to spikes evoked by current injection (f).

Overall, this study showed that the main algogenic components of lionfish venom are heat-labile proteins, which mostly cause mechanical hypersensitivity without causing thermal hypersensitivities. Furthermore, lionfish venom does not cause pain through the BK or through TRPV1 receptors and it instead targets small diameter, TRPV1-negative nociceptors, also known as nonpeptidergic nociceptors. While this study answered many open questions about the venom, it also laid the foundation for the rationale, objectives and main questions that drove this thesis, which I will discuss in the next section.

1.6 Thesis rationale and objectives

Altogether, this thesis investigates how *P. volitans* venom causes pain. Lionfish are a highly invasive species in the Gulf of Mexico that have continued to spread across the Atlantic coasts of North and South America. With increased presence of the fish come increased stings, which are notorious for the excruciating pain that they cause and have become a badge of honour for spearfisherman who experience its pain. And yet, despite this notoriously painful sting and a budding field of venomics, no efforts had been made yet to understand the venom's components, evolution, and molecular targets in its sting victims. This knowledge gap led to the work in this thesis which was driven by the main hypothesis that lionfish venom has evolved to contain components that specifically target the pain-sensing nervous system to cause pain. Three questions underlie this thesis and are addressed in each of the 3 manuscripts in the following chapters:

1. How do humans experience the pain caused by lionfish venom and what are the sting's impacts on daily life?
2. What are the components of lionfish venom and which ones cause pain?
3. What is the receptor on nonpeptidergic nociceptors that is specifically targeted by lionfish venom to cause pain?

Chapter 2

Understanding the pain experience of lionfish envenomation

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2.1 Abstract

Introduction: Stings from the lionfish (*Pterois volitans*) constitute one of the most painful wounds in the ocean. This species has invaded the Atlantic coast of the US, Gulf of Mexico, Caribbean, and Mediterranean Sea. In addition to its ecological impact on local fish populations, stings from the lionfish pose a medical problem because of the debilitating nature of the pain they produce. However, there are no studies examining the human pain experience of lionfish stings. **Methods:** We developed a pain questionnaire that includes validated scales used with patients having acute or chronic pain to characterize the various aspects of the pain experience, investigate the contribution of factors in the pain variability, and document the use of health care resources and types of treatment. **Results:** We provide the first study of the pain experience from lionfish stings. Here, we show that the pain is intense from the start and peaks approximately 1 hour after the sting, resolving itself in 7 days for most victims. Furthermore, pain intensity can be influenced by several factors including (1) age of the victim, where older victims experience significantly higher pain intensities, (2) the number of spines involved, (3) and whether infection occurred at the injury site. However, pain intensity was not different between male and female participants. **Conclusion:** These findings will inform the medical community on the pain experience and can be used by local authorities to better appreciate the impact of lionfish envenomations to develop programs aimed at curtailing the expansion of the lionfish.

2.2 Introduction

The lionfish (*Pterois volitans*) is a venomous fish endemic to the Indo-Pacific that has, over the past 30 years, invaded the Caribbean, Gulf of Mexico, Northwestern Atlantic and Mediterranean Sea. The fish has spread at an alarming rate due to its rapid reproductive capacity and its lack of natural predators in the invaded regions (Savva et al. 2020). Lionfish tend to predate any fish

smaller than them, resulting in a steep reduction in reef biodiversity in affected areas (Savva et al. 2020; Hunt et al. 2020; Santamaria, Locascio, and Greenan 2020; Norton and Norton 2021; Valdez-Moreno et al. 2012; Bryan et al. 2018). These effects on the ecosystem have led local governments to setup spearfishing initiatives to control the spread of the lionfish. This resulted in a rapid increase in the number of individuals handling lionfish and ultimately in the number of stings (Resiere, Haro, et al. 2016). While initiatives aimed at curtailing this invasion focus on limiting ecological impact, the medical aspect of this invasion must also be appreciated. This includes the pain that is experienced by victims of lionfish stings, which can affect various aspects of daily life.

Lionfish stings occur when a victim's skin is punctured by one or more of their 18 venomous spines. These cartilaginous spines are coated in an integumentary sheath, underneath which is the venom gland tissue. Once skin is punctured by a spine, the sheath is torn and venom diffuses into the victim (Morris et al. 2008; Resiere, Haro, et al. 2016; Vetrano, Lebowitz, and Marcus 2002). Lionfish stings produce painful sensations and result in swelling, redness, and many other unpleasant symptoms (Schult et al. 2017; Kizer, McKinney, and Auerbach 1985; Henn et al. 2016; Aldred, Erickson, and Lipscomb 1996; Patel and Wells 1993; Resiere, Haro, et al. 2016). However, there is no data regarding the venom's toxin composition, and little understanding of the venom's molecular mechanism of action. A prior preclinical study from our group characterized the pain following intraplantar injections of lionfish venom in mice, as well as a cellular characterization of the venom's activity on nociceptors (Mouchbahani-Constance et al. 2018). Although that study helped understand lionfish sting pain, the pain experienced by humans following lionfish stings remains poorly documented and understood. There exist numerous case studies of extreme envenomations (Schult et al. 2017; Kizer, McKinney, and Auerbach 1985; Henn et al. 2016;

Aldred, Erickson, and Lipscomb 1996; Patel and Wells 1993; Resiere, Haro, et al. 2016), but since the average lionfish sting does not usually result in hospitalization, there are no characterizations of the average human pain. Additionally, case studies mostly place a focus on the swelling, redness and non-pain symptoms of lionfish envenomations. Yet, pain is the cardinal symptom reported from lionfish stings.

There is an overall lack of pain-focused questionnaires for victims of stings from any species, and those that have been done did not take advantage of well validated scales, thus lacking standardization and making results hard to compare (Ward-Smith et al. 2020). We sought to solve this problem by assembling a pain questionnaire designed for lionfish sting victims, including validated scales/questionnaires employed with patients having acute or chronic pain from a variety of different underlying sources. The objectives of the present study were 1) to characterize various aspects of the pain experience by lionfish sting victims including its severity, qualities, and impact on various aspects of daily life, 2) to investigate contributions of various factors in the variability of pain experience including factors surrounding the sting itself, and 3) document the use of health care resources and treatments used to control the pain associated with lionfish sting.

2.3 Methods

2.3.1 Study design and setting

We assembled a questionnaire to investigate the pain caused by lionfish envenomations as well as to quantify and analyze factors surrounding the envenomations that may or may not impact pain and discomfort. This questionnaire was composed of 44 questions and hosted at the URL www.lionfishpain.org. The questionnaire was created using the survey platform Typeform and integrated into the hosting URL. To prevent duplicate submissions, responders were asked if they had completed the questionnaire before, and if they responded "Yes", their second submission was

not considered in our analysis. Furthermore, duplicate email addresses, which were voluntarily submitted by most participants, were flagged and second submissions from these individuals were not considered in our analysis.

The protocol was approved by McGill University's Faculty of Medicine and Health Sciences Institutional Review Board on February 3, 2021 (A02-M09-21B) and was conducted in conformity with the published guidelines of the Tri-Council Policy Statement 2, in compliance with the *Plan d'action ministériel en éthique de la recherche et en intégrité scientifique* (MSSS, 1998), and the Food and Drugs Act (17 June 2001), and acts in accordance with the U.S. Code of Federal Regulations that govern research on human subjects (FWA 00004545). Participants were automatically assigned a token ID number by Typeform and once data was exported, nominal data and token ID numbers were separated from the main file and kept in a separate password-protected Excel file. From that point onwards, only the token ID numbers were associated with participant data, and all nominal data was removed from the main analysis file.

Participant recruitment

The questionnaire was advertised by email and distributed through a variety of marine biologists that study the lionfish, as well as on social media accounts via lionfish hunters and within their communities. The questionnaire was also advertised at a variety of lionfish hunting events across Florida. Cards with the website URL and information on the questionnaire were distributed at a variety of lionfish hunting events, and through lionfish hunting communities to people who were interested.

2.3.2 Procedures

Using targeted questions, adapted from the standardized NIH PROMIS questionnaire (for pain intensity) (Deyo et al. 2014; Cella et al. 2010), the short-form DN4 questionnaire (for pain characteristics) (Pagé et al. 2020; Bouhassira et al. 2005) and some context-specific questions, we sought to gain insights into the characteristics of the sting sites, intensity of the victims' pain and the qualities of their pain. Furthermore, we assessed the interference of the pain on daily activities using the Brief Pain Inventory. Overall, respondents were asked to quantify pain intensity over 5 different time points, qualities of their pain, how pain interfered in their normal lives as well as a variety of different factors that we hypothesized may affect the pain caused by a lionfish sting. The questionnaire was made public online on February 23, 2021 and was available up to February 2022. We received 605 submissions within 1 year (analyses for this study were performed using data collected up until February 2022). Exclusion criteria for responses included long delayed between the sting event and completing the questionnaire (over 10 years), very incomplete filling of the questionnaire (over 1/3 of the questionnaire left unanswered), third-party reports of lionfish stings and duplicate reports of lionfish stings from the same individual. Our host URL, lionfishpain.org received 901 unique visitors between February 23, 2021 and February 25, 2022, 652 of these individuals were located in the USA, 65 in Canada, 35 in the United Kingdom, 32 in Bonaire and the rest were spread across the world (48 other countries).

Using the short-form DN4 questionnaire, we asked participants to report whether their pain matched any of the following qualities to gain insights into whether the pain caused by a lionfish sting had any neuropathic qualities. For each participant, a "yes" to each of these qualities was scored as a value of 1, and their DN4 score was tabulated based on counting how many of these qualities each patient reported, for which the maximum score was a 7.

We asked participants to complete a series of pain interference questions using a modified version of the Brief Pain Inventory (BPI) Pain Interference Scales 10 (Deyo et al. 2014; Cleeland and Ryan 1994) and indicate a score from 0 to 10 for how much each of the named activities were affected due to the pain they experienced following a lionfish sting. Items that were considered for this score included general activity, mood, walking ability, normal work, sleep, enjoyment of life and social activities. A global pain interference score was calculated for each patient by calculating the sum of their scores in each category, with a maximum possible score of 70.

To characterize the time course of the pain experienced by sting victims, we asked participants to score the intensity of their pain on the NIH PROMIS Short Form Pain Intensity Questionnaire on a scale of 0 to 10, where 0 indicated no pain, and 10 worst imaginable pain at 5 different timepoints: immediately after the sting, then 1, 2, 3, 24 hours, and 1 week after the sting.

2.3.3 Statistical analysis

Data was exported from Typeform and saved as a .csv file to reformat the data for analysis. All analyses were performed using Python. Descriptive statistics were used to depict the participants' demographics, the characteristics of their pain experience and the types of pain management modalities they used. Mean, median, standard deviation and interquartile range (IQR) values were computed for continuous variables, while percentages and frequencies were computed for categorical variables. The evolution of pain over time was analysed using a mixed model for repeated measurements (MMRM), considering participants' sex and age, history of allergies, fish status (live or dead), number of spines involved in the sting and whether the sting ultimately resulted in an infection. The type of variance–covariance matrix was compound symmetry. An analysis of covariance (ANCOVA) was performed to identify factors statistically associated with

pain at each time of measurement. MMRM and ANCOVA analyses were performed using SAS software, version 9.4 (SAS Institute Inc. Cary, NC, United States).

2.4 Results

2.4.1 Characteristics of the responders

In the 1-year period during which we collected responses to our survey, we received 605 submissions, 50 of which we had to remove due to a variety of exclusion factors (see Methods). Table 1 and Figure 1 show the demographic characteristics of the responders (N = 555). Close to 80% were males with a mean age of 46.71 years old (SD = 13.23) (Table 1 and Figure 1A-B). The median time elapsed between the sting and the completion of the questionnaire was 336 days (interquartile range: 580 days).

2.4.2 Circumstances surrounding the lionfish stings

As shown in Table 2, the most common activities performed by participants at the time of their lionfish sting were spearfishing (72.07% of participants, Figure 1C blue bar and Table 2) or while diving or snorkeling (14.95% of participants, Figure 1C purple bar and Table 2). Furthermore, the most stung body part was the hand/arm (90.63% of participants, see Table 2), consistent with common lionfish sting injuries stemming from spearfishing or diving/snorkeling. Most of the questionnaire participants were in the United States at the time of their sting (67.03% of participants, see Table 2) with the majority of the others distributed across the Caribbean, consistent with the fact that lionfish are invasive in the Gulf of Mexico and Caribbean.

For the 85.95% of participants, the lionfish was alive at the time of their sting (see Table 2) and for 86.67% of participants, the sting took place underwater (see Table 2). Finally, we found that

22.16% of participants had a history of allergies and only 3.24% of participants experienced an infection at the site of their sting in the days and weeks following the sting (see Table 2).

2.4.3 Sting characteristics and pain qualities

As shown in Table 3, all participants experienced pain after the sting. Three quarters of the responders (76.22%) reported that their pain was continuous while it was an intermittent type of pain in the other cases. Other sting characteristics included reported soreness (69.37% of the participants), redness (68.83% of the participants), swelling (85.59%), bruising (11.71%), blistering (11.53%), paleness (7.75%), and necrosis (4.68%).

Table 3 shows the results obtained on each item of the DN4 Questionnaire. The most common reported sensations were burning sensation (55.68%), tingling (46.49%) and numbness (45.95%) (Table 3). The mean DN4 score was 2.36 (± 1.61) out of a maximum possible score of 7 (Table 3).

Less common symptom experienced (and their abundances) were nausea (80/555 or 14.41% of participants), sweating (93/555 or 16.76%), trouble sleeping (54/555 or 9.73%), accelerated heart rate (74/555 or 13.33%), shock (18/555 or 3.24%) and fainting (10/555 or 1.80%) (see table 3).

2.4.4 Pain intensity

The mean (±SD) pain rating immediately after the sting was 4.97 (± 2.40) and peaked to 7.25 (± 2.69) at 1 hour after the sting (Table 3) This number dropped to 6.10 (± 3.01), 2 hours after the sting, and was still present the following day, at 2.87 (± 2.49). It eventually dissipated a week later, reaching 1.05 (± 8.99).

2.4.5 Pain interference on daily living

We sought to evaluate how the pain experience from a lionfish sting can impact an individual's normal activities. Table 4 shows the extent to which lionfish sting pain affected various aspects of daily living. In the BPI Pain Interference scales, scores at or above 4/10 indicate moderate to severe interference. In our study, most scores were lower than 4/10, with the highest scores being general activity (3.63 ± 2.49) and normal work (3.52 ± 3.09). Other categories for which responders reported low interference included self-care (2.18 ± 2.93), recreational activities (2.97 ± 2.95), mood (2.60 ± 2.33), walking ability (0.96 ± 2.31), sleep (2.55 ± 2.96), enjoyment of life (2.25 ± 2.91) and social activities (1.89 ± 2.99) (see Table 4). More than two thirds of the participants (69.37%) reported having needed to take time off from work due to their lionfish sting, reporting on average 17.98 (± 17.33) hours of work missed.

2.4.6 Past history of stings

As shown in Table 5, just under half of the participants (41.98%) had prior experiences with lionfish stings. The characteristics of the stings were similar to the one depicted for the most recent one. Almost half of the responders (47.64%) who had been stung before indicated that the pain was less intense than previous stings, while 39.48% (92/233 participants) reported that the pain was the same (see Table 5). Only 13.3% indicated that their pain was more intense than that from past lionfish stings,

2.4.7 Factors associated with pain intensity

Figure 2 and Table 6 respectively show the results of the mixed model with repeated measures (MMRM) analysis and the ANCOVAs using the GLM procedure used to identify factors influencing variability in pain intensity following lionfish stings. We observed no significant sex differences in pain from lionfish stings (see Figure 2A, Table 6 and supplemental table 1) The fact

that the lionfish was alive or dead at the time of the sting did have a significant impact on pain at specific time points. While we did not find that the fish being alive or dead had a global impact on reported pain in the MMRM analysis (although the result was close to statistical significance with $p = 0.0595$), the results of the ANCOVAs revealed that the variable did have significant influence on pain at specific time points. Immediately after the sting, there was no significant difference in pain between dead or alive fish groups, but there was a significant difference 1 and 2 hours after the sting ($p = 0.0304$ and 0.0193 respectively, ANCOVA, Figure 2B, Table 6 and supplemental table 1). Age was a variable that caused significant variation in pain experienced by sting victims, playing the largest role at 1 and 2 hours after the sting (1 hour later: $p = 0.0002$ and 2 hours later: $p = 0.0298$, repeated ANCOVAs with GLM procedure, see Table 6 and Supplemental table 1). On average, older participants reported more intense pain than younger ones with the 50-59-, 60-69- and 70–79-year-old groups reporting more pain overall than the under 30 group ($p = 0.0175$, 0.0019 and 0.0038 respectively in the MMRM analysis, see Supplemental table 1 and Figure 2C). The sting victims' history of allergies had no effect on pain at any time point (see Figure 2D, table 6 and supplemental table 1).

The number of spines from the lionfish that punctured the victim had a significant effect on the experience of pain. Overall, individuals stung by 3 or more spines experienced significantly more pain than those stung by one spine in our MMRM analysis ($p = 0.0009$, MMRM, see Figure 2E). There was also a significant difference between the 1 spine and the 3+ spines groups at all time points except for immediately after the sting ($p = 0.005$; 0.002; <0.0001; and 0.003 at 1; 2; 24 hours and 1 week after the sting, respectively. ANCOVA analyses, see Table 6 and Supplemental Table 1).

Infection was a variable that was found to be globally statistically significant in our MMRM model ($p = 0.0102$, MMRM, see Figure 2F) and was also statistically significant at all time points except for immediately after the sting ($p = 0.033$; 0.007; 0.007; and 0.004 for 1; 2; 24 hours; and 1 week after the sting, ANCOVA, see Table 6 and supplemental table 1).

Other factors that were statistically significant for certain time points in our ANCOVA model, but not in our MMRM model, included whether it was the first time that the individual had been stung and the delay between the sting and completion of the questionnaire. Prior experience with a lionfish sting proved to be a statistically significant variable 2 hours, 1 day, and 1 week after the sting ($p = 0.030$; 0.036; and 0.035, respectively. ANCOVA model, see table 6 and supplemental table 1). For these time points, prior experience with a lionfish sting tended to cause a reduction in the reported pain by the victim, whereas first-time sting victims tended to report significantly more pain. Of all the participants, 26.14% sought medical care or advice from a physician, nurse, or pharmacist (data not shown). Interestingly, individuals who needed to take time off from work because of the pain did not report significantly more pain than those who did not need time off (Table 6 and supplemental table 1).

2.5 Discussion

In this study, we provide the first large-scale study of the pain experience from lionfish stings. We assembled a questionnaire which can be modified to be used for a wide variety of other envenomations including by other fish (ex. Stonefish, scorpionfish), snakes, insects etc. to gain a wider understanding of the immediate pain and symptoms, as well as the impact of stings on the individual's life and work potential. We surveyed 555 individuals who have been stung by lionfish and obtained detailed information about the circumstances surrounding their sting, the conditions of their sting, the pain they experienced and the interference the sting had on their personal lives.

By far, the most common activity that study participants were engaged in when they were stung by lionfish was spearfishing. This is not surprising considering that spearfishing requires a hunter to get close to the fish, and to manipulate the fish with their hands to either remove them from their spear or empty their containment unit. Alarmingly, the invasion of the lionfish shows no sign of reversing soon (Norton and Norton 2021; Cortés-Useche et al. 2021), resulting in an increase in the number of individuals stung by lionfish in the future. It is therefore important to gain a better understanding of the consequences of lionfish envenomations, both from a physiological point of view and from a life-interference point of view.

Since anecdotal evidence has pointed to the fact that stings from dead lionfish produce less pain than from live ones, we asked participants to indicate whether the lionfish was alive at the time of their sting. In our ANCOVA model (Figure 2B), we show that indeed live lionfish produce stings that produce more long-lasting pain than dead fish. This would confirm our group's prior findings that the algogenic toxin in the venom may degrade quickly, suggesting it is proteinaceous in nature, since this algogenic factor seems to degrade rather quickly after the fish's death (Mouchbahani-Constance et al. 2018).

The most reported side-effects of lionfish stings are pain, swelling and redness with some experiencing paleness, bruising, blistering and in extreme cases necrosis - we sought to evaluate the frequency of these symptoms among the responders of our questionnaire. Indeed, pain, soreness, redness and swelling were experienced by the majority of our participants, with paleness, bruising, blistering and necrosis being experienced by less than 15% of our participants. This finding confirms anecdotal evidence presented in the literature based on case studies of lionfish stings (Vetrano, Lebowitz, and Marcus 2002; Schult et al. 2017; Kizer, McKinney, and Auerbach 1985; Henn et al. 2016).

The exact time course of the pain caused by lionfish stings varies immensely, with some experiencing pain for a matter of minutes and others experiencing pain for weeks. Using the NIH PROMIS Short Form Pain Intensity Questionnaire, we characterized the time course of the pain experienced by sting victims. Our results showed that lionfish stings produce a moderate to significant amount of pain immediately after the sting, increasing until it reaches its peak at approximately 1 hour after the sting and reducing for the days following, healing completely within a week for most sting victims (Figure 2). This pain varied based on the age of the victim, the number of spines they were stung by, whether the fish was alive or dead and whether the site of the sting ultimately got infected. Importantly, however, the pain reported by victims did not vary based on gender (Figure 2 and Table 6).

Since a prior study by our group indicated that lionfish venom activates non-peptidergic nociceptors to cause pain, we sought to characterize whether the pain matched symptoms associated with neuropathic pain (Mouchbahani-Constance et al. 2018). To evaluate this, we used the self-reported portion of the DN4 questionnaire. Since the traditional cut-off for patients to be considered to be 3 (Pagé et al. 2020; Bouhassira et al. 2005), we concluded that the pain caused by lionfish venom in this case did not match the qualities of traditional neuropathic pain. This was somewhat expected, since venoms are cocktails of molecules that often contain components which, in addition to components that activate nociceptors directly, trigger inflammatory reactions to amplify pain and discomfort in sting victims (Siemens et al. 2006; Brinkman et al. 2015; Campos et al. 2021; Aranda-Souza et al. 2019).

Due to the intensity of the pain caused by lionfish stings and the fact that they tend to occur on hands, we hypothesized that this would lead to a highly disruptive experience for sting victims. We sought to quantify the degree of this disruption using the BPI Interference item. The maximum

possible score for this item is 70, and is quantified from 0-10 in 7 different categories: general activity, normal work, mood, walking ability, sleep, enjoyment of life and social activities. The mean total interference score in our study was 17.40/70 (± 15.91) (Table 4). For comparison, the mean interference core associated with osteoarthritis pain is about 67 (Williams, Smith, and Fehnel 2006). These results would suggest that while lionfish stings do not completely interfere or alter one's normal activities, a lionfish sting does pose a nuisance to its victims. Additionally, more than two thirds of the participants (69.37%) reported having needed to take time off from work due to their lionfish sting, reporting on average 17.98 (± 17.33) hours of work missed. This would suggest that while lionfish stings pose a nuisance to everyday life, they also cause an average of approximately 2 missed work days.

To determine whether pain from lionfish stings decreases as an individual gets stung more and more often, we asked participants to characterize whether the pain from the latest sting was less, the same or more intense than the pain they experienced with past stings. In our ANCOVA model, we found that prior experience with a lionfish sting resulted in less pain for sting victims at the 2-hour timepoint and beyond. Thus, it seems that either prior experience with lionfish stings sets better expectations vis-à-vis the outcomes, producing less fear in sting victims and ultimately resulting in less pain, or perhaps there exists some habituation mechanism which can be built by multiple exposures to lionfish venom.

Overall, pain-related questionnaires are very uncommon for victims of stings, and those that exist lack the line of questioning seen in comparable pain questionnaires, ultimately making it hard to compare results with existing pain data. The line of questioning followed in our study can be generalized to virtually any sting experience with some adaptation and would surely be beneficial

for gaining insights into pain caused by stings and envenomations as well as the surrounding consequences of suffering from stings.

Government authorities are becoming increasingly aware of the damages caused by the invasion of the lionfish, especially on the local fish industries. Consequently, several initiatives have been set in place to help counter the impact of this invasion. Although it is known that individuals stung by the lionfish spines experience extreme pain, there remained several unknowns about the nature of the pain, and how it impacted the daily lives of the victims. In this study, we designed a clinical pain questionnaire that addressed these unknowns and have uncovered the important details on pain experience of individuals stung by lionfish, the risk factors that result in increased pain, and the extend of the interference of this pain on daily activities and work disability. These data will not only inform the general community to the risks associated with diving in lionfish-infested waters, but also in informing the medical community on the pain experience caused by a lionfish sting. Finally, these findings can also be used by local authorities to better appreciate the medical impact of the lionfish sting to further invest or develop new programs aimed at curtailing the expansion of the lionfish.

A limitation of our study is that we performed a self-reported retroactive pain questionnaire, consequently participants may not have an exact memory of the pain they experienced at the time of their sting. Furthermore, the distribution of our questionnaire (throughout groups we knew had experience with lionfish stings) may have biased the demographics of our questionnaire to individuals in the southern states of the United States. Finally, the nature of the responses we received in this questionnaire were self-reported, thus potentially increasing overall variability between questionnaire participants or slightly overestimating reported pain (Boring et al. 2022; Robinson et al. 1997).

In conclusion, we have assembled the first large-scale study of lionfish stings and created an easy to adapt line of questioning which could be adapted for other stings in the future. Data from our questionnaire has shown that lionfish sting victims experience the peak of their pain approximately 1 hour after the sting, and that most of the pain resolves itself around 7 days post-sting. A variety of factors influence the intensity of the pain a victim will experience after being stung including age, whether the fish was alive, the number of spines involved in the sting, infection and prior experience with a lionfish sting. Taking all of these into account, we have provided novel insights into the nature of the pain, as well as which groups are likely to suffer more intensely from a lionfish sting and which factors correlate with elevated pain in sting victims.

2.6 Figures and figure legends

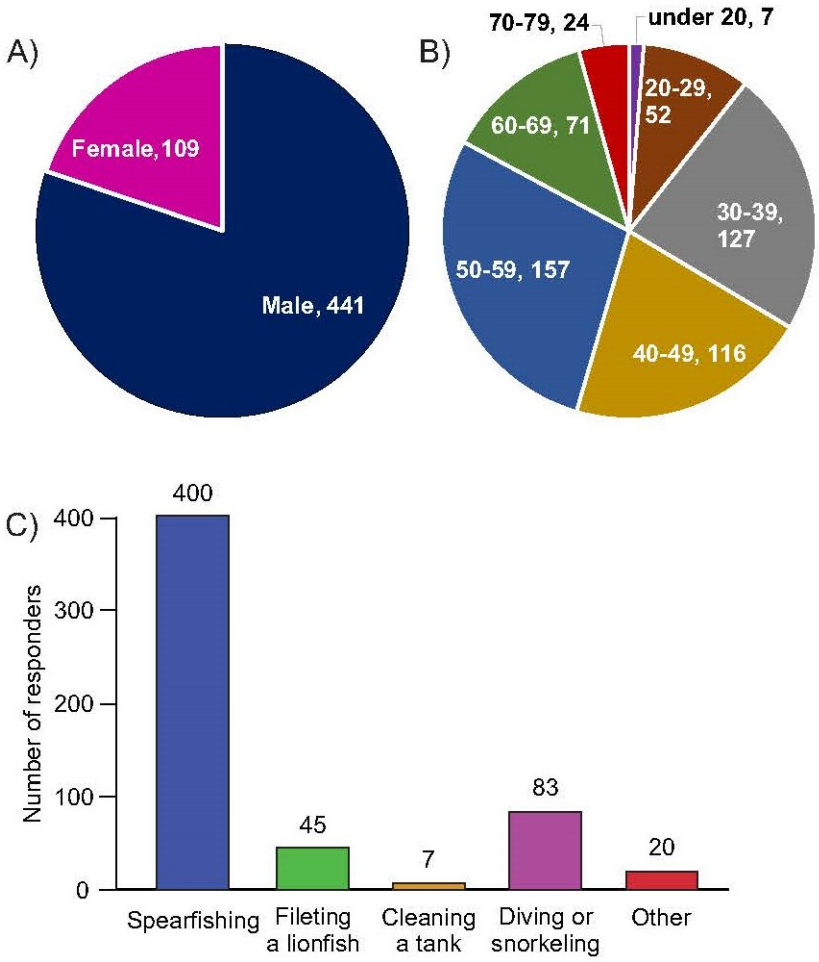

Figure 1 – Description of sting survey participants' sex, age and activity they were doing at the time of their lionfish sting. A) Pie chart depicting distribution of male vs. female participants. Number following comma indicates number of participants. B) Pie chart depicting age distribution of participants. Number following comma indicates number of participants. C) Bar chart demonstrating different activities performed by participants at the time of their lionfish sting.

Table 1.

Age	Under 20	7 (1.26%)
	20-29	52 (9.37%)
	30-39	127 (22.88%)
	40-49	116 (20.90%)
	50-59	157 (28.29%)
	60-69	71 (12.79%)
	70-79	24 (4.32%)
	Mean (SD)	46.71 (13.23)
Sex	Male	441 (79.46%)
	Female	109 (19.64%)
Race	White	509 (91.72%)
	Hispanic or Latino	26 (4.68%)
	Black or African American	4 (0.72%)
	Asian/Pacific Islander	4 (0.72%)
	Other	11 (1.98%)
Time since sting (days)	Median	336
	Mean (SD)	1854.48 (7031.49)
	Interquartile range	580

Table 1 – Demographic data describing lionfish pain questionnaire participants.

Survey responses regarding responders' age, sex and race as well as the delay in days between the sting and responding to the questionnaire. Data are expressed as counts (N) with percentage distributions indicated in brackets or means with standard deviations in brackets (SD), as indicated.

Table 2.

Body part	Hand/arm	503 (90.63%)
	Foot/leg	46 (8.29%)
	Torso	4 (0.72%)
	Face/head	2 (0.36%)
Number of spines involved in sting	1 spine	340 (61.26%)
	2 spines	128 (23.06%)
	3+ spines	81 (14.59%)
	Do not remember	5 (0.90%)
Activity at the time of the sting	Spearfishing	400 (72.07%)
	Diving or snorkeling	83 (14.95%)
	Fileting a lionfish	45 (8.11%)
	Cleaning fish tank	7 (1.26%)
	Other	20 (3.60%)
Country	United States	372 (67.03%)
	Bonaire	32 (5.77%)
	Honduras	29 (5.23%)
	Belize	18 (3.24%)
	Bahamas	16 (2.88%)
	Aruba	11 (1.98%)
	Cayman Islands	7 (1.26%)
	Curacao	7 (1.26%)
	US Virgin Islands	6 (1.08%)
	Mexico	6 (1.08%)
	Nicaragua	5 (0.90%)
	Dominica	5 (0.90%)
	Other	41 (7.39%)
	Stung underwater	481 (86.67%)
	Stung by a live lionfish	477 (85.95%)
	History of allergies	123 (22.16%)
	Stung area got infected	18 (3.24%)

Table 2 – General data describing context of reported lionfish stings.

Survey responses regarding context of lionfish stings, including the body part stung, the number of spines involved in the sting, the activity the individual was performing at the time of the sting and the country the sting took place in. Bottom 4 rows represent further details providing context to the sting including whether the sting took place underwater and by a live lionfish, whether the victim has a history of allergies and whether the stung area ultimately got infected during their recovery from the lionfish sting. Data are expressed as counts with percentage distributions indicated in brackets.

Table 3.

Characteristics of Stung Area N (%)	Redness	382 (68.83%)
	Paleness	43 (7.75%)
	Swelling	475 (85.59%)
	Bruising	65 (11.71%)
	Blistering	64 (11.53%)
	Necrosis	26 (4.68%)
Pain Intensity (Score/10) Mean (SD)	Immediately after the sting	4.973 (2.40)
	1 hour after the sting	7.25 (2.69)
	2 hours after the sting	6.10 (3.01)
	1 day after the sting	2.87 (2.49)
	1 week after the sting	1.05 (8.99)
Pain Qualities N (%)	Soreness	385 (69.37%)
	Burning	309 (55.68%)
	Cold	24 (4.32%)
	Electric shocks	54 (9.73%)
	Tingling	258 (46.49%)
	Pins and needles	198 (35.68%)
	Itchiness	211 (38.02%)
	Numbness	255 (45.95%)
	DN4 Score (Mean (SD))	2.36 (1.61)
Continuous or Intermittent Pain N (%)	Continuous	423 (76.22%)
	Intermittent	132 (23.78%(
Other Symptoms N (%)	Nausea	80 (14.41%)
	Sweating	93 (16.76%)
	Trouble sleeping	54 (9.73%)
	Accelerated heart rate	74 (13.33%)
	Shock	18 (3.24%)
	Fainting	10 (1.80%)

Table 3 – Data describing details of sting appearance, pain intensity and pain qualities.

Top: Participants self-reported visual features of their sting. Data are expressed as counts with percentage distributions indicated in brackets. Second: Participants self-reported pain intensity information in an adapted PROMIS-questionnaire format. They were required to report pain from 1-10 at the different timepoints. Data is presented as Mean (SEM). Third: Participants self-reported different qualities of their lionfish sting-induced pain based on the DN4 questionnaire, to tabulate a DN4 score. They also indicated whether their pain was continuous or intermittent. Bottom: Participants self-reported any other symptoms which have previously been reported with lionfish stings.

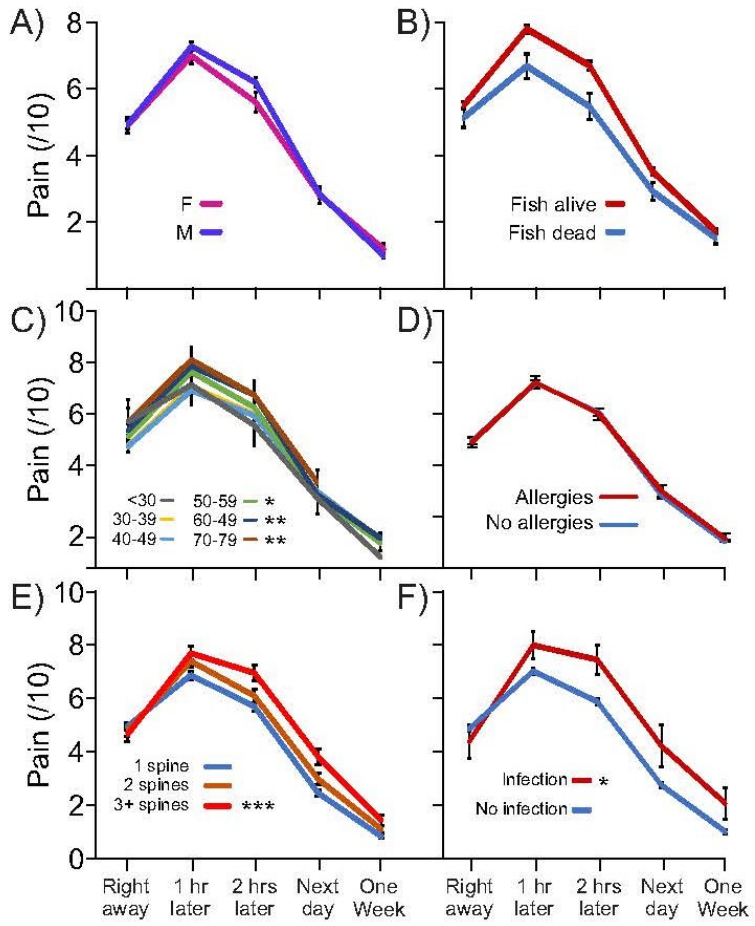

Figure 2 – Self-reported lionfish pain over the course of 1 week after a sting. Mean (±SD) self-reported pain on 10 immediately after a lionfish sting, 1 hour later, 2 hours later, one day later and one week later. A) Male (441 individuals) vs. female (109 individuals) participants B) Participants' pain divided based on whether the fish that stung them was alive (477 individuals) or dead (78 individuals) C) Participants divided based on age range (under 30: 60 individuals, 30-39: 127 individuals, 40-49: 116 individuals, 50-59: 157 individuals, 60-69: 71 individuals, 70-79: 24 individuals. All statistical comparisons are relative to Under 30 group, * $p < 0.05$, ** $p < 0.01$. D) Participants who have allergies (123 individuals) vs. do not have allergies (432 individuals). E) Participants' pain divided based on how many spines punctured the skin during the sting event (1 spine: 340 individuals, 2 spines: 128 individuals, 3+ spines: 81 individuals, unknown number of spines (not shown): 5 individuals). * (1 spine vs. 3 spines) $p < 0.001$. F) Participants' pain divided based on whether their sting ultimately got infected (infected: 18 individuals, not infected: 537 individuals), * $p < 0.05$. Mixed Model for Repeated Measures analysis for all panels

Time point	Variable	F value	P value	Time point	Variable	F value	P value
	Age	1.76	0.1191		Age	0.6	0.6986
	Sex	0.01	0.9111		Sex	0.01	0.9334
	Allergies	0	0.9626		Allergies	0.97	0.3243
	Infection	0.44	0.5082		Infection	7.34	**0.007**
Pain immediately	Delay since sting	3.23	**0.0221**	**Pain next day**	Delay since sting	0.62	0.605
	Number of spines	0.22	0.8054		Number of spines	10.74	**<.0001**
	Fish alive	1.23	0.267		Fish alive	1.58	0.2088
	1st time stung	2.63	0.1056		1st time stung	4.41	**0.0362**
	Time off of work	0.26	0.6131		Time off of work	0.49	0.4827
	Age	5.03	**0.0002**		Age	0.6	0.7032
	Sex	0.01	0.9237		Sex	1.54	0.2151
	Allergies	0.26	0.6093		Allergies	0.35	0.5568
	Infection	4.54	**0.0335**		Infection	8.36	**0.004**
Pain 1 hour later	Delay since sting	12.39	**<.0001**	**Pain next week**	Delay since sting	0.11	0.9559
	Number of spines	5.36	**0.005**		Number of spines	5.8	**0.0032**
	Fish alive	4.71	**0.0304**		Fish alive	0.52	0.4705
	1st time stung	2.7	0.1007		1st time stung	4.46	**0.0352**
	Time off of work	0.01	0.9339		Time off of work	2.01	0.157
	Age	2.5	**0.0298**				
	Sex	1.53	0.2161				
	Allergies	0.01	0.9088				
	Infection	7.32	**0.007**				
Pain 2 hours later	Delay since sting	4.84	**0.0025**				
	Number of spines	6.17	**0.0022**				
	Fish alive	5.51	**0.0193**				
	1st time stung	4.72	**0.0302**				
	Time off of work	0	0.9528				

Table 4 – Self-reported life interference details (score on 10) caused by pain and discomfort caused by lionfish stings as well as participants' time lost from work (if needed) due to lionfish sting. Data are reported as Mean (±SD) of the reported score on 10.

Altered activities (Score/10) Mean (SD)	Self-care	2.18 (2.93)
	Recreational activities	2.97 (2.95)
	General activity	3.63 (2.49)
	Mood	2.60 (2.33)
	Walking ability	0.96 (2.31)
	Normal work	3.52 (3.09)
	Sleep	2.55 (2.96)
	Enjoyment of life	2.25 (2.91)
	Social Activities	1.89 (2.99)
	Pain Interference Score	17.40 (15.91)
	Missed work (N (%))	385 (69.37%)

Table 5 - Details of past stings reported by participants who had been stung by lionfish prior to the sting reported in first part of questionnaire. Data are reported as counts with percentage distributions indicated in brackets.

	Stung in the past (N (%))	233 (41.98%)
Stung body part N (%)	Hand/arm	213 (91.42%)
	Foot/leg	17 (7.30%)
	Face/head	1 (0.43%)
Activity when sting occurred N (%)	Spearfishing	180 (77.25%)
	Diving or snorkeling	24 (10.30%)
	Fileting a lionfish	18 (7.73%)
	Other	11 (4.72%)
Number of spines involved N (%)	1 spine	174 (74.67%)
	2 spines	23 (9.87%)
	3+ spines	25 (10.73%)
Pain compared to previous sting N (%)	The same	92 (39.48%)
	Less intense	111 (47.64%)
	More intense	31 (13.30%)

Table 6 – Statistical analysis of variables influencing pain experienced by the sting victim at different time points. Analysis was performed using repeated ANCOVAs using the GLM procedure whereby the reported pain score for each time point were dependent variables, and the following variables were analyzed as independent variables: age, sex, history of allergies, sting site infection, delay since sing, number of spines, fish alive, first time stung, need for time off work. F and P values were reported.

Supplemental Table 1.

Time point	Variable	Variable value	t value	P value	Time point	Variable	Variable value	t value	P value
Pain immediately	Age	Under 30	.	.	Pain next day	Age	Under 30	.	.
		30-39	0.7	0.4832			30-39	1.06	0.2892
		40-49	0.62	0.5324			40-49	1.07	0.2869
		50-59	1.56	0.1184			50-59	0.74	0.4608
		60-69	1.91	0.0561			60-69	1.31	0.1918
		70-79	2.24	**0.0258**			70-79	1.43	0.1535
	Sex	Male	.	.		Sex	Male	.	.
		Female	0.11	0.9111			Female	0.08	0.9334
	Allergies	Yes	0.05	0.9626		Allergies	Yes	0.99	0.3243
		No	.	.			No	.	.
	Infection	Yes	-0.66	0.5082		Infection	Yes	2.71	**0.007**
		No	.	.			No	.	.
	Delay since sting	< 3 months	.	.		Delay since sting	< 3 months	.	.
		3-6 month	2.29	**0.0223**			3-6 month	0.38	0.7015
		6-12 months	1.38	0.1685			6-12 months	1.27	0.2032
		>= 12 months	-0.26	0.7967			>= 12 months	0.49	0.6275
	Number of spines	1 spine	.	.		Number of spines	1 spine	.	.
		2 spines	-0.27	0.786			2 spines	2.3	**0.0219**
		3+ spines	-0.65	0.5185			3+ spines	4.45	**<.0001**
	Fish alive	Yes	1.11	0.267		Fish alive	Yes	1.26	0.2088
		No	.	.			No	.	.
	1st time stung	Yes	1.62	0.1056		1st time stung	Yes	2.1	**0.0362**
		No	.	.			No	.	.
	Time off of work	Yes	-0.51	0.6131		Time off of work	Yes	0.7	0.4827
		No	.	.			No	.	.
Pain 1 hour later	Age	Under 30	.	.	Pain next week	Age	Under 30	.	.
		30-39	2.26	**0.0242**			30-39	0.9	0.3686
		40-49	1.69	**0.0921**			40-49	0.87	0.3871
		50-59	3.63	**0.0003**			50-59	0.5	0.6178
		60-69	3.91	**0.0001**			60-69	1.52	0.1288
		70-79	3.29	**0.0011**			70-79	0.78	0.433
	Sex	Male	.	.		Sex	Male	.	.
		Female	-0.1	0.9237			Female	1.24	0.2151
	Allergies	Yes	0.51	0.6093		Allergies	Yes	0.59	0.5568
		No	.	.			No	.	.
	Infection	Yes	2.13	**0.0335**		Infection	Yes	2.89	**0.004**
		No	.	.			No	.	.
	Delay since sting	< 3 months	.	.		Delay since sting	< 3 months	.	.
		3-6 month	2.02	**0.0437**			3-6 month	-0.01	0.9919
		6-12 months	2.23	**0.0261**			6-12 months	-0.46	0.6423
		>= 12 months	-2.47	**0.0137**			>= 12 months	-0.38	0.707
	Number of spines	1 spine	.	.		Number of spines	1 spine	.	.
		2 spines	1.99	**0.0475**			2 spines	2.04	**0.0416**
		3+ spines	3	**0.0029**			3+ spines	3.13	**0.0019**
	Fish alive	Yes	2.17	**0.0304**		Fish alive	Yes	0.72	0.4705
		No	.	.			No	.	.
	1st time stung	Yes	1.64	0.1007		1st time stung	Yes	2.11	**0.0352**
		No	.	.			No	.	.
	Time off of work	Yes	0.08	0.9339		Time off of work	Yes	1.42	0.157
		No	.	.			No	.	.
Pain 2 hours later	Age	Under 30	.	.					
		30-39	1.38	0.1685					
		40-49	1.08	0.2789					
		50-59	1.96	**0.05**					
		60-69	3.08	**0.0022**					
		70-79	2.05	**0.0411**					
	Sex	Male	.	.					
		Female	-1.24	0.2161					
	Allergies	Yes	0.11	0.9088					
		No	.	.					
	Infection	Yes	2.71	**0.007**					
		No	.	.					
	Delay since sting	< 3 months	.	.					
		3-6 month	2.22	**0.0267**					
		6-12 months	1.64	0.1025					
		>= 12 months	-0.84	0.4009					
	Number of spines	1 spine	.	.					
		2 spines	1.34	0.1809					
		3+ spines	3.47	**0.0006**					
	Fish alive	Yes	2.35	**0.0193**					
		No	.	.					
	1st time stung	Yes	2.17	**0.0302**					
		No	.	.					
	Time off of work	Yes	0.06	0.9528					
		No	.	.					

Supplemental table 1 – Further parameter details of statistical analysis of variables influencing pain experienced by the sting victim at different time points. T values and p values were reported here for more details.

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Connecting the text – Chapter 2 to Chapter 3

In Chapter 2, I performed the first large-scale study of the average human experience of lionfish stings. Once we had an idea of what an individual stung by a lionfish experiences, along with details of their pain experience, our next question was: what is in lionfish venom that activates nociceptors? We sought to answer this question in Chapter 3 by performing the first *de novo* RNA sequencing of the lionfish venom gland and using proteomics to identify every protein present in the venom. With this in hand, I identified toxin(s) that caused pain in the venom using a screening approach to identify toxins that activate nociceptors in calcium imaging experiments, detailed in Chapter 3.

Chapter 3

Detailed transcriptomic and proteomic analysis of *Pterois volitans* venom identifies a highly abundant and pain-causing Apolipoprotein-E like toxin

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In preparation

3.1 Abstract

The proliferation of invasive species has severe ecological and medical consequences. Lionfish (*Pterois volitans*), native to the Indo-Pacific, have invaded various regions including the Gulf of Mexico, Caribbean Sea, Atlantic coasts of the Americas, and Mediterranean Sea. Lionfish threaten local reef biodiversity due to their lack of predators and their capacity to consume juvenile species. Moreover, their venomous spines pose a serious medical risk to hunters due to painful stings. This study aims to comprehensively characterize the proteinaceous components of lionfish venom, shedding light on its algogenic properties. Transcriptomic and proteomic analyses were combined to identify venom components and their potential pain-inducing mechanisms. The venom gland transcriptome was sequenced, revealing a wealth of toxin-related transcripts. Mass spectrometry detected 1,550 proteins in the venom, 1,172 of which were matched to putative toxins. Analysis of these proteins revealed scoloptoxins, hyaluronidases, and Apolipoprotein-E-like toxins as the most abundant toxin families. Notably, an Apolipoprotein-E-like toxin, was identified as a major component of lionfish venom and was found to activate nociceptors and elicit pain behaviours in mice. This study provides critical insights into the complexity of lionfish venom, revealing abundant toxin families and identifying a unique algogenic toxin in the venom. Understanding lionfish venom composition and its pain-causing properties is vital for both ecological conservation and biomedical research.

3.2 Introduction

Lionfish (*Pterois volitans*) are an invasive species of fish that are endemic to the Indo-Pacific but that have invaded the Gulf of Mexico, Caribbean Sea, and Atlantic coasts of North and South America as well as the Mediterranean Sea (Perry 2022). The fish poses a threat to reef biodiversity due to its lack of natural predators in the invaded area and its ability to eat the young

of most other local species. It also poses a medical threat to those that hunt it because of its venomous spines it has evolved on its back and spine that produce one of the most painful stings in the ocean.

Lionfish stings occur when victims' skin is punctured by the cartilaginous spines which pushes down the sheath surrounding the spine and allows venom to flow into the victim. A prior study from our group showed that the most prominent symptom reported by individuals stung by the lionfish is pain, as well as redness and swelling. Victims tend to start experiencing pain almost immediately after the sting, with the peak being reach around 1 hour post-envenomation and with the majority of the pain being resolved within a few days (Vetrano, Lebowitz, and Marcus 2002; Diaz 2015; Haddad et al. 2015; Mouchbahani-Constance, Choinière, and Sharif-Naeini 2023).

A few prior studies of the lionfish transcriptome focused on identifying antimicrobial peptides from the skin of the lionfish (Houyvet et al. 2021, 2018). Some groups have attempted to identify individual components of the venom, including a 45 kDa protease, a 59 kDa hyaluronidase as well as a 150 kDa cytolysin which was identified by cDNA cloning (Balasubashini et al. 2006; Kiriake, Shiomi, and Madokoro 2014; Kiriake and Shiomi 2011; Campos et al. 2021). However, an in-depth study of lionfish venom's proteinaceous components as well as a characterization of emerging toxin families has remained unexplored.

Overall, approximately 10 different fish venoms have been studied to elucidate their transcriptomes and proteomes. The most closely related fish venom transcriptome that has been published is that of the stonefish (*Synanceia verrucosa*), another fish that produces a painful and sometimes lethal sting (Halstead, Chitwood, and Modglin 1956). The major component of this venom are the C-type lectins, a toxin family common in snake venoms (Zelensky and Gready

2005; Ogawa et al. 2005; Aranda-Souza et al. 2019), as well as many protein families that are relatively uncommon in animal venoms (Ziegman et al. 2019).

Using a combined transcriptomic and proteomic approach, we sought to identify the components of *P. volitans* venom, characterize their abundances and similarities to other known toxins and identify an algogenic (pain-causing) component of the venom to begin elucidating its pain-causing mechanism of action.

3.3 Methods

Animals

Experiments were approved by the Institutional Animal Care and Use Committee at McGill University and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and recommendations of the International Association for the Study of Pain. Male 8 to 12 weeks old C57BL/6 mice were used for all experiments, genotype: Cre-dependent GCaMP6f mice (The Jackson Laboratory Strain #024105) were crossed with Trpv1-Cre mice (The Jackson Laboratory Strain #017769). Mice were housed in a temperature-controlled room under a 12-hour light/dark cycle. Water and food were available *ad libitum*. On the day of experiments, animals were transferred into the testing room and allowed to acclimate in their cage for at least 45 minutes. Experiments were performed in a blinded fashion, so that the experimenter performing the behavioural tests was not aware of the venom concentration or the treatment conditions. Three lionfish (sizes ranging from 14 – 24 cm, purchased from a commercial pet store and originally sourced from Southern Florida) were used for venom and tissue extraction. The fish were euthanized on the day of purchase by anesthetic (MS-222, Sigma Aldrich # E10521) overdose and venom and tissue extracted according to methods described below.

Sample collection and RNA extraction

Three lionfish were used for RNA extraction and venom extraction. The lionfish were euthanized by anesthetic overdose with MS-222 (Sigma Aldrich #E10521), placed on ice, and had their 10 dorsal venomous spines removed and collected. Half of the spines were retained for RNA extraction and the other half were retained for venom protein extraction. For the RNA extraction, using forceps, the tissue in and around each groove on the venomous spines was removed and RNA was extracted from the tissue using the ReliaPrep RNA Tissue Miniprep kit (Promega #Z6111), following instructions for isolation of RNA from non-fibrous tissue. RNA was eluted in 50 μL of nuclease-free water and stored at -80 ºC until being sent for quality control and cDNA library preparation. For the venom protein extraction, all steps were based on a published protocol (Akiva S. Cohen 1989) and were performed at 4 ºC. In each of the lionfish spines, glandular tissue was collected into 2 microcentrifuge tubes containing 500 μL of 0.9% NaCl. Tissue was disrupted by homogenizing with a pestle and motorized mixer (VWR # 47747-370) for 15 seconds, followed by a 2-minute centrifugation at 2000 x*g*. The supernatant was collected in a separate tube, and the pellet was subjected to two additional homogenization and centrifugation steps, with the addition of 500 μL fresh saline solution each time. Before the final centrifugation, the pellet was triturated by passing it 10 times through a 16-gauge needle to maximize tissue disruption. All the supernatants were combined and centrifuged at 100,000 x*g* for 30 minutes. The final supernatant was collected, the protein concentration was measured and stored at -80 ºC until being used in experiments.

Library preparation, assembly, sequencing, and quality control

The mRNA library was assembled with KAPA RNA hyperprep with poly-A capture (Roche #KK8580) as per manufacturer's instructions. The quality of the purified total RNA was assessed using Agilent 2100 Bioanalyzer (RNA 6000 NanoKit) (Agilent Technologies, Waldbronn, Germany). The transcriptome was sequenced using the Illumina NovaSeq 6000 System with paired-end libraries. See Supplemental Figure 1, bottom table, for information regarding number of reads and the percentage removed at each step of quality control.

The quality of the raw reads was assessed with FASTQC v0.11.8 rCorrector v1.0.4 was used for kmer-based read error correction on fastq files. Reads that were marked as "uncorrectable" were then removed using TranscriptomeAssemblyTools. Trimming was executed with TROMMOMATIC v0.36 to remove adapters and low-quality bases from reads. *De novo* transcriptome assembly was then performed using Trinity v2.12.0 (with argument SS_lib_type = FR). The resulting assembled transcripts were then BLASTed against Uniprot venom and toxin database (reviewed and unreviewed) using blastx v2.12.0 with the "-max_target_seqs" argument to keep only the best hit for each transcript. BLAST results were filtered by percentage of identical matches > 50% and alignment length > 500 base pairs. Multiple sequence alignment was carried out on the resulting filtered sequences using MEGA X.

Bioinformatic analysis

To identify potential toxins, a comparison was made to the Uniprot animal toxin database using Blastx. Results with a BLAST bit score threshold of 50 or higher were considered. Results matching any of the following categories were also disregarded: those possessing a higher bit score to a non-toxin protein from a fish database search, those containing two or more transmembrane helices (predicted using TMHMM) and those lacking a signal peptide (predicted using SignalP). Functional annotation of the *de novo* assembled transcriptome was performed using TransDecoder

(Galaxy version 3.0.1, http://transdecoder.sf.net/) to predict Open Reading Frames (ORFs) and obtain protein sequences of at least 50 amino acids in length (Haas et al. 2013). These predicted sequences were assembled into a database, which was used as a reference for subsequent mass spectrometry experiments.

Mass spectrometry

Mass spectrometry was performed on the venom and venom fractions using an Ultimate 3000 RSLC system (Thermo Fisher Scientific) coupled to a LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific). Venom was first deactivated with 10 mM DTT and trypsinized, approximately 3 μg of each sample was used for analysis. In total, 1 550 proteins were detected in the venom with a total of 34 205 spectra.

Database searching

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.6.2). Mascot was set up to search the contaminants database and the lionfish venom gland annotated transcriptome database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 5.0 PPM. Carboxymethyl of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications.

Criteria for protein identification

Scaffold (version Scaffold_5.0.1, Proteome Software Inc., Portland, OR) was used for validating MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller et al. 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could

be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Toxin identification and classification

Proteins that were detected in the MS analysis of the venom and that were found to be matches to putative toxins in our BLAST analysis against the Uniprot animal toxin database were assembled into a separate list as putative toxins. Putative toxins were further filtered based on percent shared identity, TPM levels, number of spectra detected in MS and e-value. Proteins were then assigned to toxin families based on their BLAST matches, percent identity and conserved key functional domains.

High Performance Liquid Chromatography

A 1260 Infinity LC system (Agilent Technologies) was used with a Waters XBridge C8 (3.5 μm x 3 mm x 50 mm) column with a flow rate of 0.4 mL/min. A two-component solvent system of MilliQ water (solvent A) and acetonitrile (ACN, Sigma) (solvent B) was used. Each solvent was acidified with trifluoroacetic acid (TFA, Sigma) to a final concentration of 0.1%. Samples were also pre-acidified with 0.1% TFA and centrifuged at 10,000 xg for 10 minutes to remove precipitates. Thirty microlitres of sample were injected at a time with a gradient starting at 0% B for 5 minutes, followed by 0% increased to 75% B over 20 minutes, then increased from 75% to 100% B over 2 minutes and maintained at 100% B for 2 additional minutes. Fractions were manually collected every 30 seconds for the first 5 minutes of the gradient and then every minute

for the remaining 23 minutes of the gradient. A total of 90 μL of venom were fractionated (4.52 μg total protein) over 3 separate injections. Identical fractions were pooled across different injections, lyophilized and resuspended in PBS for physiological experiments.

Primary cell culture

Male 8–12-week-old Trpv1-Cre x GCaMP6f mice were used for dorsal root ganglia (DRG) culture. The culture of DRG neurons was performed as previously described (Mouchbahani-Constance et al. 2018; Petitjean et al. 2020). Briefly, all DRGs were dissected from the mouse and placed in sterile HBSS (Wisent Bioproducts #311-511-CL) at 4 ºC. Enzymatic dissociation was performed with 0.2% type IV collagenase (Sigma Aldrich #c1889) and 0.2% dispase (Sigma Aldrich #D4693) for 90 minutes. DRGs were dissociated mechanically by trituration and plated at low density on 96-well plates (Corning #3882) with DMEM/F12 (Wisent Bioproducts #11330-032) supplemented with 10% fetal bovine serum (Wisent Bioproducts #80150) and 1% penicillin/streptomycin (Wisent Bioproducts #450-202-EL) and were maintained in an incubator at 37 ºC with 5% CO_2, with media changes every other day. Neurons were tested 2 days after plating.

High Content Screening Imaging and Analysis

For high content calcium imaging, ImageXpressMicro automated epifluorescent imaging system (Molecular Devices) was used with a 40X S Fluor 0.90 NA objective (Nikon) and an eGFP filter set (Chroma Technology #49002). Using the MetaXpress software, the equipment was directed to focus on the plate bottom and offset by bottom thickness and then autofocus using laser with Z-offset. The 96-well plate containing cells was maintained at 37 ºC and 5% CO_2 and the sample plate containing venom and fractions was maintained at 4 ºC. For plate imaging, each well was

imaged one-at-a-time at 1 Hz for 30 seconds, and at the third acquisition, the devices robotics dispersed 10 μL of each fraction onto the well and continued imaging for the subsequent 26 acquisitions. Using Fiji for analysis, a macro was written to stitch each well's tiles together and project all timepoint images into a maximum intensity projection, this image was used to create a binary mask to overlay over all other images and detect regions of interest (ROI). The mask was applied to each timepoint of a given well, and fluorescence intensity was extracted for each ROI. Fluorescence intensity values were analyzed using Python to determine which cells demonstrated a significant (> 1.2x baseline fluorescence intensity) rise in intracellular calcium after applications of lionfish venom fractions.

Recombinant protein synthesis and purification

Plasmids encoding the proteins of interest in pET21 (+) expression vectors in were ordered from Twist Biosciences (California, USA) and used to transform BL21-DE3 cells. The BL21-DE3 were transformed in LB broth at 37 ºC up to an OD of 1.0, after which they were cooled to 16 ºC on ice, induced with 0.5 μM IPTG with 0.5% glucose and left to express protein at 16 ºC overnight. Cells were harvested by centrifugation at 3000 RPM for 20 minutes at 4 ºC, resuspended in lysis buffer (50 mM Tris, 400 mM NaCl, 0.1 mg/mL lysozyme, 25 μg/mL DNase I, 5 mM $MgSO_4$ at pH 8.0) and immediately frozen at -80 ºC for a few minutes before sonication. Lysate was applied to HisPur Ni-NTA magnetic beads (Thermofisher, #88832), washed with water and equilibrated with column wash buffer (300 mM NaCl, 50 mM HEPES) before being eluted in 300 mM imidazole, 300 mM NaCl and 50 mM HEPES. The 3C was cleaved off using a 3C protease (10 μg of 3C protease per mg of protein in 50 mM Tris, 400 mM NaCl) and left overnight for cleavage at 4 ºC with constant shaking. Samples were concentrated using the 3500 Da Amicon-Ultra concentrators and then purified using gel filtration chromatography (see supplementary figure 1).

Statistical analysis protocol

Statistical analyses were performed using the statsmodels package in python with numpy and some portions of the ToxCodAn package (Nachtigall et al. 2021). Significance was set at a $p < 0.05$ level. All graphical diagrams were made using BioRender and figures were created using CorelDraw.

3.4 Results

Pterois volitans venom contains a multitude of putative toxins

To gain insights into the components of *P. volitans* venom, and to elucidate the main groups of toxins found in its venom, we followed an established RNA sequencing and mass spectrometry workflow (Brinkman et al. 2015). We first performed *de novo* RNA sequencing of the venomous spines and subsequently used this transcriptome as a database for mass spectrometry experiments to identify the proteins in the venom (Figure 1A). Our RNA sequencing results uncovered 104 366 total Trinity 'genes', corresponding to 155 800 total contigs (consensus regions of DNA resulting from overlapping segments), with a 46.69% GC content (Figure 1B, top). To determine which of these transcripts corresponded to putative toxins, we performed a BLAST search of all assembled transcripts against the Uniprot Venom and Toxin database. Considering the abundance values we collected with our RNA sequencing, 17% of all transcripts (corresponding to 166 841 transcripts per million, TPM) were BLAST matches to putative toxins, while 83% corresponded to non-toxin, housekeeping transcripts (ex. proteins important for cellular functions or structure) (Figure 1B, bottom).

Following our RNA sequencing, we used the assembled transcriptome of the *P. volitans* spines as a database in a mass spectrometry analysis of the *P. volitans* venom. Our analysis detected a total

of 1 550 proteins, with a total of 34 205 spectra. Out of these proteins, 1 172 were matched in a BLASTp search of putative toxins. An important feature of a venom toxin is the signal peptide – which ensures the protein will be secreted. We verified the presence of signal peptides on each detected protein using the program SignalP and found that only 15% of detected putative toxins had a signal peptide (176/1172), and only 143/1172 of these had an abundance over 100 TPM (which we considered as our threshold for relevant toxins' expression level) (Figure 1C). Interestingly, two previously identified toxin transcripts from the *P. volitans* venom, called PvTx-a and -b (Kiriake and Shiomi 2011), were detected in the transcriptome, but they had very low abundances (<30 TPM), and did not contain signal peptides. This suggests that they are not major toxins in the venom, especially since they were first identified through cDNA cloning and not through studies of the venom's protein contents.

With the assembled proteome and transcriptome of *P. volitans* venom, we determined the most abundant toxin families at the transcript level and at the protein level. Based on RNA transcript abundance, the most common toxin family in the venom were ApoE-like toxins, making up more than 55% of putative toxin transcripts. Apolipoprotein-E is not a common component of venoms, and we could not find any other venoms containing an Apolipoprotein. The only exception is from a transcriptomic study that found expression of Apolipoprotein D in the venom from the Yellow Meadow Ant (*Lasius flavus*) (B. Wang et al. 2023). The second most abundant toxin family at the transcript level was the Zinc 3-finger-toxin-like peptides, followed by actitoxins, veficolin, α-conotoxins, scoloptoxins, thalatoxins and hyaluronidases (Figure 1D). At the protein level, the most abundant toxin family was the scoloptoxin-like toxins, followed by hyaluronidases, ApoE-like toxins, zinc 3-finger-toxins, neoverrucotoxin-a, cysteine-rich venom proteins and theraphotoxins (Figure 1E).

Veficolin has been observed in snake and lizard venoms and interacts with leukocytes to induce platelet aggregation and complement activation (OmPraba et al. 2010). Scoloptoxins are found in many centipede venoms and can exert a variety of functions from anticoagulant activity to K_v channel antagonism (Sunagar and Moran 2015). Actitoxins have been identified from anemone venoms and typically act as serine protease inhibitors (Tschesche, Kolkenbrock, and Bode 1987). Neoverrucotoxin-a has been detected in *Scorpaena* (scorpionfish) venom, and proposed to be the pain-causing component of the scorpionfish's venom, however this protein is a subunit of a greater complex, requiring binding to its neoverrucotoxin-b counterpart (Kiriake and Shiomi 2011; Xie et al. 2019). This counterpart was indeed detected in the transcriptome but did not possess a signal peptide (required for the cell to secrete the protein), excluding it from our analyses. Prior studies of *P. volitans* venom has demonstrated hyaluronidase activity in the venom (Saenz et al. 2017), as well as evidence of a hyaluronidase toxin at the transcript level (Kiriake, Shiomi, and Madokoro 2014). Hyaluronidase presence in the venom is likely to help digest the extracellular matrix to allow other venom toxins to reach deeper tissues or blood vessels (Kiriake, Shiomi, and Madokoro 2014). Scoloptoxin is a toxin identified from the venom of *Scolopendra subspinipes dehaani*, a centipede native to southeast Asia whose main function is platelet aggregation (Z.-C. Liu et al. 2012) but has also been shown to block potassium channels in the nanomolar concentration range (Yang et al. 2012). Theraphotoxins are typically found in spider venoms and interact with a variety of ion channels, including hERG potassium channels, voltage-gated potassium channels and others (Montandon et al. 2020; Finol-Urdaneta et al. 2022).

ApoE-like toxins and Zinc 3-finger-like toxins are the most abundant in lionfish venom

Based on the RNA sequencing transcript abundance data we collected, we determined the main families of toxins in *P. volitans* venom (Figure 2A). The most abundant toxins in the venom were

DN4547 and DN2818. DN4547 is a 31 kDa protein that is highly similar to Apolipoprotein-E from the European sea bass *Dicentrarchus labrax*, with 74.64% shared amino acid sequence identity (Figure 2B), as determined using the Clustal Omega program (Coudert et al. 2023). This putative toxin contains a lipid binding domain as well as a signal peptide, suggesting it may play a role in binding to the membranes of nociceptors and/or other cell types in the venom's path.

DN2818 is a 11 kDa protein with 60.82% shared sequence identity to a Zinc 3-finger-toxin from a snake *Micrurus altirostris* (Figure 2C). This toxin from *M. altirostris* is closely related to 3-finger-toxins that interact with the nicotinic acetylcholine receptor (nAChR), but it lacks the essential amino acids for the nAChR interactions (Silveira de Oliveira et al. 2000). Interestingly, the same is true for the *P. volitans* 3-finger-toxin DN2818 – it lacks the residues required to interact with nAChRs – so its potential binding partner and function are unknown.

Parallel fractionation of P. volitans venom using HPLC and Gel Filtration Chromatography reveals multiple fractions activating nociceptors

To identify toxins responsible for causing pain in mice, we decided to perform an unbiased and separate fractionation of *P. volitans* venom using HPLC or Gel filtration chromatography. Each fraction was tested for its ability to elicit a rapid rise in intracellular calcium on mouse nociceptors (analogous to neuronal activation). Active fractions were sent for mass spectrometry to determine their protein contents. Toxins that would be present both in positive HPLC and in gel filtration fractions would thus be considered as top contenders for being algogenic toxins and would be narrowed down in an unbiased way.

HPLC fractionation of the venom revealed a high concentration of the venom's components in the first isocratic phase of the gradient, with some proteins also being present during the early portion

of the gradient (Figure 3A). This indicates that the majority of the venom's proteins are highly polar, with a lot of small non-proteinaceous molecules as is common in many venoms (Ziegman and Alewood 2015).

To screen the fractions for their algogenic activity, we used a high content screening (HCS) approach on mouse DRG neurons obtained from Trvp1-cre x GCaMP6f mice, where all their nociceptors express the genetically encoded calcium sensor GCaMP6f. Using an HCS microscope, we screened the 31 collected fractions from HPLC on two different 96-well pates, each containing neurons pooled from 3 mice (total of 6 mice used), a total of 3 008 cells were imaged. Positive fractions were ones that elicited a rise in intracellular calcium higher than 20% of baseline within 15 seconds of the fraction application. Overall, 2 fractions were retained for subsequent MS analysis – 3 (1-1.5 minutes) and 15 (9-10 minutes). Additionally, neighbouring fractions (2 and 4 as well as 14 and 16) were retained as control fractions to ensure specificity of the toxins identified (Figure 3B). Using the same approach, we screened the fractions collected from Gel Filtration Chromatography and identified 3 active fractions that were sent for MS/MS analysis – fractions C02, E12 and F04 (Figure 3C).

Identification and recombinant synthesis of putative algogenic toxins

MS analysis of active fractions revealed a total of 12 putative toxins across all active fractions (Table 1). However, only 3 were present in both HPLC active fractions and gel filtration chromatography fractions (Table 1). A BLAST analysis of these toxins revealed that 2 of the 3 overlapping toxins were BLAST matches to housekeeping genes, leaving DN22045 as the only toxin present in active fractions from both HPLC and gel filtration chromatography that was not a BLAST match to any know proteins (Table 1).

We next examined whether the toxins DN4547 and DN22045 possessed any algogenic activity *in vitro* and *in vivo*. To synthesize enough quantities of recombinant proteins, we used the *E. coli* system (Supplemental figure 2). After recombinant protein synthesis, the proteins were purified using a His-based purification column, concentrated using molecular-weight filters and resuspended in a sterile saline solution (0.9% NaCl) for subsequent physiological experiments. To screen the toxins for their ability to elicit a rise in intracellular calcium in mouse nociceptors, we used our high content screening approach on cultured mouse DRG neurons. Each well was challenged with 30 μL of either DN4547 (diluted to 0.053 mg/mL protein concentration), DN22045 (diluted to 0.064 mg/mL protein concentration), crude lionfish venom (diluted to 0.275 mg/mL protein concentration, unfractionated, containing all venom proteins) or standard bath solution. Wells that received DN4547 showed a significant rise in intracellular calcium (Figure 4A), while those that received DN22045 showed no rise in calcium (Figure 4B) and cells that received crude lionfish venom also demonstrated a large rise in intracellular calcium (Figure 4C). Overall, in wells were DN4547 was applied, nearly 25% (43/177) of GCaMP6f-positive mouse nociceptors displayed a rise in calcium greater than 20%, while DN22045 produced a response in only 3/163 neurons. Crude venom was used as a reference and produced a response in 35% of neurons (71/205) (Figure 4D). Our experiments therefore indicate that only DN4547 can activate mouse nociceptors. We next examined whether DN4547 produced any nocifensive behaviors when injected *in vivo*. We injected 20 μL of DN4547 (0.428 mg/mL concentration, total protein 8.56 ug) or DN22045 (0.513 mg/mL concentration, total protein 10.26 μg) into the left hind paw of mice and recorded their nocifensive behaviour for 1 hour. The amount of time the mice spent licking their injected paw was quantified into bins of 5 minutes and plotted both over time (Figure 4E) or in total over the hour (Figure 4F). Overall, mice injected with DN4547 showed significant

pain behaviour during the experiment, reaching the peak of their pain roughly 45 minutes later, and spending an average of 347.58 (± 17.32) seconds licking the injected paw (n = 4 mice), while DN22045 elicited an average of only 48.53 seconds of licking behaviour (n = 2 mice). Taken together, these data show that synthesized DN4547, a highly abundant ApoE-like toxin from *P. volitans* venom, elicits both activation of mouse nociceptors in calcium imaging experiments and pain behaviour upon injection in mice, demonstrating that this peptide is an algogenic toxin from *P. volitans* venom.

3.5 Discussion

Fish venoms have been relatively understudied compared to other venomous species such as snakes, spiders and scorpions, yet their venoms are equally as complex and interesting. In this study, we performed the first ever in-depth and unbiased analysis of the components of lionfish venom, with insights into the major toxin families and the venom's potential algogenic mechanism of action. Unlike prior studies of lionfish venom, our unbiased approach allowed us to identify components without prior bias to certain toxins, like cDNA cloning-based studies of the venom (Kiriake and Shiomi 2011). Furthermore, this approach allowed us to filter putative toxins early on for the presence of a signal peptide, an essential component of any toxin (otherwise, the toxin cannot possible secreted by the venomous species into its venom) and generate a more reliable and in-depth list of the venom's components.

Due to the breadth of our proteome and the high number of different toxins in the venom, we chose to focus on the most abundant toxin and one that appeared in 2 separate fraction screens. However, there remain numerous other toxins in the venom to be studied, and the details of their sequences and relative abundances can be used by other groups to produce other specific studies of the structure and functions of other lionfish toxins. Some limitations of our study include the venom

extraction method used, which doesn't recapitulate the natural process of envenomation by a lionfish and thus may modify the quantity of venom extracted. Another limitation is that in both HPLC and Gel Filtration Chromatography, proteinaceous components can become denatured. This would limit our ability to accurately evaluate the ability of proteinaceous components to activate nociceptors.

The major component of lionfish venom is a toxin, DN4547, with high sequence similarity to ApolipoproteinE, a molecule that has been implicated in pain processing (S. Liu et al. 2023; Dhillon and Singh 2018; Tansley et al. 2022), but whose mechanism of action remains to be elucidated. ApolipoproteinE is the most abundant apolipoprotein in the central nervous system, is involved in cholesterol trafficking and lipoprotein metabolism and expressions of different ApoE polymorphisms has been directly linked to Alzheimer's disease (Huebbe and Rimbach 2017; de Bont et al. 1999; Arnold et al. 2015; Vecchio et al. 2022; Zhao et al. 2018). In a transcriptomic study of spinal cord microglia in chronic pain, Tansley et al. found that ApoE was one of top upregulated genes in these cells at chronic timepoints. Additionally, its expression in human populations was inversely correlated with Alzheimer's risk – the epsilon-2 variant, typically associated with decreased risk of Alzheimer's, was associated with an overall increased risk of developing chronic pain, while the epsilon-4 variant, typically associated with increased risk of Alzheimer's, was associated with an overall decreased risk of developing chronic pain (Tansley et al. 2022).

This study is the first of its kind to identify an ApoE-like toxin in a venom, and while puzzling, may point to an evolved pain-causing mechanism that takes advantage of a highly conserved molecule across many species. ApoE is a highly conserved protein, with isoforms conserved across mammals, reptiles, fish and insects (Huebbe and Rimbach 2017). Since the most abundant toxin

transcript in the venom is an ApoE-like toxin, it suggests that the lionfish may have evolved a toxin that mimics the function of this lipoprotein, which is linked to pain, to elicit pain in as many different predator species as possible. This highlights the evolutionary relevance of ApoE in pain across a multitude of species and highlights the importance of studying this molecule to further understand its role and mechanism in pain.

3.6 Figures

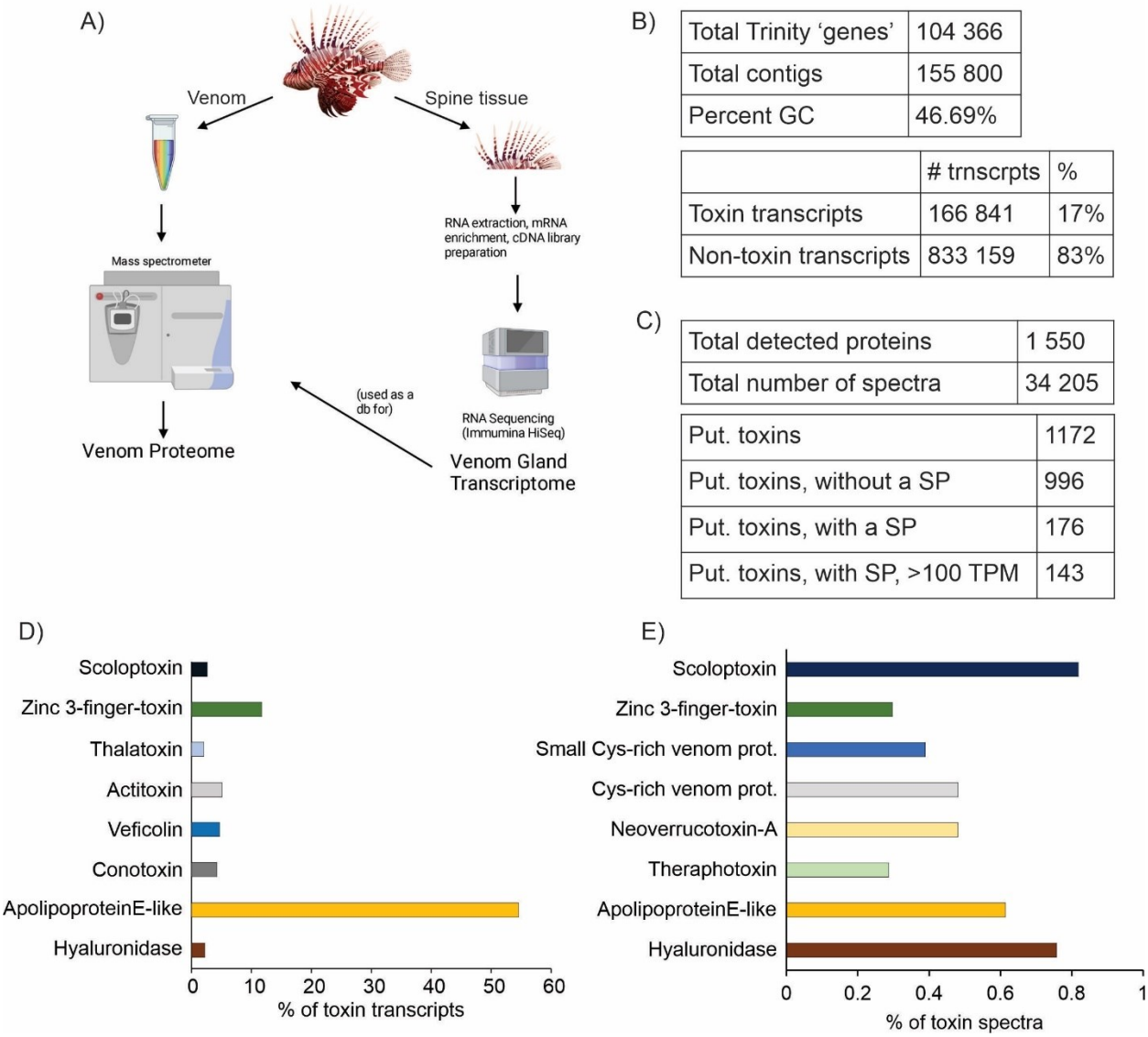
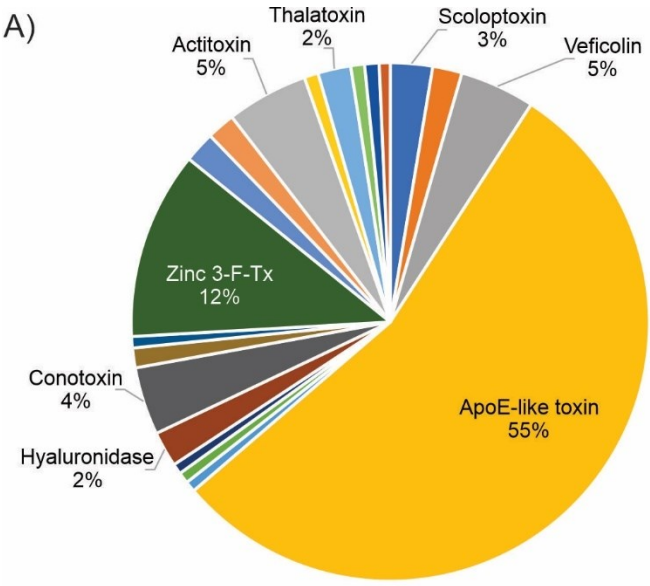


Figure 1 – The proteome and transcriptome of lionfish venom reveal a diversity of toxins. A) Schematic of the workflow followed to identify the protein components of lionfish venom. B) Summary of assembly and annotation of *Pterois volitans* venomous spine tissue. C) Summary of mass spectrometry analysis of proteins contained within the lionfish venom. D) Classification of most abundant putative toxins in lionfish venom gland transcriptome. E) Classification of most abundant putative toxins in lionfish venom proteome.

A)



B)

```
DN4547                  MQAVALILALAVITGSNARVLHQADTTPKRWEDTVDRFWQYIADLNQKADG  51
European Sea Bass ApoE  MKAVSLILALAVITGCNARAVHQTDATPNQWEETVDRFWQYVSDINQKADG  51
                        *:**:**********.***.:**:*:**::**:********::*:******

DN4547                  VLQNLKDSQITRELDTLITDSMAELNAYKADIQTKLAPYTDGTTGQLSQDL  102
European Sea Bass ApoE  VVEDIKASQLSRELDTLITDTMAELTTYRDDIQTKLTPYTDASTGQLSQDL  102
                        *::::* **::*********:****.:*: ******:****.:********

DN4547                  QLLVNKLQKDMLDAKERSTEYVGELRSMVELNADDVHTRIGTYTHKLKKRL  153
European Sea Bass ApoE  QLLANKLQKDMLDAKERSTEYLGELKTMLEQNSDDVRARINSYTHKLKKRL  153
                        ***.*****************:***::*:* *:***::**.:*********

DN4547                  DKDTEEIRSAVATYLEEIQSRASQNLDTVRANVEPYVQQASDSATKKLSDV  204
European Sea Bass ApoE  NKDTQEIHDTVATYMGELQSRTSQNLEAAREQVEPYVQQASDSATKKISDI  204
                        :***:**:.:****: *:***:****::.* :***************:**:

DN4547                  STMLKTQAEGLGQQLETQAEGIRTQLEATAQELRTSLEGKIDELTKMLSPY  255
European Sea Bass ApoE  STILKTQAEGLGQQLETQAEGIRSQLEATAQDLRTSLEGRIDELTEMFSPY  255
                        **:*******************:*******:*******:*****:*:***

DN4547                  TTQIHEQIQSIMEKVKETATN*  276
European Sea Bass ApoE  ATKIREQFEGIVDKVKETATA*  276
                        :*:*:**::.*::******* *
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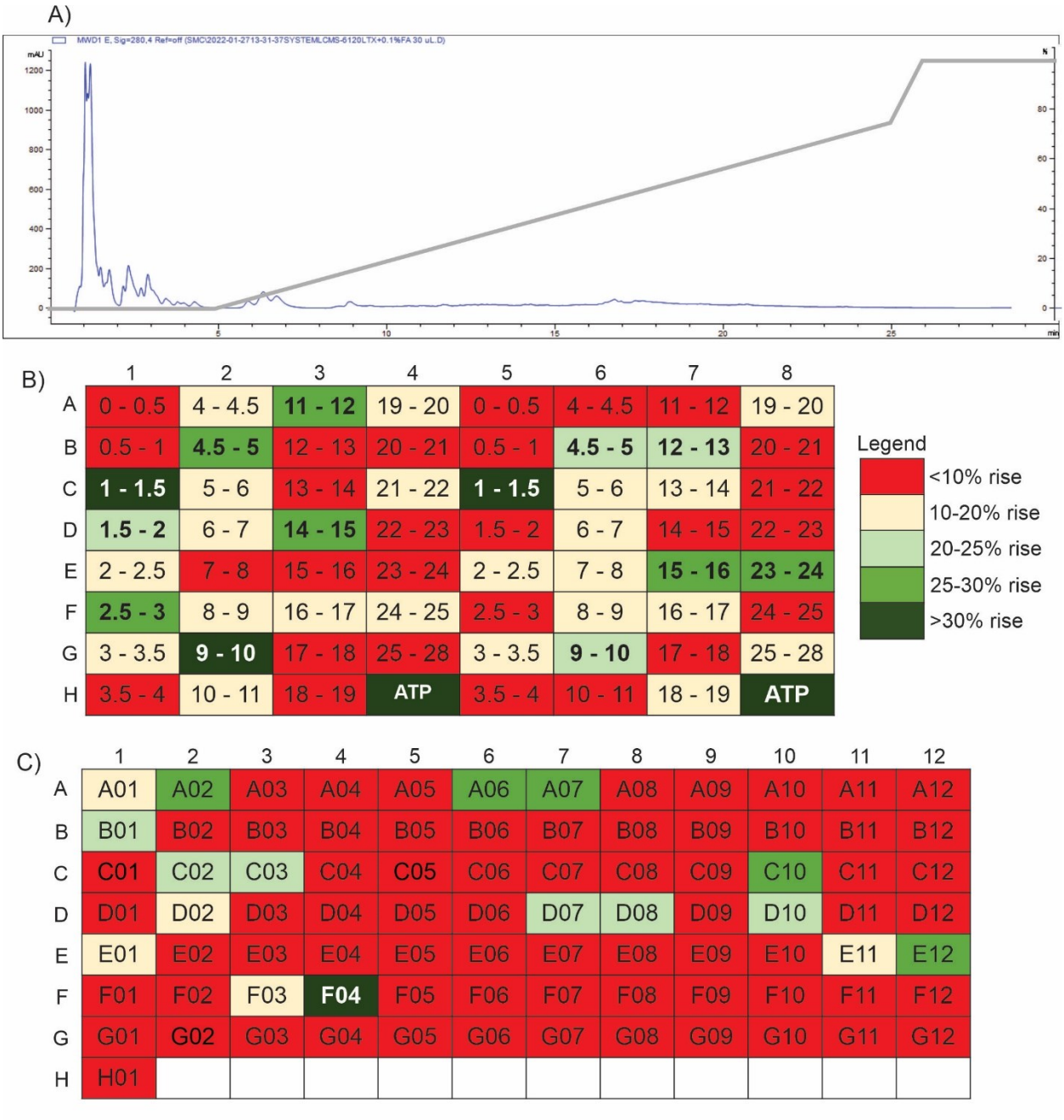
C)

```
DN2818 (Z3FTx)          MQFYGALILFMTLSAACALRCYSCSG-AACTDIITCPASPLAPLDRCSTTEVNGVVAKTC      59
Snake-Z3FTx             MHFYGALILFMTLSAAYGLKCHTCVASVGCSAVMTCPS----GLNRCASVELLGLKTKSC      56
                        *:************** .*:*::* . ..*: ::***:     *:**::.*: *: :*:*

DN2818 (Z3FTx)          MNSNLCIDPIKCCSTDLCNSAVPTGSSVLLLLVSSAIITLFL*                      101
Snake-Z3FTx             MTSQACVEPIKCCFYDLCNSAMPAGSSPLLLLLSSAIITVFL*                      98
                        *.*: *::*****  ******:*:*** ****:******:***
```

Figure 2 – ApoE-like toxins and Zinc 3-Finger-Tx-like toxins are the most abundant toxin families in the lionfish venom. A) Pie chart demonstrating relative abundance of putative toxins, classified based on matches to known putative toxins, in *P. volitans* venom. B) Amino acid sequence alignment between DN4547, a *P. volitans* toxin found to be a putative match to ApolipoproteinE

from *Dicentrarchus labrax* (European sea bass). * indicates a perfect amino acid match and : indicates amino acids with similar properties. C) Amino acid sequence alignment between DN2818, a *P. volitans* toxin found to be a putative match to known Zinc-3-finger-toxin from snake venom (*Micrurus altirostris*).



B)	1	2	3	4	5	6	7	8
A	0 - 0.5	4 - 4.5	**11 - 12**	19 - 20	0 - 0.5	4 - 4.5	11 - 12	19 - 20
B	0.5 - 1	**4.5 - 5**	12 - 13	20 - 21	0.5 - 1	**4.5 - 5**	**12 - 13**	20 - 21
C	**1 - 1.5**	5 - 6	13 - 14	21 - 22	**1 - 1.5**	5 - 6	13 - 14	21 - 22
D	**1.5 - 2**	6 - 7	**14 - 15**	22 - 23	1.5 - 2	6 - 7	14 - 15	22 - 23
E	2 - 2.5	7 - 8	15 - 16	23 - 24	2 - 2.5	7 - 8	**15 - 16**	**23 - 24**
F	**2.5 - 3**	8 - 9	16 - 17	24 - 25	2.5 - 3	8 - 9	16 - 17	24 - 25
G	3 - 3.5	**9 - 10**	17 - 18	25 - 28	3 - 3.5	**9 - 10**	17 - 18	25 - 28
H	3.5 - 4	10 - 11	18 - 19	**ATP**	3.5 - 4	10 - 11	18 - 19	**ATP**

C)	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	**C01**	C02	C03	C04	**C05**	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	**F04**	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	**G02**	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01											

Figure 3 – Fractionation of *P. volitans* and subsequent screen of fractions reveals a few fractions are capable of activating mouse nociceptors. A) Chromatogram of HPLC fractionation of *P.*

volitans venom, detected at 280 nm. B) Heat map of calcium imaging results in a high content screen of HPLC fractions on mouse nociceptors in a 96-well plate. The legend on the right indicates the colours representing the percent rise from baseline in GCaMP6f signal in nociceptors imaged in each well. A rise of 20% from baseline or greater was considered significant. C) Heat map of calcium imaging results in a high content screen of gel filtration chromatography fractions on mouse nociceptors in a 96-well plate. The legend on the right indicates the colours representing the percent rise from baseline in GCaMP6f signal in nociceptors imaged in each well. A rise of 20% from baseline or greater was considered significant.

		HPLC fractions						Gel Filtration fractions				
Lionfish Transcriptome ID	**MW**	**0.5 - 1**	**1 - 1.5**	**1.5 - 2**	**8 to 9**	**9 to 10**	**10 to 11**	**C02**	**E12**	**F04**	**BLAST match**	**Accession number**
TRINITY_DN976_c5_g1_i1	25 kDa	X	O	O	X	X	X	O	O	O	Probable n-acetyltransferase	XP_037652673
TRINITY_DN1432_c2_g4_i1	35 kDa	O	O	O	O	X	X	O	O	O	Chromobox homolog 1	XP_038593095
TRINITY_DN2028_c0_g1_i10	**50 kDa**	**O**	**X**	**X**	**X**	**X**	**O**	**O**	**O**	**X**	**Elongation factor 1-alpha**	**AEB31334**
TRINITY_DN2599_c0_g1_i9	15 kDa	O	O	O	O	X	X	O	O	O	None	
TRINITY_DN3052_c1_g1_i1	35 kDa	O	O	O	O	X	X	O	O	O	Heterogeneous nuclear ribonucleoprotein A3	XP_037646696
TRINITY_DN5052_c0_g3_i1	22 kDa	X	X	O	X	O	X	O	O	O	Peroxiredoxin	XP_034559865
TRINITY_DN6640_c0_g1_i1	**58 kDa**	**O**	**O**	**O**	**O**	**X**	**X**	**O**	**X**	**O**	**Centromere protein F**	**XP_037636201**
TRINITY_DN11131_c0_g1_i1	11 kDa	O	O	O	O	X	X	O	O	O	High mobility gropu nucleosome binding domain 6	XP_037636160
TRINITY_DN21033_c0_g1_i1	14 kDa	X	X	O	X	O	X	O	O	O	Histone H2B	XP_010754035
TRINITY_DN21033_c0_g1_i7	14 kDa	X	O	O	X	O	X	O	O	O	Histone H2B	XP_010754035
TRINITY_DN22045_c0_g2_i1	**39 kDa**	**X**	**O**	**O**	**O**	**X**	**O**	**O**	**O**	**X**	**Uncharacterized protein**	**XP_045908812**
TRINITY_DN29825_c0_g1_i1	11 kDa	X	X	O	X	X	X	O	O	O	Histone 4	EHB12217

Table 1 – MS/MS results of putative toxins identified in activating fractions identified in Figure 3. All positive fractions identified in the calcium imaging screen of HPLC and Gel Filtration fractions were sent for Mass Spectrometry analysis. 3 putative toxins were identified in active fractions of both HPLC and Gel Filtration screens. Of these 3 putative toxins, 2 were found to be matches to other proteins with cellular functions, however DN22045 did not match to any other proteins and was therefore retained for recombinant synthesis.

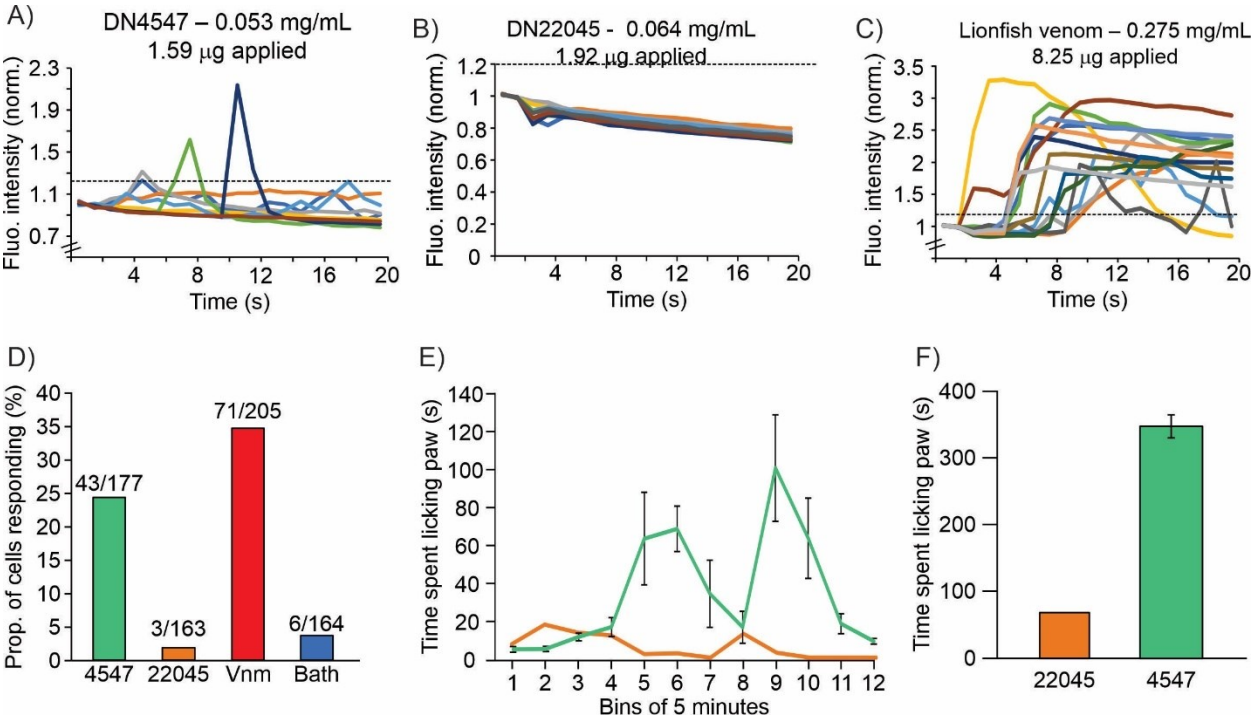
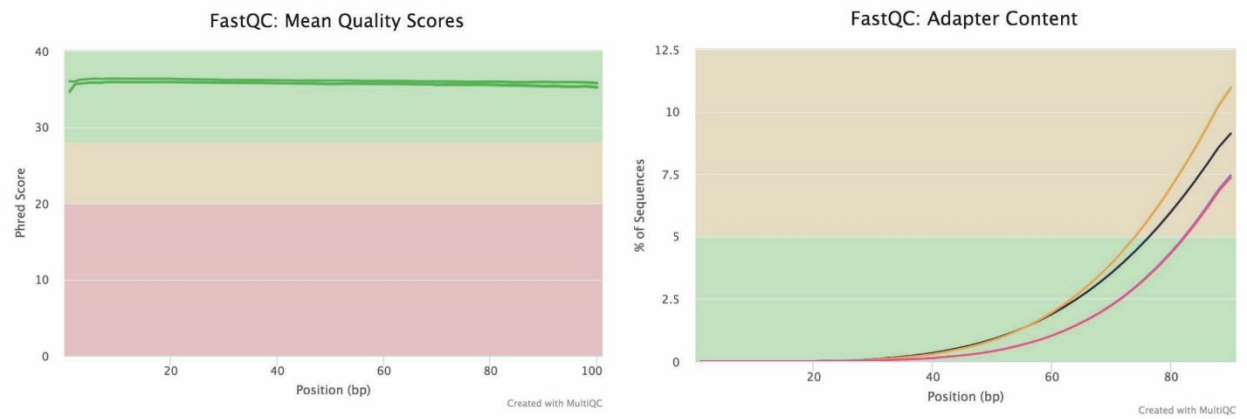


Figure 4 – *P. volitans* toxin DN4547 elicits a rise in intracellular calcium in nociceptors and pain behaviour when injected *in vivo*. A) Sample calcium response of mouse nociceptors following bath application of 1.59µg of DN4547 after timepoint 3. For panels A-C, the dotted line indicates the threshold above which calcium responses were considered positive. B) Sample calcium response of mouse nociceptors following bath application of 1.92 µg of DN22045 after timepoint 3. C) Sample calcium response of mouse nociceptor following an application of crude lionfish venom after timepoint 3. D) Quantification of cells responding (>20% of baseline fluorescence) to DN4547, DN22045, lionfish venom or a bath solution (negative control). Numbers above bars indicate raw cell counts. E) Mean (± SEM) duration of spontaneous pain behaviour during a 1-hour period after intraplantar injection of DN4547 (8.56 µg total protein injected in the mouse paw) or DN22045 (10.26 µg total protein injected in the mouse paw). The 1-hour observation period was split into bins of 5 minutes, and the time spent licking the injected paw was quantified for each bin. F) Total amount of time spent licking the injected paw over the 1-hour behaviour experiment described in panel E). Error bars indicate SEM.



QC of raw data with FASTQC			
	% dups	% GC	M Seqs
Lionfish-spine-RNA-1	68.20%	49%	43.2
Lionfish-spine-RNA-2	67.50%	49%	39.3
Lionfish-spine-RNA-3	73.80%	49%	43.5

Number of reads and percentage removed at each step					
	Untrimmed	rcorrector_unfixrm		trimmed	
Sample name	Nb reads	Nb reads	Pct removed	Nb reads	Pct removed
Lionfish-spine-RNA-1	43219863	39560273	8.47%	36073619	8.81%
Lionfish-spine-RNA-2	39304670	35530593	9.60%	31956425	10.06%
Lionfish-spine-RNA-3	43520607	39963830	8.17%	36852557	7.79%

Supplementary Figure 1 – Quality control data on RNA and RNA sequencing results. Tables indicate number of sequences (top table) and number of trimmed reads at each step (bottom table).

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Connecting the text – Chapter 3 to Chapter 4

After characterizing the pain that humans experience from lionfish stings in Chapter 2 and sequencing the venom's transcriptome and proteome to identify a pain-causing toxin in the venom in chapter 3, the major question remaining was: how does lionfish venom cause pain? Prior work in my 2018 PAIN paper, outlined in the Introduction (Section 1.5.2.1), showed that the receptor target of the venom was *not* TRPV1 or BK2. From there, I sought to determine which channel was responsible for the activation of nociceptors by the venom.

To remain unbiased and holistic in our approach to identifying how the *venom* causes pain (and not a single toxin), we decided to study the venom as a whole rather than individual toxins. The rationale behind this was that while individual toxins may or may not cause pain on their own, we know that the toxins in lionfish venom act together in concert to cause pain in victims. By studying the mechanism of action of the venom as a whole, we were able to identify a means by which blocking a single channel could significantly interfere with the activation of nociceptors by the venom and reduce pain behaviours in mice.

Chapter 4

Lionfish venom elicits pain through a P2X3-dependent mechanism

Stephanie Mouchbahani-Constance and Reza Sharif-Naeini

In preparation

4.1 Abstract

Lionfish (*Pterois volitans*), an invasive fish species, inflicts one of the most agonizing stings in the ocean. Despite its notoriety, the molecular basis of lionfish venom's pain-inducing mechanism remains elusive. While the venom comprises various toxins, including an algogenic ApoE-like toxin and Zinc 3-finger-toxin, the specific receptor targeted by the venom on nonpeptidergic nociceptors remains unidentified. Understanding this receptor's identity could unravel the pain-causing mechanism and potentially lead to therapeutic interventions. Using primary dorsal root ganglia (DRG) cultures, we demonstrated that lionfish venom triggers two types of nociceptor responses in two different cell types: a sustained influx of calcium and calcium oscillations. We showed that ATP release from large, sustained responders influences neighboring nociceptors' oscillatory responses. Through pharmacological and genetic approaches, we established that lionfish venom activates nociceptors primarily via P2X3 receptors. Pharmacological blockade and genetic knockdown of the channel significantly attenuated venom-induced calcium responses, confirming the receptor's pivotal role. Whole-cell electrophysiology experiments further demonstrated that the venom-induced inward current in nonpeptidergic nociceptors could be blocked by a P2X3 antagonist. Venom-elicited pain behaviours in mice were also attenuated when P2X3 receptors were blocked. This study sheds light on the pain-causing mechanism of lionfish venom, linking it to the activation of P2X3 receptors on nonpeptidergic nociceptors. These data not only expand our understanding of marine venom-induced pain but also propose P2X3 receptors as potential therapeutic targets for mitigating the severe pain inflicted by lionfish stings.

4.2 Introduction

The lionfish (*Pterois volitans*) is an invasive species of fish that produces one of the most painful stings in the ocean. Yet, the pain-causing molecular mechanism of that sting remains

relatively unknown. Lionfish are endemic to the Indo-Pacific, but have invaded the Atlantic Ocean and Mediterranean Sea in the past 30 years and have continued to spread due to lack of natural predators in these regions and a plethora of other factors (Perry 2022). A lionfish sting occurs when a victim's skin is punctured by one of the fish's 18 venomous cartilaginous spines, causing excruciating pain. A prior study by our group characterized the pain qualities of the sting and showed that most victims also experience redness and swelling, with the peak of the pain being reached approximately 1 hour after the sting and mostly being resolved a few days later (Vetrano, Lebowitz, and Marcus 2002; Diaz 2015; Haddad et al. 2015; Mouchbahani-Constance, Choinière, and Sharif-Naeini 2023).

Lionfish venom contains a broad range of different toxins, with an algogenic ApoE-like toxin and a Zinc 3-finger-toxin being the two most prominent transcripts in the venom along with a variety of other toxins that promote inflammatory reactions (Mouchbahani-Constance, Choinière, and Sharif-Naeini 2023). A past study from our group showed that the venom predominantly targets non-peptidergic nociceptors to cause pain (Mouchbahani-Constance et al. 2018). However, the targeted receptor on the membrane of these nonpeptidergic nociceptors remains unknown.

Identifying this receptor would elucidate the pain-causing mechanism of action of lionfish venom, provide insights into the functionality and physiological relevance of its receptor target and potentially lead to a treatment for these stings. Venom toxins have evolved to bind to their target with high efficiency and specificity, making them the perfect tools in the identification and manipulation of molecules in normal physiological processes such as synaptic transmission, haemostasis, receptor activation and action potential propagation (Sajevic, Leonardi, and Križaj 2011; French et al. 2010; Caleo and Schiavo 2009; Brodie 1999; Schmidtko et al. 2010; Dutton and Craik 2001). This target selectivity of toxins has led to discoveries resulting in Nobel prizes

and is particularly attractive in the field of pharmaceutical drugs where there are currently over 16 FDA-approved drugs or therapies derived from animal venom toxins and many more in clinical trial pipelines across the world (Bordon et al. 2020; Harvey 2014; Peigneur and Tytgat 2018; McDermott 2020; King 2011; C. R. Wang et al. 2020; Doyle et al. 1998; Gross and Mackinnon 1996; Lee and MacKinnon 2004; Rendón-Anaya et al. 2012; Swartz and MacKinnon 1997).

In this study, we sought to provide the first insights into the molecular mechanism of action of lionfish venom and showed the venom's dependence on P2X3 receptors for causing pain. P2X3 is a purinergic cationic ion channel, highly expressed on the membrane of nonpeptidergic nociceptors in mice, and is considered a valuable target for treating a variety of pain conditions as well as chronic cough (Giniatullin and Nistri 2013; Fabbretti 2017; Chen et al. 1995; Seguela et al. 1996; Dicpinigaitis, McGarvey, and Canning 2020; Bernier, Ase, and Séguéla 2018; Bele and Fabbretti 2015). Our results demonstrate the first instance of a P2X3-targeting marine venom and show the evolutionary importance of P2X3 in pain sensation.

4.3 Methods

Animals

Experiments were approved by the Institutional Animal Care and Use Committee at McGill University and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and recommendations of the International Association for the Study of Pain. Male 8 to 12 weeks old C57BL/6 mice were used for all experiments, genotype: Cre-dependent GCaMP6f mice (The Jackson Laboratory Strain #024105) were crossed with Trpv1-Cre mice (The Jackson Laboratory Strain #017769). Mice were housed in a temperature-controlled room under a 12-hour light/dark cycle. Water and food were available *ad libitum*. On

the day of experiments, animals were transferred into the testing room and allowed to acclimate in their cage for at least 45 minutes. Experiments were performed in a blinded fashion, so that the experimenter performing the behavioural tests was not aware of the venom concentration or the treatment conditions. Four lionfish (sizes ranging from 13 – 21 cm, purchased from a commercial pet store) were used for venom and tissue extraction. The fish were euthanized on the day of purchase by anesthetic (MS-222, Sigma Aldrich # E10521) overdose and venom and tissue extracted according to methods described below.

Venom preparation

All steps in the venom extraction and purification procedure were based on a published protocol (Akiva S. Cohen 1989) and were performed at 4 ºC. In each of the 4 freshly euthanized lionfish, glandular tissue from the 10 venomous spines was collected into 2 microcentrifuge tubes containing 500 µL of 0.9% NaCl. Tissue was disrupted by homogenizing with a pestle and motorized mixer (VWR # 47747-370) for 15 seconds, followed by a 2-minute centrifugation at 2000*g*. The supernatant was collected in a separate tube, and the pellet was subjected to two additional homogenization and centrifugation steps, with the addition of 500 µL fresh saline solution each time. Before the final centrifugation, the pellet was triturated by passing it 10 times through a 16-gauge needle to maximize tissue disruption. All the supernatants were combined and centrifuged at 100,000 x*g* for 30 minutes. The final supernatant was collected, the protein concentration was measured and adjusted to 2.2 µg/µL (using the Bio-Rad DC assay, Bio-Rad #500011), aliquoted and stored at -80 ºC until being used in experiments.

Primary cell culture of dorsal root ganglia

Male 8-12 week old Trpv1-Cre x GCaMP6f mice were used for dorsal root ganglia (DRG) culture. The culture of DRG neurons was performed as previously described (Mouchbahani-Constance et al. 2018; Petitjean et al. 2020). Briefly, all DRGs were dissected from the mouse and placed in sterile HBSS (Wisent Bioproducts #311-511-CL) at 4 ºC. Enzymatic dissociation was performed with 0.2% type IV collagenase (Sigma Aldrich #c1889) and 0.2% dispase (Sigma Aldrich #D4693) for 90 minutes. DRGs were dissociated mechanically by trituration and plated at low density on glass-bottom dishes (Ibidi #81156) with DMEM/F12 (Wisent Bioproducts #11330-032) supplemented with 10% fetal bovine serum (Wisent Bioproducts #80150) and 1% penicillin/streptomycin (Wisent Bioproducts #450-202-EL) and were maintained in an incubator at 37 ºC with 5% CO_2, with media changes every other day. Neurons were tested 2 to 5 days after plating.

Calcium imaging

Calcium-dependent changes in GCaMP6f fluorescence were monitored by exciting cells at a wavelength of 488 nm and imaged using a Cool-SNAP MYO camera (Photometrics, Tucson, AZ) mounted on an inverted Olympus IX71 microscope. Metafluor software (Molecular Devices Version 7.7) was used for data acquisition and analysis. In every recording, KCl was applied to neurons at the end of the session and only those that responded to KCl were analyzed for their responses to lionfish venom and other agonists. Cells were continuously perfused at a flow rate of 2 to 3 mL/minute with a bath solution composed of 150 mM NaCl, 3 mM KCl, 10 mM Glucose, 10 mM HEPES, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ in distilled water, buffered to a pH of 7.3 with 1N NaOH. Lionfish venom was prepared in bath solution to a concentration of 0.55 μg/μL and was applied to cells locally for a total of 5 seconds through a single-tip multichannel gravity-fed system. Images were acquired every second at 40X magnification. All calcium imaging traces were

background subtracted (using an adjacent region without GCaMP6f signal) and expressed as a change in region of interest fluorescence intensity normalized by baseline fluorescence intensity. Cell responses to puff applications were considered positive when the intracellular calcium concentration rose by at least 20% over baseline values within 10 seconds after the end of the application for sustained responses or between 10 and 30 seconds after the application for oscillating responses. For experiments where drugs were present in the bath solution, drugs were prepared to the below concentrations in the standard extracellular solution daily and were superfused over the cells in place of standard bath solution through the single-tip multichannel pipette and washed out with standard bath solution through another channel of the same pipette.

Compound name	Final concentration	Manufacturer and catalog number
AF-353	300 nM	Cayman Chemicals #23034-1
Thapsigargin	2 μM	Invitrogen #T7458
Apyrase	10 U	Sigma Aldrich #A6535
TNP-ATP	125 nM	Cayman Chemicals #20902-1

Electrophysiology

Neurons were recorded in whole-cell configuration using fire-polished glass pipettes (AM Systems, Glass Borosilicate, 1.5 mm OD, 0.86 ID) with resistances of 5-7 MOhms. The extracellular solution contained 150 mM NaCl, 3 mM KCl, 10 mM Glucose, 10 mM HEPES, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ in distilled water, buffered to a pH of 7.3 with 1N NaOH. Electrodes were filled with a solution containing 140 mM K-gluconate, 10 mM NaCl, 10 mM HEPES, 10

mM glucose, 5 mM BAPTA, 1 mM $CaCl_2$ (pH = 7.3 adjusted with KOH). Cells were observed on an Olympus IX71 inverted microscope with a MultiClamp 700B (Axon CNS, Molecules Devices, Sunnyvale, CA, USA) amplifier and Digidata 1440A (Molecular Devices) digitizer. Membrane current and voltage were acquired and amplified via the MultiClamp and Digidata and were sampled at 10 kHz. Signals were recorded with Clampex10 and MultiClamp 700B software, then analyzed using Clampfit10. For all recordings, pipette and membrane capacitance were compensated for with the auto function of Clampex10. Nonpeptidergic nociceptors were identified using a live cell Isolectin B4 stain conjugated to Alexa 594 (ThermoFisher Cat# I21413). Lionfish venom was applied in the same manner as in calcium imaging experiments above. In experiments with AF-353, 300 nM of AF-353 (Cayman Chemicals #23034-1) was prepared daily in extracellular solution and superfused over the cells in place of standard bath solution for 30 minutes prior to recording and during the cell's entire recording.

In vitro knockdown of P2X3 expression

In genetic knockdown experiments, DRG neurons were treated with complexes of predesigned siRNA directed against mouse P2RX3 (Qiagen Flexitube siRNA) and 2 μL of HiPerFect transfection reagent (Qiagen #301704) 24 hours after they were cultured and plated. The cells were incubated with the transfection complexes for 72 hours with addition of fresh media 24 hours post-transfection.

Spontaneous pain behaviour

Mice were placed in a Plexiglass container on a glass surface for 20 minutes and left to habituate to their environment. They then received an intraperitoneal injection of (100 μL, 2 mg/kg) AF-353 (Cayman Chemicals #23034-1) and were returned to their containers to habituate for 30 more

minutes. The mice were then injected with 20 μL of either saline or 44 μg (20 μL) of lionfish venom into the intraplantar region of their left hind paw. Immediately after the injection, they were returned to their containers and filmed for 1 hour to measure their spontaneous pain behaviour. This was quantified as the amount of time the animal spent licking its injected paw, recorded in 5-minute bins for a total of 12 bins over the hour. (Ashlee H. Rowe Joseph Scales Klaus D. Linse Matthew P. Rowe Theodore R. Cummins Harold H. Zakon 2011; Jun Chen Hui-Li Li Hui-Sheng Chen 1999)

Data analysis and diagrams

All drawings (Figure 1F) were made with Bio-Render. Statistical analyses were performed using Python with the following packages: scipy, pandas, seaborn and numpy. Significant differences were determined using either paired or unpaired t-tests, 1-way or 2-way ANOVAs or Fisher's exact test depending on the experiment with appropriate post hoc tests, described in the results section. The significance threshold was set to $p < 0.05$.

4.4 Results

Lionfish venom elicits a large and sustained influx of calcium in some nociceptors, and calcium oscillations in others

To characterize the mechanism through which lionfish venom causes pain, we first sought to identify the response properties of nociceptors in calcium imaging experiments. We performed primary cell cultures of dorsal root ganglia from Trpv1-Cre x GCaMP6f mice, whereby all nociceptors express GCaMP6f and the indicator is not expressed by non-nociceptor neurons and non-neuronal cells. Upon application of 5 μL of crude lionfish venom, many nociceptors displayed a significant rise in intracellular calcium (Figure 1A); only cells whose fluorescence level rose to

1.2x the cells' baseline fluorescence were considered as responders. These cellular responses could be divided into 2 categories – cells that displayed an oscillating response (whereby the rise in intracellular calcium began within 60 seconds of the venom application and where the fluorescence dropped rapidly following the rise, Figure 1B) and cells that experienced large and sustained responses (whereby the rise in intracellular calcium began within 10 seconds of the venom application, and remained sustained for an extended period of time, Figure 1C).

Lionfish venom elicits release of ATP from large and sustained responding nociceptors to elicits calcium oscillations in neighbouring nociceptors

A prior study on a Brazilian lancehead pit viper venom showed very similar calcium imaging response properties and showed that ATP release underlies the oscillatory nature of some responders (C. Zhang et al. 2017). If ATP was being released by the large sustained responding cells to activate oscillating cells, we hypothesized that an extracellular application of apyrase, an enzyme that catalyzes the degradation of ATP to adenosine and phosphate, would be sufficient to eliminate these oscillations while allowing the large and sustained responses to continue. We pre-incubated dishes of Trpv1-cre x GCaMP6f DRG neurons with 10U of apyrase for 5 minutes prior to imaging and maintained the enzyme in the bath solution during an application of 5 μL of crude lionfish venom. Following this application, apyrase was washed out with standard bath solution and 5 μL of venom were reapplied to the cells 8 minutes after the first application (Figure 1D). In the presence of apyrase. there was a significant reduction in the number of oscillating responders (42/98 (42.86%) in vehicle group, 12/98 (12.24%) in apyrase group) ($p < 0.001$, Fisher's exact test) while the number of sustained responders remained consistent in both the apyrase and vehicle conditions (34/89 (38.20%) in vehicle group, 31/89 (34.83%) in apyrase group) (Figure 1E). Taken together, these data suggest that lionfish venom elicits a large and sustained rise in intracellular

calcium in a subset of nociceptors, which we identified to be the nonpeptidergic nociceptors in a prior study (Mouchbahani-Constance et al. 2018), and that nociceptors then release ATP to activate their neighbouring neurons and elicit an oscillating calcium response from them (Figure 1F). This would amplify the number of neurons responding to the venom and ultimately result in a greater pain response from the sting victim.

Lionfish venom elicits an influx of calcium from the extracellular space to trigger large and sustained responses

To further understand the nature of the large and sustained responses, we sought to determine whether the rise in intracellular calcium that they showed was due to influx of calcium from the extracellular space or due to calcium release from internal stores. We performed calcium imaging experiments on primary cultures of DRG neurons in the presence of 2 μM thapsigargin in the bath solution. Thapsigargin depletes internal stores of calcium by inhibiting the sarco-endoplasmic reticulum calcium ATPase (SERCA) (Jan et al. 1999). In the first few minutes of thapsigargin incubation, we noted a rise and fall in intracellular calcium (Figure 2A), confirming that the drug has successfully depleted endoplasmic reticulum stores of calcium. Following this calcium depletion, lionfish venom was applied to the cells. There was a significant reduction in the duration of the rise in calcium (379.31 ± 21.05 seconds for venom alone (n = 36 cells) , 51.32 ±11.21 seconds for thapsigargin group (n = 43 cells), $p < 0.001$, 1-way ANOVA, Figure 2C), but no significant difference in the amplitude of the response (15.62 ± 5.92, for the venom group, 14.83 ± 3.89, for the thapsigargin group, Figure 2D), suggesting that calcium influx from outside of the cell is responsible for the large rise in intracellular calcium following venom application, but not for the sustained nature of the response. To characterize if this was due to calcium release from internal stores, we performed calcium imaging experiments on primary DRG cultures in Calcium-

free bath solution. Following venom application there was a delay before cells displayed a significant rise in intracellular calcium, which lasted for an extended time (Figure 2B). In the absence of external calcium, DRG neurons showed an extended increase in intracellular calcium (302 ± 98.23 seconds, n = 31 cells, Figure 2C), which was not significantly different from that of standard bath solution, but a significantly smaller amplitude response (2.17 ± 0.42, n = 31 cells, p = 0.0394, 1-way ANOVA, Figure 2D). Overall, this suggests that lionfish venom application onto mouse DRG neurons causes an ion channel to open, which results in a large influx of calcium into the cytoplasm, and this is followed by calcium release from internal stores (through either a second messenger cascade or calcium-induced calcium release) resulting in the sustained nature of the response.

Lionfish venom activates nociceptors in a P2X3-dependent manner

To identify the channel responsible for these large sustained responses, we elected to use a broad purinergic channel antagonist, TNP-ATP, which is a competitive antagonist of all P2X receptors (Burgard et al. 2000). In a prior study, we showed that TRPV1 and BK receptors were not responsible for the activation of nociceptors in calcium imaging experiments, so these were not considered as candidates in the current study (Mouchbahani-Constance et al. 2018). Venom was applied (5 μL) on cells in dishes pre-incubated with 125 nM TNP-ATP for 30 minutes. Eight minutes after the venom application, the drug was washed out with standard bath solution and the venom was re-applied (Figure 2E). In the presence of TNP-ATP, the proportion of nociceptors responding to lionfish venom was significantly reduced from 51.14% (245/479) to 29.59% (50/169) ($p < 0.001$, Fisher's exact test, Figure 2G). To narrow down which P2X channel was responsible for this effect, we used a specific non-competitive P2X3 and P2X2/3 antagonist, since P2X3 is the most prominently expressed P2X channel in nociceptors, AF-353 (Gever et al. 2010).

Venom was applied on cells in dishes pre-incubated with 300 nM AF-353 for 30 minutes and αβMe-ATP (an agonist of P2X3) was applied 3 minutes later to confirm the P2X3 blockade. Eight minutes after the venom application, AF-353 was washed out and the venom was re-applied to the cells (Figure 2F). In the presence of AF-353, the proportion of cells responding to lionfish venom was significantly lower than that of venom alone (from 51.14% (245/479) for venom alone to 16.67% (36/216), $p < 0.0001$, Fisher's exact test, Figure 2G), suggesting that the venom requires P2X3 receptors to activate nociceptors.

To confirm this effect, we used siRNA molecules targeting P2X3 mRNA to genetically knockdown the expression of P2X3 in mouse DRG neurons (Trpv1-cre x GCaMP6f mice). After a 72-hour incubation with the siRNA molecules, cells were imaged to measure their responses to lionfish venom and to αβMe-ATP as a control (Figure 2H). In dishes treated with P2X3-targeting siRNA molecules, there was a significant reduction in the proportion of cells responding to lionfish venom compared to cells in dishes treated with negative control scramble siRNA molecules (17.68% (64/362) in the siRNA group, 43.75% (49/112) in the scramble group, $p < 0.0001$, Fisher's exact test, Figure 2H-J). Notably, in both our genetic knockdown of P2X3 and pharmacological blockades of the channel, calcium oscillations resulting from ATP release (as shown in Figure 1B) were almost completely eliminated, suggesting that P2X3-dependent activation of nociceptors is required for subsequent ATP release and activation of neighbouring, non-P2X3 expressing nociceptors via other P2X or P2Y receptors.

Finally, we sought to identify whether the venom's application elicited a depolarization in nonpeptidergic nociceptors and whether this could be blocked with AF-353. In whole-cell, voltage-clamp electrophysiology experiments on nonpeptidergic nociceptors, we found that a 5 μL application of lionfish venom elicited a large inwards current (1410 ± 249.2 pA) (Figure 2K, left

and Figure 2L) which could be significantly reduced in amplitude in the presence of 300 nM AF-353 (342 ± 148 pA) ($p = 0.0037$, unpaired t-test, Figure 2K, right and Figure 2L).

Pain behaviours elicited by injection of lionfish venom can be reduced with a P2X3 antagonist

To quantify the pain behaviour caused by lionfish venom in mice, we used a method previously validated by our group whereby mice received an intraplantar injection of 20 μL of lionfish venom in their left hind paw and their pain behaviours were quantified over 1 hour following the injection (Mouchbahani-Constance et al. 2018). Mice that received an injection of lionfish venom spent an average of 360.22 seconds (± 33.66 sec) licking their injected paw, with the peak reached at 10 minutes following the injection and reducing over the following 50 minutes (Figure 3A and B, red line and bar). Mice that received an intraperitoneal injection of AF-353 25 minutes before the intraplantar injection of lionfish venom displayed significantly reduced pain behaviours at most time points and overall, in the hour following injection (195.4 ± 38.79 seconds) ($p = 0.0092$, unpaired t-test, Figure 3A and B, blue line and bar). These data suggest that lionfish venom not only activates nonpeptidergic nociceptors via a P2X3-dependent mechanism, but administration of a P2X3 antagonist is sufficient to significantly reduce the pain caused by lionfish venom.

4.5 Discussion

Marine venoms have been markedly understudied in comparison to venoms from snakes, spiders, and other venomous species. Herein, we demonstrate that lionfish venom, one of the most painful venoms in the ocean, elicits pain through a P2X3-dependent mechanism. In this study, we characterize the calcium response properties of mouse nociceptors to lionfish venom, demonstrate that the venom elicits a large, rapidly adapting inward current in nonpeptidergic nociceptors, and show that a P2X3 antagonist, AF-353, is sufficient to block the activation of nociceptors in calcium

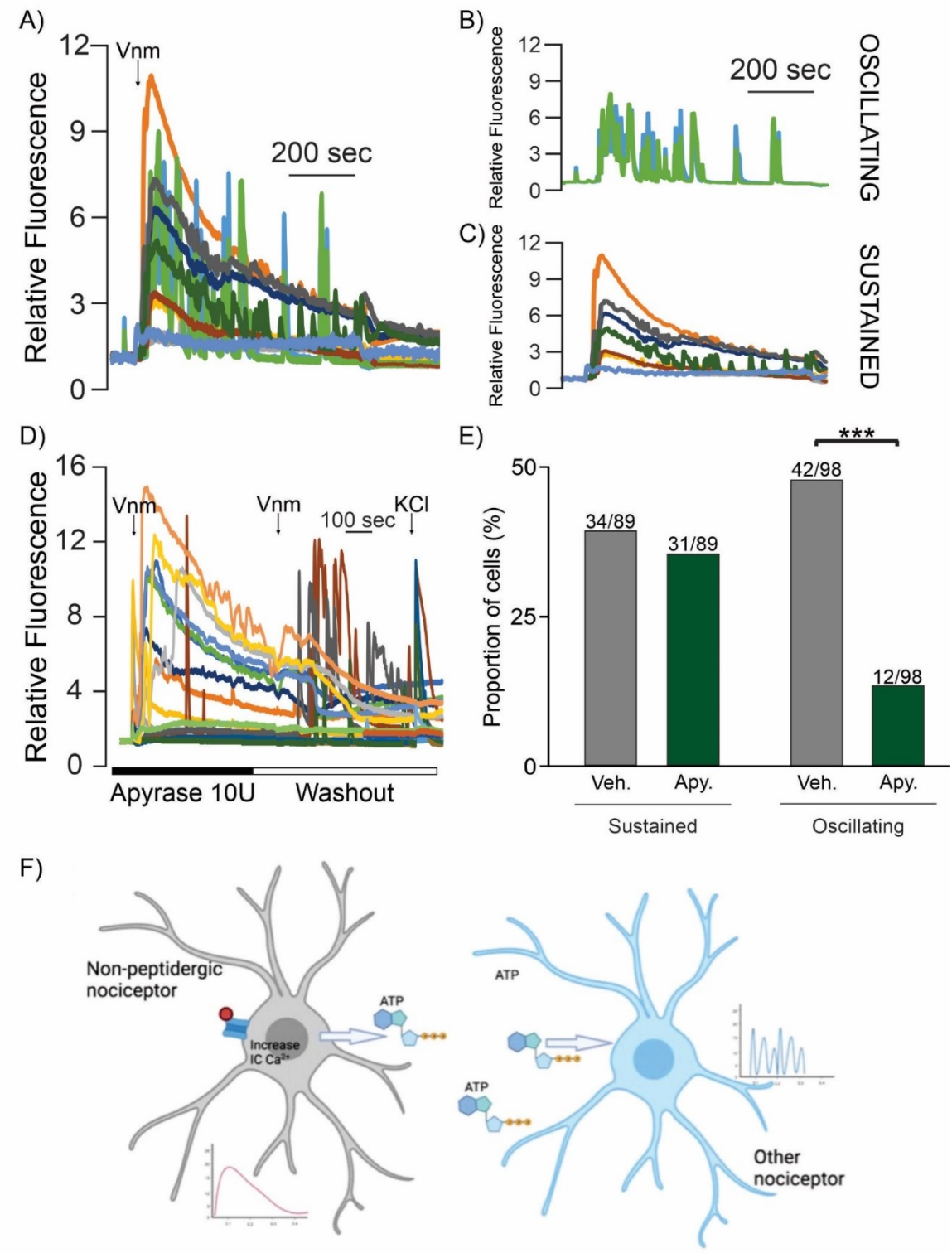
imaging, electrophysiology and behaviour experiments. These data show a clear link between the P2X3 channel and the venom's pain-causing mechanism of action, suggesting that a P2X3 antagonist could be used to block the pain caused by the venom in sting victims.

The venom also seems to have evolved a mechanism to amplify its activation of nociceptors through ATP release, very similarly to the Lys49 myotoxin studied by Zhang and colleagues (C. Zhang et al. 2017). Like our findings, they show that regulated ATP release from a subset of neurons allows for the activation of neighbouring neurons in an oscillatory calcium pattern, thus amplifying the toxin's original target. Lionfish venom first activates nonpeptidergic nociceptors, eliciting a large and sustained rise in intracellular calcium, during which these cells release ATP onto neighbouring cells to cause oscillating increases in intracellular calcium to presumably amplify the pain experienced by sting victims. The mechanism of ATP release from non-peptidergic nociceptors by lionfish venom remains unknown, however Zhang et al. found that following activation by the Lys49 myotoxin, neurons upregulated their expression of pannexin hemichannels to allow for ATP release (C. Zhang et al. 2017). This may be the mechanism of action of lionfish venom, or perhaps through vesicular release or other large channels at the membrane.

P2X3 is notable in its expression pattern in mice sensory neurons where it is reported to have high expression in nonpeptidergic nociceptors and relatively low expression in peptidergic nociceptors (Usoskin et al. 2014) and has been the target of clinical research development for pain, chronic cough, bladder incontinence and hypertension treatments (Burnstock 2002). The toxin responsible for the venom's P2X3-based activity would likely be highly valuable in informing drug design processes and in further understanding P2X3's physiological properties. Furthermore, lionfish venom is the first venom found to contain toxins that activate sensory neurons via P2X3;

only a select few toxins have been found to antagonize this channel (Kabanova et al. 2012). Finally, it is possible that the toxin responsible for this P2X3 activity is a member of a broader P2X3-targeting toxin family present in some subset of aquatic species, which would provide a compelling impetus for further study of this venom's toxins.

4.6 Figures


A)
Relative Fluorescence
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OSCILLATING
C)
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100 sec
KCl
Apyrase 10U
Washout
E)
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Proportion of cells (%)
34/89
31/89
42/98
12/98
Veh.
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Veh.
Apy.
Sustained
Oscillating
F)
Non-peptidergic nociceptor
ATP
Increase IC Ca2+
ATP
ATP
Other nociceptor

Figure 1 – Degrading extracellular ATP reduces oscillating calcium responses to lionfish venom. A) Sample trace of calcium imaging performed on mouse DRG neurons from Trpv1-Cre x GCaMP6f mice after application of 5 μL of lionfish venom. Calcium was tracked using GCaMP6f fluorescence. Each line represents a different cell within one region of interest and each cell's fluorescence was normalized to its own baseline values from the first 30 seconds of the recording. B) Cells from imaging session presented in panel A where cells showed an increase (≥ 1.2x baseline) in intracellular calcium in the 60 seconds following venom application. C) Cells from imaging session presented in panel A where cells shown had an increase (≥ 1.2x baseline) in intracellular calcium during the first 10 seconds following venom application. D) Sample trace of calcium imaging performed on mouse DRG neurons (GCaMP6f) with an application of 5 μL of lionfish venom while apyrase (10U) is present in the bath solution, followed by a washout of apyrase with regular bath solution and re-application of lionfish venom. E) Quantification of number of cells responding to lionfish venom with either a sustained or oscillating calcium response in the presence of apyrase (green bars) or in the presence of regular bath solution (grey bars). * $p < 0.05$, Fisher's exact test. F) Model of hypothesized mechanism of action of lionfish venom (created with BioRender).

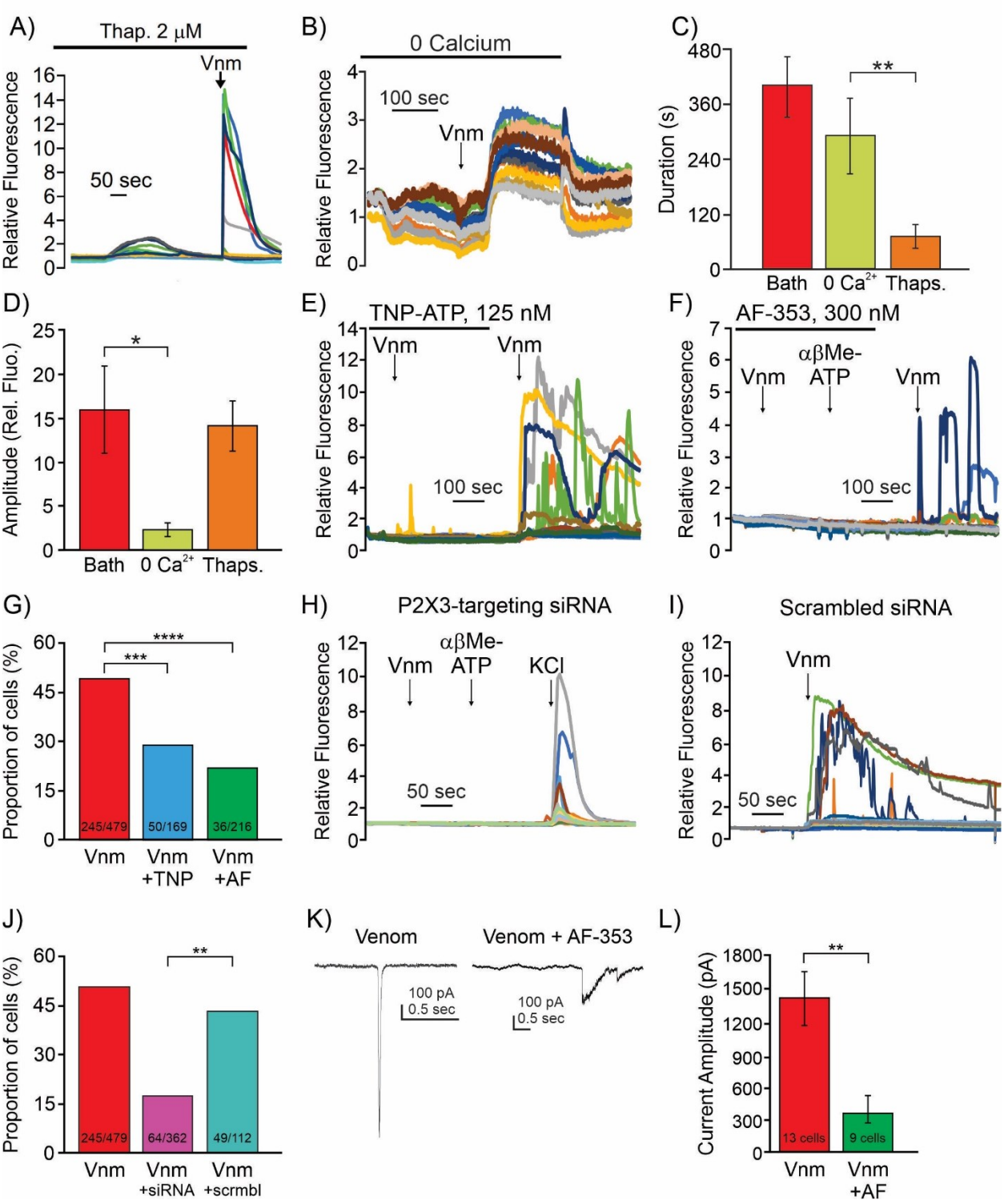

Figure 2 – Lionfish venom activates non-peptidergic nociceptors through activation of P2X3. A) Application of lionfish venom (5 μL) in the presence of thapsigargin (2 μM, prepared in bath

solution) results in a shortened rise in internal calcium in mouse DRG neurons. B) Application of lionfish venom (5 μL) in bath solution with 0 mM of calcium results in a small and delayed rise in internal calcium. Note difference in y-axis scale. C) Quantification of mean (±SEM) duration of calcium response following application of lionfish venom in normal bath solution (grey bar), 0 mM calcium solution (blue bar) and thapsigargin-containing bath solution (orange bar). D) Quantification of mean (±SEM) peak amplitude of calcium response following application of lionfish venom in normal bath (grey bar), 0 mM calcium solution (blue bar) and thapsigargin-containing bath solution (orange bar). E) Sample trace of calcium imaging on nociceptors pre-incubated (30 minutes) with 125 nM TNP-ATP (prepared in bath solution) after application of lionfish venom (5 μL). F) Sample trace of calcium imaging on nociceptors pre-incubated with 300 nM after application of lionfish venom (5 μL) and αβMe-ATP (5 μL, 100 uM). G) Quantification of the proportion of imaged nociceptors that are activated by lionfish venom in regular bath solution (red bar), in the presence of TNP-ATP (blue bar) and in the presence of AF-353 (green bar). *** $p < 0.001$. Fisher's exact test. H) Calcium trace of mouse nociceptors 72 hours after being treated with P2X3-targeting siRNA molecules with applications of lionfish venom (5 μL), αβMe-ATP (5 μL, 100 μM) and KCl (30 mM). I) Calcium trace of mouse nociceptors 72 hours after being treated with scrambled, non-targeting siRNA molecules with application of lionfish venom (5 μL). J) Quantification of the proportion of fluorescent nociceptors responding to lionfish venom in cells untreated with siRNA molecules (red bar), cells treated with P2X3-targeting siRNA molecules (green bar) and cells treated with non-targeting, scrambled siRNA molecules (blue bar). ** $p < 0.01$, Fisher's exact test. K) Example trace of voltage-clamp (-70 mV) recording in whole-cell of nonpeptidergic nociceptor after application of 5 μL of lionfish venom (left) and after application of lionfish venom on a cell pre-incubated with 300 nM AF-353. L) Mean (±SEM)

current amplitude of currents elicited by lionfish venom alone (green bars) or in the presence of 300 nM AF-353 (red bars). ** $p < 0.01$, Unpaired t-test.

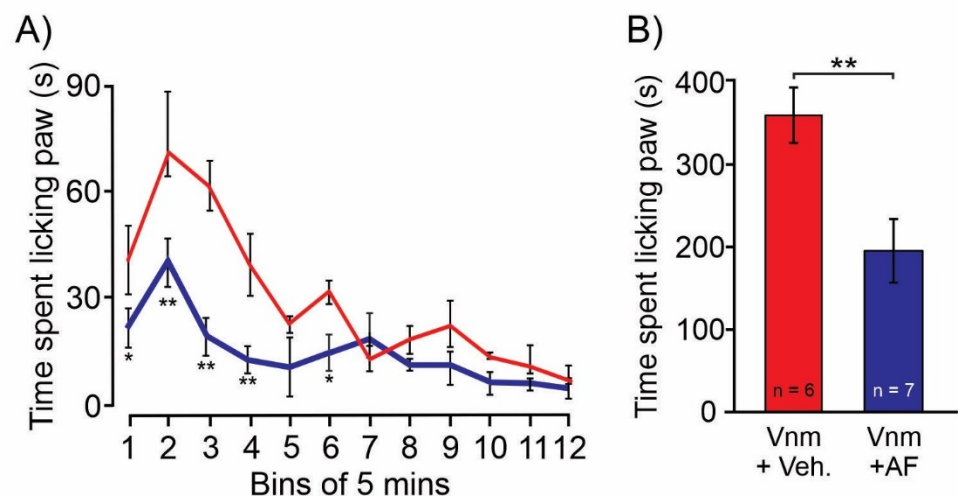

Figure 3 – Local administration of AF-353 can significantly reduce the pain behaviour elicited by lionfish venom. A) Mean (±SEM) duration of spontaneous pain behaviour assessed for 1 hour (divided into bins of 5 minutes) after intraplantar injection of venom (red) and after intraplantar injection of AF-353 immediately after intraplantar injection of venom (blue). * $p < 0.05$, ** $p < 0.01$, 2-way ANOVA. B) Sum of the time spent displaying spontaneous pain behaviour in the 1 hour immediately after venom injection. ** $p < 0.01$, unpaired t-test.

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Chapter 5 – General discussion

5.1 Discussion of Chapter 2

5.1.1 Study limitations

In the literature surrounding lionfish stings, there exist numerous case studies of extreme lionfish stings in a variety of different geographical areas where the lionfish is invasive. Across all these studies, there was a clear area that lacked exploration – what is the average experience of a lionfish sting? Having spoken to nearly 100 victims of lionfish stings prior to this study, I was struck by the similarities across their experiences of lionfish stings and sought to quantify the experience that *most* people have once being stung by a lionfish, since it is rarely as extreme and those described in case studies. With this in mind, I designed a clinical pain questionnaire with Dr. Manon Choinière to capture this data from lionfish sting victims.

Our study sought to quantify as many variables as possible surrounding a lionfish sting: pain intensity, pain duration, pain qualities, location of the sting, the activity performed while the victim was stung, the victim's age and gender, interference with daily activities and past experiences with lionfish stings. Due to the nature of our study, we had to depend on the ability of each of our participants to accurately recall their sting experience, which may have introduced some recall bias into our data. For this reason, we limited study participants to those having experienced a lionfish sting within the past 10 years. However, there may still have been some bias in recalling pain intensities, duration, and qualities.

An analysis that could have added value to our study, and that a future study may aim to do, would be to try and develop a model for predicting the most severe lionfish sting reactions. This could be done by looking at the top 10% of most severe lionfish sting cases and develop a regression model of the risk factors we analyzed to see which may correlate with a high likelihood for a severe sting reaction. Such a model could be used clinically to screen patients presenting in

emergency rooms with severe cases of lionfish envenomations to determine which may be at risk for highly severe reactions (such as tissue necrosis or shock) and begin treatment earlier.

5.2 Discussion of Chapter 3

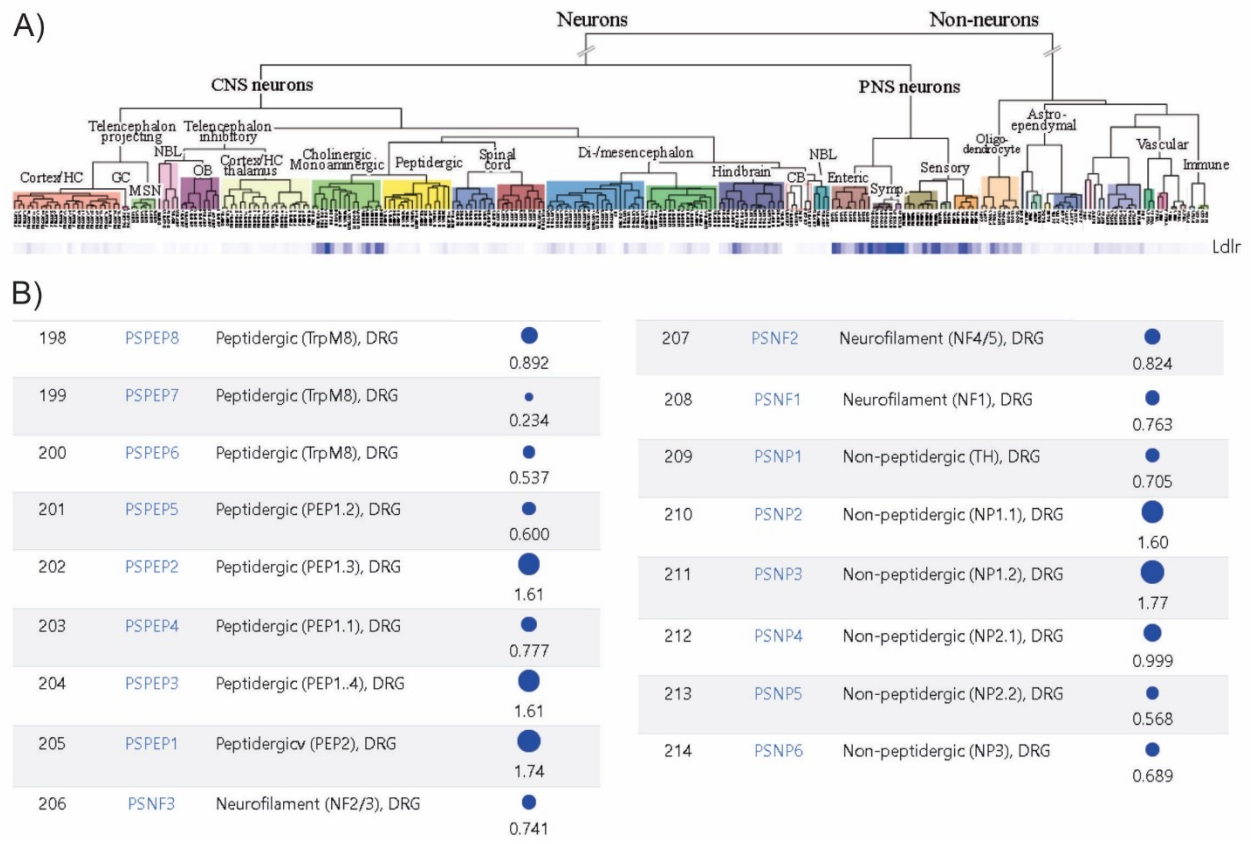
5.2.1 Insights into ApoE and the potential mechanism of action of the ApoE-like toxin

ApoE is a molecule that has been shown to be intrinsically linked with chronic pain, and yet, the mechanism through which it is involved in pain has remained quite unknown (Tansley et al. 2022). It is the top upregulated gene in spinal cord microglia after peripheral nerve injury, and isoforms of the APOE gene are associated with chronic pain (Tansley et al. 2022). The fact that one of the most abundant toxins in lionfish venom is such a close match to this molecule is, in my opinion, likely not a coincidence. Lionfish venom specifically evolved to cause pain and not to induce predatory outcomes such as paralysis or death that some other venoms produce (Walker 2020). I hypothesize that perhaps because ApoE is so intrinsic to pain sensation, lionfish evolved a toxin to mimic this molecule to exert maximal pain in its victims. This evolution may have taken place through a number of processes, be it convergent evolution, the "accidental" appearance of a toxin with a very similar sequence to ApoE due to the necessity to include ApoE in lionfish venom but with no common evolutionary ancestor, or some sort of adaptive evolution, whereby there is a common ancestor between this ApoE-like toxin and ApoE itself from fish (via neofunctionalization, described in the Introduction Section 1.4.1). ApoE is a highly conserved molecule, with orthologs even being identified in insects and bacteria, so the latter hypothesis may not be such a stretch (Huebbe and Rimbach 2017).

In terms of a potential mechanism of action for this ApoE-like toxin, it is clear to me that the toxin does not activate nociceptors via the P2X3-dependent mechanism identified in chapter 4 (which I discuss in section 5.3.1 of this discussion). So how might this toxin activate nociceptors

and cause pain in sting victims? As I mentioned in the introduction of this thesis, ApoE is an amphipathic plasma protein whose main function is to bind to membrane receptors to mediate lipid transfer between circulating lipoproteins and target tissues. Once it binds to lipids, it undergoes a major conformational change and becomes a ligand for the LDL receptor family, which is highly expressed on all DRG neuron types and lowly expressed on satellite glia (Huebbe and Rimbach 2017; Usoskin et al. 2014). The interaction between ApoE and the LDL receptor family has been well characterized, but downstream effects of this interaction remain poorly understood (Sheikh et al. 2019). Discussion Figure 1 is a screenshot from the searchable single-cell sequencing database of the mouse nervous system from Sten Linnarsson's group, Mousebrain.org, which indicates high expression levels of LDLR across PNS neurons and cholinergic monoaminergic neurons in the CNS (Discussion Figure 1A) and high expression levels across all DRG neuron subtypes, but especially high in some nociceptor subtypes (PEP1.3, PEP1.4, PEP2, NP1.1 and NP1.2) (Discussion Figure 1B). Given this high expression of the LDL receptor on nociceptors, I would then hypothesize that there may be some interaction with the ApoE-like toxin and some lipid, either present in the tissue or present in the venom, allowing the toxin to bind to the LDL receptor. Upon LDL receptor binding, there would be downstream activation of second messengers due to either the imported lipid itself or due to some downstream cascades triggered by the LDL receptor. The mass spectrometry analyses that were performed as part of my thesis were all focused on identifying protein components, but an interesting experiment that could be performed would be a lipidomic analysis of lionfish venom using mass spectrometry to characterize any lipid components in the venom. An *in silico* analysis of the lipid structures could also be performed to determine whether it would be possible for them to interact with ApoE and allow it to bind to an LDL receptor. Interactions have been characterized between LDL receptors

and NDMA subunits through their cytoplasmic domains with the scaffold protein Psd95, which is broadly expressed across the entire nervous system (Beffert, Stolt, and Herz 2004; Gotthardt et al. 2000; Kornau et al. 1995). It may be that this ApoE-like toxin results in indirect activation of NMDA receptors, ultimately causing depolarization of nociceptors and initiation of the pain signal.



Discussion Figure 1. The LDL receptor is highly expressed across PNS neurons, including high expression in DRG neurons, screenshotted from Mousebrain.org and which uses data from (Usoskin et al. 2014). A) Heat map of LDLR gene expression across all neurons in the nervous system, dark blue indicates high expression and lighter shades of blue indicate lower levels of expression. B) Relative expression levels of LDLR in specific subtypes of sensory neurons in the DRG, notice highest expression in PEP1.3, PEP1.4, PEP2, NP1.1 and NP1.2 subtypes, all of which are nociceptors.

5.2.2 Other toxins of interest in *P. volitans* venom

Venoms are known to be cocktails of hundreds and sometimes thousands of toxins, with each toxin performing a unique but essential role for the venom's overall effect on its victims. In chapter 3, I chose to focus on a few individual toxins to try and synthesize and study them in isolation, but the reality is that we found 143 putative toxin proteins in our analysis of *P. volitans* venom, and much remains to be understood about the role of these toxins in the venom.

Other toxins of interest that were detected included the lionfish's homolog of stonustoxin and neoverrucotoxin, respectively from *Synanceia* (stonefish) and *Scorpaena* (scorpionfish). This toxin in lionfish venom has been reported to likely play a role in the pain and discomfort that lionfish sting victims experience, but our findings contradict this hypothesis. The lionfish's homolog of these toxins does not contain a signal peptide. Signal peptides are essential components of a secreted protein – they are short amino acid sequences on the amino terminus of the protein which targets the protein into the membrane or across it (de Oliveira et al. 2018; Ziegman et al. 2019; Silva et al. 2018; Stern et al. 2007). An important step in filtering detected proteins in a venom is to filter based on whether they contain a signal peptide, since toxin proteins must be secreted by venom gland cells in order to find themselves in the venom. One may ask themselves, why would a toxin without a signal peptide ever have been considered a toxin in the first place? The answer lies in the technique used to identify this toxin. The group used a biased cDNA cloning-based approach to identify this toxin in lionfish venom, using primers matching conserved domains of stonustoxin and neoverrucotoxin to clone a homolog in lionfish venom (U. C. de Oliveira et al. 2018; Ziegman et al. 2019; Silva et al. 2018; Stern et al. 2007). However, there was never an attempt to confirm the protein's functionality, presence in crude venom or its ability to cause pain. This was a motivation for our unbiased transcriptome/proteome-based approach to identifying toxins in lionfish venom. By holding back any biases on identifying particular toxins in the venom

and simply identifying all the ones present in the venom and later analyzing their potential functions, we avoided identifying "red herring"-type toxins in our study.

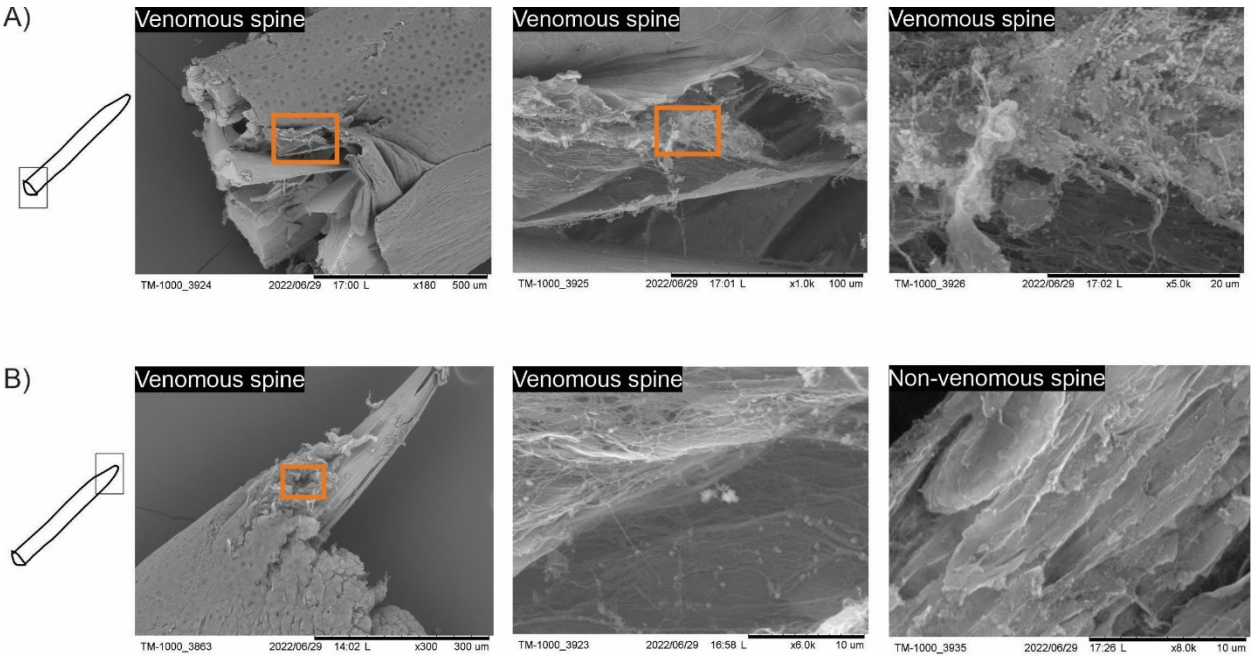
Interestingly, we identified hyaluronidases as being a major component of *P. volitans* venom at both the protein and transcript level. Typically, hyaluronidase plays an important role in a venom to help break down hyaluronan present in the extracellular matrix, reducing the extracellular matrix's viscosity and allows toxins to more easily penetrate tissues and bind to their targets to exert their final effects (D. Oliveira et al. 2018; Ziegman et al. 2019; F. Silva et al. 2018; Stern et al. 2007). Our detection of relatively large amounts of hyaluronidase in lionfish venom confirms findings from a study performed by Kiriake et al. on, ironically, cloned hyaluronidases from lionfish venom, and shows that not only does the lionfish venom contain functional hyaluronidases, but they may play an important role in lionfish venom (Kiriake, Shiomi, and Madokoro 2014). This finding is also in agreement with prior studies of hyaluronidases in stingray venom, which hypothesized that fish venoms have likely undergone a convergent evolution of hyaluronidase toxins (F. Silva et al. 2018).

5.2.3 How is lionfish venom produced?

An interesting consideration that I did not explore in this thesis is how the lionfish produces its venom. Typically, venomous animals have clear venom glands or venom-producing structures, but these structures have yet to be identified in the lionfish. There are many ways one could begin by answering this question, the first is using scanning electron microscopy to examine the structures of lionfish spines and to identify whether structures exist within venomous spines that are not present within non-venomous spines; these structures might then play a role in venom production or stabilization. I fixed and dehydrated a few venomous and non-venomous lionfish spines to image them with a scanning electron microscope and did in fact identify a structure

present in venomous but not in non-venomous spines. However, this experiment was only performed with spines from one lionfish, so the data was not included in any manuscript, but it is interesting to discuss, nonetheless. Discussion Figure 2 shows some images taken from the spines. In panel A, images were taken of a cross section of skin and underlying tissue and cartilage from the base of a venomous lionfish spine. At 1000x resolution, it is clear that there seems to be some granules tangled with the tissue fibers, which I assume to be collagen or collagenous in nature (due to its high content in the transcriptome and venom extract, but it may be another protein). At 5000x resolution, these granules become more apparent – they are roughly 1 μm in diameter and are extremely ubiquitous throughout the tissue within the venomous spine. They are also present throughout the spine, as can be seen in the left and middle panels of Discussion Figure 2B, which show a 300x and 6000x image of the cross section of the tip of a lionfish spine. However, in the right panel of Discussion Figure 2B, one can clearly see the difference in the composition of the tissue within the non-venomous spine. It is much less fibrous and contains no granules. Whether these granules truly play a role in the production or storage of lionfish venom is still unknown, but I hypothesize that these granules may be a production and storage mechanism that the lionfish evolved for its venom. It has been shown before that some snake venoms are secreted through snake venom gland secretory vesicles, which are specialized structures forms of lysosomes (Nicolau et al. 2017). I hypothesize that the venom may be produced by lionfish venom gland cells and secreted in these highly stable vesicle-like structures, which remain intact until the lionfish stings a victim, and the shear stress on the skin surrounding the venomous spine pushes down on the membrane of these vesicles, bursting them and allowing their contents to be freed within the venom. This hypothesis is supported in part by our findings in a prior study (and confirmed by others) that the lionfish venom is highly heat-sensitive and in fact breaks down quite quickly

(within a few hours) at room temperature (Mouchbahani-Constance et al. 2018). Furthermore, as reported in Chapter 2, victims of lionfish stings who were stung by dead lionfish instead of live ones experience much less pain. The time the fish spends at room temperature (and during which it is not producing new venom vesicles since it has died) may result in the degradation of some of these vesicles and ultimately a lower quantity of venom proteins being injected into the victims of dead lionfish stings.



Discussion Figure 2. The venomous spines of *P. volitans* contain granules, which are not present in the fish's non-venomous spines. A) Illustration of image location relative to lionfish spine. Left, 180x zoom image of base of a venomous *P. volitans* spine, orange box indicates zoomed-in area for next picture. Middle, 1000x zoom image of the venomous spine tissue, orange box indicates zoomed-in area for picture on the right. Right, 5000x zoom image of the venomous spine tissue, with granules visible. B) Illustration of image location relative to lionfish spine. Left, 180x zoom image of tip of a venomous *P. volitans* spine, orange box indicates zoomed-in area for next picture. Middle, 5000x zoom image of the venomous spine tissue. Right, 5000x zoom image of the tissue within the tip of a non-venomous *P. volitans* spine.

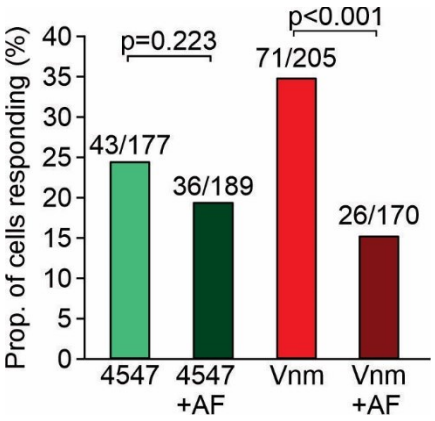
Another experiment which could shine a light on the lionfish's venom-producing structures would be a simple hematoxylin and eosin stain of a cross-section of a lionfish spine. This could indicate cellular densities at a particular height within the spine (for example, at its base) and may point to an area where venom-producing cells cluster. Using RNAScope, one could even design probes against the RNA sequences encoding the most abundant toxins in lionfish venom and perform an RNAscope experiment on these cells to conclusively see which express the transcripts to produce toxins and thus, the venom itself.

5.3 Discussion of Chapter 4

5.3.1 The ApoE-like toxin does not activate nociceptors via P2X3

An obvious question I asked myself once identifying the ApoE-like toxin in lionfish venom and observing the pain responses it elicited in mice was whether this was *the* toxin responsible for activating nociceptors via P2X3. To answer this question, I designed a simple experiment where I created a plasmid expressing both GCaMP7b and human P2X3, whereby cells transfected with this plasmid would express both P2X3 and GCaMP7b, allowing them to be used as an easy screen for a toxin's ability to activate P2X3 in calcium imaging experiments. Using the same High Content Screening system used in Chapter 3, I screened HEK293T cells transfected with my plasmid for their ability to be activated by lionfish venom, venom in the presence of AF-353, the ApoE-like toxin (4547 in Discussion Figure 3) and the ApoE-like toxin in the presence of AF-353. Ideally, if the toxin were responsible for the cells' activation via P2X3, many cells exposed to the toxin alone would show a rise in intracellular calcium, but a significantly smaller proportion of them would be activated by the toxin in the presence of the P2X3 antagonist AF-353. Unfortunately, this was what I observed in the cells exposed to crude venom – many cells that were exposed to venom alone showed a rise in intracellular calcium and significantly fewer

responded in the AF-353 group (Discussion Figure 3, bright and dark red bars) – but this was not the case in the 4547-toxin group. Many cells that were exposed to the toxin did show a rise in intracellular calcium, albeit a smaller proportion than those exposed to crude lionfish venom, but there was no significant reduction in the proportion of cells responding in the presence of AF-353 (Discussion Figure 3, bright and dark green bars). This would suggest that the ApoE-like toxin does not activate cells via P2X3, but rather through another still unknown mechanism.



Discussion figure 3. The *P. volitans* ApoE-like toxin does not activate nociceptors via P2X3. Quantification of number of mouse DRG neurons that showed a significant rise in intracellular calcium (1.2x baseline or greater) following application of DN4547 toxin (light green bar), and application of the toxin on cells pre-incubated with 300 nM AF-353 (dark green bar), or following application of crude lionfish venom (light red bar) and application of the venom on cells pre-incubated with AF-353 (dark red bar). $P < 0.001$, Fisher's exact test

5.3.2 How might activation of P2X3 by *P. volitans* venom lead to ATP release?

One of the findings that surprised me the most in this thesis is the fact that somehow, activation of nonpeptidergic nociceptors by lionfish venom induces ATP release and subsequent activation of neighbouring sensory neurons (and most likely also non-neuronal cells) via other P2X and P2Y receptors. This phenomenon is not unique to lionfish – in fact I first heard of it due to findings published in a paper from David Julius' group studying a toxin names Lys49 from a

Brazilian pit viper (C. Zhang et al. 2017). In their study, Zhang et al. found that the Lys49 toxin activated nociceptors (through some still unidentified pathway), these cells then experienced a large and sustained rise in intracellular calcium and recruited Pannexin1 hemichannels to their membrane to release ATP onto neighbouring cells. Whether the release of ATP is a process unique to the toxin's target, or a coincidence is unclear, but I think that this is a mechanism whereby venoms can amplify their effect and activate more cells.

Another question that arises is how does the cell release ATP? Is it through recruitment of Pannexin hemichannels, as was observed in Zhang et al.'s paper, or is it through vesicular ATP release? This question could be answered through a rather simple experiment using the dye molecule YO-PRO-1. This dye molecule is too large to cross the cell membrane alone unless a large pore is opened on the membrane, so having YO-PRO-1 present in the bath solution while lionfish venom is applied to a primary cell culture of DRG neurons would, in the case of a hemichannel like Pannexin1 opening on the membrane, flow into nonpeptidergic neurons right before ATP is released to activate neighbouring cells. Experimentally, one would observe the activated nonpeptidergic nociceptors turning green (the colour of the YO-PRO-1 dye) under a fluorescent microscope following their activation when the pore opens and ATP flows out of the cell (C. Zhang et al. 2017). If the cell does *not* take up the YO-PRO-1 dye, then it is in an indication that no pore has opened on the cell's membrane and most likely, the cell is releasing ATP via vesicular release. To confirm whether ATP is being released via vesicles, one could block or knock down the vesicular nucleotide transporter V-NUT, which has been shown to mediate the loading of ATP into synaptic vesicles of neurons in the hippocampus (Larsson et al. 2012).

If it is through Pannexins, then how would the Pannexin channel be recruited to the membrane by P2X3? The P2X3 receptor scaffold CASK stabilized P2X3 receptors at the

membrane and controls their turnover. However, upon repeated stimulations with the specific P2X3 agonist αβMe-ATP, the P2X3 receptor becomes dissociated from CASK, and CASK recruits Pannexin1 to the membrane to release ATP onto neighbouring cells and amplify the P2X3-mediated response to other cells (Gnanasekaran et al. 2013; Bele and Fabbretti 2015). With this in mind, I think that strong activation of P2X3 by lionfish venom results in the dissociation of P2X3 from CASK at the membrane, causing the recruitment of Pannexin1 by CASK to release ATP onto neighbours.

5.3.3 Potentiation of venom-induced P2X3-mediated currents

While there were no specific venom components identified that directly potentiate P2X3 currents, there may have been some potentiation of P2X3-mediated currents, and ultimately pain, by other inflammatory components that may be released due to the venom's activity. Prostaglandin E2 (PGE2) is a well characterized marker of inflammation that is known to enhance the excitability of DRG neurons and would likely be released by affected tissue following lionfish envenomation. PGE2 potentiates P2X3 through the following mechanism: PGE2 binds to and activates EP3 receptors (PGE2 receptor 3) which results in the activation of the cAMP/PKA signaling pathway, PKA increases calcineurin activity which dephosphorylates P2X3 and ultimately enhances P2X3-mediated responses (C. Wang, Li, and Huang 2007; C. Wang et al. 2007; Nair et al. 2010). Taken together, I think it is likely that the venom's inflammatory components trigger PGE2 release from envenomated tissues, potentiating P2X3-mediated currents and ultimately increasing the amount of pain the sting victim experiences.

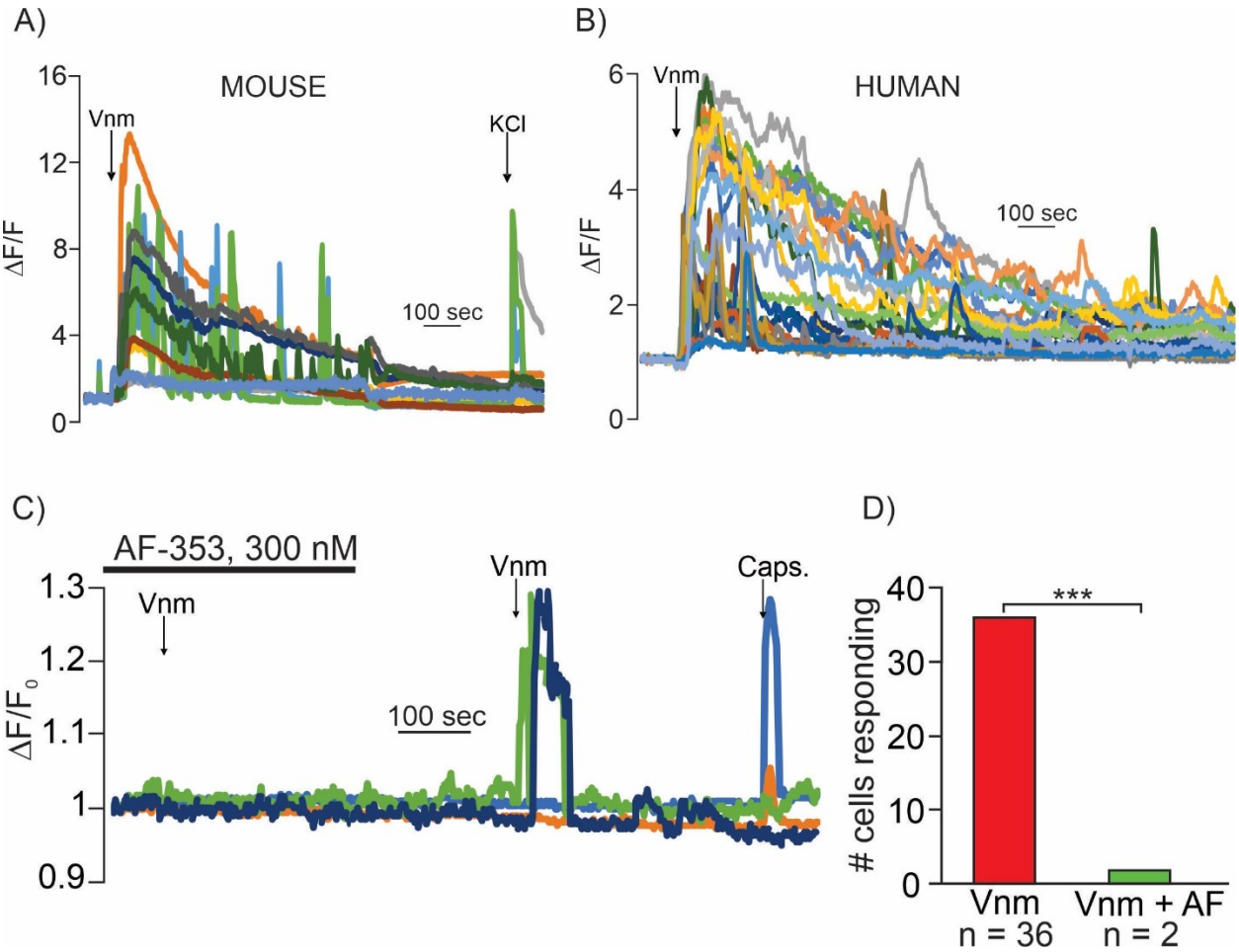
5.3.4 Could P2X2/3 heterotrimers be another candidate for *P. volitans* venom activation?

The main antagonist I used in my experiments was AF-353, which is known to block both P2X3 channels and P2X2/3 heteromeric channels (Gever et al. 2010). Upon successfully blocking the venom-elicited responses with AF-353, I focused specifically on the P2X3 channel, and not the P2X2/3 channel for the following reasons.

Firstly, in order for P2X2/3 heterotrimers to form, a cell must express both the P2X3 and P2X2 gene, and upon translation of the subunits, they will associate and form P2X2/3 heterotrimers (as opposed to the canonical P2X3 or P2X2 homomers) (Brederson and Jarvis 2008). In rats, for example, this is the case in a subset of sensory neurons. However, evidence from *in situ* hybridization and electrophysiology studies of primate DRG tissue has shown that cells expressing P2X3 tend to express little to no P2X2 mRNA and the currents elicited in these cells matched the rapidly desensitizing currents of P2X3 channels and not the slowly desensitizing currents of P2X2/3 heteromeric channels (Serrano et al. 2012). Taken together, these data suggest that these P2X2/3 heteromers may not exist in primates (Serrano et al. 2012) and since humans are very clearly in pain following lionfish envenomations, I decided to focus on the only candidate that was reliably expressed in humans – P2X3.

Furthermore, over the course of my PhD, I adapted and implemented a technique in our lab of culturing human DRG neurons with tissue obtained from organ donors and sought to confirm my mouse findings on human nociceptors. On a few dishes of primary cell cultures of human DRG neurons in two experiments, I applied lionfish venom and observed very similar neuronal response profiles in human DRG neurons to those that I observe in mouse nociceptors (see Discussion figure 4A and B). Upon incubation with AF-353, I was able to significantly reduce the number of responding neurons (see Discussion Figure 4C and D), indicating that the venom activates human

nociceptors in P2X-dependent mechanism that can be blocked using AF-353, leaving only 1 candidate: P2X3.



Discussion Figure 4: *P. volitans* venom activates human nociceptors via P2X3. A) Sample calcium trace of a primary culture of mouse DRG neurons (Trpv1-Cre x GCaMP6f mice). B) Sample calcium trace of a primary culture of human DRG neurons incubated with the calcium indicator dye Fura-2AM. C) sample calcium trace of human DRG neurons with application of *P. volitans* venom (and capsaicin) pre-incubated with 300 nM AF-353. D) Quantification of number of human DRG neurons that showed a significant rise in intracellular calcium (1.2x baseline or greater) following application of *P. volitans* venom (red bar) and pre-incubated with AF-353 and following application of *P. volitans* venom (green bar). *** $p < 0.001$ Fisher's exact test.

5.3.5 How are the lionfish's predators resistant to its venom?

In nature, prey animals tend to develop adaptations that will allow them to avoid being eaten, while predators will develop adaptations that make them more effective at catching prey. Similarly, prey species tend to develop venoms that protect them for predators, typically inflicting significant pain to prevent the animal from being eaten and for the predator to remember not to try and eat the animal in the future, while predatory venoms tend to aim to immobilize prey by either killing or paralyzing it (Calvete 2017; Dawkins and Krebs 1979). The lionfish seems to have evolved venom to protect itself from being eaten, and this strategy has been highly successful in the areas it has become invasive such as the Caribbean, Gulf of Mexico and Mediterranean, since the fish has no predators there. However, in the Indo-Pacific, the lionfish's natural home, the lionfish is not invasive because it does have predators, who have somehow evolved a resistance mechanism to the lionfish's venom. There are a few ways that a species can become resistant to another species' venom: target site insensitivity, a toxin sponge, off-target repurposing or a physical resistance to being penetrated by a venomous spine. In this section of the discussion, I will explore these possibilities, and my efforts to determine whether target site insensitivity is at play for one of the lionfish's predators, *Gymnothorax favagineus* (honeycomb moray eel).

In the case of a toxin sponge, the stung species would possess a molecule in its body (these can appear anywhere, but typically are molecules present in the circulatory system) that can bind to toxin molecules and sequester them, preventing them from binding to their target and causing damage. An example of this is the saxiphilin molecule from *Dendrobates tinctorius*, a poisonous frog, which is able to rescue voltage-gated sodium channels from saxitoxin poisoning (saxitoxin blocks voltage-gated sodium channels) by binding to saxitoxin directly and sequestering it from the channels, preventing them from being blocked (Abderemane-Ali et al. 2021).

Off-target repurposing would require the stung species to have evolved a *new* target for the venom's toxin with a higher affinity than the original one. Grasshopper mice have developed a resistance to the painful venom of bark scorpions using this strategy. Typically, the venom from bark scorpions causes pain by activating $Na_v1.7$, but grasshopper mice have evolved a mutation in the extracellular and pore regions of $Na_v1.8$ providing the channel with a higher affinity for the toxin than its $Na_v1.7$ channel, and upon binding, the toxin instead *inactivates* $Na_v1.8$ instead of activating it, providing the mouse with pain relief instead of pain (Rowe and Rowe 2015; Rowe et al. 2013; Ashlee H. Rowe Joseph Scales Klaus D. Linse Matthew P. Rowe Theodore R. Cummins Harold H. Zakon 2011; Rowe and Rowe 2008, 2006).

In the case of a physical resistance, the stung species would have some sort of physical property of their skin or mouth tissue preventing it from being penetrated by a venomous spine (or other envenomating apparatus). This has been well documented in the case of the shark in a study by Galloway and Porter, whose braincase is incapable of being punctured by a lionfish spine (K. A. Galloway and Porter 2021). In their study, only 2 out of the 15 venomous spines tested were able to puncture the shark's upper jaw region due to the high density of dermal denticles, which are V-shaped scales made out of enameloid and dentine, very similar to scale-like teeth (K. A. Galloway and Porter 2021; Katherine A. Galloway and Porter 2019). However, lionfish spines were always able to easily puncture the mouth tissue of the grouper, another predator that was tested. This would suggest that sharks, one of the common predators of the lionfish in the Indo-Pacific, are able to eat the lionfish because the venomous spines do not puncture their mouth during the eating process, and stomach acids likely destroy the venom's toxins during digestion.

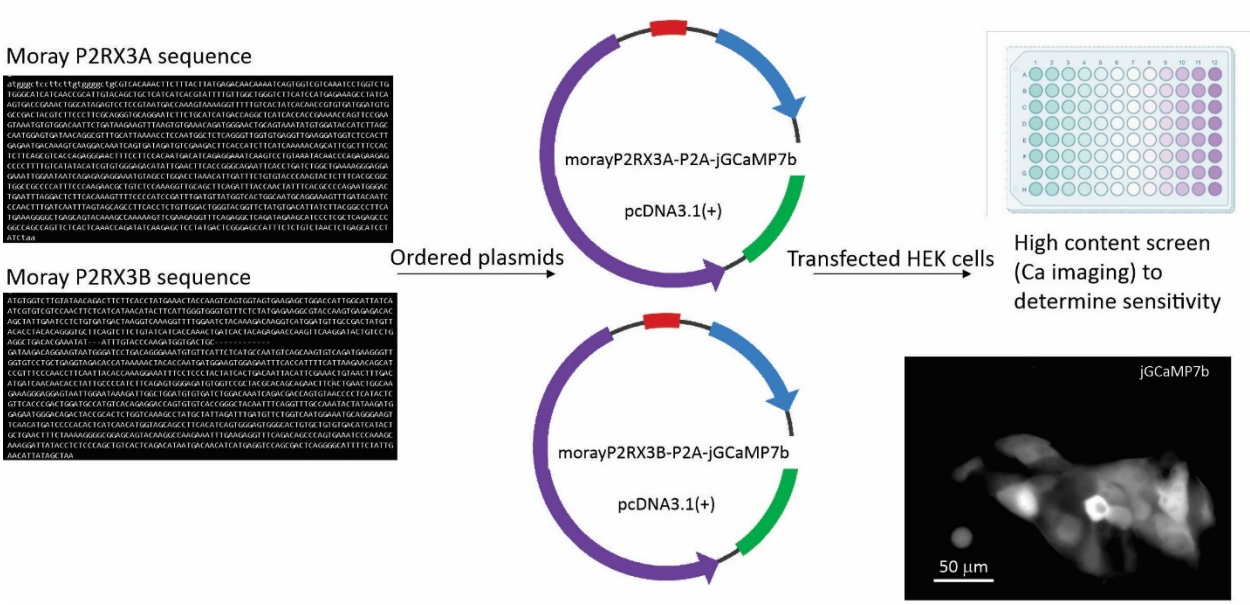
In the case of target site insensitivity, the stung species would have a specific mutation on the protein that is targeted by the toxin, making the target now insensitive to the toxin and the

species resistant to negative effects caused by such toxin. Classic examples of this are the resistance mechanisms of the mongoose and the honey badger to snake bites – their nicotinic acetylcholine receptors are specifically mutated to prevent binding of snake toxins to their surface, but the channels are still physiologically functional for the animals (Drabeck, Dean, and Jansa 2015; Barchan et al. 2006; Dellisanti et al. 2007). I was curious to determine whether the reason that one of the lionfish's predators, *G. favagineus* (honeycomb moral eel) (Discussion Figure 5, top), was able to eat the lionfish was due to some specific mutation on its P2X3 channel, making it insensitive to the negative effects of the venom.

To answer this question, I spent 4 months in Dr. Makoto Tominaga's lab at the National Institutes for Physiological Science (NIPS) in Okazaki, Japan, to learn how to clone a channel from a species whose genome has yet to be annotated (which is the case for the lionfish). Since fish have duplicated genomes, I had to clone both copies of the P2RX3 gene, P2RX3A and P2RX3B, to determine whether one, both, or neither of the channels was sensitive to lionfish venom. Using a technique based on identifying and taking advantage of conserved regions of the P2RX3 gene, I was able to amplify fragments of the *G. favagineus* P2RX3A and B genes, and then design primers against these regions and use 3' and 5' RACE to amplify the remaining portions of the genes. Once the full sequence of each gene was identified (Discussion Figure 5, bottom), we ordered plasmids expressing these channels along with jGCaMP7b, a newer and brighter version of the genetically encoded calcium sensor GCaMP6f (used in the body manuscripts of this thesis) to transfect HEK293T cells with and screen the channels for their sensitivity to lionfish venom in high content calcium imaging screening experiments (see Discussion Figure 6 for a diagram describing this workflow).

Discussion Figure 5. Top: Photo of *Gymnothorax favagineus* (Honeycomb moray eel), the species of moray eel used to cloned P2X3 receptors. This species of moray eel has been shown to be able to eat lionfish, and anecdotally seems to have no behavioural pain response to the fish's spines and venom. Bottom: Alignment of the cloned P2X3 receptors from *G. favagineus* aligned with human P2X3. Green boxed areas are the transmembrane domains of the channel. Purple boxed areas

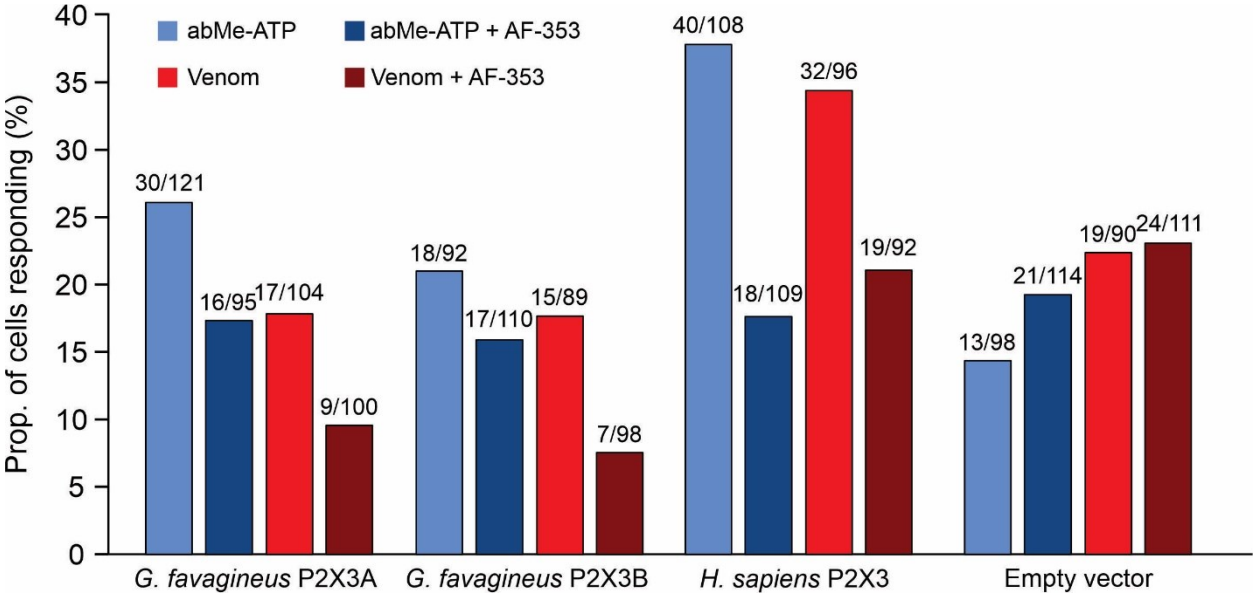
indicate the 10 conserved Cysteine residues that are essential for proper folding of P2X3's extracellular domain and ATP binding site.



Discussion Figure 6. Diagram demonstrating the workflow followed to screen the cloned P2X3 channels from *G. favagineus* for their sensitivity to lionfish venom. Bottom right inset: sample fluorescence image showing cells transfected with P2RX3A-P2A-jGCaMP7b plasmid.

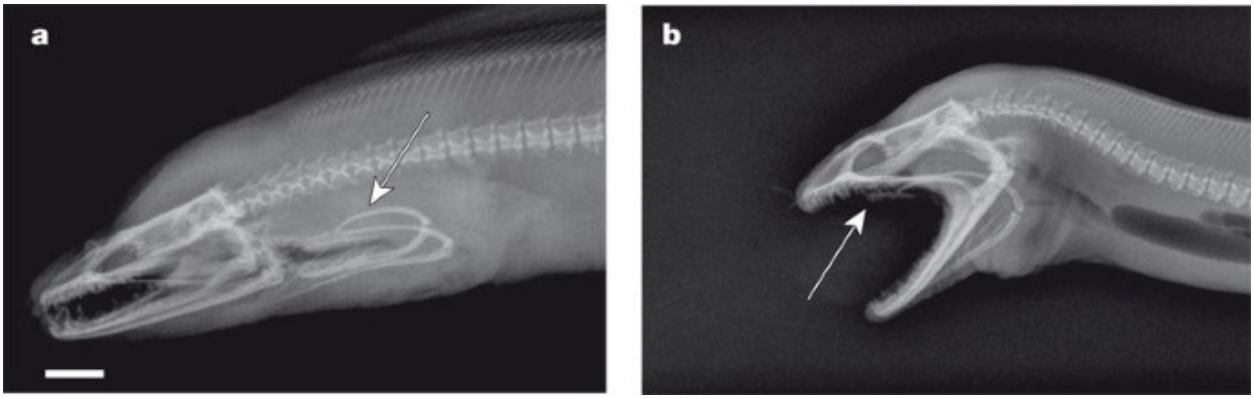
Interestingly, in our HCS experiments on HEK293T cells expressing the *G. favagineus* P2X3A and P2X3B channels, many cells responded to the application of the venom, and this activation was able to be reduced in the presence of the P2X3 antagonist AF-353 (Discussion figure 7, bright and dark red bars). Similarly, αβMe-ATP was able to activate these cells, but could also be blocked with AF-353 (Discussion figure 7, light and dark blue bars), which would suggest that the channels are still functional since they can be activated by canonical agonists of P2X3.

Taken together, these data seem to suggest that *G. favagineus* is not resistant to the lionfish's venom due to some mutation in its P2X3 channels, but rather via some other resistance mechanism.



Discussion Figure 7. Cloned *G. favagineus* P2RX3 channels are both sensitive to lionfish venom and to αβMe-ATP. Quantification of the number of transfected HEK293T cells responding to applications of αβMe-ATP (light blue bars), αβMe-ATP and AF-353 (dark blue bars), lionfish venom (bright red bars) and lionfish venom and AF-353 (dark red bars). Numbers above bars indicate the number of cells responding and the total number of cells imaged.

Notably, moray eels have a second cartilaginous jaw, called their raptorial pharyngeal jaw, which reaches up from behind their skull to bite and swallow very large prey that they capture with their primary jaw in their oral cavity (see Discussion figure 8A-B) (Mehta and Wainwright 2007; Koçak et al. 2022). It is possible that this second jaw destroys lionfish spines before they are even capable of puncturing the skin of the moray eel's mouth, rendering it resistant to lionfish venom due to a physical resistance mechanism, but this remains to be validated experimentally.



Discussion Figure 8. Image from (Mehta and Wainwright 2007). Radiographs in the left lateral view of the mouth of *M. retifera* depicting the two extreme positions of the pharyngeal jaws. A) With mouth closed, the pharyngeal jaws (arrow) are located posterior to the skull. B) With mouth open and pharyngeal jaw engaged, the jaw comes out (arrow) to the main mouth cavity to assist the primary jaw in its bite.

5.4 Conclusion

This thesis provides a comprehensive body of work characterizing the pain experience of a lionfish sting, the venom's proteinaceous contents and the venom's algogenic mechanism of action. Furthermore, I explored some potential resistance mechanisms that predators of the lionfish may have evolved to its venom and identified a pain-causing toxin within the venom with a high percent of shared identity with a known molecule, ApoE, that has been implicated in pain sensation. While lionfish have been an invasive, venomous species for over 20 years in the Gulf of Mexico, Caribbean, Western Atlantic and Mediterranean areas, there have been very few studies of the lionfish's venom. This thesis provides the first characterization of the lionfish venom's components, the first identification of a pain-causing toxin in the venom and the first insights into the venom's pain-causing mechanism of action. Venoms are increasingly becoming a topic of interest for basic science research, as they have been evolutionarily honed to exert their effects on

their victims through highly efficient and specialized means. I hope that this work can be a basis for more investigation into lionfish venom, and that this may motivate more groups to investigate the mysterious and beautiful world of venoms.

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