The noble false widow *Steatoda nobilis*:

Ecology, Venom and Potential Medical Importance

A thesis submitted to the National University of Ireland Galway for the Degree of Doctor of Philosophy

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Subadult male *Steatoda nobilis*

Photo by Andy Wilson

Declaration

I, John P. Dunbar, certify that this thesis is all my own original work and I have not previously obtained a degree in the National University of Ireland Galway, or anywhere else, based on the work presented in this thesis.

Signature: 

Date: 14/08/2020

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Achievements

The research undertaken for this PhD has resulted in a total of eight manuscripts, of which six are published, one is in review and one is ready for submission. Aspects of this research have been presented at one national and five international conferences (5 oral & 1 poster presentations). Media have shown a keen interest in this work and this has led to over a dozen radio interviews, one TV appearance for the Irish National Broadcaster RTE, and >30 printed news articles citing our research. Parts of this research were also presented six times to four universities as an invited guest speaker. Finally, I discussed my research with David Chambers (the RubberBandits) in front of an audience of 400 as part of a live podcast at the Galway Town Hall Theatre.

Abstract

The noble false widow spider *Steatoda nobilis* (Thorell, 1875) has expanded its range globally in recent years and may represent a potential threat to native ecosystems and public health. Our data shows that *S. nobilis* is an extremely prolific, resilient species with distinct synanthropic affinities. In Ireland, this species appears to be currently restricted to urbanised habitat across at least 17 counties with the largest populations observed along the eastern and southern coastal urban corridors. While our findings suggest *S. nobilis* is currently absent from natural, undisturbed habitats such as woodlands, bogs, and grassland, distribution trends in other parts of the world indicate that eventually this species may invade natural habitats. *S. nobilis* is a generalist predator capable of preying on a diverse range of prey including invertebrates and small reptiles. In addition to its comparatively fast reproductive rate, long life span and year-round activity, *S. nobilis* appears capable of having a detrimental impact on native species.

With a preference for synanthropic habitats in temperate climates, this has led to an increase in human encounters resulting in bites with medically important outcomes. *S. nobilis* envenomation symptoms have similarities to *Latrodectus* and using a combination of transcriptomic and proteomic cutting-edge approaches we have characterised the venom composition of *S. nobilis*. We demonstrate that two-thirds of venom toxins produced by *S. nobilis* are also present in *Latrodectus*, including α-latrotoxins, δ-latroinsectotoxins and α-latrocrustotoxins. Also present are the enzymatic machinery that presumably facilitate the spread of venom into the prey (metallo and serine proteases, chitinases). In high concentrations, α-latrotoxin can cause localised cell death and is potentially potentiated by the presence of enzymes. This may induce necrosis and provide substrate that could facilitate bacterial virulence. Indeed, the medical assessment of 20 case reports of confirmed bites by *S.*

nobilis demonstrate that the venom is highly active and that symptom presentation can overlap with those caused by *Latrodectus*. The most common symptoms are prolonged moderate to intense pain, erythema, swelling and pruritus. Others include radiating and debilitating pain, tremors, fatigue, nausea, local sweating, a sensation of heat, tenderness, piloerection, inflammation, irritation, raised skin, lump/pimple and blister formation, and paraesthesia around the bite site, muscle contractions in the affected area, reduced mobility in the affected limb, vasodilation of the capillaries, and necrosis. Other pathologies can include vectored bacterial infections including cellulitis and dermatitis. We conclude from this extensive study that steatodism is a rare, but potentially severe, clinical syndrome when caused by the noble false widow spider *S. nobilis*.

Irrespective of the media sensationalism, the continued rise of *S. nobilis* may legitimately present as an emerging public health issue, with potential serious outcomes for some victims. In addition to having potential invasiveness, this species deserves close monitoring and research by both the scientific and medical community and updated public awareness by public health authorities.

Chapter 1
General Introduction

Adult female *Steatoda nobilis* *Photo by Andy Wilson*

1.1 - INTRODUCTION

1.1.1 - Rationale for this study

When I started this project in the fall of 2016, the noble false widow spider *Steatoda nobilis* had been regularly headlining the media throughout Ireland and the United Kingdom for over a decade, with highly alarming and controversial claims, which attracted little scientific interest. These articles included symptoms of necrosis, bacterial infections resulting in some deaths following bites by *S. nobilis*, which seemed in complete contrast to what was known about their nearest cousins, the true black widow spiders from the genus *Latrodectus*. Since the first ever recorded bite by *S. nobilis* in 1991 (Warrell et al., 1991), and despite the growing number of reports, no study has focused on bites or the venom of *S. nobilis* even though the topic has been heavily debated among scientists and the general public alike on social media platforms. This resulted in a large knowledge gap on this species that needed to be addressed. Although no study was carried out on the Irish population since the first reported sighting in 1998 (Nolan, 1999), anecdotal reports indicated that *S. nobilis* was present in large numbers in and around Dublin. A population was therefore easily accessible to study and with potentially enough specimens to maintain captive populations for lab-based studies. As *S. nobilis* are quite small spiders with a body length (prosoma + opisthosoma) reaching 14 mm (Snazell and Jones, 1993), large numbers of specimens could be maintained in captivity to conduct research.

Due to the recent global range expansion, concerns of the potential invasiveness and human conflict has caused much debate, but many important knowledge gaps remain on this species. To focus this research entirely on one main aspect would, on one hand, result in extremely in-depth knowledge of a focussed area, but on the other hand, many important aspects would remain neglected. For this reason, a classical interdisciplinary scientific approach was used to tackle questions on occurrence and ecological aspects, venom composition, and potential medical importance.

1.1.2 - A HISTORY OF SPIDERS

1.1.3 - Introduction to Spiders

The radiation of the class Arachnida occurred sometime during the late Cambrian - early Ordovician period approximately 500 million years ago (mya). With approximately 112,000 extant species, arachnids have diversified to include the subclass Acari (ticks and mites) and several orders: Scorpiones (scorpions), Pseudoscorpiones (pseudoscorpions), Solifugae (camel spiders), Thelyphonida (vinegaroons), Amblypygi (whip spiders), and one of the most diverse and successful groups, Araneae (true spiders) (Fernández et al., 2018, Lozano-Fernandez et al., 2020). Spiders (Order Araneae) appear in the fossil record approximately 400 mya and along with their sister groups the Amblypygi (whip scorpions) and the Thelyphonida (vinegaroons), they share a common ancestor with true scorpions (Lozano-Fernandez et al., 2019). Spiders have two suborders: Mesothelae, consisting of one family, and the Opisthothelae (117 families), which is divided further into two infraorders: Mygalomorphae (20 families) and Araneomorphae (97 families) (Herzig et al., 2019). Currently there are over 48,804 extant species of spiders (World Spider Catalog, 2020). Spiders can be characterised by having two distinct body parts: the cephalothorax (prosoma) and the abdomen (opisthosoma), which are joined by a flexible structure called the pedicel. As with all arachnids, spiders have eight walking legs, a pair of pedipalps, a pair of chelicerae and usually six or eight eyes. With the notable exception of the family Uloboridae (287 species), all spiders possess a venom apparatus consisting of a pair of venom glands prolonged anteriorly by a pair of ducts opening on the outer subterminal part of the chelicerae. The venom glands are either encased within the chelicerae (Mygalomorphae) or extend into the prosoma (Araneomorphae). Venom is produced as a primary means to subdue prey (Herzig et al., 2019), but can also aid in the pre-digestion of prey and defence against predators (Vassilevski et al., 2009). However, perhaps the most defining characteristic of spiders is the production of silk for building webs, egg sacs and chambers, wrapping prey, predatory defence, lining of burrows, and ultimately to facilitate in navigation and communication (Selden et al., 2008).

1.1.4 - Spider venom

Spiders are predatory arthropods abundant in every terrestrial habitat except Antarctica. Their total global biomass is estimated to be approximately 25 million tons, which makes them especially important in controlling arthropod populations as they are thought to consume 400–800 million tons of prey each year (Nyffeler and Birkhofer, 2017). Spider venoms are complex cocktails of biologically active compounds of diverse chemical types. These include proteins, peptides, amino acids, salts, organic compounds, and neurotransmitters (Vassilevski et al., 2009). The venom is secreted within the epithelium of the glands and excreted using muscle contraction. Spiders use venom as their primary means to subdue prey. However, the abundance of enzymes including metalloproteases, serine proteases, and chitinases allow for the breakdown of tissue to facilitate the spread of venom in prey (Haney et al., 2014). This suggests that some degree of pre-digestion occurs from the venom prior to the expulsion of digestive enzymes from the spider's digestive tract into the prey, as spiders digest prey externally before sucking up the liquefied meal.

Venom is a specialised poison that must be delivered into the flesh of prey or predators using weaponry such as fangs, teeth, stings, spurs, and forcipules to name a few. Venom is composed of a toxin cocktail, specifically produced in specialised glands, that is usually directly attached to a delivery device, with some exceptions (e.g. slow loris). In contrary, poison is a singular toxin compound that can be produced in either specialised glands (e.g. the parotid glands of toads) or single cells distributed around the organism but are not associated with a delivery mechanism (Brodie, 2009). Poison can enter the bloodstream through inhalation, ingestion, or absorption through the skin (Harris and Arbuckle, 2016). This is because the molecules are typically small enough to pass through the membrane barriers. However, venom molecules are too large to pass through the membrane and therefore requires these specialised sharp piercing devices to cause an opening in the flesh so the venom can penetrate the body.

According to the venom optimisation hypothesis, animals regulate the use of their venom to minimise the metabolic cost associated with its use (Dugon and Arthur, 2012, Morgenstern and King, 2013, Wigger et al., 2002). When tackling prey, the predator can gauge its prey and increase venom delivery appropriately. After depleting venom stock, typically smaller sized and defenceless prey are selected. Larger, potentially more dangerous prey are avoided for a period when venom replenishes. When attacked, they can also increase venom load to the perceived predator to avoid predation. This likely plays a role in bites to humans where the level of severity in symptom onsets can vary (Dunbar et al., 2019).

Typically, the venom of spiders is deemed neurotoxic (Brodie, 2009) as most envenomations induce pain and an array of neurotoxic symptoms. For example, some of the most notable neurotoxins isolated from spider venoms are α-latrotoxins (α-LTX) and Phoneutria toxins (PhTx), which target ion channels inducing severe pain and symptoms of paralysis. However, of the approximately 47,000 species of spiders, only roughly 100 species (0.3 - 0.4%) have proteins and peptides characterised from their venom (Kuhn-Nentwig et al., 2011, Herzig et al., 2019, Peigneur et al., 2018). Collectively as of 2018, these include 1946 toxins from just 28 of the 118 currently recognised families (Herzig et al., 2019). Therefore, the study of spider venomics is in its infancy. Those already studied include large and charismatic species popular in the pet trade, and those of medical importance (Hauke and Herzig, 2017). The study of spider venoms has also revealed the antimicrobial and other targeted drug potential for pharmaceutical interests making spider venoms an ideal and untapped source for bio prospecting (Escoubas and King, 2009).

Many prey species can be fast and potentially escape or be aggressive and potentially turn from hunted to hunter. Therefore, the most effective way to immobilize prey safely and efficiently is by inducing rapid paralysis. However, the composition of spider venoms can be complex and hugely diverse between species (Paiva et al., 2019). The venom of a single species can contain hundreds of different types of proteins, which target a variety of biochemical pathways. As such, a single drop of venom can have multiple targets. For example, the venom gland transcripts of

Latrodectus hesperus include the presence of metalloproteases, which degrade extracellular matrix components, serine proteases, which target peptide bonds in proteins, and hyaluronidases, which cleave hyaluronic acid of the extracellular matrix. These actions facilitate the spread of venom toxins (Haney et al., 2014). These toxins may lead to minor destruction of cells/tissues that do not contribute to internal or superficial necrosis, but cause enough damage to facilitate the spread of the venom, thus increasing the impact of neurotoxins (Haney et al., 2014). The presence of these same toxins in snake venoms is known to produce wide-ranging effects, including haemorrhage and necrosis, and contribute to high numbers of morbidity and mortality (Giribaldi et al., 2020, Whiteley et al., 2016). In spiders however, their role is not yet fully determined but it remains plausible that in some spiders, the presence of these toxins provide the enzymatic machinery allowing the venom to more easily spread into the prey to induce more severe symptoms.

1.1.5 - Some notable medically important spiders

The most medically important spiders are the black widows from the genus *Latrodectus*, which are found in most parts of the world and thrive in and around human habitats. *Latrodectus* species possess a highly potent neurotoxic venom that can induce intense pain, diaphoresis, paresthesia, hypertension, fasciculations of muscles, and neuromuscular paralysis (Haney et al., 2014). Their venom contains the highly potent neurotoxin α-latrotoxin that targets the neuromuscular junctions and form calcium ion channels on the presynaptic membrane that triggers extensive exocytosis of acetylcholine, a neurotransmitter involved in muscle contraction, subsequently resulting occasionally in human deaths (Figure 1). However, only 1.4% of envenomations develop as severe cases (Hauke and Herzig, 2017).

The recluse spiders (genus *Loxosceles*) found in North and South America are synanthropic and are implicated in bites to humans that can result in necrosis. The venom contains Phospholipases D, which hydrolyses sphingomyelin (Ribeiro et al.,

2015). The resulting breakdown product ceramide phosphate, a proinflammatory mediator, which can induce an inflammatory response, which in turn may trigger a regulated process of cell death (Dunbar et al., 2019). In some rare cases the venom can cause systemic symptoms of acute renal failure, but more commonly the symptoms are non-life threatening. Superficial localised necrosis occurs in over 50% of envenomation cases and lesions heal after several weeks. *Loxosceles* species are the only spiders whose envenomation symptoms commonly include necrosis (Hauke and Herzig, 2017).

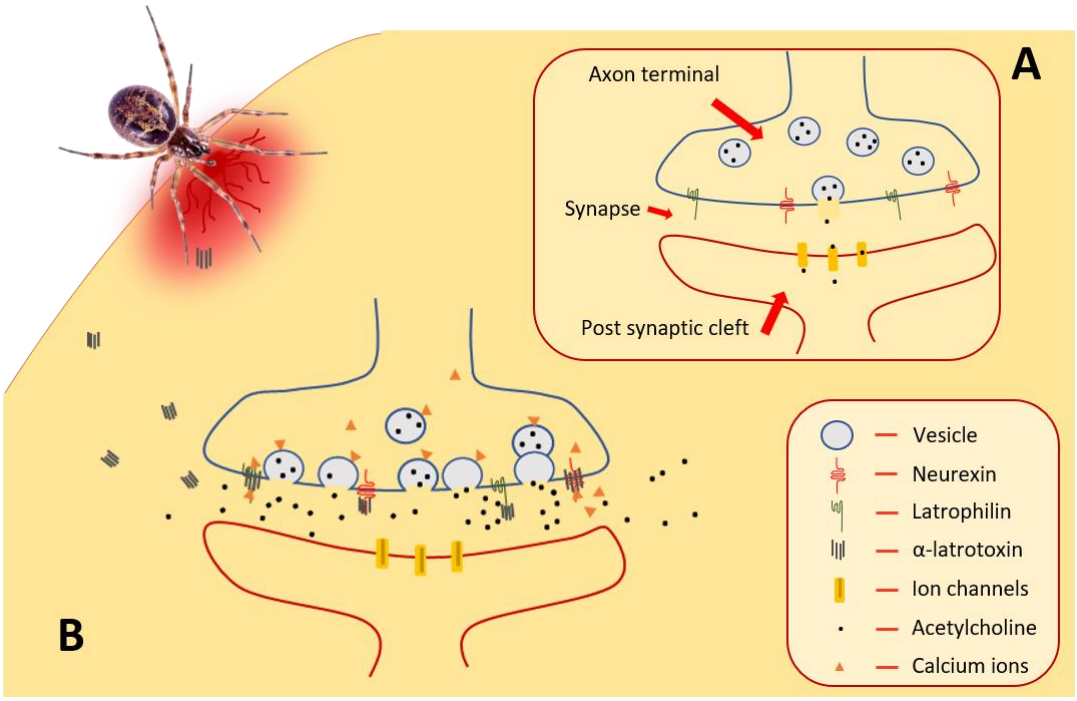

Figure 1. The mechanism of action by α-latrotoxin at the neuromuscular junction: A) Normal nerve function – acetylcholine released from vesicles into the synapse and transported into the post synaptic cleft through membrane bound ion channels. **B)** α-latrotoxin binds to surface receptors neurexins & latrophilins and inserts into the plasma membrane, forming a Ca^{2+} permeable channel. When Ca^{2+} enters the axon terminal they trigger the synaptic vesicles to release acetylcholine neurotransmitters into the synapse.

In tropical South America the Brazilian wandering spider (genus *Phoneutria*) has a highly potent venom (Valenzuela-Rojas et al., 2019). The venom contains approximately 150 proteins and peptides. Several neurotoxins (e.g. Phoneutria toxins (PhTx)) target mainly sodium, calcium, and TRPA channels, while others show activity on NMDA, cannabinoid, or opioid receptors (Peigneur et al., 2018).

Symptoms range from salivation, lachrymation, convulsions, flaccid and spastic paralysis (Richardson et al., 2006) to priapism. The latter has led to the study of two peptides (PhTx2-5 and PhTx2-6) recovered from the venom of *Pheunotria nigriventer* for their potential role in treating erectile dysfunction (Peigneur et al., 2018). Envenomations from this genus can sometimes result in death. These spiders are typically aggressive, and their venom is considered one of the most medically important to humans, although only 0.5% of cases are severe (Hauke and Herzig, 2017).

1.1.6 - BACKGROUND

1.1.7 - Introduction to black widows (Genus *Latrodectus*) and false widows (Genus *Steatoda*)

The family Theridiidae (Sundevall, 1833) includes over 2,510 species of spiders distributed in 124 genera (World Spider Catalog, 2020). The monophyly of the clade Theridiidae is supported by both morphological (Agnarsson, 2004) and molecular data (Arnedo et al., 2004). Although Theridiid spiders exhibit wide variations in morphology and behaviour, all members of the clade are small to medium in size. Theridiids are characterised by the presence of serrated setae on the tarsus of the fourth pair of legs and the production of three-dimensional irregular cobwebs, hence their vernacular names of cobweb spiders or comb-footed spiders. The family is distributed worldwide with some examples of cosmopolitan clades, particularly within the genus *Parasteatoda* and the subfamily Latrodectinae.

The subfamily Latrodectinae (Petrunkevitch, 1928) accommodates three genera (Garb, 2003), two of which are known for the potency and medical importance of their venom, i.e. *Latrodectus* (Walckenaer, 1805) and *Steatoda* (Sundevall, 1833). The venom composition and potency of the third genus, *Crustulina* (Menge, 1868), has yet to be investigated. *Latrodectus* ("true widows"– 30 species worldwide) envenomations are considered medical emergencies, for which antivenom therapy is often required. The

venom of *Latrodectus* affects primarily the parasympathetic nervous system and inducing symptoms as described earlier.

Despite being widely reported by local media wherever they occur, envenomations by *Steatoda* ("false widows" – approximately 220 species worldwide) remain under-documented in the scientific literature. The rare cases of systemic steatodism (envenomation symptoms characteristic of *Steatoda* species) ever reported in the literature have been described as being similar to latrodectism but with a milder onset and a faster resolution of symptoms (Isbister and Gray, 2003, Warrell et al., 1991). This is consistent with the presence of α-latrotoxins in the venom of both *Latrodectus* and also in *Steatoda grossa* (Graudins et al., 2004). However, to date, there has not been any study carried out to determine if these highly potent neurotoxins are present in the venom of *S. nobilis* as this could account for the range of symptoms.

Theridiidae spiders are the most diverse non-native spiders in Europe with at least 13 alien species already successfully established across the continent (Kobelt and Nentwig, 2008). The spread of these alien species is facilitated by globalisation and the increase in international import and export of goods and services. This is especially true for species that thrive in and around human habitats (Nentwig et al., 2017).

Many latrodectine spiders hitchhike along human transport routes and successfully establish colonies outside their native range, mainly in the tropical and temperate climatic zones (Hänggi and Straub, 2016, Vink et al., 2011). In Ireland, *Latrodectus* are occasionally found in shipments of imported goods (O'Connor and Holmes, 1993, Ross, 1988). There is however no record of *Latrodectus* establishing colonies anywhere in Northern Europe.

Four species of *Steatoda* are present in Ireland and it is quite likely that all four are recent additions to the Irish fauna. *Steatoda bipunctata* (Linnaeus, 1758) was recorded for the first time in 1929 (Pack Beresford, 1929), and until now, its distribution is thought to be restricted to the Dublin metropolitan area. *Steatoda grossa* (Koch, 1838) has been established at least since the late nineteen century (Carpenter, 1898). *S. grossa* has been previously recorded from Cos Cork, Kerry, Galway, Sligo, Down, Dublin,

Wicklow, Kilkenny and Waterford (Fern, 2010). *Steatoda phalerata* (Panzer, 1801), which is originally distributed in Western mainland Europe and the UK, has been recently recorded from a single location in County Down (McFerran, 1993, Merrett, 1989). The noble false widow *S. nobilis* (Thorell, 1875) was recorded for the first time in Bray, Co Wicklow, almost two decades ago (Nolan, 1999) and remains largely understudied.

1.1.8 - The noble false widow spider *S. nobilis*

Steatoda nobilis is a species of particular interest in Ireland and Great Britain as it has been involved in the only case of systemic envenomation reported in these islands (Warrell et al., 1991). As such it is the only potentially medically significant spider established in Ireland. The geographical expansion of the noble false widow has been fairly well documented for over a century, thanks to its relatively large size, conspicuous markings and superficial resemblance to the black widows of the genus *Latrodectus*. *S. nobilis* is thought to originate from the Macaronesian archipelago of Madeira (Thorell, 1875) and the Canaries (Bristowe, 1929). In 1879, *S. nobilis* was recorded for the first time outside its native range: Rev. Pickard-Cambridge identified a sub-adult female collected a few years earlier by Rev. Hamlet Clark near Torquay, Devon (Cambridge, 1879). This confirms that *S. nobilis* reached the British Isles–at least as an occasional visitor–well before the turn of the twentieth century. Further afield, the distribution range of *S. nobilis* has been continuously expanding since the turn of the 21st century. *S. nobilis* has become widespread in coastal urban centres in France (Kovoor and Munoz-Cuevas, 2000), Belgium (Van Keer, 2010), Italy (Kulczycki et al., 2012), Spain (Déjean, 2013), Portugal (Cardoso, 2000), California (Vetter et al., 2015, Vetter and Rust, 2012) and Chile (Faúndez and Téllez, 2016, Taucare-Ríos et al., 2016). Eastward, *S. nobilis* has been observed in Germany (Reiser, 2013), Turkey (Türkeş and Mergen, 2007) and Iran (Zamani et al., 2015).

When first discovered in Ireland in 1998, the author described well established and thriving colonies in a residential setting (Nolan, 1999). Observations of tolerance to cold conditions and a high reproductive rate led to the author to conclude that its future in Ireland was secured. Considering *S. nobilis* is closely related to true black

widows, which possess highly potent venom, and how prey and competitive species in subtropical habitats may differ to native species in Ireland, it is completely unknown how *S. nobilis* would endure against Irish native spiders and prey species. While *S. nobilis* has continued to expand its range, data on its diet has not been reported. However, Kulczycki et al. (2012) reports on its potential to replace native species after establishing colonies in Italy, where *S. nobilis* appears to be displacing native *Zygiella* species. Vetter et al. describes *S. nobilis* as a well-dispersed resident of California (Vetter et al., 2015) after only a few years since first reported in the region (Vetter and Rust, 2012). Even though *S. nobilis* has been present in the UK for 140 years, for more than a century they remained relatively static. The recent expansion across the UK correlating with its sudden appearances in several other parts of the world over the past couple of decades is intriguing. Shifts in global transport of goods and services, in addition to changes in bio control measures for fruit and garden centre imports, general work, and holiday travel may affect the patterns of introductions. However, the long-recorded history of *S. nobilis* in the UK since its first arrival in 1879 shows that the species has changed its behaviour in recent years.

The recent advances in the global distribution of *S. nobilis* has coincided with complaints by the general public of bites. In addition to the reported case in the UK in 1991 (Warrell et al., 1991), a case in Chile in 2016 (Faúndez and Téllez, 2016) suggests that *S. nobilis* do find themselves in close contact with people and that those contacts sometimes result in bites. The increasing numbers of *S. nobilis* in and around residential settings suggest bites may also increase in the future. In both previous envenomation cases, symptoms were typically neurotoxic, and in one case systemic (Warrell et al., 1991). To date, bites by *Steatoda grossa* are reported, and studies into the venom of *S. grossa, Steatoda capensis*, and *Steatoda paykulliana* suggest the venom is similar to black widows (Atakuziev et al., 2014). Some of the most severe cases of envenomation by *S. grossa* are reported as indistinguishable from *Latrodectus* and intravenous redback spider (RBS) antivenom has proven effective in neutralising symptoms (Isbister and Gray, 2003, Graudins et al., 2002). The venom of *Steatoda* contains α-latrotoxins that are likely responsible for the symptom onsets that are comparable to *Latrodectus* by their actions on nerve terminals (Figure 1). Considering

1) the *Latrodectus-like* symptoms experienced by *S. nobilis* envenomation victims, 2) the range expansion of *S. nobilis* and 3) the likelihood for an increase in bites as *S. nobilis* becomes more prevalent in urbanised areas, it seems very plausible that some severe envenomation may occur in the future.

1.2 - CONCLUSION

Despite its rapid range expansion in North America, Western Europe, and West Asia, since the early 2000's, there is a fundamental lack of studies addressing the true distribution, ecological impact, physiology, and medical importance of this species both at the national and international levels. Considering the potential of *S. nobilis* as a prolific coloniser (Locket, 1979, Vetter et al., 2015), its possible medical importance (Warrell et al., 1991), synanthropic inclination and potential detrimental effect on local arthropod fauna (Kulczycki et al., 2012), further studies on all aspects of this species are urgently needed. This is especially because we are amidst a global catastrophe with insect decline (Hallmann et al., 2017) and the prospects of a highly invasive spider disrupting native ecosystems is extremely concerning.

1.3 - AIMS OF THIS THESIS

The first chapter provides an overview of the occurrence and ecology of *S. nobilis* in Ireland, including development, distribution maps, notes on predation events involving other arthropods including insects and spiders and a predation event on a native lizard. The focus in this chapter is to determine the potential of *S. nobilis* as an invasive species. Also, establishing its occurrence, preferred habitat, and likelihood of increasing population size and range expansion will help determine how likely humans are to encounter these spiders and the risk of being bitten.

Steatoda nobilis is a sister group to *Latrodectus*, which possesses the highly potent neurotoxins called α-latrotoxins that can induce neuromuscular paralysis and is responsible for human fatalities. However, and despite this close relationship, the venom composition of *S. nobilis* has never been investigated. As such, the second

chapter on venom provides a full characterisation of the venom composition and highlight the toxins that are likely to be most important in envenomation symptoms.

Finally, the third chapter focuses on three medical aspects related to bites by *S. nobilis*: First, a case series is compiled, and new symptoms are described. Second, a systematic review is carried out to investigate the potential for necrosis to occur from an immune response induced by inflammatory pathways of regulated necrosis. Third, the surface microbiome of *S. nobilis* is investigated to determine if post-bite bacterial infections can occur from bacteria directly vectored by the spiders.

Chapter 2
Methods

In situ adult female *Steatoda nobilis* *Photo by JP Dunbar*

2.1 - The noble false widow spider *Steatoda nobilis* – a model research species

2.1.1 - Field Sites & Sampling

The first sighting of *Steatoda nobilis* in Ireland was in Bray Co Wicklow in 1998 (Nolan, 1999) where a considerably well-established colony was described along iron railings in a housing estate. Following the author's lead, the general area of Lucan Co Dublin was searched. *S. nobilis* can be initially identified from their large body size (approx. 14mm) (Dugon et al., 2017, Snazell and Jones, 1993), overall dark colourations contrasting with the presence of a bright cream crescent on the antero-lateral part of the opisthosoma, and the species typical way of hanging upside down on webs. In younger specimens, an intricate pentagonal or hexagonal cream-coloured pattern on the dorsal aspect of the opisthosoma is observable (Fig. 1). A search after dark showed that *S. nobilis* was extremely common on street furniture, signposts, traffic lights, bus shelters, boundary railings of parks, and graveyards and garden furniture, garden walls, pillars and gates, easily out numbering native spiders. In Esker graveyard, Lucan *S. nobilis* were found on dozens of large Yew trees (Fig. 3h), potentially up to 5 or 6 on a single tree from ground level up to at least 2.5 meters high. As we extended the sampling sites to other areas of Dublin, and beyond to Maynooth Co Kildare, *S. nobilis* were found in the same typical habitats.

Figure 1: A young specimen of *Steatoda nobilis*. Red arrow indicates the bright cream crescent on the antero-lateral part of the opisthosoma. Blue arrow indicates the intricate pentagonal or hexagonal cream-coloured pattern on the dorsal aspect of the opisthosoma.

Over the course of four years, Lucan (Fig. 2) remained the main sampling area, due to the high abundance of suitable habitats. Other sampling sites in Dublin included Tallaght, Clondalkin, Rathfarnham, Dun Laoghaire, Dublin City centre, boundary wall/iron fence of Glasnevin cemetery in North Dublin, and Maynooth Co Kildare. The most productive habitats were east facing iron railings, especially the point where railings connected to brick and stone walls, and wooden fences (Fig. 3).

Ideal times of the year for catching *S. nobilis* in high numbers is between August and November. In addition to the warmer days, the earlier nightfall increases sampling times, resulting in more spiders. Overall, *S. nobilis* can be found throughout the year; from spring throughout the summer. *S nobilis* is mainly active after dark.

2.1.2 - Capture and housing

The spiders were initially captured using a Katcha™ Bug Buster Spider Vacuum (Fig. 4a). The device was quite useful to reach otherwise inaccessible specimens especially in high up in signposts etc. However, with more experience, catching the spiders directly with forceps was proven more successful, especially as their webs are complex with supporting strands that are difficult to see and can easily trigger when approaching, therefore alerting the spider. Using a 25 cm pair of forceps (Fig. 4b) proved to be the most successful technique. Typically aiming for a leg, one quickly learns to catch specimens efficiently while applying only enough force to secure the spider without causing any injury to the legs. If positioned openly on their webs, or on a wall, they can simply be scooped up using a tube.

Once captured they can be placed directly into 50ml falcon tubes, maintained at a constant 20°C, with a piece of paper towel at the base and small airholes at the top (Fig 5). A single drop of water is added to each tube and specimens are fed commercially available crickets (*Gryllus assimilis*, Fabricius, 1775) and fruit flies (*Drosophila melanogaster*, Meigen, 1830) on a weekly basis.

Figure 2: Sampling locations in the general area of Lucan Co Dublin. Red lines indicate the path taken during sampling. **A)** Residential estate, *S. nobilis* are collected from the outside of garden walls and pillars. **B)** The boundary railings of Woodies DIY store where specimens are collected from holes in the iron railings, **C)** Iron railings that joins to concrete pillars every 5 meters along main road (red arrows), wooden fence with steel posts (blue arrow). **D)** Iron railings that joins to concrete pillars along main road, and **E)** the boundary wall and steel gated entrance to Esker graveyard. Inside the main graveyard entrance are large Yew trees, which are also found growing along part of the boundary, which appear to be suitable habitat for *S. nobilis*.

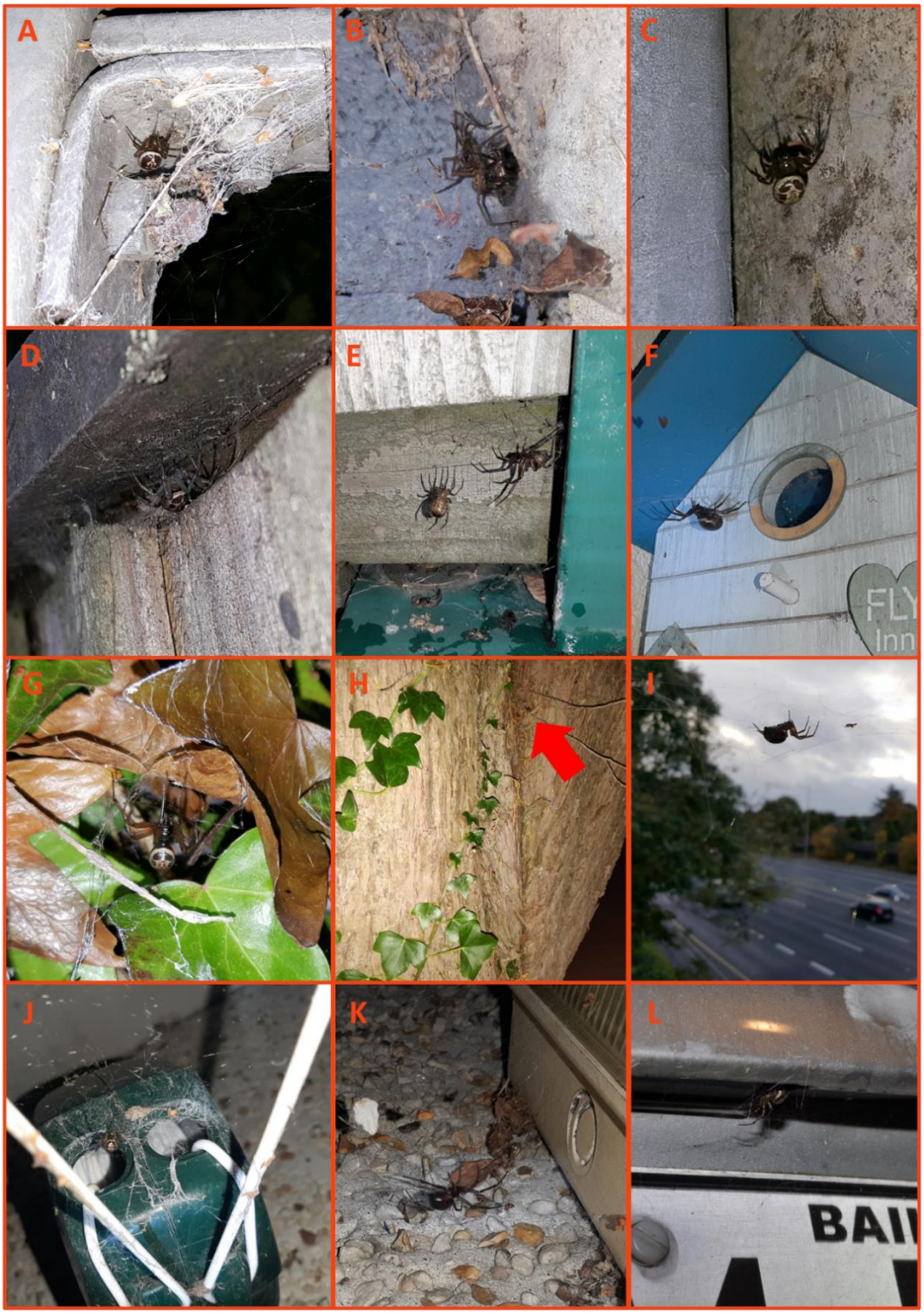

Figure 3: Types of habitat favoured by *Steatoda nobilis*. A) The joint of an iron railing. **B)** concrete wall meets a concrete pillar. Here you can see *S. nobilis* feeding on a large *Eratigena atrica*. **C)** Steel railings post meets a concrete wall. **D)** Wooden fence meeting a wooden cap. **E)** Wooden fence meeting a Steel post bolted on to a concrete wall. **F)** a garden bird box. **G)** Ivy growing on a concrete wall just as it joins an iron railing. **H)** A yew tree in a graveyard. **I)** A web extending out from steel railings on a footbridge over a dual carriageway. **J)** A wind-up clothesline. **K)** An outdoor garden light over a backdoor to the garden. **L)** Car registration plate.

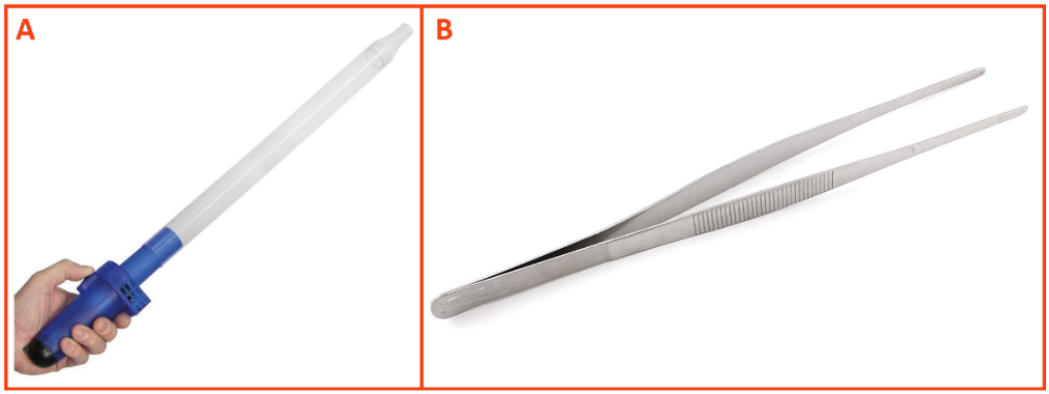

Figure 4: Some of the tools used in the field for catching *Steatoda nobilis*. A) A Katcha™ Bug Buster Spider Vacuum. **B)** A 25 cm forceps.

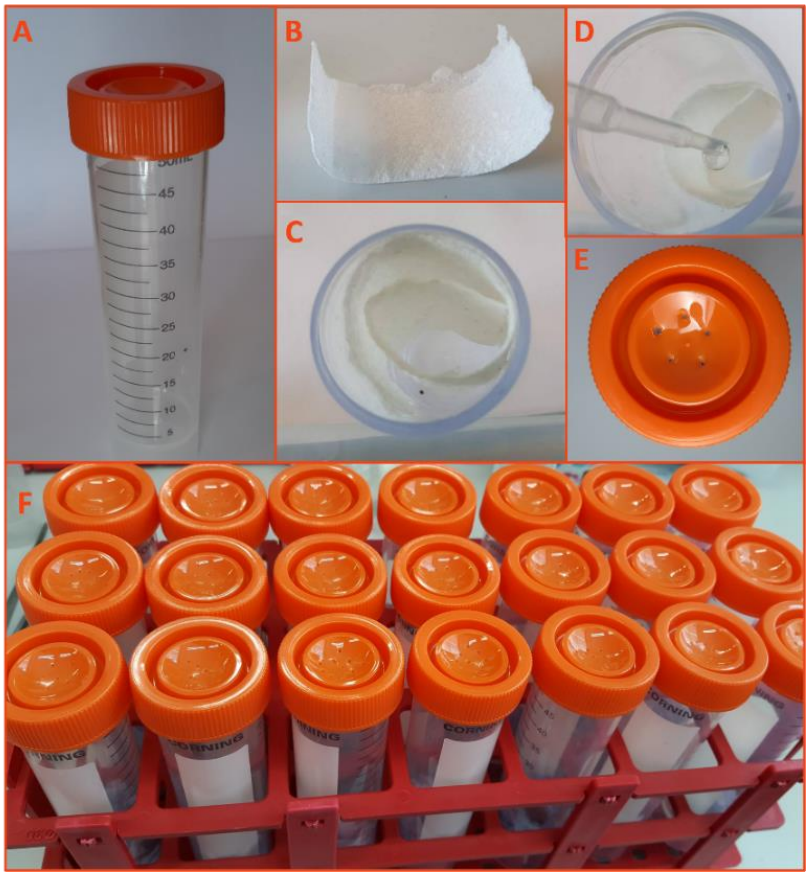

Figure 5: The stages of housing specimens of *Steatoda nobilis*. A) A standard 50 ml falcon tube. **B)** tissue cut to size to occupy ¼ of the tube. **C)** Tissue is rolled and placed at the base of the tube. **D)** Once a week 1 to 2 drops of water is added to increase humidity. **E)** Tiny airholes are added on the lid. **F)** Rack system for housing individually multiple *S. nobilis*.

2.1.3 - Venom Extraction and Storage

The spiders were placed in a clear plastic beaker and anesthetized using CO_2 (Fig 6a), which is pumped through a diffusion plate placed over the beaker for 2 mins (Fig. 6b). Once fully anesthetized, the spider is placed on the venom extraction platform (Fig 6c). The platform is based on (Garb, 2014) and arranged with a pair of soft tweezers mounted onto a soldering hand secured to the stage of a stereo microscope and a positively charged electrode connects the tweezers to a pulse stimulator (Fig. 7b). To increase conductivity, a sewing thread is thoroughly wrapped around the lower tweezer blade. Using a syringe with a blunted needle tip, an electrode connects the electrostimulation device to the syringe to facilitate the negative charge (Fig 7c). The electrostimulation device is linked to a foot pedal, which delivers repeated pulses at 15-20V when pressure is applied. The thread is soaked with 0.01 M Phosphate Buffer Saline (PBS) solution to facilitate the flow of electrical charge.

To begin venom extractions, the anesthetized spider is placed ventral side up between the forceps blades (Fig 6c). The thorax is secured between the tweezer blades (Fig. 8a), then the negatively charged electrode is placed against the base of the chelicerae (Fig. 8b). Pressure is applied to the foot pedal and once electricity passes through the spider; muscle contractions causes the release of the venom. The venom droplets were collected from the venom pores located on the outer subterminal part of the fangs (Fig. 8d) using 5µl microcapillary tube modified with a tapered end for maximum efficiency (Fig. 8c). Microcapillary tubes are tapered by holding both extremities of a tube with forceps, the tube is held over the flame of a Bunsen burner and the operator gently pulls apart both extremities as the tube heats up and melts. The end of the tube should be clipped less than 4 mm from the point it begins to taper to ensure that the diameter (and therefore the volume) of the inner space remains intact. Indeed, a narrower inner diameter would erroneously translate to a higher venom yield (Fig. 8c). For smaller spiders it may be necessary to have a longer and narrow tapered end in order to reach the fang tips and collect venom without extremity of a tube with forceps, the tube is held over the flame of a Bunsen burner and the operator gently pulls apart both extremities as the tube heats up and melts.

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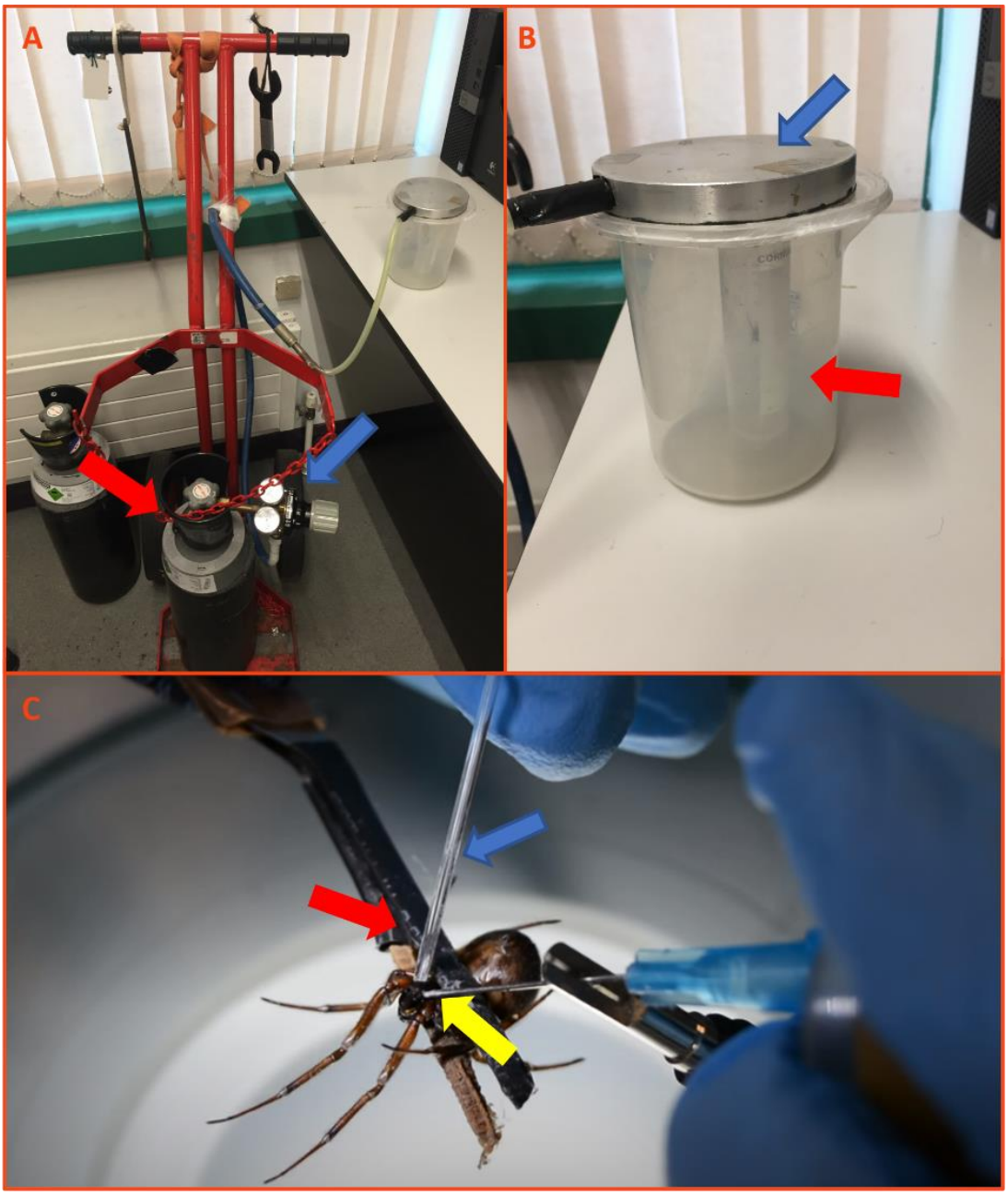

Figure 6: The initial stages of anesthetizing spiders for venom extractions. A) CO_2 canister (red arrow) connected to a flow regulator (blue arrow). B) beaker (red arrow) for holding the spider and a gas diffusion plate on top (blue arrow). C) shows the spider secured between the tweezer blades (red arrow), the negative electrode (yellow arrow) and the microcapillary tube (blue arrow).

contamination. However, caution should be taken on record collecting and venom should then be measured using a pipette.

Each venom droplet is collected into a graded microcapillary. The venom-filled length of the tube is measured (in mm) using a Vernier callipers for each spider. The spider's gender and body size (prosoma + opisthosoma) are also measured and recorded. The venom is converted to µl using the formula (Y = 5x/60), where Y is the volume of venom in µl, x is the venom measured in mm, 5 is the full volume capacity of the tube (in µl) and 60 is the full length (in mm) of the tube . Venom from all specimens are pooled in a 0.5ml eppendorf (Fig. 8e) kept on ice. It is essential to spin down the tube in a centrifuge for 5 secs after adding venom from each extraction to assure all venom collected is accumulated at the bottom of the tube. The venom is then flash-frozen in liquid nitrogen and freeze dried in a lyophilizer (Fig. 8f), then crystalized venom (Fig. 8g) can be stored at -20°C.

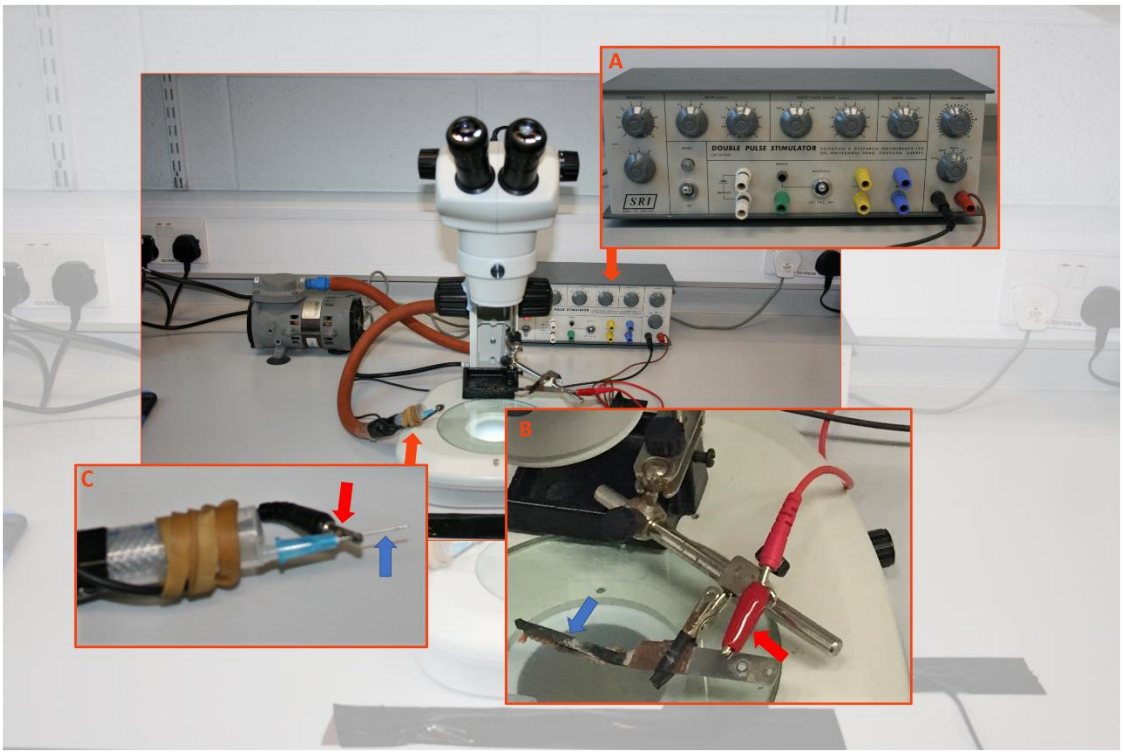

Figure 7: The venom extraction platform. A) A double pulse stimulation devise. B) shows the soldering hand secured to the stage of a stereo microscope. Connected to it is the soft forceps (blue arrow) and the positive electrode (red arrow). C) A clipped syringe tip connected to the negative electrode.

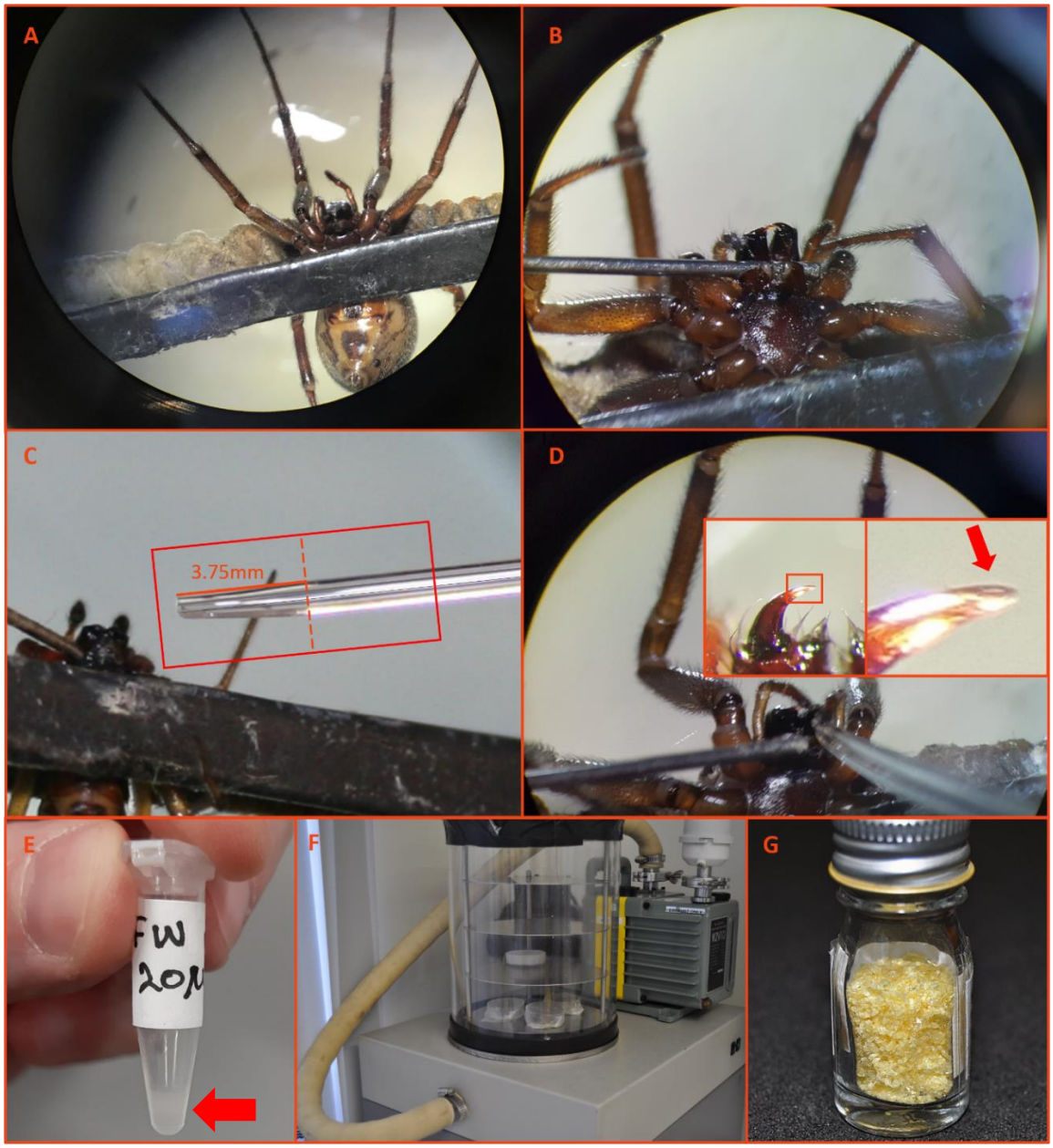

Figure 8: The venom extraction process. A) A *Steatoda nobilis* specimen secured by the thorax in the forceps blades. B) positioning the negative electrode at the base of the chelicerae. C) Approaching the fangs with a 5µl microcapillary tube that has been tapered for easier access to the fang tips. Note the broken line marks the point when the tapered end begins, and the end is clipped to 3.75mm to avoid narrowing the inner flow tube. D) microcapillary tube approaches the fangs, the insert shows the fang manipulated into position where the fang tip is exposed and the venom pore (red arrow) is accessible to collect the venom. E) 0.5 ml eppendorf containing 20µl of venom, after spinning down the tube in the centrifuge, venom accumulates at the bottom of the tube (red arrow). F) once venom is flash frozen it is placed in the lyophilizer and venom is freeze dried overnight. G) Crystalized venom after the lyophilizing process, it's now stabilized and can be stored at -20°C for decades.

2.1.4 - Average Venom Yields and Body Lengths

During four nights of sampling in August 2019, 550 *Steatoda nobilis* specimens were captured and housed in the lab. Immature or moulting specimens were discarded from the venom extraction process. As a result, venom extractions were carried out on a total of 522 individuals separated by gender based on the presence of swollen pedipalps or the epygine. A total venom yield of 113.48µl was collected and processed. Individual venom yields and the specimen's gender and body sizes were recorded to determine the relationship between the size, gender, and venom availability. The mean body size (prosoma + opisthosoma) for females (N=403) was 9.78 mm with a standard deviation of 1.49 mm, and the largest size reaching 13.9mm. On average, females produced 0.22µl of venom with a standard deviation of 0.11µl, and a maximum yield of 0.56µl. The mean body size for males (N=119) was 9.14mm with a standard deviation of 1.23 mm, and the largest specimen measured 12.5mm. Males produced 0.18µl on average with a standard deviation of 0.09µl and a maximum yield of 0.5µl.

Overall, the abundance, size, and hardiness of this species make *S. nobilis* an excellent model organism for research. Our general methods show that, although labour intensive, field sampling wild caught specimens and electrostimulation-driven venom extractions are viable methods to obtain sufficient quantities of venom to facilitating large scale research projects.

2.2 - Distribution study

New distribution records were compiled either directly by the authors during habitat surveys, or by screening wildlife-recording and social media re-sources for photographic records posted online by amateur arachnologists.

2.2.1 - Field inspection and spider collection

Surveys took place between September 2014 and February 2017 as part of a series of student-led arthropod surveys held by the National University of Ireland Galway Discipline of Zoology. Urbanised areas, woodlands, coastal habitats, bogs, meadows,

and a cave system were inspected across nine Irish counties for the presence of alien arthropods, including *S. nobilis*. Depending on habitats, survey methods included visual inspection, net sweeping, beating trays, pitfall traps, and cryptozoic traps. The latter consisted of 40cm-wide pieces of cardboard wrapped around tree trunks and checked weekly for a period of three weeks. When sightings were made, the subsequent area was extensively searched by the authors and *S. nobilis* were collected and locations were systematically recorded. In those instances when they occurred, predation events involving *S. nobilis* were recorded and photographed.

2.2.2 - Online-based records

Records of *S. nobilis* sightings were compiled following a web search on four wildlife recording websites (brc.ac.uk/irecord/; biodiversityireland.ie; ispotnature. org; nbnatlas.org/) and two online social media groups (facebook.com/groups/BritishSpiderIdentification/;facebook.com/groups/insectsinvertebratesire/).

Webpages were searched using the following key words: *Steatoda, nobilis,* false widow, spider, Theridiidae, cobweb, combfooted. A list of messages containing positive photographic identification of *S. nobilis* was compiled. Photographers were contacted by the authors to confirm recorded sightings and to obtain photographic voucher material. Additionally, the authors posted public messages on the two social media webpages aforementioned, inviting group members to submit photographic material of new sightings. Records were subsequently compiled, and locations were mapped to their nearest 10km grid using ArcGIS v10 (ESRI, California, USA).

2.2.3 - Reproductive Study

Previous studies (Hann, 1990) suggest that the high reproductive rate of the invasive *Steatoda capensis* (Hann, 1990) in New Zealand is a contributing factor in the displacement of the native *Latrodectus katipo* (Powell, 1871). Following Hann's lead, we investigated the reproductive rate of *S. nobilis* in captivity and compared it with existing data on the reproductive rate of the missing-sec-tor orb weaver *Zygiella x-notata* (Clerck, 1757), a common urban dweller in Ireland (Wherry and Elwood, 2009).

S. nobilis caught in Co Dublin were kept individually and egg sacs were either left to hatch or processed for further developmental investigations. Hatchlings were kept communally for the first week after emerging from the sac. They were then separated, kept individually in 1ml Eppendorf tubes and fed *Drosophila melanogaster* (a stock of the *wingless* mutant for ease of handling) twice weekly. The number of eggs or hatchlings for each egg sac was recorded.

2.2.4 - Photography

Photographs of *S. nobilis* were taken using a Nikon AF-P DX NIKKOR 18–55mm f/3.5– 5.6G and a Nikon AF-S DX Micro Nikkor 40mm f 2.8G lenses mounted on a Nikon D5200 DSLR camera. Macro-photographs were taken using a Sigma 105mm Macro lens mounted on a Canon 5DS camera. Pictures were manually adjusted for light, contrast and colour using IrfanView 3.92 (1996–2004 Irfan Skijan; Vienna University of Technology).

2.3 - Molecular techniques

2.3.1 - DNA extractions

Spiders specimens were euthanized by overdose of CO2, and/or acquired remains were used for DNA extraction. To avoid contamination with other arthropod species from gut contents, the spider's legs were removed, and DNA extraction was carried out using the NucleoMag Plant kit (744,400.1) protocol following the manufacturer's instructions.

Spiders legs were flash-frozen in liquid nitrogen and ground to a fine powder using a bead mill (Qiagen Tissue Lyser II). 500 µl of Buffer MC1 was added and mixed by vigorous shaking for 15–30 s. The sample was centrifuged briefly for 30 s at 1,500 x g and incubated at 56 °C for 30 min. The samples were then centrifuged for 20 min at a full speed (5,600–6,000 x g). 400 µl of the cleared lysate (equilibrated to room temperature) was transferred to a Square-well Block. 30 µl of NucleoMag® C-Beads

and 400 µl of Buffer MC2 was added to each well of the Square-well Block. The samples were mixed by pipetting up and down 6 times and shaken for 5 min at room temperature. 600 µl of wash Buffer MC3 was added to each well and resuspended with the beads by repeated pipetting up and down 15 times. 600 µl of wash Buffer MC4 was added to each well and resuspended with the beads by repeated pipetting up and down 15 times. 600 µl 80% ethanol was added to each well and resuspend with the beads by repeated pipetting up and down 15 times. 600 µl of wash Buffer MC5 was added to each well and incubated for 45–60 s while the beads were still attracted to magnets, then aspirated and the supernatant was discarded. 200 µl of elution Buffer MC6 was added to each well of the Square-well Block and resuspended with the beads by repeated pipetting and incubated for 5–10 min at 56 °C. We then separated the magnetic beads by placing the Square-well Block on the magnetic separator for 2 min until all the beads have been attracted to the magnets. Then the supernatant containing the purified DNA was transferred to the Elution Plate.

2.3.2 - PCR

DNA was extracted using magnetic beads as per (Fort et al., 2018) and PCR amplification of the Cytochrome Oxydase Subunit 1 gene was done using the primers coxf3 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and coxFR3 (5'-TAA ACT TCA GGG YGA CCA AAA AAT CA-3'). PCR samples contained 5µl MyTaq Red mix (Bioline), 3.5 µl H2O, 0.125 µl of each primer (20 uM stock) and 1 µl DNA. We used the following PCR program: 95 ºC for 3 min, followed by 35 cycles of 95 ºC for 30 s, 58 ºC for 30 s and 72 ºC for 30 s, and a final extension of 7 min at 72 ºC. Amplified material was sent to LGC Genomics GmbH, Germany, for Sanger sequencing.

To determine the species identities, the COX1 genes were isolated from the mitochondrial genome sequence of *S. nobilis* (unpublished data). *Steatoda* and *Crustulina* specimens were acquired from online public databases including National Center for Biotechnology Information (NCBI) and http://www.boldsystems.org/index.php/Public_BarcodeCluster?clusteruri=BOLD:ABA5272 (Bauer et al., 2019). The sequences were aligned using MAFFT (Katoh et al., 2009). The best evolutionary model for the COX1 alignment was chosen using

jModeltest2 (Darriba et al., 2012) based on Akaike and Bayesian information criteria (AIC and BIC, respectively), and HKY + G + I model was found to be the most appropriate. Next, a Maximum Likelihood tree was generated using RaXML-NG (Kozlov et al., 2019), HKY + G + I model and 1,000 replicate trees. In parallel, a Bayesian inference tree was generated using MrBayes with the same evolutionary model, four Markov Chain Monte–Carlo chains and 1 million generations. Finally, we tested for species delimitation as per (Fort et al., 2019) using a General Mixed Yule Coalescent (GMYC) model in BEAST (Bouckaert et al., 2014) and the R packages Splits and Rncl (Fujisawa and Barraclough, 2013).

2.3.3 - RNA extractions

Adult *S. nobilis* specimens were collected from the same location as above. Venom extraction was carried out on 25 females, and three days later the spiders were euthanised with an overdose of CO_2 and once dispatched, using micro-dissection tweezers the dorsal exoskeleton was removed exposing the venom glands. The glands were removed and pooled together in a 2 ml tube containing RNA later (Ambion), with three biological replicates. Venom glands were then flash-frozen in liquid nitrogen and ground to a fine powder using a bead mill (Qiagen Tissue Lyser II). Immediately after grinding, 0.5 ml of TRIzol was added before samples thawed. Samples were shaken for 15 seconds, incubated at room temperature for 5 minutes and then centrifuged (25,000 g) at 4°C for 10 minutes. The supernatant was collected and transferred into new tubes. Nucleic acids were separated by the addition of 100 µL of chloroform, followed by 30 seconds of shaking, and 3 minutes incubation at room temperature. Then, the two phases were separated by centrifugation for 10 minutes at 4°C. Nucleic acid precipitation was performed on 200µL of the upper aqueous phase with 200µL of isopropanol, mixed for 10 seconds, incubated in ice for 15 minutes and centrifuged for 15 minutes. The pellets were washed twice with 75 % EtOH (prepared in DEPC water). Finally, the pellets were air dried at 37°C and resuspended in 50 µL of RNAse-free water. Following extraction, DNA was removed

from the nucleic acids using DNAse 1 (Sigma-aldrich AMPD1), following manufacturer's instructions.

Finally, DNA-free total RNAs were cleaned up and concentrated using a silica column based kit (Zymo Research RNA Clean & Concentrator).

2.4 - Venomics techniques

2.4.1 - Transcriptomics assembly and analysis

For preparation of RNA transcript libraries and sequencing, samples were sent to *Novogene Company Limited, Cambridge Science Park, Milton Road, Cambridge, CB4 0FW, UK*. Libraries were generated using mRNA enrichment, and sequencing was performed using Illumina technology (150 bp paired-end reads). For all three libraries, >50 million 150 bp paired-end reads were obtained.

Raw reads were corrected using Rcorrector (Song and Florea, 2015) and leftover Illumina adapters removed using trimmomatic (Bolger et al., 2014). Then, reads mapping to ribosomal RNAs (using bowtie2 (Langmead and Salzberg, 2012) mapping against *Latrodectus* and *Steatoda* ribosomal RNAs sequences available in NCBI) were removed to ensure good representation of reads belonging to mRNA transcripts in the transcriptome assembly. The transcriptome of one of the three venom gland samples was assembled using the Trinity pipeline (Grabherr et al., 2011, Haas et al., 2013). A single sample was used for assembly to limit the hardware requirements of such method. Transdecoder (Haas and Papanicolaou, 2015) was used to identify Open Reading Frames (ORFs) originating from the transcripts, with a minimum protein length of > 75 amino acids. We intentionally lowered the minimum ORF prediction from Transdecoder (100) to 75 amino acids in length, to account for the presence of potentially relevant small proteins, such as those described in ref (Rokyta and Ward, 2017) The assembly was annotated using Trinotate (Bryant et al., 2017) based on ORFs homology using BLASTp (e-value cutoff of $1e^{-3}$) against i) SwissProt curated database (Consortium, 2019) and ii) TrEMBL Arachnids database

(also (Consortium, 2019)). In addition, ORFs were compared to the Protein family (Pfam) database (El-Gebali et al., 2019).

Gene expression analysis was performed as part of the Trinity pipeline using Kallisto (Bray et al., 2016) on the three venom gland libraries, using the assembled transcriptome generated by Trinity. The gene expression matrix obtained was used to extract genes with high expression level (>100 Transcripts per Million (TPM)) for Gene Ontology (GO) analysis. GO terms associated with the annotated genes were retrieved using Trinotate's extract_GO_assignments_from_Trinotate_xls.pl script. GO analysis was performed using GOseq (Young et al., 2012), with the genes > 100 TPM as input (~900 genes), against the background of all ~17,000 genes with GO annotations. Over-representation of Gene Ontologies was analysed using ReviGO (Supek et al., 2011), and plotted in R (R Development Core Team, 2011). For venom-encoding genes, relative abundance of enzyme, and toxin-producing genes was generated using the Treemap package in R (Bederson et al., 2002).

2.4.2 - SDS-PAGE of *S. nobilis* female venom

10µg of *S. nobilis* venom was diluted in Laemlu buffer, heated for 3min to 100°C, before being separated using 1D SDS-PAGE NuPage® (ThermoFisher Scientific) in MES SDS buffer. 6µg of a standard composed of insulin beta-chain (3kDa), aprotinine (6kDa), lysozyme (14kDa), red myoglobin (17kDa), carbonic anhydrase (28kDa), alcohol dehydrogenase (38kDa), glutamic dehydrogenase (49kDa), bovine serum albumin (62kDa), phosphorylase (98kDa) and myosin (188kDa), were used as molecular weight markers. The electrophoresis was performed by applying 200V during 40min to the system. The resulting gel was firstly dehydrated with 50% EtOH and phosphoric acid 3% during 3 hours, then rehydrated by the mean of a 20min bath of ultrapure water (MilliQ). Le coloration of the proteins was performed overnight with Coomassie blue (360 g/L, in an aqueous buffer with 34% MeOH, 3% phosphoric acid and 17% ammonium sulphate). The gel was finally conserved at 5°C in 5% of acetic acid for further experiments.

2.4.3 - Shotgun Proteomics of *S. nobilis* female venom

0.2mg of lyophilized venom was dissolved in 100µL of pure water (MilliQ). 3µl corresponding to roughly 6µg was lyophilized and dissolved into 20µL of 50 mM NH_4HCO_3 pH 7.8. The sample was then reduced with 5µL of 500mM dithiothreitol (DTT) for 45min at 56°C under shaking at 300 rpm. The reduced venom was then alkylated with 6µL of 500 mM iodoacetamide for 30 min, at room temperature, in the dark. The venom was then submitted to enzymatic digestion with trypsin at a ratio of 1:50, incubated overnight, at 37 °C, under shaking at 300 rpm. Reactions were stopped by acidifying the medium using 10% TFA. The digested sample was finally dried on speed vacuum. Before the mass spectrometry analysis, the samples were suspended in 20 µL of 0.1% TFA for desalting on ZipTip™ pipette tips with C18 resin. The elution was made by 18µl of TFA 0.1%/ACN (50/50), to reach a theoretical concentration de ~3µg/9µL, suitable for LC-MS analysis (9µL injected, 100min run). The efficiency of the digestion was controlled by MALDI-TOF, using saturated CHCA (70/30 ACN/FA 0.1 %) as the matrix.

The purified material was analysed using an Acquity UPLC® M-Class (Waters, Milford, MA, USA) coupled to the Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, Bremen, Germany). The trap column is a Symmetry C18 5µm (180 µm x 20 mm) and analytical column is a HSS T3 C18 1.8 µm (75 µm x 250 mm) (Waters, Corp., Milford, USA). The samples were loaded at 20 µL/min on the trap column in 98% solvent A (water/0.1% formic acid) during 3 minutes, and subsequently separated on the analytical column. Peptides were eluted using a gradient of 2–85% of solution B in 73min (B: acetonitrile/0.1% formic acid), at a flow rate of 0.6 mL/min. Regarding mass spectrometry, all the analyses were performed in data dependent analysis (DDA) mode that automatically triggers the MS/MS experiments. The automatic gain control (AGC) target values were 3.10^6 for MS spectra and 2.10^5 for MS/MS spectra. The maximum injection times were set at 200 ms for the MS step and 1000 ms for MS/MS events. For MS/MS, a "Top 12" experiment was applied, meaning that the twelve most intense ions of each MS scan have been selected for fragmentation. Singly charged ions, ions with undetermined

charge (for example, electronic noise) and ions with signal intensities below the AGC threshold set at 1e3 were excluded from the selection. For precursors ions, the selection windows were 2.0 m/z, the AGC target was 1e5 (or 50 ms as a maximum of injection time) and the resolving power of 17,500 @m/z 200. Normalized collision energy was 25. A dynamic exclusion of 10s was also applied to avoid the redundancy of MS/MS spectra of the same ions. 2.6.

Bioinformatic analysis of proteomic data PEAKS Studio X+ (Bioinformatics solutions, Waterloo, ON, Canada) a *de novo* assisted database software was chosen to analyse MS/MS data from *S. nobilis* venom. The database chosen for analysing the proteomics data is composed of the translated sequences obtained from the assembled transcriptome. PEAKS studio initially produces de novo sequences from MS/MS spectra without relying on database. The confidence of each peptide sequence obtained by this process is given by an ALC (Average Local Confidence) score. These de novo sequences are then corrected by comparing them to the database to provide additional information about post-translational modifications (PTM's), mutations, homologous peptide and novel peptides. Carbamidomethylation was set as fixed modification, while oxidation (M) was set as variable modifications, with maximum missed cleavages at 3 for trypsin digestion. Parent mass and fragment mass error tolerance were set at 5ppm and 0.015 Da, respectively. False discovery rate (FDR) of 1% [29,30] and unique peptide ≥2 were used for filtering out inaccurate proteins. A−10lgP > 120 indicates that the detected proteins by enough reliable peptides MS/MS spectra.

2.4.4 - Statistical analysis of venom yields, body sizes & seasons

A bivariate Pearson Correlation was used to estimate the relationship between spider body size and venom yield using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA). Scatter plots and bar charts were generated using the same software. Spider body sizes (overall, females & males) were arranged into three categories: <9 mm, 9-11 mm, and >11 mm. A one-way analysis of variance (ANOVA) coupled with a Tukey's post-hoc test was performed to estimate 1) the difference in

venom yield amongst the spiders arranged in groups of size and 2) the difference in venom yield depending on seasonality (i.e. spring, summer and autumn). Significance was noted when $p \leq 0.05$.

2.5 - Case studies

12 adult females, six adult males, and parents of a seven and a five-year-old children reported envenomation symptoms caused by *S. nobilis*. Medical charts were obtained for two cases. In all cases, the victims provided the authors with either photographs, live specimens, or physical remains of the offending spiders. All specimens were identified using Roberts (1995), Dugon (2017), or genetic barcoding techniques (Bauer et al., 2019, Hambler, 2019).

2.6 - Online web search for a systematic review

ISI Web of Science databases were searched using the terms "spider bite necrosis", "arthropod envenomation necrosis", "venom necrosis", "venom immune response", "loxoscelism", "arachnidism", "necroptosis venom", "necroptosis dermatitis", "tumour necrosis factor TNF venom", "scorpionism", "scolopendrism", "centipede necrosis", "NETosis venom", "NETosis necrosis". All citations (N¼2,680) were exported to EndNoteTM version X7. The citation list was inspected and all duplicate entries (N¼843) were manually removed. Searches produced a total of 1,737 nonduplicate citations, of which 74 were considered relevant to this manuscript. Because of the large volume of unsubstantiated reports on venom-induced necrotic lesions, non-peer-reviewed sources were excluded. Historical reports of suspected venom-induced necrosis published without voucher material clearly identifying the organism involved in the envenomation were also discarded.

2.7 - Aseptic techniques for microbiological analysis of bacterial on body and fang surfaces of spiders

2.7.1 - Spider and venom collection

Specimens of *Amaurobius similis, Eratigena atrica, Steatoda grossa, S. nobilis* and *Zygiella x-notata* were collected in Ireland, from garden walls and park railings in Lucan, Co. Dublin, Edgeworthstown, Co. Longford, Galway city, Co. Galway and Ferrybank, Co

Waterford. Specimens were collected using sterile forceps, placed immediately into sterile tubes, and transported to the lab. Species identities were confirmed using identification guides specific to *S. nobilis* (Dugon et al., 2017) and Collins Field Guide for all other spiders (Roberts, 1995).

Using aseptic technique, the specimens were dispatched, and the fangs were either clipped or swabbed. For whole body cultures, spiders were either submerged in media or swabbed. For surface colonisation analysis, spiders walked directly on Brain Heart Infusion (BHI) agar. The most common method for euthanising arthropods is dispatchment. A select number of spiders were euthanised using CO_2, to determine if bacteria was recoverable.

For venom extractions, *S. nobilis* specimens were anesthetized using CO_2 for 2 min and venom was extracted by electrostimulation with repeated pulses delivered at 15-20V. Venom droplets were collected from the venom pores located on the outer subterminal part of the chelicerae using 5 µl microcapillary tubes modified with a tapered end for maximum efficiency. Venom from approximately 100 specimens was pooled and then flash-frozen in liquid nitrogen and stored at -80°C.

2.7.2 - Preliminary testing for microbiomes from *A. similis, E. atrica, S. nobilis, S. grossa* and *Z. x-notata* and 16S rRNA gene amplification, sequencing, and analysis:

Whole bodies or fangs from five species of spiders: *A. similis*, *E. atrica*, *S. grossa*, *S. nobilis*, and *Z. x-notata* were transferred into 750 µl (10% dilution) of Luria Bertani (LB) broth, Nutrient broth (NB), Tryptic Soy broth (TSB), MRS broth and BHI broth, and incubated at both 37°C and 10°C. Whole culture from each spider or fang were pelleted, DNA was extracted collectively from each sample using the QIAGEN Dneasy Blood & Tissue Kit and V3-V4 region of 16S rRNA was amplified using 341F 5'-CCTACGGGAGGCAGCAG-3' (Lane, 1991), and 806R 5'-GGACTACHVGGGTWTCTAAT-3' (Caporaso et al., 2011). The amplified product was then sent to GATC Biotech for sanger sequencing. A BLAST search was carried out with the obtained sequence using the NCBI rRNA/ITS database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7.3 - Bacterial isolation from *S. nobilis* and 16S rRNA gene amplification, sequencing and analysis:

For isolating surface bacteria, *S. nobilis* spiders were washed individually with 5 ml BHI broth for 5 min. The wash media was then incubated at 37°C overnight. For isolating bacteria from fangs, clipped fangs from each individual spider were inoculated into BHI broth and incubated at 37°C. After 24 h incubation, the cultures were diluted and plated on BHI agar and incubated 48 h to 72 h at 37°C. Selective media, Baird-Parker agar and TS-blood agar supplemented with colistin and nalidixic acid, were also inoculated with overnight cultures and incubated 48 h to 72 h at 37°C. Colonies with different morphologies were selected for further analysis.

The 16S rRNA gene was amplified using *Taq* polymerase (Bioline) and universal primers, 27F (5′-AGAGTTTGATCATGGCTCAG-3') and 1492R (5′-GGTTACCTTGTTACGACTT-3′) using Colony PCR (Senthilraj et al., 2016). The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced using primers, 27F 1492R (Eurofins Genomics, Germany).

A BLAST search was carried out with the obtained sequence using the NCBI rRNA/ITS database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Closest bacterial species were identified using Blast tree view produced by Blast pairwise alignment.

2.7.4 - Inhibitory effects of *S. nobilis* venom against pathogens

Venom extracted from *S. nobilis* was assessed for its inhibition of bacterial growth by determining the Minimum Inhibitory Concentration (MIC). Due to the limited amount of venom available, dilutions were carried out on the samples to achieve usable volumes. The antibacterial potential of the venom was assessed against clinical isolate *E. coli* DSM1103 (aka ATCC 25922, NCIB 12210) (DSMZ, Germany) *E. coli* DSM10973, Methicillin Resistant *Staphylococcus aureus* (MRSA) BH1CC and *L. monocytogenes* EGD-e. An overnight culture was adjusted with LB broth to an inoculum density of 1×10^6 cfu ml^{-1}. 1:10 dilution of the venom was tested against *E. coli* DSM1103 and 1:100 against other pathogens in a final inoculum of 5×10^5 cfu ml^{-1}, and were incubated for 24 h at 37°C. After 24 h incubation at 37°C,

absorbance at 590 nm was measured using a microplate reader (Tecan) with Magellan software.

2.7.5 - Antibiotic susceptibility testing

Disk diffusion assays were carried out to determine antibiotic susceptibility. Experiments were done according to the CLSI guidelines. 6 mm discs preloaded with each antibiotic (Oxoid) were placed onto Mueller-Hinton agar plate that had been spread with 100 µl overnight bacterial culture (1×10^8 cfu/ml). Plates were incubated at 37°C for 18 h and the clear zone around each disc was measured.

Chapter 3
Ecology

In situ adult female *Steatoda nobilis* in a brooding chamber with an eggsac

Photo by JP Dunbar

3.1 - Occurrence, reproductive rate, and identification of the non-native noble false widow spider *Steatoda nobilis* (Thorell, 1875) in Ireland

JPD contributed to the conceptualization, methodology, data acquisition, software, original draft preparation, and review and editing. This section is published in a peer reviewed journal and available at the following reference: DUGON, M. M., DUNBAR, J. P., AFOULLOUSS, S., SCHULTE, J., MCEVOY, A., ENGLISH, M. J., HOGAN, R., ENNIS, C. & SULPICE, R. Occurrence, reproductive rate and identification of the non-native noble false widow spider *Steatoda nobilis* (Thorell, 1875) in Ireland. *Biology and Environment: Proceedings of the Royal Irish Academy*, 2017. JSTOR, 77-89.

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3.1.1 - ABSTRACT

The noble false widow *Steatoda nobilis* (Thorell, 1875) has established thriving populations in urban centres throughout England and Wales since it was accidentally imported from the Canary Islands and Madeira to Britain over a century ago. In recent years, *S. nobilis* has colonised parts of Western Europe, California, Chile and the Middle East. In Ireland, *S. nobilis* was first recorded in 1999 from a single location in Co Wicklow. The present study examines the current range and main habitats of *S. nobilis* in Ireland and assesses its potential as an invasive species by documenting its reproductive rate. Additionally, we present photographic material illustrating the intraspecific phenotypic variations exhibited by *S. nobilis* to assist in correct identification of this species by the public. Our data shows that *S. nobilis* is an extremely prolific, resilient species with distinct synanthropic affinities. This species currently occurs in at least sixteen Irish counties with the largest populations observed in the greater Dublin area, where it has become widespread in buildings

and on street furniture. *S. nobilis* seems to be currently absent from natural, undisturbed habitats such as woodlands, bogs, and grassland. We suggest that due to its comparatively fast reproductive rate, long life span, and year-round activity, *S. nobilis* might have a detrimental impact on native urban-dwelling spiders.

3.1.2 - INTRODUCTION

The first Irish record for the noble false widow spider *S. nobilis* (Thorell, 1875) occurred in Bray, Co Wicklow, in 1998 (Nolan, 1999). This alien species is of particular interest in Great Britain and in Ireland as it has been involved in the only case of systemic envenomation (steatodism) reported in these islands (Warrell et al., 1991). Additionally, studies suggest that *S. nobilis* might have a negative impact on populations of native arthropods in areas it has colonised (Kulczycki et al., 2012).

The geographical expansion of *S. nobilis* has been fairly well documented for over a century, thanks to its relatively large size, conspicuous markings, and superficial resemblance to the black widows of the genus *Latrodectus* (Walckenaer 1805). *S. nobilis* is thought to originate from the Atlantic archipelagos of Madeira (Thorell 1875) and the Canaries (Bristowe, 1929). In 1879, *S. nobilis* was recorded for the first time outside its native range; Rev. Pickard-Cambridge identified a sub-adult female collected a few years earlier by Rev. Ham-let Clark near Torquay, Devon (Cambridge, 1879). This confirms that *S. nobilis* reached the British Isles—at least as an occasional visitor—well before the turn of the twentieth century. In 1906, A.R. Jackson came into the possession of at least one adult female collected by a third party on a cliff in southern England, away from man-made structures (Jackson, 1907). Jackson concluded that the specimen was unlikely to be a mere alien 'visitor', and implied that a population may have already been established in southern England by the early 1900's. Referring to Pickard-Cambridge's work, Bristowe (1929) suggested that *S. nobilis* may have been imported occasionally from the Canary Islands in shipments of bananas but rejected the idea that *S. nobilis* had established sustainable colonies at the time of publication. *S. nobilis* was later recorded at closer intervals in the southern half of Britain: Hampshire (Jones, 1979, Jones, 1987), Dorset (Snazell and Jones, 1993), Essex

(Merrett, 1989, Smith, 1992), Sussex (Warrell et al., 1991), Warwickshire (Bate, 2005), Glamorgan-shire (Jones, 2006), Leicestershire (Daws, 2008) and Lincolnshire (Binding, 2014). Further afield, the distribution range of *S. nobilis* has been continuously expanding since the turn of the twenty-first century. *S. nobilis* has become widespread in coastal urban centres in France (Kovoor and Munoz-Cuevas, 2000), Belgium (Van Keer, 2010), Italy (Kulczycki et al., 2012), Spain (Déjean, 2013), Portugal (Cardoso, 2000), California (Vetter et al., 2015, Vetter and Rust, 2012) and Chile (Faúndez and Téllez, 2016, Taucare-Ríos et al., 2016). Eastward, *S. nobilis* has been observed in Germany (Reiser, 2013), Turkey (Türkeş and Mergen, 2007) and as far as Iran (Zamani et al., 2015).

Although *S. nobilis* is thought to have spread in many parts of Ireland since Nolan's original report (1999), the occurrence and the ecological impact of this species at the national level have not been assessed yet. The aims of the present study are threefold: 1) assess if *S. nobilis* has expanded its Irish range since it was first recorded; 2) document the reproductive rate of *S. nobilis*; 3) document the intraspecific phenotypic variations exhibited by *S. nobilis* in Ireland to assist in correct identification by the public.

3.1.3 - MATERIALS AND METHODS

3.1.3.1 - Distribution study

New distribution records were compiled either directly by the authors during habitat surveys or by screening wildlife-recording and social media resources for photographic records posted online by amateur arachnologists.

3.1.3.2 - Field inspection and spider collection

Surveys took place between September 2014 and February 2017 as part of a series of student-led arthropod surveys held by the National University of Ireland Galway Discipline of Zoology. Urbanised areas, woodlands, coastal habitats, bogs, meadows, and a cave system were inspected across nine Irish counties for the presence of alien arthropods, including *S. nobilis*. Depending on habitats, survey methods included

visual inspection, net sweeping, beating trays, pitfall traps, and cryptozoic traps. The latter consisted of 40cm-wide pieces of cardboard wrapped around tree trunks and checked weekly for a period of three weeks. Coordinates, habitats, dates and sampling methods for each surveyed location are detailed in Table 1. When sightings were made, the subsequent area was extensively searched by the authors and *S. nobilis* were collected using either a pair of flexible tweezers or a Katcha™ Bug Buster Spider Vacuum and then placed in 50ml falcon tubes. Locations were systematically recorded. In those instances when they occurred, predation events involving *S. nobilis* were recorded and photographed. The collection of live specimens for lab-based experiments took place after sunset (when *S. nobilis* is most active) in the larger Dublin area between September 2015 and October 2016.

3.1.3.3 - Online-based records

Records of *S. nobilis* sightings were compiled following a web search on four wildlife recording websites (brc.ac.uk/irecord/; biodiversityireland.ie; ispotnature. org; nbnatlas.org/) and two online social media groups (facebook.com/groups/BritishSpiderIdentifi cation/; facebook.com/groups/insectsinvertebratesire/).

Webpages were searched using the following key words: *Steatoda, nobilis,* false widow, spider, Theridiidae, cobweb, combfooted. A list of messages containing positive photographic identification of *S. nobilis* was compiled. Photographers were contacted by the authors to confirm recorded sightings and to obtain photographic voucher material. Additionally, the authors posted public messages on the two social media webpages aforementioned, inviting group members to submit photographic material of new sightings. Records were subsequently compiled, and locations were mapped to their nearest 10km grid using ArcGIS v10 (ESRI, California, USA).

3.1.3.4 - Reproductive Study

Previous studies (Hann, 1990) suggest that the high reproductive rate of the invasive *Steatoda capensis* (Hann, 1990) in New Zealand is a contributing factor in the displacement of the native *Latrodectus katipo* (Powell, 1871). Following Hann's lead,

we investigated the reproductive rate of *S. nobilis* in captivity and compared it with existing data on the reproductive rate of the missing sector orb weaver *Zygiella x-notata* (Clerck, 1757), a common urban dweller in Ireland (Wherry and Elwood, 2009).

S. nobilis caught in Co Dublin were kept individually in 80mm x 50mm cylindrical plastic containers with shredded paper as substratum. Spiders were stored in chest incubators at a constant 20°C, watered and fed weekly on a diet of commercially available crickets (*Gryllus assimilis*, Fabricius, 1775) and fruit flies (*Drosophila melanogaster*, Meigen, 1830). Each spider was individually coded to facilitate records of egg-laying. Egg sacs were consistently removed from the mother in the 24 hours following egg-laying. Each egg sac was individually coded, measured and incubated at 20°C in a 25ml falcon tube containing wet cotton to avoid dehydration. Egg sacs were either left to hatch or processed for further developmental investigations. Hatchlings were kept communally for the first week after emerging from the sac. They were then separated, kept individually in 1ml Eppendorf tubes and fed *Drosophila melanogaster* (a stock of the *wingless* mutant for ease of handling) twice weekly. The number of eggs or hatchlings for each egg sac was recorded.

3.1.4 - Identification Charts

3.1.4.1 - Photography and carapace measurements

Photographs of *S. nobilis* were taken using a Nikon AF-P DX NIKKOR 18–55mm f/3.5– 5.6G and a Nikon AF-S DX Micro Nikkor 40mm f 2.8G lenses mounted on a Nikon D5200 DSLR camera. Macro-photographs were taken using a Sigma 105mm Macro lens mounted on a Canon 5DS camera. Pictures were manually adjusted for light, contrast and colour using IrfanView 3.92 (1996–2004 Irfan Skijan; Vienna University of Technology).

To investigate the mean size of individuals within the Irish population of *S. nobilis*, we measured 102 mature specimens collected between September 2015 and October 2016 in Co Dublin. Specimens were placed in a gas chamber and exposed to CO2 for two minutes until anaesthetised. Spiders were then placed lying flat on their ventral aspect. Carapace length was measured using a precision analogue calliper and

measurements rounded to the closest 0.1mm. Carapace length was determined by measuring each specimen from the proximal article of the chelicerae (in 'normal' resting position) to the base of the spinnerets. Eggs and hatchlings were measured with a ruler reticle mounted on an Olympus SZ61 stereomicroscope.

3.1.5 - RESULTS

3.1.5.1 - County distribution of *S. nobilis*

During the study that took place between September 2014 and February 2017, *S. nobilis* was identified in 54 geographically distinct locations across 16 Irish counties, of which a total of 15 are new county records (Fig. 1). The social media-based inquiries produced 36 of the new geographic locations (67%) across 13 counties, some dating back as far as 2009 (Table 2). The remaining eighteen locations (33%) were registered by the authors between 2014 and 2016 across six counties (Table 1). As of 1 April 2017, our search on online wildlife recording websites did not return any record for Ireland.

A total of 23 sighting locations (43%) were in Co Dublin, where *S. nobilis* was found in the highest number and appears to be the most common species in and around street furniture. Thirty of the 37 social media-based records were of a single specimen. Four of the 32 locations surveyed by the authors yielded single specimens and 14 yielded multiple specimens. The remaining 16 locations surveyed did not yield any specimen. At the national level, a majority of specimens were sighted in coastal areas as opposed to the midland counties (Fig. 1). The authors observed mature *S. nobilis* throughout the year regardless of ambient temperatures, both outdoors and indoors (Table 1).

3.1.5.2 – *S. nobilis* habitat

Urbanised areas, woodlands, coastal habitats, bogs, meadows, turlough shores, and a cave system were investigated for the presence of *S. nobilis*. Adult specimens were exclusively found on steel, concrete, or timber structures in heavily urbanised areas. Such habitats included sheds, steel fencing, concrete walls, cellars, room ceilings, skirting boards, and window ledges. All locations surveyed in and around Dublin yielded at least one specimen on each visit. Over a period of 18 months, 620

specimens were obtained from a single row of semi-detached houses in Lucan, Co Dublin. Meanwhile, not a single *S. nobilis* was found in the ten non-urbanised locations we surveyed.

A total of six juveniles were sighted on vegetation in two locations. In Lucan, Co Dublin, two specimens were collected in a public park, on webs built amongst the branches at *c*. two meters high and less than ten meters away from the parks' outer boundary railings, which contained large numbers of adult specimens. An additional four juveniles were observed in Oranmore, Co Galway on the leaves of a large Hydrangea contiguous to the glass wall of a heated swimming pool located in the courtyard of a hotel complex.

3.1.5.3 - Predation records

While sampling, several predation events were observed, consisting of nine diet items and two predators of *S. nobilis*. Diet items included a variety of Irish native invertebrates belonging to three arthropod subphyla. The subphylum Insecta was most represented with six, comparatively medium to large prey items, including a Coleopteran (*Ocypusolens* Müller, 1764), a Dermapteran (*Forficula auricularia* Linnaeus, 1758), a Hemipteran (*Palomena prasina* Linnaeus, 1761), a Hymenopteran (*Vespula vulgaris* Linnaeus, 1758), and two Dipterans, (*Calliphora vomitoria* Linnaeus, 1758 and a smaller nonidentified specimen). Two additional prey items belonged to the class Arachnida (subphylum Chelicerata) (*Eratigena atrica* Koch, 1843 and *Araneus diadematus* Clerck, 1758). Woodlice (subphylum Crustacea) seem to be the commonest prey of all. In two instances we recorded adult *S. nobilis* falling prey to common suburban spiders: the cellar spider *Pholcus phalangioides* (Fuesslin, 1775) and the lace-webbed spider *Amaurobius fenestralis* (Ström, 1768).

Table 1—Habitats surveyed for the presence of the noble false widow spider *Steatoda nobilis*. Surveys took place between September 2014 and February 2017 across six Irish counties. Coordinates were obtained from the National Biodiversity Data Centre, Waterford (http://maps. biodiversityireland.ie/#/Map). *Cryptozoic traps* refer to 40cm wide pieces of cardboard wrapped around ten tree trunks and checked weekly for a period of three weeks. *Pitfall traps* consisted of five plastic cups of 7.7cm diameter and 10.6cm height filled with a saline solution. Traps openings were covered with plastic lids secured into the ground with two nails, leaving a 1cm gap between the rim of the cup and the lid. Pitfall traps were relieved weekly for a period of three weeks. The *Transect belt* consisted in a 20m long ribbon laid on the floor. The survey area extended one meter on each side of the ribbon, vegetation up to approximately 1.8m in height was searched. Surveys were typically two to two and a half hours long and involved one to six surveyors. (*) Surveys / collection performed after sunset; all other surveys were performed during daytime.

Location	*Coordinates*	*Habitats*	*Sampling methods*	*Number of visits / Sampling dates*	*Specimens caught*
Co Galway - Galway City Docks	130127, 224953	Apartment complex outdoors	Visual inspection	First sighting in Galway November 2011	1
Co Galway – Galway City City centre	129986, 225191	Parking lots	Visual inspection	Three visits September 2014	4
Co Galway - Galway City Southpark	130005, 224475	Sandy shore / coastal meadow	Visual inspection Sweeping net	Three visits (weekly) October 2014	0
Co Galway - Galway City NUI Galway campus	129340, 225689	Mix trees / buildings	Visual inspection Cryptozoic traps	Three visits (weekly) September–October 2014	0
Co Galway – Galway City Terryland Forest Park	129863, 226223	Peri-urban mixed deciduous woodland	Cryptozoic traps Pitfall traps Visual inspection Sweeping net	Seven visits (weekly) September–November 2014	0
Co Galway – Knocknacarra Rusheen Bay	125847, 223820	Sandy shore / coastal meadow	Visual inspection Sweeping net	Three visits (weekly) September–October 2014	0
Co Galway – Barna Barna Woods	124380, 223775	Deciduous woodlands	Cryptozoic traps Pitfall traps Visual inspection	Three visits (weekly) September–October 2014	0
Co Galway - Barna Barna bog	122467, 227026	Blanket bog	Pitfall traps Visual inspection Visual inspection	Three visits (weekly) September–October 2014	0
Co Galway - Monivea Monivea Woods	153980, 236403	Mixed woodlands	Cryptozoic traps Pitfall traps	Three visits (weekly) September–October 2014	0
	154284, 236034	Thickets of *Prunus laurocerasus* exclusively	Visual inspection Sweeping net		0

Table 1 (*Continued*)

Location	*Coordinates*	*Habitats*	*Sampling methods*	*Number of visits / Sampling dates*	*Specimens caught*
Co Galway – Coole Coole Park	143731, 205117	Mixed deciduous and coniferous woodlands	Cryptozoic traps Pitfall traps	Three visits (weekly) September – October 2014	0
	143387, 204757	Turlough shores / meadows	Visual inspection Sweeping net 20m transect belt		0
Co Galway - Salthill Quincentenary Rd	128344, 223833	Apartment complex Indoors	Report from the public – collected by MD	One visit (collection) November 2014	1
Co Galway - Galway City Newcastle Street	128915, 226361	Semi-detached houses Indoors and outdoors	Report from the public – collected by MD	One visit (collection) March 2015	1
Co Galway - Galway City Terryland Retail Park	129980, 226058	Industrial estate Indoors and outdoors	Visual inspection	Three visits (weekly) September 2015	0
Co Galway - Oranmore Gurraun North	137019, 226188	Peri-urban bungalow	Visual inspection Sweeping net	Three visits (weekly) September– November 2015	0★
Co Galway – Oranmore Claregalway Rd	138500, 227034	Peri-urban bungalow	Visual inspection Sweeping net	Three visits (weekly) September– December 2015	0★
Co Galway – Oranmore Tudor Vale	137936, 225423	Hotel complex Outdoors		One visit September 2016	4
Co Mayo – Ballina Belleek Castle Park	125127, 320744	Mixed deciduous and coniferous woodlands	Visual inspection Sweeping net 20m transect belt	One visit May 2015	0
Co Sligo – Ballimote Kesh cave system	170587, 312149	Limestone cave system	Visual inspection	Two visits June 2016 & January 2017	0
Co Roscommon - Ballymurray Mote Park	190563, 261296	Mixed deciduous and coniferous woodlands	Visual inspection Sweeping net 20m transect belt	One visit September 2015	0
Co Tipperary – Clogheen	195749, 113229	Farmland, inside dry shed	Visual inspection	One visit October 2016	1
Co Longford – Edgeworthstown	224682, 273918	Across the dashboard on the registration plate, wing	Visual inspection	Two visits October 2015	5

Table 1 (*Continued*)

Location	*Coordinates*	*Habitats*	*Sampling methods*	*Number of visits / Sampling dates*	*Specimens caught*
Co Westmeath – Mullingar	245211, 253612	Indoors, inside a garden shed	Report from the public – collected by JD	One visit (collection) October 2016	3
Co Kildare – Maynooth NUI Maynooth Campus	292997, 238528	Indoors on walls, outdoors on windows, concrete and steel structures	Visual inspection	Four visits August 2015 to September 2016	34★
Co Dublin – Tallaght	310309, 227410	Indoors under kitchen units, inside garden shed, greenhouse and outdoors around windows	Visual inspection	One visit February 2016	20
Co Dublin – Tallaght Tallaght Business Park	308322, 227041	Indoors	Report from the public Collected by MD and JD	One visit February 2016	20
Co Dublin – Dublin City Merrion St	316434, 233550	Metallic railing	Visual inspection	One visit June 2016	2
Co Dublin - Clondalkin, Green Isle hotel	306471, 229989	Outdoors on boundary railings	Visual inspection	One visit Junes 2016	4
Co Dublin – Lucan public park	304694, 234684	Public park boundary wall and railings	Visual inspection	One visit July 2016	54★
Co Dublin - Stephens Green	315880, 233507	Public park boundary railings	Visual inspection	One visit July 2016	3
Co Dublin - Raheny	321695, 238343	Indoors on a blanket and outdoors on garden wall	Visual inspection	Two visits August 2016	1
				September 2016	4
Co Dublin - Lucan	305265, 234054	Inside and outside of garden shed, along garden walls and furniture	Visual inspection	Eight visits	
				August 2015	15
				October 2015	71★
				February 2016	50★
				March 2016	1★
				September 2016	150★
				October 2016	172★
				December 2016	1★
				February 2017	160★
Co Dublin – Bawnogue, Clondalkin	305363, 231769	Outdoors - Inside and outside a wheelie bin	Visual inspection	One visit October 2016	7

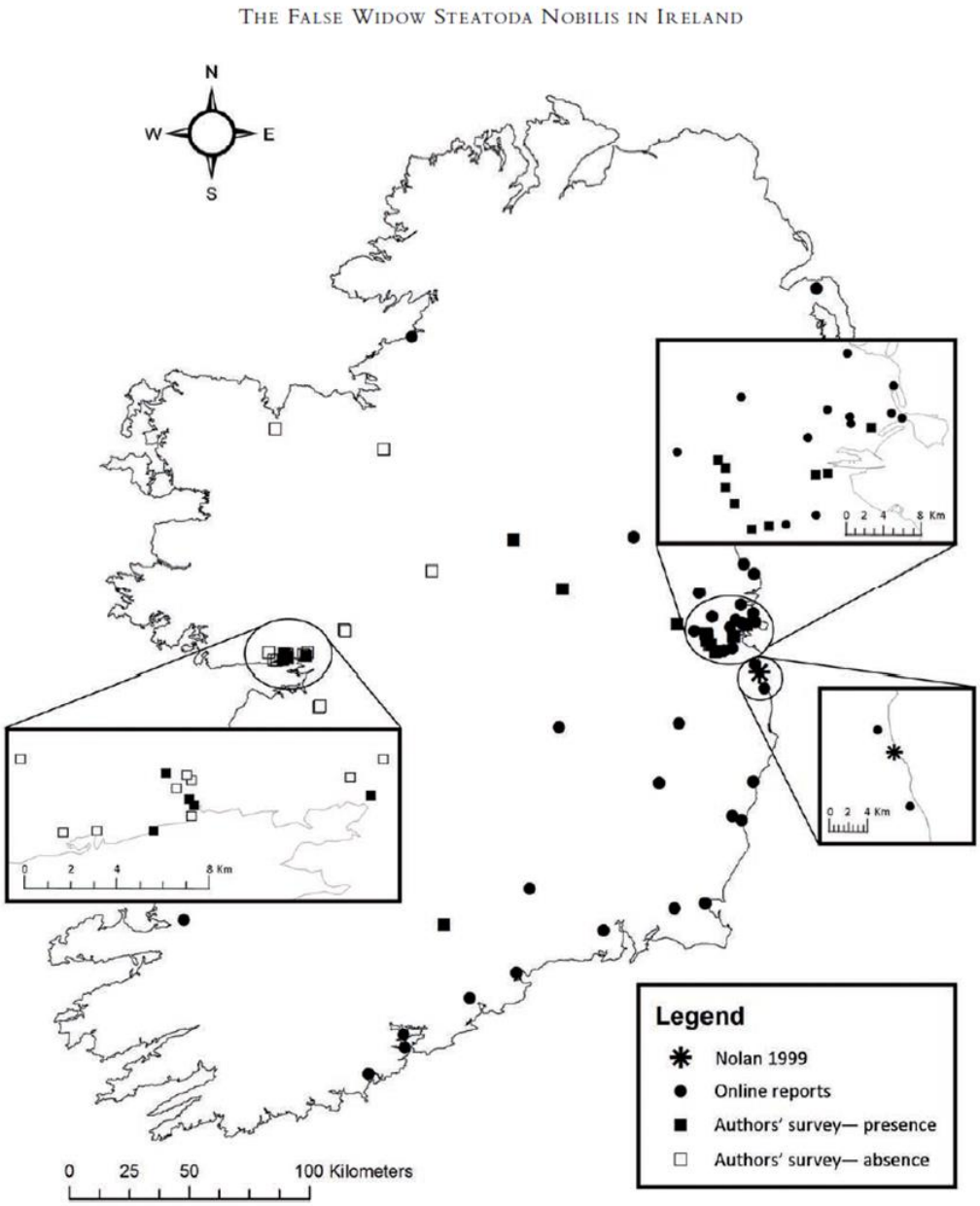

Figure 1—Occurrence of the noble false widow *S. nobilis* in Ireland. 36 positive reports across 13 counties were obtained from online social media. 32 additional locations across nine counties were surveyed by the authors between September 2014 and February 2017. A total of 14 locations in six counties yielded at least one specimen of *S. nobilis*. The first Irish sighting in Bray, Co Wicklow (Nolan 1999) is marked by a star.

Table 2—Noble false widow spider *S. nobilis* sightings in Ireland based on online reports retrieved from two social media interest groups (facebook.com/groups/BritishSpiderIdentification/; facebook.com/groups/insectsinvertebratesire/). All reporters were contacted to obtain precise location, details on habitat, dates of observation and photographic voucher material. Only reports accompanied with unambiguous photographic material were retained.

Location	*Coordinates*	*Habitats*	*Observation dates*
Co Dublin – Tyrellstown	307202, 241771	On a garden fence	July 2009
Co Dublin – Balbriggan	320305, 263918	Outdoors - Garden wall	October 2014
Co Dublin – Balbriggan	320371, 263938	Outdoors - On a flower pot	September 2015
Co Dublin – Portmarnock	324224, 243176	Indoors	September 2015
Co Dublin – Santry	316959, 240469	Outdoors - On stone pavement	2016
Co Dublin – Baldoyle	323986, 239991	Indoors - Window frame	May 2016
Co Dublin - Glasnevin	314638, 237366	Outdoors - on garden wall	May 2016
Co Dublin - Churchtown	315625, 228650	Indoors	June 2016
Co Dublin - Skerries	324632, 259875	Indoors	July 2016
Co Dublin - Coolock	319396, 239360	Outdoors	August 2016
Co Dublin - Knocklyon	312220, 227546	Indoors - On wooden structure	September 2016
Co Dublin - Shankill	324975, 221856	Outdoors - On a garden rock	September 2016
Co Dublin - Sutton	325135, 239555	Outdoors - In garden	September 2016
Co Dublin - Swords	319036, 246777	Outdoors - Garden wall	October 2016
Co Donegal - Bundoran	182121, 358977	In arm pit of wet suit	March 2016
Down – Bangor	350722, 378692	Indoors	December 2016
Meath Kells	274762, 275309	Indoors - On a wall	July 2014
Meath – Ratoath	302166, 251766	Indoors - Window frame	October 2016
Kildare – Leixlip	300209, 235770	Outdoors	November 2015
Laois – Clonkeen	243768, 196040	Indoors	2016
Wicklow – Hollywood /Donard	293708, 197630	Indoors on skirting board	2016
Wicklow – Greystones	328949, 212314	Indoors	June 2016
Wicklow – Arklow	324208, 173379	Indoors	October 2016
Wexford – Gorey	315525, 159179	Inside garden shed	October 2010
Wexford – Wexford town	304477, 122145	Indoors	November 2015
Wexford – Taghmon	291884, 119945	Indoors on a blanket	September 2016
Wexford – Courttown	319446, 157427	Indoors - On window frame	October 2016
Tipperary – Kilcash, Clonmel	231489, 128155	Indoors - On a wall	July 2016
Waterford – Waterford City	262304, 110758	Indoors - On wooden floor	September 2015
Waterford – Cherrymount	206328, 82579	Indoors	March 2016
Waterford – Dungarvan	225932, 93016	Outdoors	2016
Carlow – Tullow	285544, 172950	Outdoors - on stone	2016
Kerry – Tralee	86865, 115194	Outdoors, on concrete	February 2015
Cork – Cobh	178777, 67245	Indoors	May 2015
Cork – Curraghbinny Woods	179281, 62036	Indoors - Window frame	October 2016
Cork – Glanbeg	164119, 50847	Indoors	2016

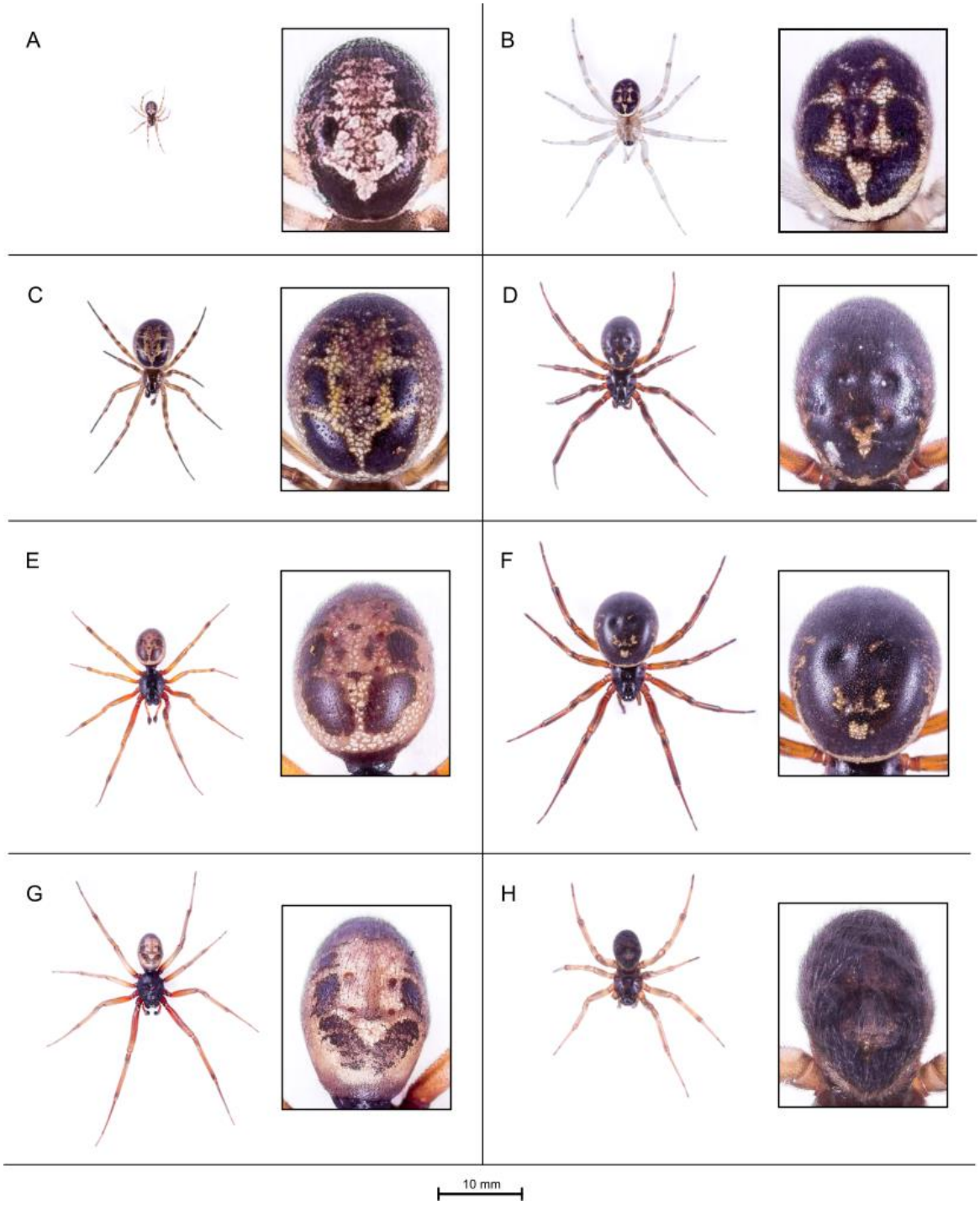

Figure 3: Identification chart for various life stages of *Steatoda nobilis* collected in Ireland. Variations in opisthosoma markings are presented in each frame. **(A)** Unsexed second instar juvenile specimen; **(B)** Unsexed sub-adult specimen hours after ecdysis. Notice the pale colour of the prosoma and legs in contrast to other specimens **(C)** Male specimen at the penultimate developmental stage; **(D)** Mature female with reduced opisthosomal markings; **(E)** Mature male displaying mature palpal bulbs on the distal end of the pedipalps; **(F)** Large mature female with round, bulbous abdomen and reduced opisthosomal markings; **(G)** Mature male with slender, discoloured opisthosoma and mature palpal bulbs on the distal end of the pedipalps; **(H)** Old mature female, with a missing frontal leg, slender, shrivelled abdomen. The opisthosomal markings are barely visible.

3.1.5.4 - Identification chart

Male and female *Steatoda nobilis* were overall photographed at various stages of their development to help with identification. *Steatoda nobilis* displays a wide range of variation in overall size, opisthosomal shape, colouration, and dorsal markings, making identification very difficult to the untrained eye (Fig. 2). Those variations do not seem to correlate with Geographical origins or relatedness: specimens collected centimetres apart and siblings born from the same egg sac can display widely different dorsal patterns (Figs. 3A–3D).

In Ireland, *Steatoda nobilis* can be consistently distinguished from other local spiders (including other members from the genus *Steatoda*) by the presence of a cream crescent on the antero-lateral part of the opisthosoma (Figs. 2B and 2E), an intricate pentagonal or hexagonal cream-coloured pattern on the dorsal aspect of the opisthosoma (Figs. 2A–2H) and sturdy reddish to orange legs banded with black markings (Figs. 2D and 3E). The prosoma is consistently solid black in mature specimens, but it may be light yellow to cream colour in immature and freshly moulted specimens (Fig. 2B). The cuticle of the opisthosoma bears four small depressions on the dorsal aspect (Figs. 2D and 2F). Mature males are easily distinguished from mature females due to the presence of a pair of palpal bulbs on the distal end of the pedipalps (Figs. 2C, 2E and 2G). The size and shape of the opisthosoma varies from short and slender (Figs. 2E and 2G) to large and bulbous (Fig. 2C). Old specimens—particularly females—may present a small and shrivelled opisthosoma with faded or absent dorsal and antero-dorsal markings (Figs. 2G, 2H).

The average body length (prosoma + opisthosoma) was 10.5mm for mature females (N=90) and 9.4 mm for mature males (N=12). The largest mature female measured 13.7mm and the largest mature male measured 11.6mm. When hatching, juveniles measure 1.7mm (N=15) and the sexes are indistinguishable from each other.

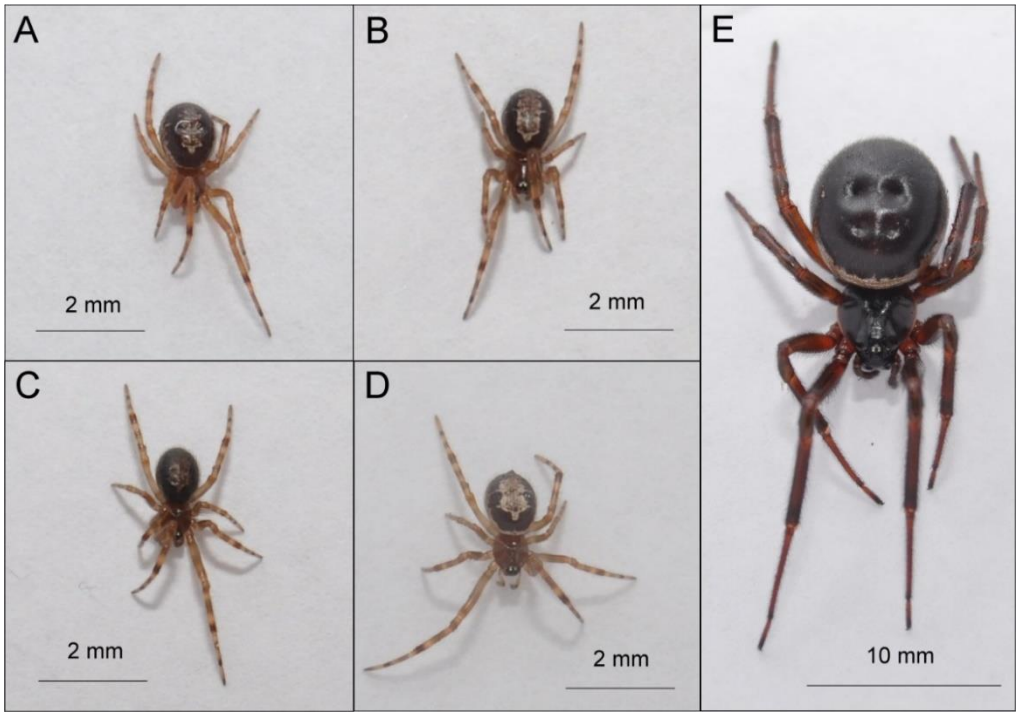

Figure 4: (A-D) Sibling second instar *Steatoda nobilis* hatched from the same egg sack. Note the wide variations in the intensity and shape of the opisthosomal markings **E:** mature female *Steatoda nobilis* with four prominent dimples on the dorsal aspect of the opisthosoma, characteristic of the genus *Steatoda*. Note the reduced dorsal markings but the presence of a cream band on the antero-lateral aspect of the opisthosoma.

3.1.5.5 - Reproductive rate

For the present study, females and males were housed separately in the lab; over a period of 11 months, 40 wild-caught females produced a total of 50 egg sacs in captivity. Each egg sac contained on average 94 eggs (range 34 to 208 eggs). The most prolific spider produced four egg sacs within four months.

Prior to oviposition, the female produces an irregular silky brooding chamber approximately 3–4cm in diameter, which does not possess any entrance. After two to ten days spent in the brooding chamber, a spherical or pear-shaped egg sac is produced. Eggs are laid in quick succession and clumped together into a spherical mass. A first layer of loose silk is produced around the egg mass, followed by a second layer of denser silk. Ultimately, the egg sac resembles a spherical or pear-shaped mass 5–12mm in diameter, suspended by threads inside the brooding chamber (Fig. 4). After an average of 18 days at 20°C, spiderlings emerge from their chorion, and remain within the egg sac, where they undergo a first ecdysis. Two to four days after the first moult, spiderlings emerge from the egg sac and collectively produce a long clump of silk threads along which they settle as a group. At this stage, the spiderlings are capable of capturing and consuming small live prey (e.g. *Drosophila melanogaster*). Dispersal occurs several days later, at which point the spiderlings display increasing cannibalistic behaviour against their siblings.

Figure 5: Captive mature female *Steatoda nobilis* guarding her egg sac suspended in a brooding chamber made for that purpose. The egg mass is visible through the silk as the faint yellow circular shape at the centre of the egg sac.

3.1.6 - DISCUSSION

3.1.6.1 - Irish distribution

Considering its conspicuous dorsal markings, overall body size and synanthropic affinities, it is unlikely (but not impossible) that Irish populations of *Steatoda nobilis* passed unnoticed for several years prior to Nolan's report (1999). Van Helsdingen did not include the species in his extensive literature review on Irish spiders (Van Helsdingen, 1996). Rapid colonisation event by *Steatoda nobilis* has been reported before from California, where the species has become widespread within three years of the first sighting (Vetter et al., 2015, Vetter and Rust, 2012).

Although we did not survey each of the 32 Irish counties, the present study shows that since Nolan's report (1999), *Steatoda nobilis* has expanded its range that now includes at least sixteen counties. Within the last two decades, *Steatoda nobilis* has established thriving colonies in major port towns on the eastern, southern and western coasts of Ireland and, to a lesser extent, in the midland counties. Most sightings recorded during the course of this study occurred in cities or along major transport routes across Ireland, with the exception of a single specimen captured in a bungalow located in Curraghbinny Woods, near the coastal village of Crosshaven,

Co Cork. Surveys of coastal habitats, meadows, and woodlands in Cos Galway, Mayo, and Roscommon did not yield any additional specimen. There is no doubt however that a comprehensive, nationwide survey would yield many more additional positive records.

The evident distribution pattern that emerges from our survey is the distinct preference of *Steatoda nobilis* for man-made structures: all sightings were made in and around houses, or on street furniture. This is in contrast to its native range where *Steatoda nobilis* occupy mixed habitats consisting of natural features (e.g. plants and rock crevices) and man-made structures. In Italy, *Steatoda nobilis* occurred first in urban areas and then spread to the surrounding countryside (Kulczycki et al., 2012) while in the northern part of its new range, *Steatoda nobilis* appears to be restricted to man-made habitats. This pattern suggests a climate-dependent colonisation: *Steatoda nobilis* is an exclusively synanthropic species in Ireland, and its current long-range expansion is highly dependent on human activity rather than natural dispersal methods such as ballooning. Unintentional human-led introductions are believed to have been the main factor in the introduction of over 87 species of non-native spiders in Europe between 1850 and 2000, of which 71% live synanthropically (Kobelt and Nentwig, 2008).

3.1.6.2 - Intraguild competition, reproductive rate and potential impact

The impact of alien spiders on native Irish ecosystems has not been investigated yet but some observations can be drawn from our dataset. *Steatoda nobilis* appears to be a generalist feeder, preying on a wide range of arthropods, as long as the prey can be immobilised and consumed. The few interactions between *Steatoda nobilis* and native (or naturalised) spiders recorded here show that large numbers of *Steatoda nobilis* might have a negative impact on local populations of common urban dwellers such as the missing sector orb weaver *Zygiella x-notata.* This species occurs in habitats which are also favoured by *Steatoda nobilis* (e.g. street furniture, railings, and stone walls) and competition between both species is therefore likely. Although *Zygiella x-notata* and *Steatoda nobilis* have been observed side by side during our site inspections, it seems that *Zygiella x-notata* is not as widespread in those areas colonised by *Steatoda nobilis* than in locations where we did not observe the presence of *Steatoda nobilis.* Inter-specific competition for prey items is a possible factor for this observation, as it has been shown that prey availability is a major factor for the successful establishment or the displacement of *Zygiella x-no-tata* colonies (Wherry and Elwood, 2009).

Differences in reproductive rates may bring additional stress on local species that are unable to compete with the larger, more productive *Steatoda nobilis. Zygiella x-notata* has been shown to produce one–three egg sacs per year, each containing *c.*10–70 eggs (Wherry and Elwood, 2009). In comparison, our data shows that *Steatoda nobilis* can produce much larger egg sacs containing between 34 and 208 eggs (98 eggs in average

per clutch), every month, over a period of several months. Previous reports show that *Steatoda nobilis* can still produce viable clutches 18 months following fertilisation (Locket, 1979). Additionally, *Zygiella x- notata* does not lay eggs during the coldest month of the year (Wherry and Elwood, 2009) while female *Steatoda nobilis* are cold tolerant (Jones, 1979) and active throughout the year. In our study, *Steatoda nobilis* captured outdoors in the middle of winter were capable of producing viable egg sacs days only after being established in the lab. In addition, *Steatoda nobilis* has a remarkable life span: a specimen kept in a sheltered environment free of predators can live over five and a half years (Snazell and Jones, 1993) against twelve months on average for *Zygiella x-notata* (Yoward, 1999). Considering the preference of *Steatoda nobilis* for sheltered habitats where it has few to no predators, it is likely that 'wild' specimens are almost as productive and long-lived as the specimens kept in captivity. If this is the case, *Steatoda nobilis* may well have the potential to outcompete native urban dwelling spiders. Further field-based studies and long-term surveys will be needed to monitor the range expansion of *Steatoda nobilis* and to assess if this occurs at the expense of native species.

3.1.7 - ACKNOWLEDGEMENTS

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3.2 - New Irish record for *Steatoda triangulosa* (Walckenaer, 1802), and new county records for *Steatoda nobilis* (Thorell, 1875), *Steatoda bipunctata* (Linnaeus, 1758) and *Steatoda grossa* (C.L. Koch, 1838)

JPD contributed to the conceptualization, methodology, data acquisition, molecular techniques, original draft preparation, and review and editing. This section is published in a peer reviewed journal and available at the following reference: DUNBAR, J., SCHULTE, J., LYONS, K., FORT, A. & DUGON, M. 2018b. New Irish record for *Steatoda triangulosa* (Walckenaer, 1802), and new county records for *Steatoda nobilis* (Thorell, 1875), *Steatoda bipunctata* (Linnaeus, 1758) and *Steatoda grossa* (CL Koch, 1838). The *Irish Naturalists' Journal*, 36, 39-43.

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3.2.1 - INTRODUCTION

The family Theridiidae (Sundevall, 1833) comprises over 2,200 species of ecribellate spiders worldwide, of which many have synanthropic affinities. It has been estimated that Theridiidae spiders are the most diverse non-native spiders in Europe, with 13 alien species having successfully established colonies across the continent between 1850 and 2000 (Kobelt and Nentwig, 2008). Within the family Theridiidae, several species of synanthropic false widows (genus *Steatoda* Sundevall, 1833) have recently expanded their range across most of Europe and beyond (Dugon et al., 2017, Nedvěd et al., 2011, Nyffeler et al., 1986). Members of the genus *Steatoda* have been involved in envenomations in Ireland (Dugon et al., 2017), Britain (Dunbar et al., 2018, Warrell et al., 1991), France (Pommier et al., 2006), Chile (Faúndez and Téllez, 2016) and Australia (Isbister and Gray, 2003). Additionally, *Steatoda* may compete with, predate on, and displace native species of spiders (Dugon et al., 2017, Hann, 1990, Kulczycki et al., 2012, Nyffeler et al., 1986). It is therefore essential to monitor range expansion by *Steatoda* species. Here we provide new records for four *Steatoda* species: a new Irish record for *Steatoda triangulosa* (Walckenaer, 1802), a new county record for *Steatoda*

nobilis (Thorell, 1875), *Steatoda bipunctata* (Linnaeus, 1758) and two new county records for *Steatoda grossa* (Koch, 1838). All specimens were identified using Roberts (Roberts, 1995).

3.2.2 - New Irish Record for *Steatoda triangulosa* (Walckenaer, 1802)

In February 2017, a live juvenile spider was discovered by JS in a tub of commercially available crickets (*Gryllus assimilis* Fabricius, 1775) bought from a major pet shop in Co. Galway. The specimen measured 3 mm (prosoma and opisthosoma). Inspection revealed morphology and abdominal patterns consistent with *Steatoda triangulosa* (Walckenaer, 1802) (Figs 1a, 1b) as presented by Roberts (1995). However, a reliable diagnosis could not be obtained through the observation of the epigyne. The specimen was subsequently used for DNA extraction and PCR amplification of the Cytochrome Oxydase Subunit 1 using the primers coxf3: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and coxFR3: 5'-TAA ACT TCA GGG YGA CCA AAA AAT CA-3'. Amplified material was sent Amplified material was sent to LGC Limited, Middlesex, UK for sequencing. The sequence was then aligned and compared with CO1 sequences retrieved from Genbank (https://www.ncbi.nlm.nih.gov/genbank, sequence accession number MF860399). The specimens' identity was subsequently confirmed as *Steatoda triangulosa*. A comprehensive online and literature review revealed that *Steatoda triangulosa* is absent from the historical Irish records and Van Helsdingens' review (Van Helsdingen, 1996). We therefore conclude that this is the first recording of *Steatoda triangulosa* in Ireland.

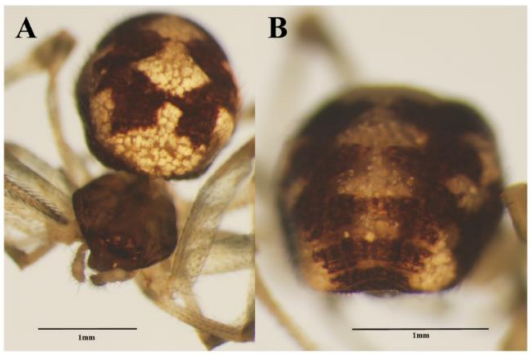

Figure 1. Immature *Steatoda triangulosa* found in Galway City, Ireland. **A)** Anterodorsal view of the cephalothorax and abdomen. The anteromedian white band is typical of the genus *Steatoda* **B)** Posterodorsal view of the abdomen showing the triangular patterns running along the median line of the opisthosoma.

3.2.3 - New County Record for *Steatoda nobilis* (Thorell, 1875)

On 3 October 2017, a spider was found living inside a van in Daingean, Co. Offaly by L. Kelly Hensey. A photograph (Fig. 2) was sent to the authors for identification. The specimen was identified as a subadult female *Steatoda nobilis* (Thorell, 1875) based on opisthosomal dorsal patterns (Dugon et al., 2017). A subsequent search of the house and garden yielded no further specimens.

Steatoda nobilis was recorded in Ireland for the first time in a housing estate in Bray, Co. Wicklow in 1998 (Nolan 1999). Since then, this species has spread across Ireland and has been recently recorded from 16 counties (Dugon *et al*. 2017). Our new record brings the total to 17 counties.

Figure 2. Subadult female *Steatoda nobilis* found in Co Offaly. Identified from the presence of a cream crescent on the antero-lateral part of the opisthosoma and the intricate pentagonal cream-coloured pattern on the dorsal aspect of the opisthosoma.

3.2.4 - New County Record for *Steatoda bipunctata* (Linnaeus, 1758)

On 12 June 2017, a mature female *Steatoda bipunctata* (Linnaeus, 1758), was found by KL in a commercial tractor garage in Skehana, Co. Galway (Figs 3 a–c). The building was subsequently searched and a small but thriving population (>100 encompassing all developmental stages) was discovered occupying window, door frames, shelving

and benches within close proximity to the windows (Fig. 3d). Further search of the garage and neighbouring houses did not yield additional specimens. The doors and windows were also occupied by other spider species (*Eratigena atrica* (C. L. Koch, 1843), *Amaurobius fenestralis* (Ström, 1768), *Amaurobius similis* (Blackwall, 1861), *Nuctenea umbratica* (Clerck, 1757) and *Zygiella x-notata* (Clerck, 1757)), but these were greatly outnumbered by *Steatoda bipunctata*. Several dead prey items were present on the webs of *Steatoda bipunctata*, including *Calliphora vomitoria* (Linnaeus, 1758), *Oniscidea* sp., *Coleoptera* sp. and *Musca domestica* Linnaeus, 1758.

Steatoda bipunctata was recorded from Ireland for the first time by Pack-Beresford (Pack Beresford, 1929) and to this day, its distribution was thought to be restricted to the Dublin metropolitan area (Van Helsdingen, 1996). This report is the first population recorded on Irelands' west coast.

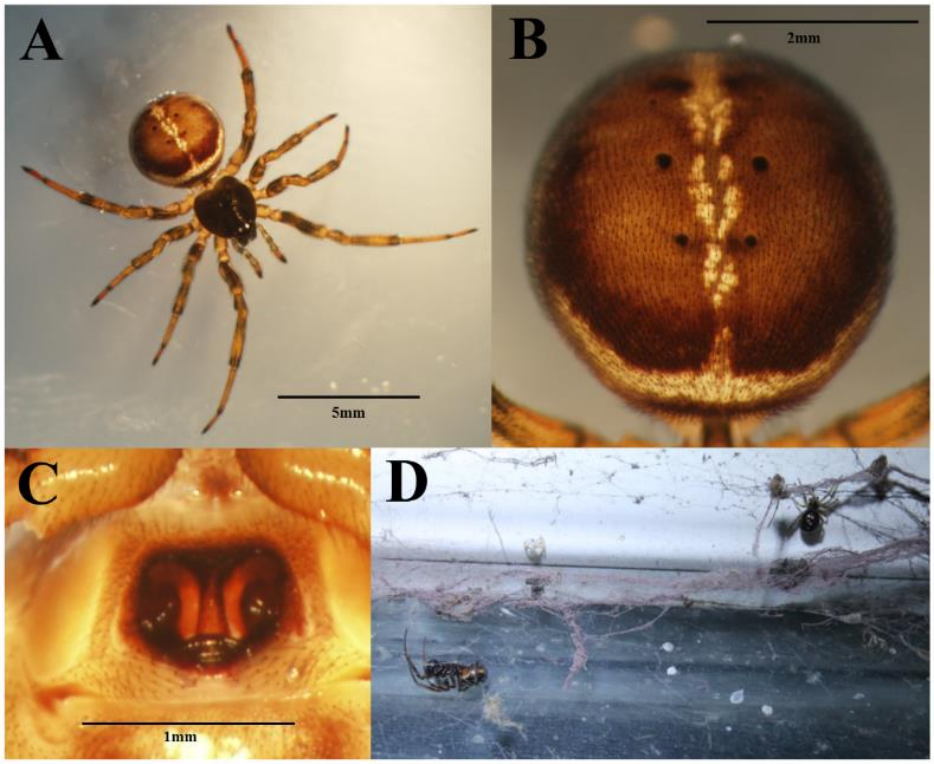

Figure 3. *Steatoda bipunctata* found in Skehana, Co Galway **A)** Dorsal aspect showing the cream crescent typical of the genus *Steatoda* on the anterior aspect of the opisthosoma. **B)** Dorsal aspect of the opisthosoma showing the median line and apodemal depressions characteristic of *Steatoda bipunctata*, **C)** ventral micrograph of a mature female showing the epigyne, **D)** In-situ picture of specimen habitat in a disuse garage.

3.2.5 - New County Records for *Steatoda grossa* (Koch, 1838)

In April 2017, two subadult female *Steatoda grossa* (Koch, 1838) were brought to the attention of the authors by members of the public (Fig. 4). The first specimen (Figs 4 a–c) was observed walking across a kitchen floor and collected by P. Evans in Edgeworthstown, Co. Longford. A subsequent search of the house and garden at the time by JPD yielded no further specimens. However, in October 2017, during renovation work on the kitchen, several specimens from various developmental stages were collected and>20 egg sacs were found. The second specimen (Figs 4 d–f) was collected by M. Taite, on top of a kitchen cupboard in a house located in the outskirts of Castlerea, Co. Roscommon.

Steatoda grossa is established in Ireland at least since the late nineteenth century (Carpenter, 1898) and has been previously recorded from Cos Cork, Kerry, Galway, Sligo, Down, Dublin, Wicklow, Kilkenny, and Waterford (Fern, 2010). Our observations are the first reports from Irish midland counties.

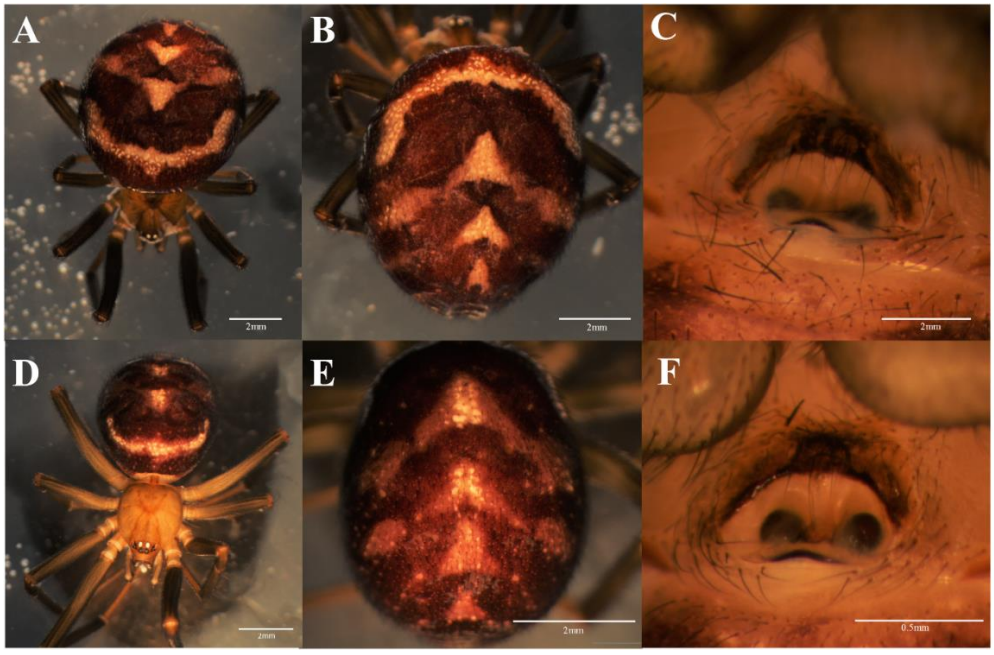

Figure 4. Subadult females *Steatoda grossa* collected in Co Longford **(A-C)** and Co Roscommon **(D-F)**. **A)** Dorsal aspect showing the anterior cream crescent typical of *Steatoda*. **B)** Posterodorsal aspect of the opisthosoma. **C)** Ventral micrograph showing the epigyne. **D)** Subadult female *Steatoda grossa* collected in Co Roscommon. **E)** Dorsal aspect showing the anterior cream crescent typical of *Steatoda*. B) Posterodorsal aspect of the opisthosoma. **F)** Ventral micrograph showing the epigyne.

3.2.6 - CONCLUSION

Four species of *Steatoda* have previously been recorded from Ireland. The inclusion of *Steatoda triangulosa* brings this figure to five species, which is in par with the number of *Steatoda* present in Great Britain (6 species). *Steatoda grossa* and *Steatoda bipunctata* have been present for most of the 20th century (Pack Beresford, 1929) while *Steatoda phalerata* (Merrett, 1989) and *Steatoda nobilis* (Nolan, 1999) are recent additions to the Irish fauna. *Steatoda phalerata* is the only local *Steatoda* to favour undisturbed grassland habitats; the remaining four species are confined to man-made structures. In the case of *Steatoda triangulosa*, it should be noted that the only specimen recovered was found in a box mailed from Britain merely days before; there is therefore no indication that this species has established colonies anywhere on the island. It is however quite likely that all five species have been inadvertently brought to Ireland through the movements of commercial goods and people rather than through natural dispersal means such as ballooning. Due to their synanthropic affinities, *Steatoda bipunctata* has a Holarctic distribution (Topçu et al., 2005), and *Steatoda nobilis*, *Steatoda grossa*, and *Steatoda triangulosa* are now distributed globally (Dugon et al., 2017, Graudins et al., 2002, Pommier et al., 2006), which further obscures the true biogeography and original distribution patterns of the genus *Steatoda*. Further studies using molecular data may shed some light on the movement and colonisation history of these intriguing and exceptionally successful spiders.

3.2.7 - ACKNOWLEDGEMENTS

We thank Pr. Grace McCormack, Belinda Longakit and James Dunbar.

3.3 - Biting off more than one can chew: first record of the non-native noble false widow spider *Steatoda nobilis* (Thorell, 1875) feeding on the native Viviparous lizard *Zootoca vivipara* (Lichtenstein, 1823) in Ireland

JPD contributed to the conceptualization, methodology, original draft preparation, and review and editing. This section is published in a peer reviewed journal and available at the following reference: DUNBAR, J., ENNIS, C., GANDOLA, R. & DUGON, M. Biting off more than one can chew: first record of the non-native noble false widow spider *Steatoda nobilis* (Thorell, 1875) feeding on the native Viviparous lizard *Zootoca vivipara*. *Biology and Environment: Proceedings of the Royal Irish Academy*, 2018a. 45-48.

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3.3.1 - ABSTRACT

As the noble false widow spider *Steatoda nobilis* (Thorell 1875) continues to expand its range across Europe, Asia, and the Americas, its potential as an invasive species has not yet been fully assessed. Latrodectinae spiders are remarkably adaptable and possess fast-acting neurotoxic venom that can cause neuromuscular paralysis in vertebrates, and occasionally feed on small reptiles. We describe here a predation event by a mature female *Steatoda nobilis* on a juvenile *Zootoca vivipara* lizard in suburban Dublin. This is the first report of *Steatoda nobilis* preying on a vertebrate, and the first report of a terrestrial vertebrate organism falling prey to an arachnid in Ireland. *Zootoca vivipara* is a protected species in both the Republic of Ireland and

Northern Ireland and may increasingly fall prey to *Steatoda nobilis* as urbanisation encroaches on lizard habitat. Therefore, *Steatoda nobilis* should be closely monitored outside of its original native range to assess its status as an invasive species.

3.3.2 - INTRODUCTION

The noble false widow *Steatoda nobilis* (Thorell, 1875) is a medium sized araneomorph spider of the family Theridiidae, measuring up to 13.7mm (prosoma + opisthosoma) in length (Dugon et al., 2017). It is characterised by conspicuous dorsal markings and produces a three-dimensional tangled web. *Steatoda nobilis* is thought to originate from the Atlantic archipelagos of Madeira (Thorell, 1875) and the Canaries (Bristowe, 1929) but the species is now distributed throughout Western Europe, Western Asia, and parts of the American Pacific coast (Dugon et al., 2017). In the Republic of Ireland, *Steatoda nobilis* was first recorded in 1998 in Bray, Co Wicklow (Nolan, 1999). By 2017 the species occurred in at least seventeen counties and appeared to be spreading rapidly in urban and suburban habitats throughout the country (Dugon et al., 2017, Dunbar et al., 2018a).

Outside of its native range, *Steatoda nobilis* is proving to be a remarkably adaptable species with distinct synanthropic affinities (Kulczycki et al., 2012, Vetter et al., 2015). *Steatoda nobilis* is cold tolerant, active year-round, has a high reproductive rate, an exceptional longevity, and possesses a fast-acting neurotoxic venom (Dugon et al., 2017, Dunbar et al., 2018b, Isbister and Gray, 2003, Warrell et al., 1991). In Ireland, *Steatoda nobilis* has been observed preying on a range of comparatively large native arthropods including beetles, hymenopterans, and spiders (Dugon et al., 2017). Previous studies suggest that *Steatoda nobilis* may have a detrimental impact on arthropod fauna (Kulczycki et al., 2012), although this has not been confirmed for native Irish fauna.

The viviparous, or common lizard, *Zootoca vivipara* (Lichtenstein, 1823), is the only native terrestrial reptile in Ireland. This species has a widespread distribution on the

island and is present in all 32 counties (data held by National Biodiversity Data Centre, Herpetological Society of Ireland, Centre for Environmental Data and Recording (CEDaR) Northern Ireland and National Biodiversity Network (NBN) Gateway, UK). It is protected under the Wildlife Act (1976, and amendments 2000, 2012) in the Republic of Ireland and the Wildlife Order (1985) in Northern Ireland. Predators of common lizards in Ireland comprise raptors and other avifauna, small carnivores (e.g. stoats), and domestic animals (cats). However, it is likely that juvenile individuals may also be predated upon by predatory invertebrates.

A comprehensive review by O'Shea and Kelly, indicates that reptiles occasionally fall prey to *Latrodectus* species and although less frequently reported (O'Shea and Kelly, 2017), they are also preyed upon by *Steatoda* species (Petrov and Lazarov, 2000, Zamani, 2016). In this report we describe a predation event by a mature female *Steatoda nobilis* on a juvenile *Zootoca vivipara* in suburban Dublin. This is the first report of *Steatoda nobilis* preying on a vertebrate, and the first report of a terrestrial vertebrate organism falling prey to an arachnid in Ireland.

3.3.3 - CASE REPORT

On 18 May 2017 in Killiney, Co Dublin, a member of the public discovered a spider alongside a small

dead lizard entangled on a thick, three-dimensional web between a garden wall and a contiguous wooden crate. Upon closer inspection, it appeared that the spider had covered the anterior half of the lizard with silk, positioned itself on the antero-dorsal aspect of the lizard and was seemingly feeding on it. Once disturbed, the spider fled to its retreat. Both the spider and the lizard were later photographed *in situ* using a camera phone (Fig 1), subsequently captured and sent to the authors for identification. Body lengths and weights of both the spider and the lizard as well as the spiders legspan were recorded using an analogue Vernier calliper and a precision digital scale. Both specimens were observed and photographed using a Nikon AF-S DX Micro Nikkor 40mm f 2.8G lense mounted on a Nikon D5200 DSLR camera. Detailed micrographs were taken using an Olympus DP25 camera mounted to an

Olympus SZX 16 stereomicroscope and using the Olympus Cell D software package (Fig 2).

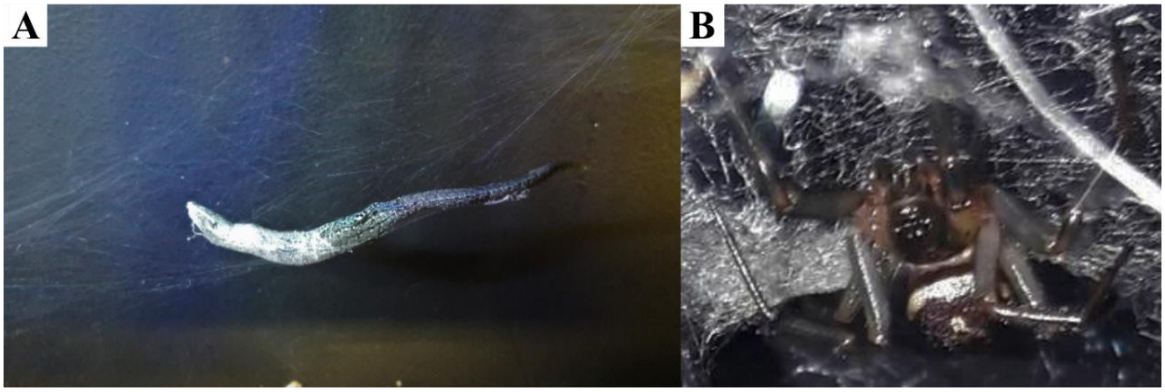

Figure 1—Insitu smartphone images of (A) juvenile *Zootoca vivipara* entangled on web with the anterior half densely wrapped in silk, and (B) mature female *Steatoda nobilis* in its retreat.

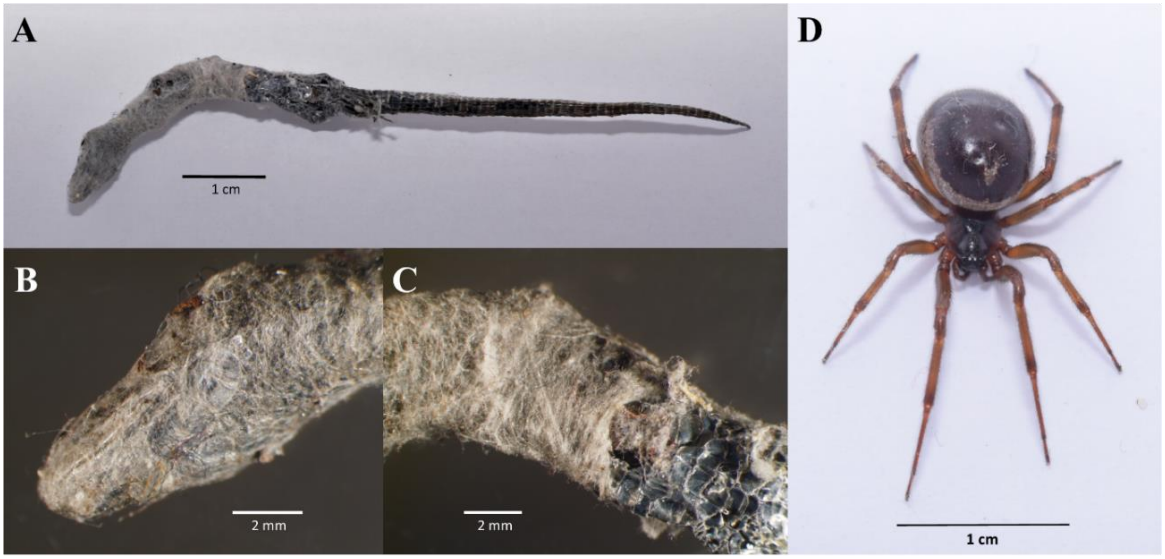

Figure 2—Detailed micrographs of the (A - C) *Zootoca vivipara* and (D) *Steatoda nobilis* specimens.

The spider was identified by the authors (JD and MD) as a mature female *Steatoda nobilis*, based on typical opisthosomal dorsal patterns (Dugon et al., 2017). Body length was 11mm, leg span was 33.8mm and body weight was 0.3g. The lizard was identified by the authors (CE and RG) as a juvenile *Zootoca vivipara*. Total body length was 8.5cm, and the weight of the desiccated specimen was 0.4g.

The lizard was densely wrapped in silk with the limbs aligned along the anterior to posterior axis of the body, suggesting that the spider actively wrapped the lizard in silk with the intent to trap it. When discovered, the spider was positioned over the lizard's head, where soft tissues can be accessed (Petrov and Lazarov, 2000). This suggests that the spider was actively feeding on the lizard rather than just defending against a potential predator. As the event was not witnessed, we cannot definitively conclude that the spider hunted down and envenomed a healthy lizard or whether the spider fed opportunistically on an already weakened, possibly dead lizard.

3.3.4 - DISCUSSION

The diet of Latrodectinae (*Latrodectus* and *Steatoda*) spiders typically consist of arthropods, however, they have been documented capturing and feeding on snakes and lizards (O'Shea and Kelly, 2017, Petrov and Lazarov, 2000, Zamani, 2016). Latrodectinae of the genera *Latrodectus* and *Steatoda* produce the vertebrate-specific neurotoxin 'α-latrotoxin', which facilitates the release of neurotransmitters leading to neuromuscular paralysis (Isbister and Gray, 2003, Warrell et al., 1991). While predation events by Latrodectinae on small vertebrates have only been observed on rare occasions, the presence of α-latrotoxin may suggest that small vertebrates are preyed upon more frequently than previously thought.

Zootoca vivipara is a ground-dwelling species that occupies bogs, rocky outcrops, old stone walls, sand dunes, and are frequently found around man-made structures and close to human dwellings (Farren et al., 2010, Marnell, 2002). In much of its northerly range including Ireland, *Steatoda nobilis* are exclusively synanthropic, building webs around man-made objects from roof tops down to ground level. Considering the continuous range expansion by *Steatoda nobilis* in Ireland and Great Britain, encounters with *Zootoca vivipara* are likely to become more frequent in the future. This is likely to be exacerbated in Dublin and other urban areas with increasing urbanisation and encroachment into lizard habitat. *Steatoda nobilis* should be closely

monitored outside of its original native range to assess its status as an invasive species.

3.3.5 - ACKNOWLEDGEMENTS

This work has been financed through an Irish Research Council postdoctoral fellowship held by Michel Dugon and a NUI Galway College of Science PhD scholarship held by John Dunbar.

Chapter 4
Venomics

The fangs of an adult female *Steatoda nobilis* extending from the chelicerae

Photo by JP Dunbar

4.1 - Venomics approach reveals a high proportion of *Lactrodectus-Like* toxins in the venom of the noble false widow spider *Steatoda nobilis*

JPD contributed to the conceptualization, methodology, data acquisition, molecular techniques, original draft preparation, and review and editing. This section is published in a peer reviewed journal and available at the following reference: DUNBAR, J. P., FORT, A., REDUREAU, D., SULPICE, R., DUGON, M. M. & QUINTON, L. 2020. Venomics Approach Reveals a High Proportion of *Lactrodectus*-Like Toxins in the venom of the noble false widow spider *Steatoda nobilis*. *Toxins*, 12, 402.

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4.1.1 - ABSTRACT

The noble false widow spider *Steatoda nobilis* originates from the Macaronesian archipelago and has expanded its range globally. Outside of its natural range, it may have a negative impact on native wildlife, and in temperate regions it lives in synanthropic environments where it frequently encounters humans, subsequently leading to envenomations. *S. nobilis* is the only medically significant spider in Ireland and the UK, and envenomations have resulted in local and systemic neurotoxic symptoms similar to true black widows (genus *Latrodectus*). *S. nobilis* is a sister group to *Latrodectus*, which possesses the highly potent neurotoxins called α-latrotoxins that can induce neuromuscular paralysis and is responsible for human fatalities. However, and despite this close relationship, the venom composition of *S. nobilis* has never been investigated. In this context, a combination of transcriptomics and proteomics cutting-edge approaches has been used to deeply characterise *S. nobilis* venom. Mining of transcriptome data for the peptides identified by

proteomics revealed 240 annotated sequences, of which 118 are related to toxins, 37 as enzymes, 43 as proteins involved in various biological functions, and 42 proteins without any identified function to date. Among the toxins, the most represented in numbers are α-latrotoxins (61), δ-latroinsectotoxins (44) and latrodectins (6), all of which were first characterised from black widow venoms. Transcriptomics alone provided a similar representation to proteomics, thus demonstrating that our approach is highly sensitive and accurate. More precisely, a relative quantification approach revealed that latrodectins are the most concentrated toxin (28%), followed by α-latrotoxins (11%), δ-latroinsectotoxins (11%), and α-latrocrustotoxins (11%). Roughly two thirds of the venom are composed of *Latrodectus*-like toxins. Such toxins are highly potent towards the nervous system of vertebrates and likely responsible for the array of symptoms occurring after envenomation by black widows and false widows, and thus, caution should be taken in dismissing *S. nobilis* as harmless. This work paves the way towards a better understanding of the competitiveness of *S. nobilis* and its potential medical importance.

4.1.2 - INTRODUCTION

Animal venoms are complex cocktails of toxic proteins that evolved as a primary means to immobilize and subdue prey (Lyons et al., 2020, Whiteley et al., 2016) and potentially assist in predigesting the tissues of prey (Bottrall et al., 2010, Vassilevski et al., 2009, Zobel-Thropp et al., 2012). However, venoms are also often extremely effective weapons for defence against perceived predators, including humans. Virtually all of the 48,000 species of spiders described so far are venomous (Kuhn-Nentwig et al., 2019). Among these, black widow spiders from the genus *Latrodectus* represent a significant risk to human health due to the synanthropic habits of some species and their highly potent neurotoxic venom (Garb and Hayashi, 2013).

In recent years, another spider from the widow family (Latrodectinae), the noble false widow *S. nobilis* (Thorell, 1875), which looks superficially like true black widows (**Figure 1**) has extended its range globally and may represent a potential risk to native ecosystems and human health (Bauer et al., 2019, Dunbar et al., 2018c, Faúndez and

Téllez, 2016, Warrell et al., 1991). *S. nobilis* is now regarded as potentially being one of the world's most invasive species of spiders (Bauer et al., 2019). This species originates from the Macaronesian archipelago (Dugon et al., 2017) and has established populations across Western Europe including Ireland and Great Britain (Bauer et al., 2019, Dugon et al., 2017, Dunbar et al., 2018a, Dunbar et al., 2018b, Nolan, 1999), through Western Asia (Turkey and Iran) (Türkeş and Mergen, 2007, Zamani et al., 2015), and North and South America (Faúndez et al., 2018, Faúndez and Téllez, 2016, Taucare-Ríos et al., 2016, Vetter et al., 2015b). *S. nobilis* has an exceptional longevity (up to five years) (Snazell and Jones, 1993), a fast-reproductive rate, is cold tolerant with year-round activity (Dugon et al., 2017), and has a fast-acting venom that allows it to subdue a broad range of invertebrate and even vertebrate prey (Dugon et al., 2017, Dunbar et al., 2018a). Outside of its native range *S. nobilis* has been demonstrated to have a negative impact on native species (Bauer et al., 2019, Dugon et al., 2017, Dunbar et al., 2018a, Kulczycki et al., 2012). In temperate regions, *S. nobilis* has a typical synanthropic lifestyle, which brings them within close contact with humans (Dugon et al., 2017).

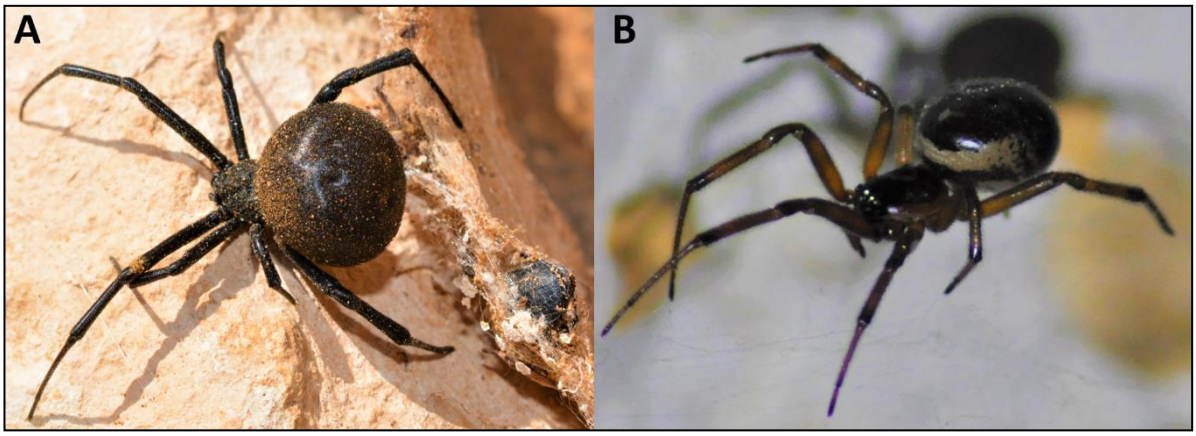

Figure 1. Similarities between black widow and false widow spiders. (**A)** Mature female black widow *Latrodectus lilianae*, Morocco (Photo taken by M. Dugon), (**B)** Mature female false widow *Steatoda nobilis*, Ireland (Photo taken by JP. Dunbar).

In Europe and South America, *S. nobilis* has been involved in envenomations (Dunbar et al., 2018c, Faúndez and Téllez, 2016, Warrell et al., 1991) commonly resulting in prolonged moderate to intense pain, swelling and erythema. Other symptoms can include piloerection, diaphoresis, facial flushing, feverishness, vasodilation of the blood capillaries and minor necrosis localised at the bite site (Dunbar et al., 2018c).

Although the venom of *S. nobilis* has never been investigated before, it has been suggested that symptoms may be triggered by neurotoxins present in their venom (Warrell et al., 1991). This is because members of the genus *Latrodectus*, the sister genus to *Steatoda*, possess a fast-acting neurotoxic venom (**Table 1**) that can induce extreme pain and neuromuscular paralysis in humans, which can occasionally result in death (Gendreau et al., 2017). The toxicity of *Latrodectus* venom towards vertebrates is mainly due to the presence of α-latrotoxin, a large (130kDa) neurotoxin, which binds to receptors on pre-synaptic neurons, then forms a pore that allows an influx of Ca^{2+}, which triggers an efflux of neurotransmitters (Haney et al., 2014, Orlova et al., 2000, Ushkaryov et al., 2008). α-latrotoxin was first documented in *Latrodectus* and has recently been described in *Steatoda grossa* (Garb and Hayashi, 2013). It seems likely that it is the presence of α-latrotoxin that allows Latrodectinae spiders (black widows and false widows) to subdue and feed on vertebrate prey (Dunbar et al., 2018a, O'Shea and Kelly, 2017).

Table 1. Main toxins identified in the venom of true black widow spiders by Haney et al., (2014).

***Latrodectus* toxins**	**Abbreviation**	**Function/Activity**
α-latrotoxins	α-LTX	Toxic to vertebrates, forms calcium channels on pre-synaptic neurons, triggers neurotransmitter release.
α- latrocrustotoxins	α-LCT	Toxic to crustaceans, forms calcium channels on pre-synaptic neurons, triggers neurotransmitter release.
α,δ-latroinsectotoxins	α-LIT / δ-LIT	Toxic to insects, forms calcium channels on pre-synaptic neurons, triggers neurotransmitter release.
Latrodectins	α-LTX LMWPs	Enhances potency of latrotoxins
Cystein Rich Secretory Proteins	CRISPs	Block Calcium channels
Metalloproteases	MPs	Tissue lysis, facilitates spread of latrotoxins
Serine proteases	SPs	Tissue lysis, facilitates spread of latrotoxins
Hyaluronidases	--	Tissue lysis, facilitates spread of latrotoxins
Chitinase	--	Degrades arthropod exoskeletons
Inhibitor cystine knot	ICK	Alters ion channel function

As this species continues to expand its range, it is inevitable that encounters with humans and subsequent envenomations will increase. Therefore, it has become important to characterise the venom of *S. nobilis* and determine the true medical

impact of envenomations. Here, we carried out the first in-depth investigation into the composition of *S. nobilis* venom using a venomics approach, combining venom gland transcriptome and crude venom proteome. The advances of next generation sequencing of mRNA, combined to accurate tandem mass spectra provided by cutting-edge mass spectrometers, represent the current gold standard method to characterize venom from various species, from small animals such as cone snails (Degueldre et al., 2017) to larger ones such as snakes (Giribaldi et al., 2020). In recent studies, such approaches led to the unambiguous identification of hundreds of proteins from single specimens. Sticking to these previsions, our results describe with accuracy the molecular composition of the venom of *S. nobilis*, providing a more resolved picture of its potency and a better understanding of its toxic effects. This knowledge is of prime interest to help in the treatment of envenomations, and to understand the competitiveness of *S. nobilis* against native species where it has become invasive.

4.1.3 - RESULTS

To investigate the diversity and expression of venom proteins in *S. nobilis*, the assembly of the transcriptome obtained from the venom gland mRNAs was used as a database to filter the proteomics results.

4.1.3.1 - Protein identification from transcripts.

The transcriptome assembly yielded 113,803 genes and 157,469 transcripts (**Figure 2A**). Out of those, 49,494 contain Open Reading Frames (ORFs) encoding proteins of > 75 amino acids in length. Homology searches between the predicted proteins and proteins found in Uniprot database found 30,097 proteins with homology against the high-quality manually curated SwissProt database. Out of the remaining 19,397 predicted proteins, 9,749 had matches against the computationally analysed TrEMBL database (restricted to Arachnids). The set of ~40,000 transcripts encoding proteins with BLASTp hits against either database likely represent the biologically relevant transcriptome of *S. nobilis* venom glands, and is encoded by 17,675 genes. Indeed, the

expression level of genes coding for predicted proteins with BLASTp hits is generally higher than the expression level of genes without ORFs/BLASTp hits (Figure 2B).

Among the protein-coding genes, we found 380 genes associated with toxin activity, 37 genes with toxin transport, and 907 genes associated with secretion, indicating that our assembly likely represents a good overview of the venom gland transcriptome of *S. nobilis*. Indeed, gene ontology analysis of the most expressed genes (arbitrarily chosen as > 100 Transcripts per Million (TPM), 872 genes) compared with the entire set of protein-coding genes with Gene Ontologies (28,406 genes) showed enrichments for toxin activity, exocytosis, myosin filament, toxin transport, and peptidase activity (False Discovery Rate (FDR) < 0.05, (**Figure 3)**. The full list of GO enrichment can be found in **Supplementary Dataset 1**.

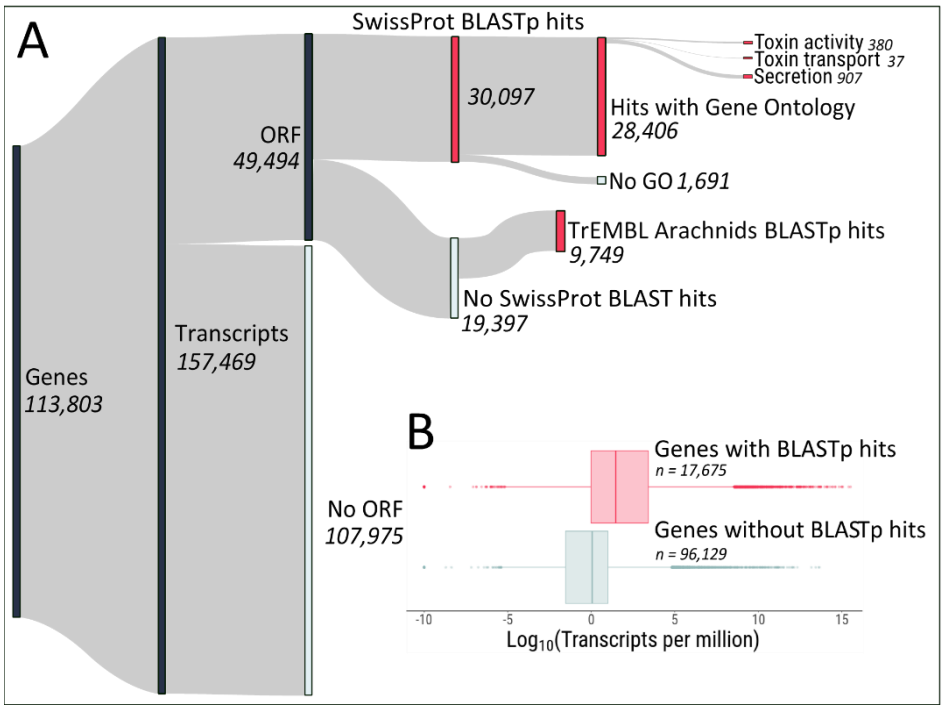

Figure 2. Transcriptomic analysis pipeline of *S. nobilis* venom glands**.** (**A**) Trinity assembly and annotation metrics. Nodes represent features and their associated numbers found in the assembly. (**B**) Expression level of predicted protein-coding genes versus non-coding genes.

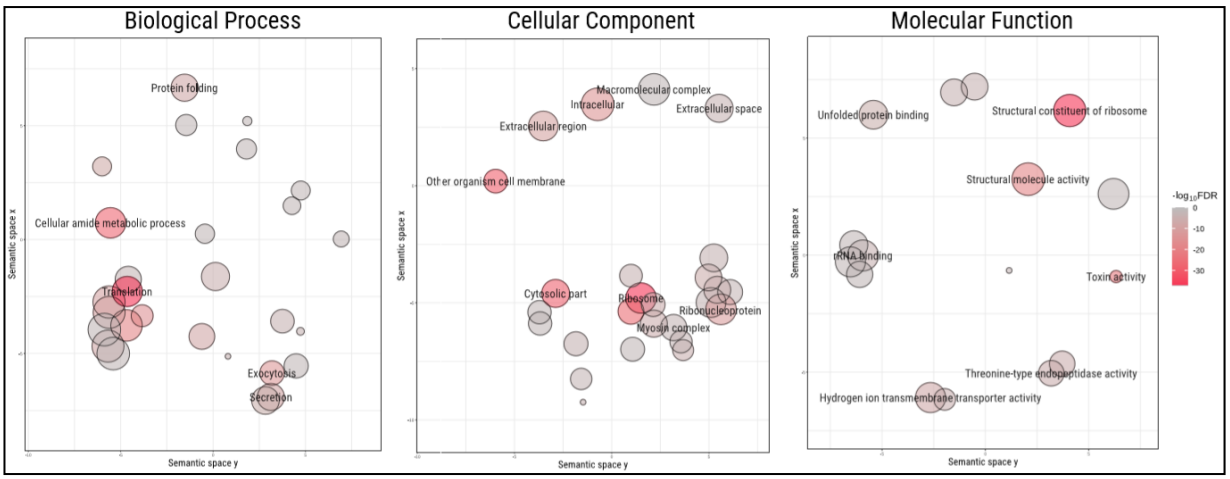

Figure 3. Enriched Gene Ontologies among highly expressed genes in *S. nobilis* venom glands. Circles represent significant Gene Ontologies, arranged according to their semantic space. Size of the circles represent the number of genes associated to the given ontology in the dataset, while colour represent the -$\log_{10}$ False Discovery Rate for the ontologies' enrichment.

Potential genes encoding venom proteins were identified based on homology with known toxin/enzymes identifies in previous venom studies. The list of putative venom-encoding genes was further reduced by retaining only those genes with an expression level > 1 TPM across three biological replicates. We found 228 and 127 genes encoding for enzymes and toxins, respectively (**Figure 4A**). Among the enzymes, Peptidases, and Serine Proteases are the most abundant (85 and 40 genes, respectively), with the most expressed classes of enzymes being Pancreatic lipases and Chitinases (**Figure 4B**). The toxin genes are mainly comprised of α-latrotoxin and δ-Latroinsectotoxins (67 and 39, respectively), (**Figure 4A**), with a generally higher expression for Latrodectin, CRISP/Allergen/PR1, and Theritoxin genes (**Figure 4B**). Altogether, the transcriptomic data indicates a diverse arsenal of venom enzymes and toxins produced by the venom gland of *S. nobilis*.

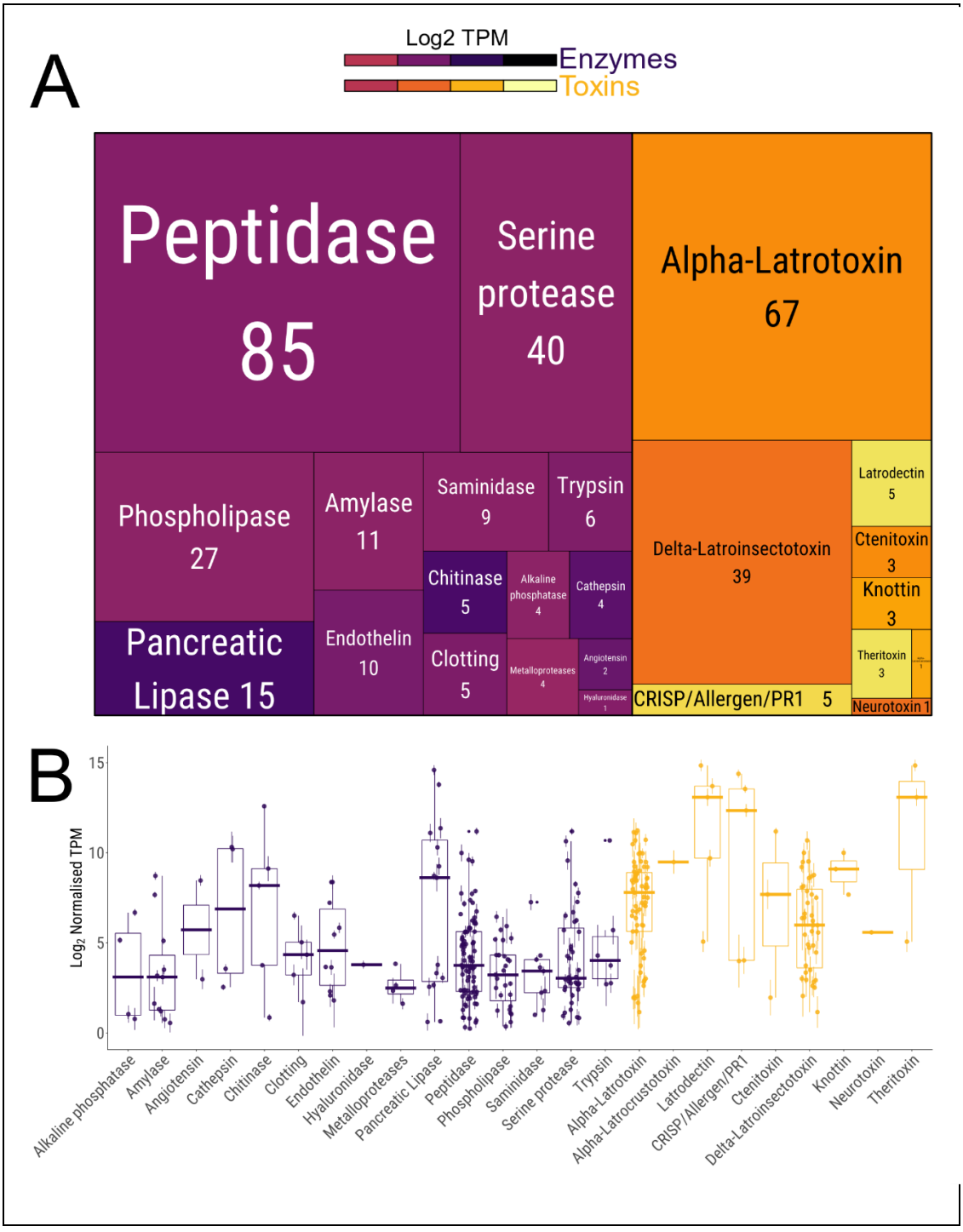

Figure 4. Relative abundance and expression level of genes encoding predicted venom enzymes and toxins. (A) Treemap chart of the main classes of venom-related enzymes and toxins present in *S. nobilis* venom gland transcriptome. The size of the rectangles is proportional to the number of genes in each category, indicated under the labels. The colour represents the $\log_2$ Transcripts per Millions (TPM) of the median expression of the genes in each category. (B) Expression levels of each gene in each enzyme/toxin category. Dots represent the mean expression of individual genes across three biological replicates ± s.d. Purple: enzymes, orange: toxins.

4.1.3.2 - Shotgun proteomics of *S. nobilis*.

Crude venom extracted from female *S. nobilis* was reduced, alkylated, and digested with trypsin before being separated using micro-HPLC hyphenated to Q-Exactive, leading to the acquisition of 10,225 MS and 34,343 MS/MS scans. High resolution mass spectrometers such as Orbitraps ensure both high efficiency in fragmentation and accurate mass measurements of parent and fragment ions. In these conditions, *de novo* sequencing of peptides, performed with Peaks X+, becomes more powerful and accurate. Imposing a precursor mass tolerance < 5ppm and fragment ions mass tolerance < 0.015Da, 7,759 peptides were sequenced with a *de novo* score >50 among, of which 2,974 (38.3%) displayed a score above 80. *De novo* score is a criterion of quality, expressed in percentage, linked to residue local confidence (the higher the score, the higher the confidence in the sequencing). The sequence tags contain from 6 to 23 amino-acids, and have masses ranging from 799.4 to 2524.24 Da.

4.1.3.3 - Qualitative data analysis.

To evaluate the quality of the proteomics data, the sequencing was compared to two databases of protein sequences, extracted from Uniprot (3rd March 2020, https://www.uniprot.org/). The first database was obtained by collecting all the protein sequences returned using the keyword "Spider"(n=317,450 sequences). The second database contained 2,778 sequences selected from Uniprot using the keywords "Spider AND Toxins". For each analysis, a false discovery rate of 0.1% was applied, so to only keep the best matches. With these parameters, the database « Spider » identified 53 proteins, with the help of 773 peptides from the 7,759 *de novo* sequences (10.0%). 23 of those proteins were identified as various hemocyanins (43%). The presence of these proteins may result from the venom extraction process, when haemolymph is accidently drawn into the venom duct and excreted with the venom. Hemocyanins participate in the formation of the arthropod cuticles and in wound healing process. The best identification however is a δ-latroinsectotoxin-Lt1a (AN=Q25338) from *Latrodectus tredecimguttatus*, the Mediterranean black widow. The toxin family is unambiguously identified with 16 unique peptides describing 10% of the whole protein sequence (-10lgP=208.57). U11-Theriditoxin-Lha1d, from the

Australian black widow *Latrodectus hasselti* is also identified within the best matches (4 unique peptides, 26% of sequence coverage, -10lgP=112.71, AN=A0A482ZCV4). These two toxins clearly confirm *S. nobilis* as a close relative to the species of the *Latrodectus* genus. The database "Spider+Toxin" aimed at focusing the search on spider toxins, excluding additional proteins such as hemocyanins. Using the same parameters, only six proteins were identified from 476 peptides (6.1%). These proteins are two isoforms of δ-latroinsectotoxin-Lt1a, an α-latrocrustotoxin-Lt1a, and an α-latrotoxin-Lt1a from *Latrodectus tredecimguttatus*, an α-latrotoxin-Lh1a from *Latrodectus hasselti*, and finally a toxin from *Cupennius salei* named Toxin 21. Even if *Ladrodectus* toxins are mainly identified, the results of this qualitative analysis collected with the help of Uniprot database are relatively poor. This clearly highlights the need to combine proteomics and transcriptomic data for unsequenced organisms, and in our case, to gain a better overview of the venom of *S. nobilis*.

4.1.3.4 - Integration of transcriptomics and proteomics data

4.1.3.4.1 - Qualitative data analysis

Transcriptomic data were used as a sequence database for analysing proteomics *de novo* sequences. Using the same search parameters, 240 proteins were identified with significant peptides, from 4,161 of the 7,759 de novo sequenced peptides (53.6%). This first result was already very encouraging as much more proteins are identified from both approaches, validating the quality of not only the trancriptomics data but also the proteomics-based sequencing. A BLAST of each of the 240 sequences identified 118 of these as toxins (49.2%), 37 as enzymes (15.4%), 43 as proteins with other functions (17.9%), such as cysteine-rich secretary proteins (CRISPs, x8), hemocyanines (x5), or histones (x2). 42 were also identified from mRNA and from the venom, but those proteins present unknown biological activities (17.5%) (**Figure 5A**). The 118 toxin-annotated sequences were classified into 9 different families (**Figure 5B**). Among these, a large number of α-latrotoxins, δ-latroinsectotoxins and latrodectins, commonly expressed in the venom of *Latrodectus* species, are again identified. Importantly, the 118 identified toxin proteins using proteomic data to

mine transcript data closely match the 127 predicted toxin transcripts from the venom gland transcriptome (identified against SwissProt and TrEMBL databases), highlighting the power of the approach. Altogether they represent 94% of the identified sequences; other toxins are a minority in the venom.

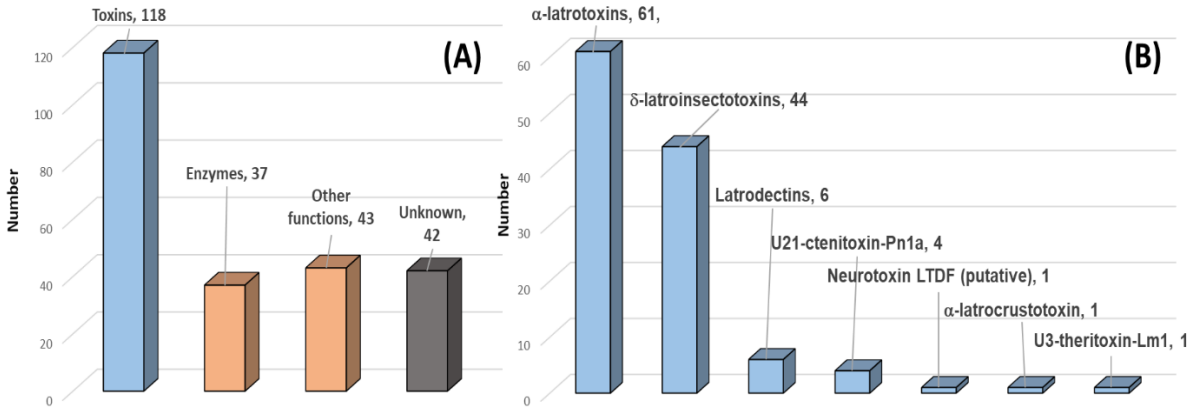

Figure 5. (**A**) Distribution of the 240 proteins identified from *S. nobilis* venom gland transcriptome and proteome, classified into four major protein groups showing the predominant presence of toxins and enzymes in the venom. (**B**) Distribution of the 118 toxin sequences grouped into seven different families, where *α*-latrotoxin, *δ*-latroinsectotoxins and latrodectins combined make up 94% of the identified toxins and more than 46% of the 240 identified protein sequences.

This data suggests that the venom of *S. nobilis* contains plenty of highly bioactive toxins. However, it is difficult to fully appreciate these results without quantifying each kind of toxins. A 1D SDS-PAGE analysis of crude *S. nobilis* venom provided a rough idea of the abundance of each family of proteins (Figure. 6). The electrophoretic separation of the crude venom led to a large number of intense bands, which were interpreted based on their molecular weights. The most intense band (90 and 140kDa) corresponds to the presence of *α*-latrotoxin, *δ*-latroinsectotoxins, and *α*-latrocrustotoxin, which have molecular masses in this range. The brightness of the band tends to indicate that together, these toxins constitute the most concentrated group of proteins in the venom. Two other intense bands are detected at 40 kDa and 10kDa respectively, and are associated to hemocyanins and latrodectins. Others proteins such as CRISPs are also potentially among the most abundant compounds present in the venom. Although all of these observations are consistent with our previous results, the 1D SDS-PAGE gel does not provide any information on the

relative abundance of each kind of protein family present in a single band (for example, the band around 100kDa). In this context, a relative quantitative approach is necessary to get a better overview of the molecular composition of this venom.

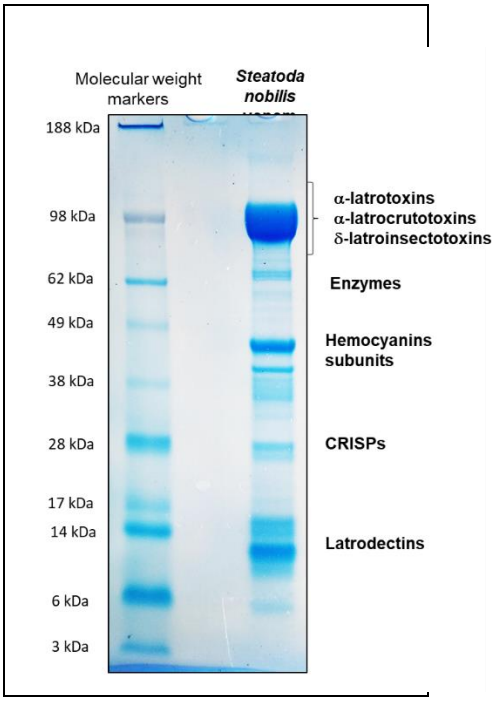

Figure 6. 1D-SDS Page analysis of *S. nobilis* crude venom showing the most abundant protein families of this venom.

4.1.3.4.2 - Relative quantitative analysis

Absolute protein quantification by mass spectrometry is usually done by adding isotopically labelled internal standards to the sample. However, here it would have been very impractical to use this method to consider each of the 240 proteins identified in the venom of *S. nobilis*. Instead, we use relative quantification of venom proteins. Quantifying the proteins based on the signal intensities of their three most intense ions provides a good evaluation of protein abundances, even in complex mixtures (Silva et al., 2006). This approach has demonstrated its usefulness to quantify totally different classes of proteins, with low sequence identity and without common peptides. Unfortunately, in the case of venoms, this way of quantification can create a bias due to the presence of many isoforms. For example, each α-latrotoxins sharing a high sequence identity with others would be quantified according to the three most intense ions of the α-latrotoxin population. In other words, if one α-latrotoxin isoform is very abundant in the venom, all the isoforms,

even those with low concentration would be quantified at the same quantity because they share the same three most intense ions. To avoid this issue, here the relative quantification has been expressed from the three most intense signals coming from *unique peptides* for each toxin. A "unique peptide" is a peptide that does not share its sequence with any other toxin identified in the experiment. The use of unique peptides greatly improves the selectivity of the quantification. The drawback of this approach is the loss of some identifications based on relevant peptides shared between sequences. Here, only 199 out of the 240 identified proteins displayed at least one unique peptide. As a result, only those were considered for relative quantification. It is however an acceptable compromise regarding the complexity of the venom. Out of these 199 proteins, 118 possess at least three unique peptides, 37 only two and 44 one unique peptide. To normalize the data, the mean intensity was set to quantify the 155 proteins possessing two or three unique peptides. **Figure 7A** presents the quantified proteins expressed in percentage and classified according to their families. Even if the quantitation is relative and must be interpreted with care, the figure clearly shows that two thirds of the venom is composed of toxins (66% of the total intensity of the signal). Interestingly, the most concentrated toxins in the venom of *S. nobilis* are *Latrodectus*-like toxins. This finding is in good agreement with the transcriptomic data, suggesting that venom composition is largely controlled at gene expression level. **Figure 5(B)** displays the genera from which the 199 proteins are the closest. While the genera *Steatoda* and *Parasteatoda* are the two closest (32% and 29%, in relative signal intensity), the genus *Latrodectus* represents more than a quarter of the whole venom, thus confirming a close relationship between the "false" and the "true" widow spiders.

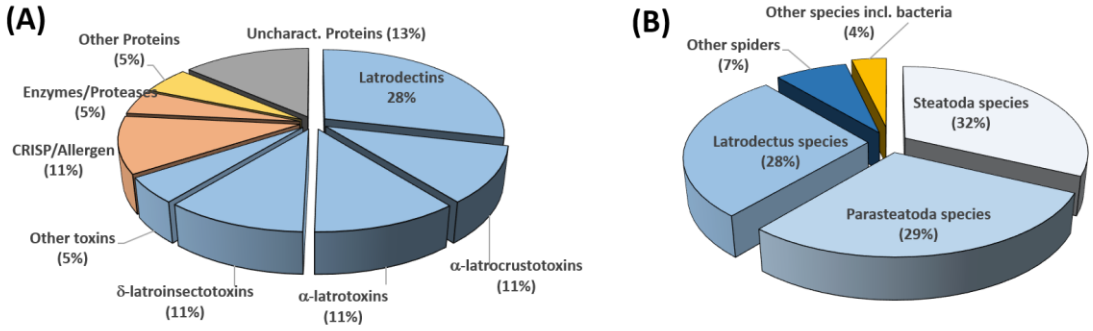

Figure 7. (**A**) Pie-chart showing the relative abundance of the 199 proteins quantified in the venom of *S. nobilis*, arranged into families. The blue slices detail the toxin content of the venom. The orange slices are non-toxin proteins that are important to venom activity. The yellow slice represents the proteins not directly related to venom (e.g. hemocyanins). The grey slice comprises all the proteins with undetermined biological activity. (**B**) Pie-chart showing the relative abundance of the 199 quantified proteins, arranged by genera with matching sequences (determined by BLAST).

The three most abundant individual toxins represent 43.5% of the overall quantified venom and the twelve most abundant account for more than 70% of the quantified content. The main toxin families are latrodectins, α-latrocrustotoxins, CRISPs, α-latrotoxins, and α, δ-latroinsectotoxins. The most abundant single toxin, Latrodectin-Sno1a, accounts for 23.5% of the quantified toxins. Also, two of the most abundant compounds have uncharacterized activities. If these compounds are secreted in such an important proportion, their function, which has yet to be determined, is probably essential to the potency of the spider's venom.

Table 2. Most twelve abundant toxins quantified in the venom.

Steatoda nobilis proteins	Best match (Blast)	Accession number	Species	Quantification in %	Sequence coverage %	-10lgP
Latrodectin-Sno1a	Latrodectin; Alpha-latrotoxin associated LMWP-2	V9QFH8	*Steatoda grossa*	**23.5**	63	210.63
Alpha-latrocrustotoxin-Sno1a	Alpha-latrocrustotoxin-Lt1a	Q9XZC0	*Latrodectus tredecimguttatus*	**10.6**	70	447.19
CRISP-Sno1a	CRISP/Allergen/PR-1-like	XP_015912134	*Parasteatoda tepidariorum*	**9.4**	81	274.42
Uncharacterized Protein-Sno2	Uncharacterized protein LOC107442339	XP_015911366	*Parasteatoda tepidariorum*	**6.1**	66	242.45
Alpha-latrotoxin-Sno1a	Alpha-latrotoxin-Lhe1a	P0DJE3	*Latrodectus hesperus*	**4.7**	60	295.77
Putative neurotoxin-Sno1a	Putative neurotoxin LTDF 06-01	A0A0K1D8C3	*Dolomedes fimbriatus*	**3.7**	48	167.43
Delta-latroinsectotoxin-Sno1a	Delta-latroinsectotoxin-Lt1a	Q25338	*Latrodectus tredecimguttatus*	**2.8**	55	369.06
Latrodectin-Sno1b	Latrodectin; Alpha-latrotoxin associated LMWP-2	V9QFH8	*Steatoda grossa*	**2.5**	60	222.56
Uncharacterized Protein-Sno2	Uncharacterized protein LOC107437515	XP_015905073	*Parasteatoda tepidariorum*	**2.4**	56	175.17
Latrodectin-Sno1c	Latrodectin; Alpha-latrotoxin-associated LMWP	AHC13266.1	*Steatoda grossa*	**2.1**	63	178.46
Delta-latroinsectotoxin-Sno1b	Delta-latroinsectotoxin-Lt1a	Q25338	*Latrodectus tredecimguttatus*	**1.2**	47	352.89
CRISP-Sno1b	CRISP/Allergen/PR-1-like	XP_015912134	*Parasteatoda tepidariorum*	**1.1**	69	209.5

4.1.4 - Discussion

We investigated the venom composition of the noble false widow spider *S. nobilis* using transcriptomics and proteomics. The ability of venomous animals to target various pathways of multiple prey types is facilitated by a diverse toxin repertoire (Lyons et al., 2020, Whiteley et al., 2016). As seen with *Latrodectus* species, *S. nobilis* is also capable of subduing a diverse range of invertebrates but also vertebrate prey (Dugon et al., 2017, Dunbar et al., 2018a). The ability for *Latrodectus* to subdue vertebrates is due to α-latrotoxin, which subsequently can be highly potent to humans (Garb and Hayashi, 2013). Advanced venomics techniques combining transcript libraries produced from next generation sequencing of venom gland RNA with tandem mass spectrometry of crude venom allowed us to identify 240 molecules representing four protein groups, of which 49% are toxins, 15% are enzymes, 18% are proteins with other functions and 18% are proteins with unknown biological functions. Comparison of the transcriptome of *S. nobilis* (presented here) with the transcriptome/proteome of other spiders using spider-specific tools such as ArachnoServer (Pineda et al., 2018) will represent an important follow-up study to compare the evolution of spider venom systems. We confirm for the first time the presence in the venom of *S. nobilis* of various toxins already described from *Latrodectus* species, such as α-latrotoxin, which is among the most dominantly expressed toxins. Unsurprisingly, and in addition to arthropod-specific neurotoxins such as latrodectins, α-latrocrustotoxin, and δ-latroinsectotoxins, these *Latrodectus-like* toxins made up over two thirds of overall toxin composition.

4.1.4.1 - Toxins

From a biological point of view, when α-latrotoxin binds to receptors such as neurexins and latrophilins on pre-synaptic neurons, it permeates the membrane by penetrating the lipid bilayer and forming a pore that allows an influx of Ca^{2+}, which triggers an important efflux of neurotransmitters. Once the vesicles are depleted, the signals between nerve and muscles are blocked, leading to neuromuscular paralysis (Casas, 2011, Orlova et al., 2000). Latrodectins also known as α-latrotoxin associated LMWPs are suspected of enhancing the potency of latrotoxins by altering ion balance

near different channel types, thus regulating Ca^{2+} influx and neurotransmitter release. However, they are not known to be toxic to insects or mammals in their purified form (McMahon et al., 1990). Other proteins detected in the venom that may target neurons include CRISP/Allergen/PR-1, which block Ca^{2+} channels (Mackessy, 2016), although U21-ctenitoxin-Pn1a is a serine protease, which has activity through the hydrolysis of peptide bonds (Langenegger et al., 2018), as well as a Putative neurotoxin LTDF 06-01, and U3-theritoxin-Lm1, which have a neurotoxin-like activity. Collectively, these toxins act simultaneously to facilitate targeting and disrupting various aspects of normal nerve function. *S. nobilis* is a very generalist predator. In addition to producing strong 3-dimensional cobwebs (Dunbar et al., 2018a), they also use a very effective "attack wrap" strategy to immobilize would-be prey or predators alike (Forster, 1995). Consequently, as an opportunist, they are often faced with tackling invertebrate, and/or vertebrate prey that can be strong, fast, aggressive, and many times larger than them. Therefore, the most effective way to immobilize captured prey safely and efficiently is by inducing rapid paralysis. We previously observed wild caught specimens of *S. nobilis* biting insects and spiders and causing a rapid reduction in motor function (*unpublished data*). The mechanical bite from an adult *S. nobilis* is almost painless, however, the rapid release of neurotransmitters induces intense pain and is therefore also an effective weapon for defence. Victims of *Latrodectus* and *Steatoda* bites typically experience immediate sharp pain, which are attributed to the effects of α-latrotoxins (Haney et al., 2014).

While necrosis has not been reported as a symptom of envenomation by *Latrodectus* species (Vetter et al., 2015a), in high doses (>10 nM), α-latrotoxin can reduce ATP levels in the nerve terminal, compromising plasma membrane integrity and releasing cytoplasmic markers including glutamate, y-aminobutyric acid, aspartate and α-aminoisobutyrate. This can result in morphological alterations, swelling of mitochondria and subsequent cell death (Casas, 2011, Südhof, 2001, Ushkaryov et al., 2008). As such, α-latrotoxin may indirectly result in cell death. In *Latrodectus hesperus*, transcriptomic studies show that 39 Latrotoxin sequences account for 16% of venom gland expression (Haney et al., 2014), whereas our study shows that 61 α-latrotoxin

sequences, accounting for 52% of all proteins annotated as toxins, are detected in *S. nobilis* venom. This higher concentration of α-latrotoxins in *S. nobilis* venom may explain the minor necrosis localised around the bite site previously described in the literature (Dunbar et al., 2018c). The high concentration of α-latrotoxin in *S. nobilis* venom is significant as it most likely plays a primary role in prey immobilization, defence and subsequent medical envenomations. The venom of *Latrodectus* can induce intense pain, diaphoresis, paresthesia, hypertension, fasciculations of muscles and ultimately neuromuscular paralysis occasionally leading to death (Haney et al., 2014). In the first case of envenomation by *S. nobilis* (Warrell et al., 1991), some neurotoxic symptoms were reported as systemic. While severe neurotoxic symptoms have not yet been reported from *S. nobilis*, increasing reports of envenomation by the latter should be a concern, especially given their close relatedness to *Latrodectus*. In the overwhelming majority of envenomations by *Latrodectus*, the victims do not require medical attention; only a small percentage result in severe symptoms and death. Only seven medically assessed case reports of *S. nobilis* envenomations have been reported so far. As this species is becoming more widespread, envenomation occurrences are likely to rise, and reports of severe envenomations, although rare, should be expected. Therefore, we recommend caution on dismissing these spiders as harmless until larger scale reporting of case studies is compiled, and clinical assays are carried out to further verify the medical potential this highly neurotoxic venom has on human health.

4.1.4.2 - Enzymes

A range of enzymes making up 15% of *S. nobilis* venom suggests that the venom not only plays a role in prey immobilization, but also assists in the pre-digestion of the prey's tissue. These include pancreatic lipase-related protein, which hydrolyze both phospholipids and galactolipids. Chitinase, also represented, is involved in the breakdown of the exoskeletons of arthropods (Haney et al., 2014), Carboxypeptidase is thought to remove the C-terminal Arg-residues from immature venom peptides (Kuhn-Nentwig et al., 2019). Astacin-like metalloprotease are proteases that function in the metabolism of extracellular matrix components. The astacins are proteases that

may aid as a spreading factor for other venom toxins (Morgon et al., 2016), proclotting enzyme is most likely involved in promoting coagulation of haemolymph in prey, which may aid as an immobilizing toxin. Cathepsin has protease activity and endothelin-converting enzyme homolog degrades large endothelins into smaller forms, which display vasoconstriction activity. Pancreatic α-amylase is involved in endohydrolysis of (1->4)- α-D-glucosidic linkages in polysaccharides such as cellulose and chitin suggesting a role in the breakdown of cytoskeleton. β-hexosaminidase subunit β is involved in the degradation of gangliosides on the cellular surfaces of neuronal cells, this may indicate a toxin function for immobilization. Angiotensin-converting enzymes interact with metabolic pathways causing disturbances of the cellular homeostasis and thus contributing to prey immobilization (Kuhn-Nentwig et al., 2019). Alkaline phosphatase has a proteolytic activity, peptidylglycine α-hydroxylating monooxygenase-like is involved in electron transport and anionic trypsin-2-like cleaves peptide bonds in proteins. Altogether, this array of enzymatic proteins suggests that like other venomous animals such as snakes (Bottrall et al., 2010), the venom of *S. nobilis* does likely play a role in pre-digestion of prey (Vassilevski et al., 2009, Zobel-Thropp et al., 2012) and may possibly contribute towards cell death at the bite site.

4.1.5 - CONCLUSIONS

This study proposes for the first time an in-depth investigation of the venom of a *Steatoda* species, a close relative of *Latrodectus*. We reveal the striking similarity between the toxins found in *S. nobilis* venom and that of black widow spiders (**Table 1**). The most powerful toxin classes (α-LTX, α-LCT, α,δ-LIT) and the enzymatic machinery allowing the venom to more easily spread into the prey (Metallo and serine proteases, chitinases) are both present in large quantities. This however does not mean that *Steatoda* is as dangerous to human beings as some members of the genus *Latrodectus*. If isoforms of potent toxins are present, our study does not provide information about their potency. Evaluation of toxin toxicity would need to be performed before any conclusion can be reached. Nevertheless, given the composition of the venom depicted in this study, *S. nobilis* should be considered a

species of medical importance and it highly likely that *S nobilis* (with *Latrodectus tredecimguttatus)* is one of the most dangerous spiders in Western Europe. Moreover, as *S. nobilis* continues to expand its range, its impact on native wildlife needs to be monitored and its potential invasiveness assessed. In temperate regions where it occupies synanthropic habitats, envenomations of medical importance will undoubtedly rise as the species becomes more prevalent in and around human habitations. The work carried out in this study will hopefully help researchers across disciplines to better understand the evolution of the Latrodectinae family, the competitiveness of *S. nobili*s, and the potential medical importance of envenomations.

4.1.6 - MATERIALS & METHODS

4.1.6.1 - Spider collection and venom extraction

All specimens of *S. nobilis* used in this study were collected in the Republic of Ireland, from street furniture, garden walls, and park railings in the general area of Lucan, Co. Dublin. In total, 80 specimens were collected and identified as female from the presence of the epigyne. The spiders were anesthetized using CO_2 for 2 mins and venom was extracted by electrostimulation with repeated pulses delivered at 15-20V. Venom droplets were collected from the venom pores located on the outer subterminal part of the fangs using 5 µL microcapillary tubes modified with a tapered end for maximum efficiency. The venom was pooled, then flash-frozen in liquid nitrogen, lyophilized, and stored at -20°C.

4.1.6.2 - Venom gland removal and RNA extraction

Adult *S. nobilis* specimens were collected from the same location as above. Venom extraction was carried out on 25 females, and three days later the spiders were euthanised with an overdose of CO_2 and once dispatched, using micro-dissection tweezers the dorsal exoskeleton was removed exposing the venom glands. The glands were removed and pooled together in a 2 ml tube containing RNA later (Ambion), with three biological replicates. Venom glands were then flash-frozen in liquid nitrogen and ground to a fine powder using a bead mill (Qiagen Tissue Lyser II). Immediately after grinding, 0.5 ml of TRIzol was added before samples thawed.

Samples were shaken for 15 seconds, incubated at room temperature for 5 minutes and then centrifuged (25,000 g) at 4°C for 10 minutes. The supernatant was collected and transferred into new tubes. Nucleic acids were separated by the addition of 100 µL of chloroform, followed by 30 seconds of shaking, and 3 minutes incubation at room temperature. Then, the two phases were separated by centrifugation for 10 minutes at 4°C. Nucleic acid precipitation was performed on 200µL of the upper aqueous phase with 200µL of isopropanol, mixed for 10 seconds, incubated in ice for 15 minutes and centrifuged for 15 minutes. The pellets were washed twice with 75 % EtOH (prepared in DEPC water). Finally, the pellets were air dried at 37°C and resuspended in 50 µL of RNAse-free water. Following extraction, DNA was removed from the nucleic acids using DNAse 1 (Sigma-aldrich AMPD1), following manufacturer's instructions.

Finally, DNA-free total RNAs were cleaned up and concentrated using a silica column based kit (Zymo Research RNA Clean & Concentrator).

4.1.6.3 - Transcriptomics assembly and analysis

For preparation of RNA transcript libraries and sequencing, samples were sent to *Novogene Company Limited, Cambridge Science Park, Milton Road, Cambridge, CB4 0FW, UK*. Libraries were generated using mRNA enrichment, and sequencing was performed using Illumina technology (150 bp paired-end reads). For all three libraries, >50 million 150 bp paired-end reads were obtained.

Raw reads were corrected using Rcorrector (Song and Florea, 2015) and leftover Illumina adapters removed using trimmomatic (Bolger et al., 2014). Then, reads mapping to ribosomal RNAs (using bowtie2 (Langmead and Salzberg, 2012) mapping against *Latrodectus* and *Steatoda* ribosomal RNAs sequences available in NCBI) were removed to ensure good representation of reads belonging to mRNA transcripts in the transcriptome assembly. The transcriptome of one of the three venom gland samples was assembled using the Trinity pipeline (Grabherr et al., 2011, Haas et al., 2013). A single sample was used for assembly to limit the hardware

requirements of such method. Transdecoder (Haas and Papanicolaou, 2015) was used to identify Open Reading Frames (ORFs) originating from the transcripts, with a minimum protein length of > 75 amino acids. We intentionally lowered the minimum ORF prediction from Transdecoder (100) to 75 amino acids in length, to account for the presence of potentially relevant small proteins, such as those described in Rokyta and Ward, (2017) The assembly was annotated using Trinotate (Bryant et al., 2017) based on ORFs homology using BLASTp (e-value cutoff of $1e^{-3}$) against i) SwissProt curated database (Consortium, 2019) and ii) TrEMBL Arachnids database (also (Consortium, 2019)). In addition, ORFs were compared to the Protein family (Pfam) database (El-Gebali et al., 2019). A summary of the annotation results is shown in **Figure 2**.

Gene expression analysis was performed as part of the Trinity pipeline using Kallisto (Bray et al., 2016) on the three venom gland libraries, using the assembled transcriptome generated by Trinity. The gene expression matrix obtained was used to extract genes with high expression level (>100 Transcripts per Million (TPM)) for Gene Ontology (GO) analysis. GO terms associated with the annotated genes were retrieved using Trinotate's extract_GO_assignments_from_Trinotate_xls.pl script. GO analysis was performed using GOseq (Young et al., 2012), with the genes > 100 TPM as input (~900 genes), against the background of all ~17,000 genes with GO annotations. Over-representation of Gene Ontologies was analysed using ReviGO (Supek et al., 2011), and plotted in R v.3.6.3 (R Development Core Team, 2011). For venom-encoding genes, relative abundance of enzyme and toxin-producing genes was generated using the Treemap package in R (Bederson et al., 2002).

4.1.6.4 - SDS-PAGE of *S. nobilis* female venom

10µg of *S. nobilis* venom have been diluted in Laemlu buffer, heated for 3min to 100°C, before being separated using 1D SDS-PAGE NuPage® (ThermoFisher Scientific) in MES SDS buffer. 6µg of a standard composed of insulin beta-chain (3kDa), aprotinine (6kDa), lysozyme (14kDa), red myoglobin (17kDa), carbonic

anhydrase (28kDa), alcohol dehydrogenase (38kDa), glutamic dehydrogenase (49kDa), bovine serum albumin (62kDa), phosphorylase (98kDa) and myosin (188kDa), were used as molecular weight markers. The electrophoresis was performed by applying 200V during 40min to the system. The resulting gel was firstly dehydrated with 50% EtOH and phosphoric acid 3% during 3 hours, then rehydrated by the mean of a 20min bath of ultrapure water (MilliQ). Le coloration of the proteins was performed overnight with Coomassie blue (360 g/L, in an aqueous buffer with 34% MeOH, 3% phosphoric acid, and 17% ammonium sulphate). The gel is finally conserved at 5°C in 5% of acetic acid for further experiments.

4.1.6.5 - Shotgun Proteomics of *S. nobilis* female venom

0.2mg of lyophilized venom was dissolved in 100µL of pure water (MilliQ). 3µl corresponding to roughly 6µg was lyophilized and dissolved into 20µL of 50 mM NH_4HCO_3 pH 7.8. The sample was then reduced with 5µL of 500mM dithiothreitol (DTT) for 45min at 56°C under shaking at 300 rpm. The reduced venom was then alkylated with 6µL of 500 mM iodoacetamide for 30 min, at room temperature, in the dark. The venom was then submitted to enzymatic digestion with trypsin at a ratio of 1:50, incubated overnight, at 37 °C, under shaking at 300 rpm. Reactions were stopped by acidifying the medium using 10% TFA. The digested sample was finally dried on speed vacuum. Before the mass spectrometry analysis, the samples were suspended in 20 µL of 0.1% TFA for desalting on ZipTip™ pipette tips with C18 resin. The elution was made by 18µl of TFA 0.1%/ACN (50/50), to reach a theoretical concentration de ~3µg/9µL, suitable for LC-MS analysis (9µL injected, 100min run). The efficiency of the digestion was controlled by MALDI-TOF, using saturated CHCA (70/30 ACN/FA 0.1 %) as the matrix.

The purified material was analysed using an Acquity UPLC® M-Class (Waters, Milford, MA, USA) coupled to the Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, Bremen, Germany). The trap column is a Symmetry C18 5µm (180 µm x 20 mm) and analytical column is a HSS T3 C18 1.8 µm

(75 μm x 250 mm) (Waters, Corp., Milford, USA). The samples were loaded at 20 μL/min on the trap column in 98% solvent A (water/0.1% formic acid) during 3 minutes and subsequently separated on the analytical column. Peptides were eluted using a gradient of 2–85% of solution B in 73min (B: acetonitrile/0.1% formic acid), at a flow rate of 0.6 mL/min. Regarding mass spectrometry, all the analyses were performed in data dependent analysis (DDA) mode that automatically triggers the MS/MS experiments. The automatic gain control (AGC) target values were 3.10^6 for MS spectra and 2.10^5 for MS/MS spectra. The maximum injection times were set at 200 ms for the MS step and 1000 ms for MS/MS events. For MS/MS, a "Top 12" experiment was applied, meaning that the twelve most intense ions of each MS scan have been selected for fragmentation. Singly charged ions, ions with undetermined charge (for example, electronic noise) and ions with signal intensities below the AGC threshold set at 1e3 were excluded from the selection. For precursors ions, the selection windows were 2.0 m/z, the AGC target was 1e5 (or 50 ms as a maximum of injection time) and the resolving power of 17,500 @m/z 200. Normalized collision energy was 25. A dynamic exclusion of 10s was also applied to avoid the redundancy of MS/MS spectra of the same ions. 2.6.

Bioinformatic analysis of proteomic data PEAKS Studio X+ (Bioinformatics solutions, Waterloo, ON, Canada) a *de novo* assisted database software was chosen to analyse MS/MS data from *S. nobilis* venom. The database chosen for analysing the proteomics data is composed of the translated sequences obtained from the assembled transcriptome. PEAKS studio initially produces de novo sequences from MS/MS spectra without relying on database. The confidence of each peptide sequence obtained by this process is given by an ALC (Average Local Confidence) score. These de novo sequences are then corrected by comparing them to the database to provide additional information about post-translational modifications (PTM's), mutations, homologous peptide, and novel peptides. Carbamidomethylation was set as fixed modification, while oxidation (M) was set as variable modifications, with maximum missed cleavages at 3 for trypsin digestion. Parent mass and fragment mass error tolerance were set at 5ppm and 0.015 Da, respectively. False discovery rate (FDR) of

1% [29,30] and unique peptide ≥2 were used for filtering out inaccurate proteins. A−10lgP > 120 indicates that the detected proteins by enough reliable peptides MS/MS spectra.

4.1.7 - ACKNOWLEDGEMENTS

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Chapter 5
Medical Importance

In situ adult female *Steatoda nobilis* hanging from her web in urbanised Dublin

Photo by JP Dunbar

5.1 - Envenomation by the noble false widow spider *Steatoda nobilis* (Thorell, 1875) – five new cases of steatodism from Ireland and Great Britain

JPD contributed to the conceptualization, methodology, data acquisition, original draft preparation, and review and editing. This section is published in a peer reviewed journal and available at the following reference: DUNBAR, J. P., AFOULLOUSS, S., SULPICE, R. & DUGON, M. M. 2018c. Envenomation by the noble false widow spider *Steatoda nobilis* (Thorell, 1875)–five new cases of steatodism from Ireland and Great Britain. *Clinical Toxicology*, 56, 433-435.

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5.1.1 - ABSTRACT

The noble false widow *Steatoda nobilis* is the only medically significant spider known to occur in the British Isles and Ireland, with a single case of steatodism ever reported from Great Britain. We present here five new cases of envenomations by *S. nobilis*, three from Ireland and two from Great Britain and describe symptoms not previously reported for the genus *Steatoda*. Four adult males and one adult female with confirmed *S. nobilis* bites reported their symptoms to the authors. General practitioner chart was obtained for case #3. In all five cases, envenomations were immediately followed by a sharp and prolonged onset of pain, mild to extensive erythema, and localised to extensive swelling around the bite site. Additional symptoms include moderate to intense pruritus, vasodilation of the capillaries around the bite site, and a possible minor necrotic wound. In all cases, symptoms subsided within 48–72 h and no further complications were reported. Envenomations by *S. nobilis* seem to produce symptoms similar (but not identical) to those previously

reported from other *Steatoda* sp. Considering their benign outcome, envenomations by *S. nobilis* should still be regarded as of moderate medical importance, requiring monitoring and pain management strategies.

5.1.2 - INTRODUCTION

The noble false widow spider *Steatoda nobilis* (Thorell, 1875) (Figure 1(A)) has been involved in the only case of systemic araneism reported in Ireland and Great Britain (Warrell et al., 1991). As such, it is the only known medically significant spider established in these islands. Envenomations by *S. nobilis* (steatodism) have been described as "far more severe than a bee or a wasp sting" with symptoms similar (albeit less severe) to envenomations by true black widows of the genus *Latrodectus* (latrodectism). Steatodism include intense pain, diaphoresis, piloerection, facial flushing, raised temperature, and general discomfort lasting several hours (Faúndez and Téllez, 2016, Isbister and Gray, 2003, Warrell et al., 1991). Additional claims of toxic shock, dermal necrosis, and subcutaneous haematoma have never been confirmed. Envenomations by *S. nobilis* are therefore referred to as of moderate medical importance but have become a major concern for members of the public since the rapid spread of the species in heavily urbanised areas globally (Dugon et al., 2017). In this report, we present and describe five new cases of envenomations by *S. nobilis* from Ireland and Great Britain.

5.1.3 - Case presentation

5.1.3.1 - Case 1

A 48-year-old male from Gloucestershire, Great Britain, woke after experiencing a sharp pain in the right forearm. Erythema and swelling developed around the bite site within minutes (Figure 1(B)). A crushed spider was subsequently found in the bed, sent to the authors and identified as a mature female *S. nobilis*. Pain and discomfort steadily increased to peak (7/10) 12–15 h post-bite; the whole right forearm was swollen, inflamed and clammy. A general practitioner prescribed antibiotics, pain killers and antihistamine. The pain dropped in severity overnight (5/10). On the second day, swelling and erythema had reduced but pain (3/10) and discomfort

persisted. The bite site felt prickly and itchy. By the third day, pain, swelling and erythema had subsided. The bite site was observable, and a minor wound had developed (Figure 1(C)). Mild irritation and pruritus persisted for several days around the bite site.

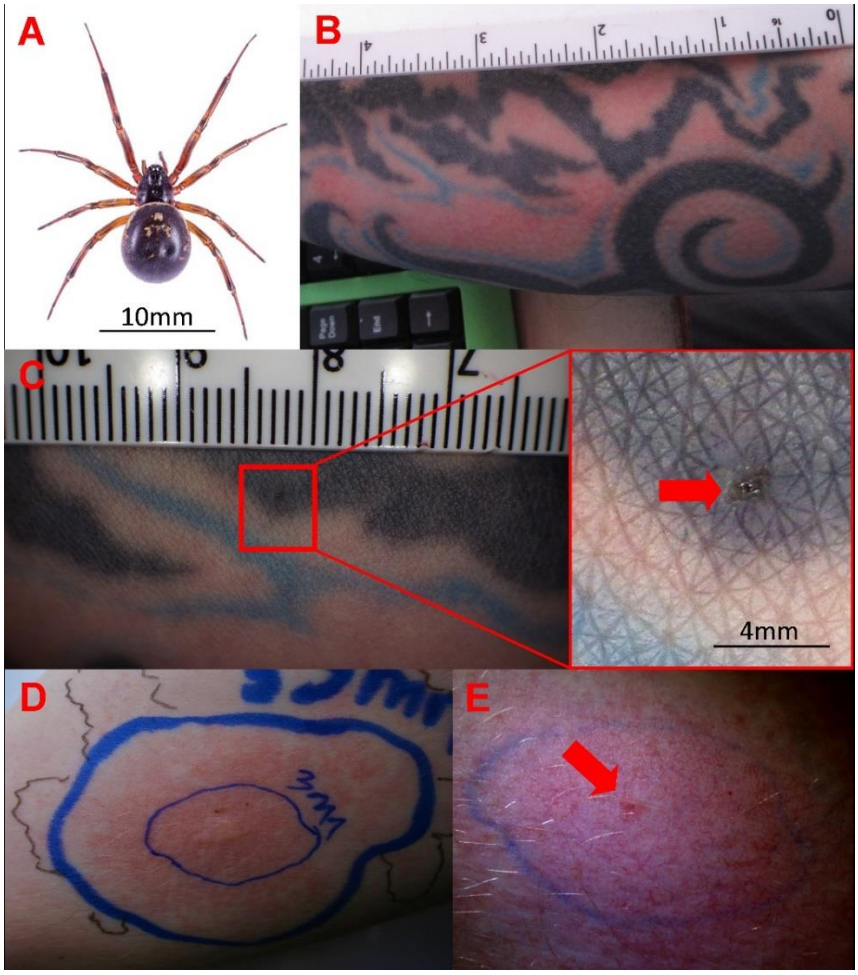

Figure 1. **(A)** Mature female *Steatoda nobilis* from Co Dublin, Ireland. Dorsal markings are highly variable between individuals but generally consist of a cream or white crescent on the antero-dorsal aspect of the abdomen and a pentagonal pattern on the dorsal aspect of the abdomen (Dugon et al., 2017, Vetter and Rust, 2012). **(B–C)** Envenomation by a mature female *S. nobilis* on the right forearm (Case #1). **(B)** Piloerection, erythema and swelling around the envenomation site; ruler is in inches. **(C)** Possible dermo-necrosis at the envenomation site 72 h post-bite (red arrow). **(D–E)** Envenomation by a mature female *S. nobilis* on the inner part of the right biceps (Case #3). **(D)** Erythema surrounding the envenomation site. Blue lines show extent of erythema 3 min post-bite (inner) and 52 min post-bite. **(E)** Vasodilation of blood capillaries surrounding the envenomation site (red arrow) 24 h post-bite.

Table 1. Clinical symptoms and bite circumstances of Steatoda nobilis ($N = 7$, including data from Warrell et al., 1991 and Faúndez & Téllez, 2016) and Steatoda sp. ($N = 23$, Isbister & Gray, 2003). Steatoda sp. refer to Steatoda grossa ($N = 3$), Steatoda capensis ($N = 23$) and other unidentified Steatoda species ($N = 4$).

		Steatoda sp. *(N = 23)*	*Steatoda nobilis* ($N = 7$)						
Symptoms		Isbister & Gray (2003) Australia	Warrell (1991)	Faúndez (2016)	Case 1 Gloucestershire, UK	Case 2 London, UK	Case 3 Galway, Ire	Case 4 Cork, Ire	Case 5 Laoise, Ire
Location	# of cases	Upper/lower limbs, trunk	Shoulder	Face	Rright forearm	Left forearm	Right bicep	Arms, palm	Right arm
Pain level /10	30	Yes	> Wasp sting	Burning sensation	3–7	3–4	1–4	Cigarette burn	4
Erythema	28	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Swelling	6	Yes	Yes	No	Yes	No	No	Yes	Yes
Nausea	4	Yes	No	No	No	No	No	No	No
Headache	4	Yes	No	No	No	No	No	No	No
Pruritus	3	No	No	No	Yes	Yes	No	No	Yes
Raised skin	2	No	No	No	No	Yes	Yes	No	No
Piloerection	2	No	Yes	No	Yes	No	No	No	No
Vasodilation	2	No	No	Yes	No	No	Yes	No	No
Malaise	2	Yes	No	No	No	No	No	No	No
Lethargy	2	Yes	No	No	No	No	No	No	No
Necrosis	1	No	No	No	Possible	No	No	No	No
Facial flushing	1	No	Yes	No	No	No	No	No	No
Feverishness	1	No	Yes	No	No	No	No	No	No
Diaphoresis	1	No	Yes	No	No	No	No	No	No

5.1.3.2 - Case 2

A 52-year-old male from south London, Great Britain, felt a sharp prick on the left forearm while in bed. Upon inspection, the victim found a squashed spider at his side. As an experienced arachnologist working with the NHM London, the victim immediately identified the spider as an adult male *S. nobilis*. After a few minutes, pain level increased (4/10) and an intense pruritus developed. By the morning, the area of the bite was raised with erythema covering ca. 2 cm around the bite site. The pruritus extended to the whole arm, wrist and down to the base of the thumb. Twenty-six hours post-envenomation, the bite site was still raised and remained itchy for several days with constant mild to minor pain.

5.1.3.3 - Case 3

On 18 April 2017 at 13:52, a 37-year-old male in County Galway, Ireland, was bitten twice on the inner part of the right bicep by a mature female *S. nobilis* wedged between the skin and clothing. The bite was immediately followed by a throbbing pain. By 14:00 a 5 mm-diameter discoloured raised bump was observed at the bite site with erythema spreading 3 cm from the epicentre. Pain level escalated to 4/ 10 over the next hour. By 14:45 erythema 7–8 cm across surrounded the bite site (Figure 1(D)). By 15:00 pain had reduced to 1/10. At 15:20 a medical examination was carried out by a general practitioner. Temperature reading from the ear was 37.2 °C, breathing was deemed normal, with slightly elevated blood pressure (138/93). Blood samples returned normal. At 17:15, pain level suddenly escalated to 5/10 momentarily and eased after c.1 min. By 17:43, the erythema and the raised lump had partly subsided, but the area remained sensitive to touch. A mild dull ache spreading from the biceps down to the fingers was felt overnight, with short onsets of more intense pain. Mild erythema 48mm across and vasodilation of the capillaries persisted for 48 h, after which the bite zone became visible, as a raised, dark brown 3mm patch of skin (Figure 1(E)).

5.1.3.4 - Case 4

While in bed, a 54-year-old woman from Cork, Ireland, felt a succession of sharp pricks described as being similar to cigarette burns on her left arm, right mid-forearm and the palm of her right hand. The victim inspected the bed when the pain started to travel up her arm and into her shoulder. A spider was found between the sheets and sent to the authors who identified it as a mature female *S. nobilis*. The pain remained severe for 12 h with extensive erythema and swelling on the whole right arm. The victim described the pain as dull and throbby, with noticeable loss of mobility of the arm for 24 h.

5.1.3.5 - Case 5

A 33-year-old male from County Laois, Ireland, woke up after feeling a sharp prick on his right arm and found a spider on the bedside. The spider was collected, photographed for identification purposes and then released in the garden. The specimen was identified as an adult male *S. nobilis* by the authors. The victim experienced slight swelling at the bite site with moderate pain (4/10), localised erythema and pruritus. By 18:44 the next day, symptoms had receded, and pain was described as similar to a nettle sting. A mild pruritus persisted for the following 24 h.

5.1.4 - CONCLUSION

Prolonged moderate to intense pain, erythema, swelling around the bite site, and pruritus seem to be common symptoms of envenomation by *S. nobilis*. These symptoms partially overlap with those previously reported from envenomations by *Steatoda grossa* and *Steatoda capensis* in Australia (Isbister and Gray, 2003) (Table 1). Previously unreported symptoms include vasodilation of the capillaries around the bite site (case #3), and a minor, potentially necrotic wound (case #1). Despite the many alleged *Steatoda*-related necrosis reported by the media, extensive necrotic wounding (due to either envenomation or secondary infection) seems extremely unlikely to occur. In all instance's symptoms subsided within 48–72 h and no further complications were reported. Considering their benign outcome, envenomations by *S. nobilis* should still be regarded as of moderate medical importance, requiring at most, monitoring and pain management strategies.

5.2 - Envenomations by the noble false widow spider *Steatoda nobilis*: Venom yield, symptomatic and implications for public health

JPD contributed to the conceptualization, methodology, data acquisition, original draft preparation, and review and editing. This section is ready to submit to a peer reviewed journal for publication.

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5.2.1 - ABSTRACT

In recent years, the noble false widow spider *Steatoda nobilis* (Thorell, 1875) has expanded its range globally and may represent a potential threat to native ecosystems and public health. Increasing numbers in synanthropic habitats has led to an increase in human encounters resulting in envenomations. *S. nobilis* bites are currently classed as medically significant with similarities to *Latrodectus* but milder in onset, with symptoms generally ranging from mild to moderate, lasting from several hours to several days. In this manuscript we present 14 new cases of *S. nobilis* envenomations and provide additional information on one previously reported case, bringing the total number of globally reported cases to 23. We report a range of pathologies including necrosis, *Latrodectus*-like symptoms that include debilitating pain, tremors, fatigue, nausea, and hypotension, and vectored bacterial infections including

cellulitis and dermatitis. Furthermore, because the quantity of venom injected undoubtedly reflects envenomation outcomes, we provide venom extraction data from 520 wild caught specimens of *S. nobilis* and demonstrate a statistically significant correlation between spider body size and venom yield. Extraction data show that on average adult *S. nobilis* produce 0.22 µl of venom, with a maximum yield of 0.51 µl. Overall, the venom quantities were extremely variable and may reflect the overall variation in symptom onsets. The continued rising numbers of *S. nobilis* will undoubtedly result in further bites and this study will help provide the medical community with a better understanding of the potential medical outcomes from bites by this species and alert them to the possibility of medically important outcomes.

5.2.2 - INTRODUCTION

The noble false widow spider *Steatoda nobilis* originates from the Macaronesian archipelago and has recently expanded its range globally (Bauer et al., 2019, Dugon et al., 2017, Dunbar et al., 2018b, Faúndez and Téllez, 2016, Kulczycki et al., 2012, Nolan, 1999, Snazell and Jones, 1993, Taucare-Ríos et al., 2016, Türkeş and Mergen, 2007, Vetter et al., 2015b, Vetter and Rust, 2012, Warrell et al., 1991, Zamani et al., 2015). Outside of its native range the species establishes in synanthropic habitats where close contact with humans is unavoidable (Bauer et al., 2019, Dugon et al., 2017, Hambler, 2019). Consequently, 10 cases of envenomation have previously been reported in the scientific literature: four in Ireland (Dunbar et al., 2018d; O'Keeffe & Dugon, *In Review*), three in Great Britain (Dunbar et al., 2018d, Warrell et al., 1991), two in Chile (Faúndez et al., Faúndez and Téllez, 2016) and one possible case from Columbia (Porras-Villamil et al., 2020). The first case reported in 1991 described symptoms of local and systemic neurotoxicity similar to a mild form of latrodectism (spectrum of symptoms of *Latrodectus* envenomations) (Warrell et al., 1991). The authors warned of the potential medical importance of *S. nobilis* and called for further research on this species.

Despite its presence in England for 140 years, it is only in the past decade that this species has significantly increased in numbers and expanded its range across

southern England and continues to spread northwards (Bauer et al., 2019, Hambler, 2019). This may be linked to an ecological niche expansion following the recent arrival of individuals with adaptive genetic traits (Bauer et al., 2019). In 1998, the first sightings of *S. nobilis* was recorded in Ireland (Nolan, 1999) and since then the species has flourished, becoming one of the most common urbanised spiders in some counties (Dugon et al., 2017, Dunbar et al., 2018c). *S. nobilis* is currently regarded as naturalised in Great Britain, but this species' recent change in occurrence has revealed the potential to be invasive and cause medically significant bites to humans (Bauer et al., 2019, Dugon et al., 2017, Dunbar et al., 2018a, Dunbar et al., 2018d, Faúndez et al., 2020, Faúndez and Téllez, 2016, Hambler, 2019, Kulczycki et al., 2012, Warrell et al., 1991).

Over the past decade, the Irish and British media have taken a keen interest in reporting on bites by *S. nobilis* with alarming stories of debilitating tissue necrosis and bacterial infections that resulted in amputations and fatalities (Hambler, 2019). With the exception of a single case of envenomation in 1991, until 2016, scientific research on many aspects of this species, including symptom onsets, venom and bacterial carriage has been completely absent, leaving an important knowledge gap to competently advise the medical community on the true potential risk to public health. 90% of bite cases were reported (N=9) between 2016 and 2020, and revealed additional symptoms (Dunbar et al., 2018d, Faúndez et al., 2020, Faúndez and Téllez, 2016, Warrell et al., 1991). A recent study focusing on venom composition revealed that two thirds of the toxins produced by *S. nobilis* are also found in *Latrodectus* (Dunbar et al., 2020). In addition, microbiological analysis revealed the potential for *S. nobilis* to vector antibiotic resistant strains of pathogenic bacteria during bites (Dunbar et al., *In Review*).

Further geographical expansion and increase in population density by *S. nobilis* will inevitably lead to an increase in bites. Consequently, potentially severe outcomes can be expected. It is therefore crucial that envenomation cases continue to be compiled and reported to the medical community to alert clinicians to the potential range of symptoms resulting from envenomations by *S. nobilis*.

In this manuscript, we present 14 new cases of *S. nobilis* envenomations bringing the total number of confirmed bite cases globally to 23 and provide additional information on one previously reported case (Case 15) (O'Keeffe & Dugon, *In Review*). Eight of the victims are adult females and seven are males (five adults & a seven and a five-year-old children). Bites occurred while the victims were asleep or resting (N=7), when the spider was trapped in clothing (N=5), or while performing DIY activities (N=3). We provide further evidence of necrosis and previously unreported pathologies including cellulitis and dermatitis involving infectious microbes likely vectored during the bite (Dunbar et al., *In Review*). We also reveal *Latrodectus*-like symptoms including severe debilitating pain, tremors, hypotension, nausea, muscle contractions and impaired mobility of the affected limb.

It is fundamentally important that the offending spiders are either seen within close proximity at the time of the bite, or found trapped in clothing, or bed sheets and accurately identified by specialists to meet the rigorous standards required by the scientific community to maintain the necessary accurate reporting of verified spider bites (Vetter and Isbister, 2008). The inconspicuous body shape and markings make *S. nobilis* easily identifiable to the trained eye (Dugon et al., 2017). In the case where specimens are squashed during the incident, the remains can still be identified to species level using genetic sequencing (Bauer et al., 2019, Dunbar et al., 2018c, Hambler, 2019).

Overall, symptoms from *S. nobilis* bites range from benign to severe, with a majority in the moderate range. The range of symptoms and severity are likely related to several factors that may include from the spider side the total amount of venom present in the glands, which might change due the size of glands, or the level of replenished toxins since last used, regulated venom delivery (Venom Optimization Hypothesis), and from the victims side their immune status, and pathophysiological response to the venom, (Dunbar et al., 2019). There are currently no studies addressing any of the above for *S. nobilis*, therefore a fundamental aspect to start with is to know how much venom these spiders produce on average and how variable that amount/volume is within wild populations. We provide information from venom

extractions on 520 *S. nobilis* that offers an insight into variation in available venom quantities in wild populations that may reflect variation in symptom onsets.

5.2.3 - METHODS

5.2.3.1 - Spider collection, measurements, and venom extraction

Specimens of *S. nobilis* were collected in Lucan, Co. Dublin between May 2018, and October 2019. In total, 520 specimens were collected; 401 were identified as female (based on the presence of the epigyne) and 119 as male (based on the presence of swollen pedipalps). The venom extraction process was performed as described in (Dunbar et al., 2020) and carried out within 24 hrs of capture. Previous studies on other spider species (Herzig et al., 2002, Vapenik and Nentwig, 2000, Binford, 2001) demonstrate that factors such as gender and body size can influence the quantity of available venom, which can directly affect the symptom onset. Therefore, the body sizes (prosoma + opisthosoma) of *S. nobilis* specimens were measured using an analogue Vernier callipers, and individual venom yields, the specimen's gender and the months of capture/extraction were recorded to determine the relationship between the size, gender, season and venom yield.

5.2.3.2 - Statistical analysis

A bivariate Pearson Correlation was used to estimate the relationship between spider body size and venom yield using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA). Scatter plots and bar charts were generated using the same software. Spider body sizes (overall, females & males) were arranged into three categories: <9 mm, 9-11 mm, and >11 mm. A one-way analysis of variance (ANOVA) coupled with a Tukey's post-hoc test was performed to estimate 1) the difference in venom yield amongst the spiders arranged in groups of size and 2) the difference in venom yield depending on seasonality (i.e. spring, summer and autumn). Significance was noted when $p \leq 0.05$.

5.2.3.3 - Case studies & spider identification

11 adult females, two adult males, and parents of two male minors reported envenomation symptoms caused by *S. nobilis*. Medical charts were obtained for case #15. In all cases, the victims provided the authors with either photographs, live specimens, or physical remains of the offending spiders. All specimens were identified using Roberts (1995), Dugon (2017) (Dugon et al., 2017, Roberts, 1995), or genetic barcoding techniques (Bauer et al., 2019, Hambler, 2019). In three of the cases (1, 9 & 13), reliable identification was achieved using genetic sequencing. DNA was extracted using magnetic beads as per (Fort et al., 2018) and PCR amplification of the Cytochrome Oxydase Subunit 1 gene was done using the primers coxf3 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and coxFR3 (5'-TAA ACT TCA GGG YGA CCA AAA AAT CA-3'). Amplified material was sent to LGC Genomics GmbH, Germany, for Sanger sequencing.

To determine the species identities, the COX1 genes were isolated from the mitochondrial genome sequence of *S. nobilis* (unpublished data). *Steatoda* and *Crustulina* specimens were acquired from online public databases including National Center for Biotechnology (NCBI) Information and http://www.boldsystems.org/index.php/Public_BarcodeCluster?clusteruri=BOLD:ABA5272 (Bauer et al., 2019). The sequences were aligned using MAFFT (Katoh et al., 2009). The best evolutionary model for the COX1 alignment was chosen using jModeltest2 (Darriba et al., 2012) based on Akaike and Bayesian information criteria (AIC and BIC, respectively), and HKY + G + I model was found to be the most appropriate. Next, a Maximum Likelihood tree was generated using RaXML-NG (Kozlov et al., 2019), HKY + G + I model and 1,000 replicate trees. In parallel, a Bayesian inference tree was generated using MrBayes with the same evolutionary model, four Markov Chain Monte–Carlo chains and 1 million generations. Finally, we tested for species delimitation as per (Fort et al., 2019) using a General Mixed Yule Coalescent (GMYC) model in BEAST (Bouckaert et al., 2014) and the R v.3.6.3 packages Splits and Rncl (Fujisawa and Barraclough, 2013).

5.2.4 - RESULTS

5.2.4.1 - Spider size and venom yield

In total, 520 (401 female and 119 male) records of spider body size and associated venom yield were obtained (Table 1). The mean body size (prosoma + opisthosoma) for females (N=401) was 9.82 (±1.42) mm, with the largest specimen measuring 13.90 mm. On average, females produced 0.22 (±0.11) µl of venom, with a maximum yield of 0.51 µl. The mean body size for males (N=119) was 9.15 (±1.24) mm, with the largest specimen measuring 12.50 mm. Males produced 0.19 (±0.09) µl of venom on average, with a maximum yield of 0.50 µl (Table 1).

Table 1. Characteristics of overall, female and male *S. nobilis* groups.

Group		Body size (mm)	Venom yield (µl)
Overall	Min	5.40	0.01
N=520	Mean ±SD	9.67 (±1.40)	0.22 (±0.11)
	Max	13.90	0.51
Female	Min	5.70	0.01
N=401	Mean ±SD	9.82 (±1.42)	0.22 (±0.11)
	Max	13.90	0.51
Male	Min	5.40	0.03
N=119	Mean ±SD	9.15 (±1.24)	0.19 (±0.09)
	Max	12.50	0.50

The relationship between the overall, female, and male body sizes and associated venom yield, although significant, was found to be weak ($r^2<0.2$) (Fig. 1). Pearson's correlation showed a weak to moderate correlation between the overall ($r=0.363$, $p<0.001$, $N=520$) and female ($r=0.355$, $p<0.001$, $N=401$) body size and venom yield, and

a weak correlation between the male (r=0.298, p=0.001, N=119) body size and venom yield.

The results of average venom yield comparison amongst size classes and seasons are presented in Table 2. On average, venom yield of all groups increased with increasing body size (Fig. 2 a-c) and was the lowest during the summer period (Fig. 2d).

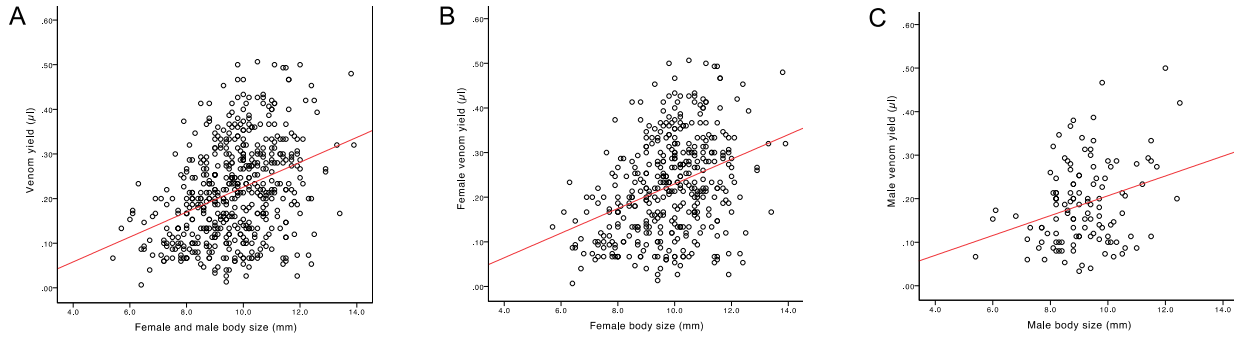

Figure 1. Relationship between body size and venom yield. (A) A weak relationship was observed between female and male *S. nobilis* (N=520, r^2=0.132) body size (mm) and venom yield (µl). Similarly, a weak relationship was observed between the two variables in female (N=401, r^2=0.126) (B) and male (C) (N=119, r^2=0.089) groups.

Table 2. Venom yield comparison amongst size classes and seasons. Asterisk (*) marks cohorts that significantly differ in mean venom yield (p≤0.05, One-way ANOVA, followed by Tukey's post-hoc).

Group	Cohorts comparisons		
	<9 mm & 9-11 mm	9-11 mm & >11 mm	<9 mm & >11 mm
Female & male	p<0.001*	p=0.008*	p<0.001*
Female	p<0.001*	p=0.192	p<0.001*
Male	p=0.710	p=0.003*	p=0.001*
	Spring & Summer	Summer & Autumn	Spring & Autumn
Female & male	p=0.040*	p<0.001*	p=0.797

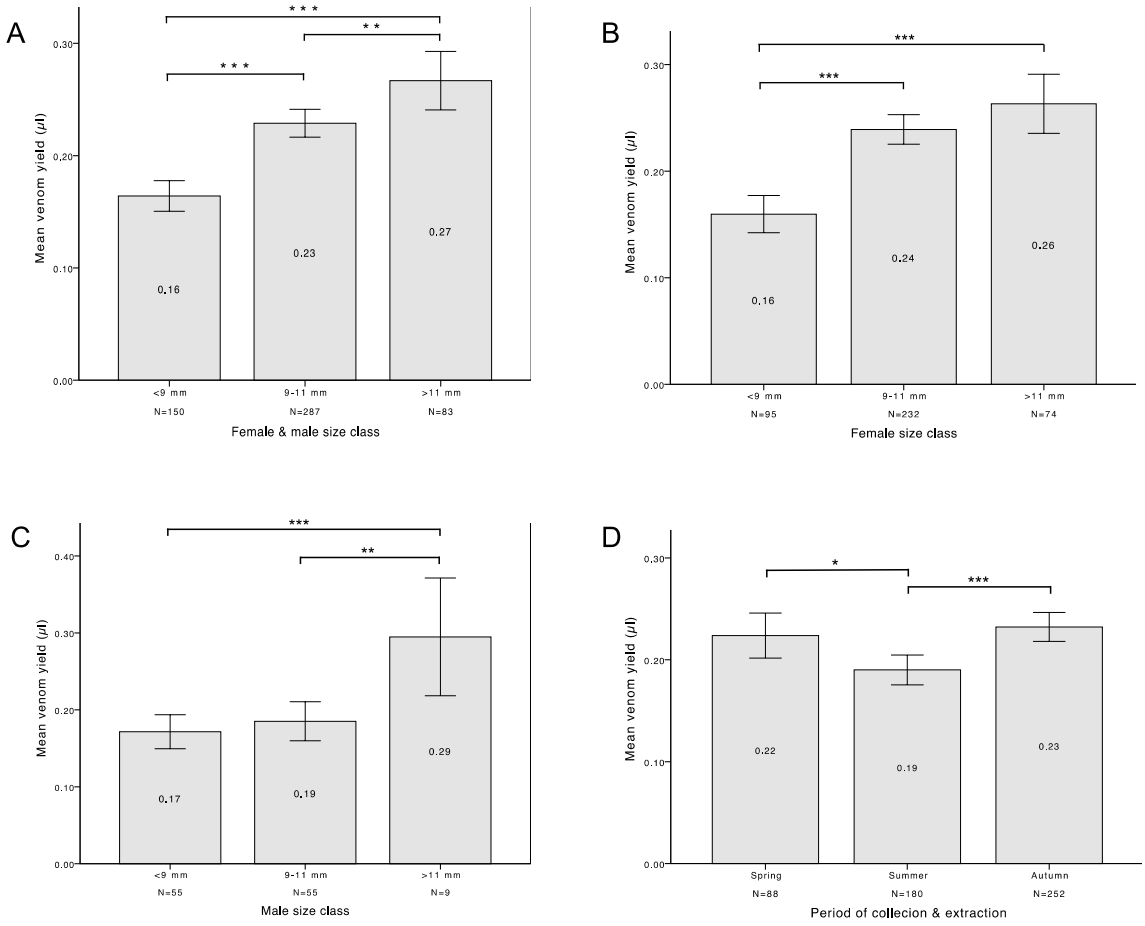

Figure 2. Venom yield variability amongst different size classes and seasons. Venom yield variability between the three female and male (A), female (B) and male (C) *S. nobilis* size classes. (D) Male and female venom variability during spring, summer, and autumn. The bars show mean venom yield values and associated standard errors of the mean (SEM). Asterisk (*) marks the groups that are significantly different (*p≤0.05, **p≤0.01, ***p≤0.001, One-way ANOVA, followed by Tukey's post-hoc).

5.2.4.2 - Case Series

5.2.4.2.1 - *Case 1*

A 37-year-old female from Co Louth, Ireland, woke during the night after experiencing a sharp pain in the upper right arm, and felt a spider crawling up the

shoulder. A small raised bump was observed at the bite site with erythema spreading 4 cm from the epicentre. The victim experienced localised heat, ache, dull pain, and stinging pain (3/10) for 15 hrs, at which time the victim began to experience pruritus. 11 hrs post bite, antihistamine cream was applied, and ibuprofen and antihistamine tablets were taken but no improvement was felt. By the next day all symptoms had resolved. The spider was captured, photographed, and the specimen sent to the authors. The slightly squashed specimen was identified as a mature male *S. nobilis* through genetic sequencing (Fig. 7).

5.2.4.2.2 - *Case 2*

An adult female from Co Wexford, Ireland, found a spider in her bed during the night. She was bitten on the finger of her left hand while capturing it. The victim experienced a very sharp pain, and the whole finger became red, swollen and was throbbing. These symptoms eased over the next 48 hrs and by the third day the finger was tender and only a slight tingle was felt. The spider was captured and sent to the authors, which was identified as a mature male *S. nobilis*.

5.2.4.2.3 - *Case 3*

A 35-year-old female in Co Dublin, Ireland, was changing the battery in the smoke alarm. When she opened it up, a spider fell out and dropped down the back of her top. As the spider was moving towards her arm it became trapped. While trying to remove it, the spider was slightly squashed and bit her in the region of the armpit. The victim described the pain as similar to a wasp sting, and erythema radiated from the bite site. After 24 hrs, the pain resided but the area felt sensitive. Intense pruritus and vasodilation of the capillaries persisted for 48 hours post bite. By 72 hrs the symptoms had fully resolved. The spider was captured, photographed, and sent to the authors, and identified as a mature male *S. nobilis*.

5.2.4.2.4 - *Case 4*

While sleeping during the day on the sitting room couch, a 26-year-old female from Co Dublin, Ireland, felt a pinch on her cheek below the left eye. Approximately a minute later she experienced a throbbing pain (3/10). A small raised bump appeared

followed by erythema radiating from the bite site. After 1 hr the left side of the victim's face had swollen, piloerection was felt in both arms. 3 hrs later the victim felt nausea and unwell. The pain continued into the next day but resolved within 24 hrs with a pimple sized blister remaining around the bite site. The spider was captured, photographed, and collected by one of the authors (JPD), and identified as a mature female *S. nobilis*.

5.2.4.2.5 - *Case 5*

A 52-year-old female in Co Louth, Ireland, was bitten above the wrist on the right arm while gardening. The spider, still alive, was photographed, and identified by the authors as a mature male *S. nobilis.* Immediately after the bite, the victim began to feel sharp pain (5/10), with erythema and intense swelling of the whole lower arm (excluding the fingers). After 48 hrs a medical examination was carried out by a general practitioner who determined the victim had developed cellulitis and prescribed a course of antibiotic (Co-amoxiclav 500mg/125mg), anti-inflammatories and antihistamines. After 24 hrs the pressure on the elbow and wrist eased and swelling reduced. The victim had fully recovered after 7 days.

5.2.4.2.6 - *Case 6*

A 44-year-old female in Co Wicklow, Ireland was sitting on her couch holding her 7-month-old baby when she felt a sharp pain down her leg. When she stood up, she felt the pain spread around her left side. Upon inspection, a spider was found trapped in her clothing. The bite site was located on the hip as it was apparent with sharp pain (6/10) and a 1 cm raised bump, piloerection, and erythema radiating 5 cm from the epicentre. The victim began to feel the pain radiating to her neck and jaw line, and down to the ankle for 24 hrs before it eased. Throughout the day the pain levels increased and was like a constant stinging sensation. The victim took antihistamines. The squashed spider was collected, photographed, and the specimen sent to the authors and identified as a mature female *S. nobilis*. In the subsequent weeks when the bite victim felt very tired, or run down, the bite area became itchy, and a mark from the bite was still visible.

5.2.4.2.7 - *Case 7*

While changing clothes, a 56-year-old female in Co Cork, Ireland, was bitten twice on her left breast by a spider. After feeling a sharp pain, she removed her top for a closer inspection. A large spider fell from her clothing. The spider was photographed before being released. The spider was identified by the authors as an adult female *S. nobilis*. Immediately after the bites, two raised bumps developed, and erythema radiated from the epicentre. The victim described the pain experienced in the first hour post bite as (1/10), which then escalated to (3/10) over a 48-hr time period. The victim cleaned the area and applied Sudocrem and took over-the-counter antihistamines (Zirtec). On the third day minor necrosis began to develop at the bite sites and a photo was taken at day 6 (Fig. 3b). By day 14 ulceration of the skin are observable (Fig. 3c). The wounds healed with no complication, but marks remained visible 23 weeks after (Fig. 3d).

5.2.4.2.8 - *Case 8*

A 56-year-old male in Co Galway, Ireland, felt a sharp pain under his right armpit. As he touched the area, he felt further "stings" in the same location. As he removed his clothing, he discovered a large spider, which he collected and sent to the authors for identification. The spider was identified as a mature female *S. nobilis*. The victim experienced multiple bites and immediately felt stinging pain (3/10). An area of raised skin covering ca. 4 cm, and erythema radiated ca. 8 cm from the area of the bite sites. A tingling sensation was felt around the bite area throughout the day and sensations were felt down the whole arm extending to the fingers. By the evening of the same day, all redness and raised area had resolved and all that remained was a dark spot around the bites. By 24 hrs post bite, the dark spot had disappeared and all symptoms had completely resolved.

5.2.4.2.9 - *Case 9*

While in bed, a 47-year-old male in Co Wexford, Ireland, woke to sharp pains in four different locations on the posterior compartment of his right shoulder and elbow. A spider was found squashed under the blanket near the pillow. The squashed spider

was collected, photographed, and sent to the authors. The spider was identified via genetic sequencing as a mature female *S. nobilis*. Within a minute, pain localised to the bite sites was described as intense (8/10), and blisters began to develop at the bite sites. Intense swelling and erythema were observed radiating from the bite sites. The pain further progressed and radiated through the whole arm. The victim also experienced tightness around the elbow region when bending his arm. The victim began perspiring in the arm and shoulder (particularly inner arm) regions. The overall pain was described as excruciating by the victim. The victim visited the Accident & Emergency department where he was given steroids and morphine intravenously, which did not reduce the pain. Subsequent peroral treatment with Pregabalin Clonmel 75 mg, Vimovo 500 mg/20 mg and Paracetamol 500 mg helped to slightly reduce the pain. 48 hrs post bite, the pain had mostly subsided. However, the elbow was still very tender, with tightness and tingling sensations, and mild pain similar to that of a constant nettle sting. Perspiration continued around the shoulder and elbow regions of the affected limb. The victim felt groggy and drowsy, however this was deemed likely to be attributed to the medication. 72 hrs post bite most of the symptoms had resolved apart from intense and persistent pruritus.

5.2.4.2.10 - *Case 10*

A 46-year-old female in Co Wexford, Ireland, woke up with a pain in the big toe on her right foot. Upon closer inspection a spider was found in between the bed sheets. The spider was captured, sent to the authors and identified as a mature female *S. nobilis*. The victim's toe became red, swollen, and was painful throughout the day. The pain increased (7/10) by the next night and she was woken twice during the night by the pain. Pain, swelling, and erythema persisted in the foot including all toes for 72 hrs. On the fourth day all symptoms had resolved.

5.2.4.2.11 - *Case 11*

A 34-year-old female in Co Dublin, Ireland, felt sharp pain on the upper right thigh shortly after putting on a pair of jeans. When she removed her jeans, she found a large spider. The spider was collected, and photographs were sent to the authors. The

spider was identified as an adult female *S. nobilis*. The victim was bitten twice, and experienced erythema radiating from the bite sites. She also suffered from intense pain (7/10) with painful sensations spreading throughout her legs and torso. The victim felt nauseous for one hour and body tremors and intense pain lasted up to three hours. By the end of the day all symptoms had resolved.

5.2.4.2.12 - *Case 12*

A 5-year-old male in Co Dublin, Ireland, was woken by his mother by pulling back the child's blanket. Just as his mother noticed a spider in the bed, the boy accidently rolled over on the spider and was subsequently bitten on the right side of his upper back. The spider was captured, photographed, and was identified by the authors as an adult female *S. nobilis*. The victim felt immediate pain after the bite (2/10). Within a minute, erythema developed and spread approximately 2.5cm from the bite site, and a small raised lump appeared. The victim complained that it was painful to use his whole right arm to pick up his toothbrush. The bite area was washed, and ice was applied. Antihistamine cream (Hydrocortisone 1% w/w) was also applied to the area of the bite site. By the next day, erythema and the raised lump had reduced to a pimple sized red mark. By 48 hours all symptoms had fully resolved.

5.2.4.2.13 - *Case 13*

A 33-year-old female in Co Dublin, Ireland was woken during the night by a sharp intense pain on the posterior compartment of her upper right arm above the arm pit. As she was pulling back the bed sheets, she observed a large spider that was squashed in the bed next to her. The squashed spider was collected, photographed, and the specimen was sent to the authors. The spider was identified as a mature male *S. nobilis* via genetic sequencing. Immediately, a raised lump appeared at the bite site with erythema radiating approximately 7 cm from the bite site. The victim described the pain as intense and sharp. The victim also described constant stinging in the area of the bite and that it felt hot to touch. The victim took Nurofen plus but found no relief from the pain and discomfort. The victim tried to go back asleep, but the pain kept breaking her sleep. By the next morning, the swelling and erythema began to subside,

but the pain remained intense and was radiating down her arm and armpit. For all of the next day the victim felt fatigued. By the end of the day the pain began to subside but continued to come and go as sharp stinging sensations with the area remaining tender to the touch. After 48 hrs the symptoms had significantly reduced but the bite site remained tender to touch. All symptoms had completely resolved 72 hrs after the bite.

5.2.4.2.14 - *Case 14*

In Cornwall, Great Britain, a 7-year-old male was handling a small spider that he found inside his house whilst getting ready for school. While handling, he was bitten on the back of his left hand just above the wrist. The victim immediately complained of pain (4/10), and erythema radiated from the bite site. The symptoms were monitored by the boy's teacher at his school. The teacher reported that the boy described the pain as similar to that of nettle stings. This pain completely resolved after 2 hrs but pruritus remained. One week later the victim complained that the bite was becoming sore with evidence of redness. A medical examination was carried out by a general practitioner who determined an infection had set in and recommended using Fucidin cream. Immediately after the bite occurred, the spider was captured, photographed, and the specimen sent to the authors. The spider was identified as a juvenile *S. nobilis*. Due to the small size of the specimen, micrographs were taken (Fig. 5).The specimen was raised in lab conditions until reaching maturity. Once mature, the spider was determined as male.

5.2.4.2.15 - *Case 15*

While walking across her sitting room, a 43-year-old female from Co Waterford, Ireland, felt a sharp pain in her lower left leg. Whilst inspecting the cause of the pain, she found a spider trapped in the leg of her trousers. As the victim rolled up her trousers, she observed a spider, which continued to run farther up her leg. Each time she tried to catch it, she was bitten, three times in total. The spider was immediately captured, and photographs were taken and sent to the authors (Fig. 6f). The spider

was identified as an adult male *S. nobilis*. Approximately 1-minute post bite the victim felt an intense sharp pain (8/10). The pain was described as similar to boiling water being poured onto her skin in the affected area. Erythema radiated extensively from the epicentre of the bite sites. After approximately one hour, swelling and large blisters developed at the bite sites. The next morning (12 hrs post bite) the victim drew a blue line around the erythema (Fig. 6a) and visited a general practitioner for a medical examination. The victim's blood pressure was recorded as low (90/50) and she was prescribed Neoclarityn and Flucloxacillin. Erythema spread further and was later marked again with a second blue line. Edema of the leg was evident after three days. On the fourth night the victim visited an out-of-hour clinic and was prescribed antibiotic creams. On the sixth day the victim was admitted to the Accident & Emergency department with cellulitis (Fig. 6b). The erythema continued to spread, blistering and swelling of the full lower leg between the knee and ankle had intensified. The victim described the pain level as (10/10). Scattered fluid filled blisters formed and fused and the colouration of the leg was reddish purple. The victim spent six nights in hospital where she was administered Benzylpenicillin and Flucloxacillin intravenously. The victim was prescribed OxyContin for two weeks for pain relief and returned home with a 10-day course of oral Flucloxacillin. The victim developed secondary dermatitis (Fig. 6c, 6d) and was admitted to hospital a further two times over following two months and remained on antibiotic treatment. On day 73 post bite, the victim received a decreasing course of Prednisone. On day 80 (1 week later) the victim's condition began to improve and by day 95 she had fully recovered and only some visible marks remained (Fig. 6e).

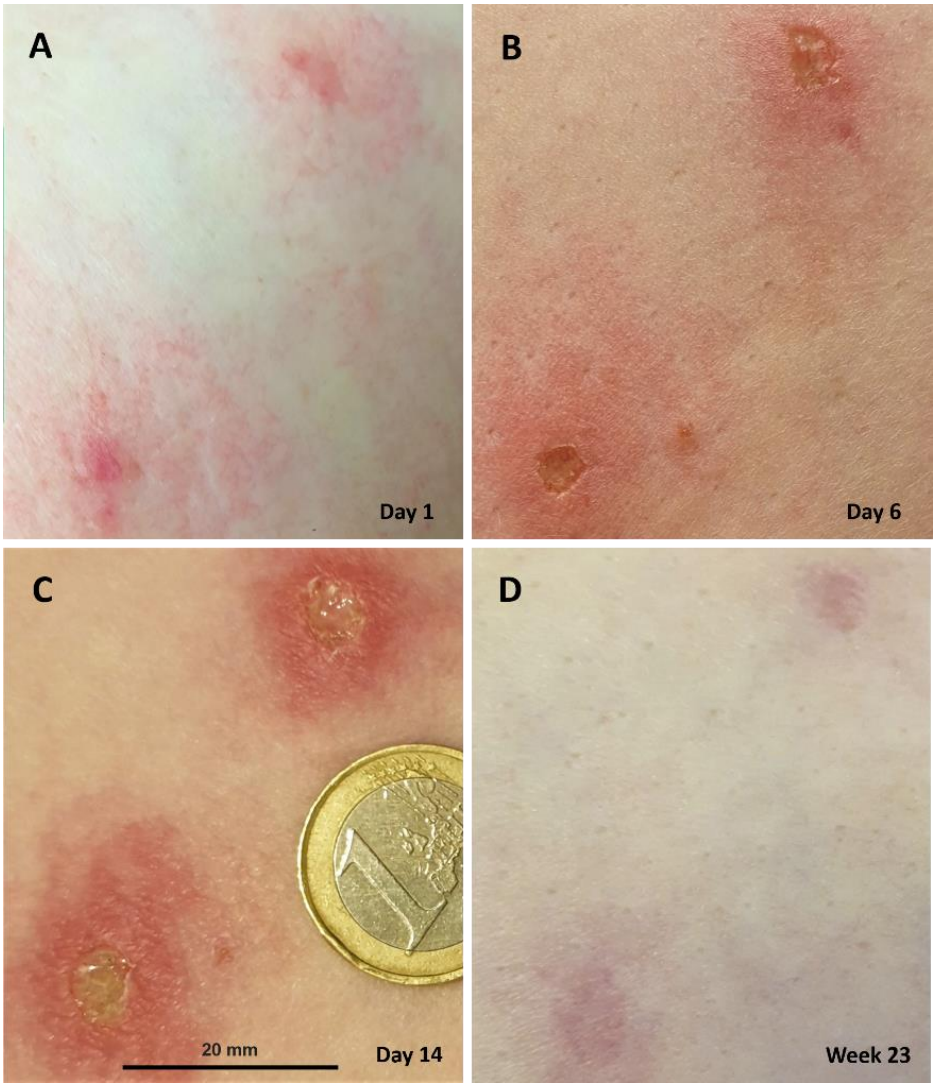

Figure 3. Cutaneous necrosis in a 56-year-old female bitten twice on the left breast by *S. nobilis* trapped in clothing (Case 7). (A-D) representative images of wound progression from day 1 to week 23.

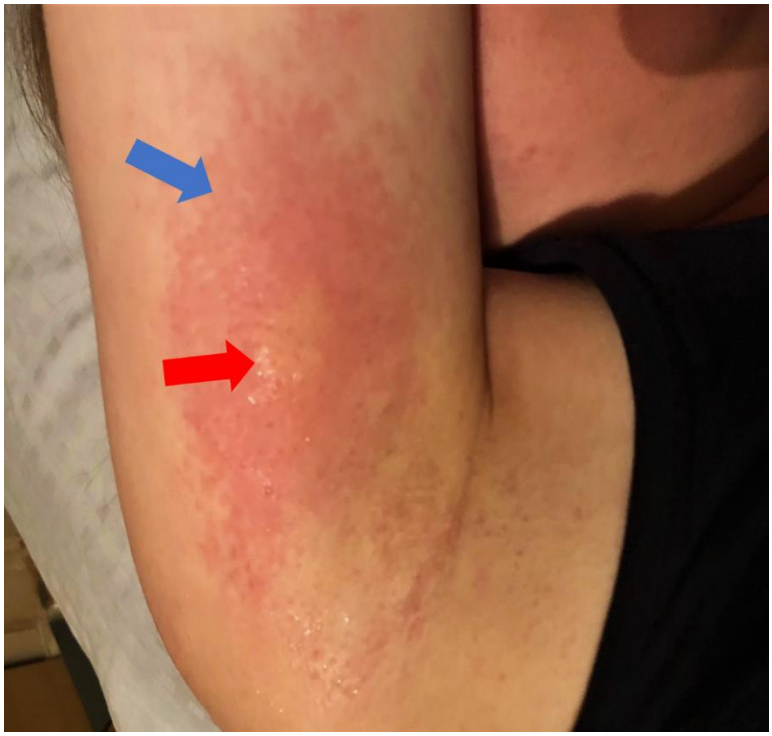

Figure 4. The right arm of a victim (Case 13) after being bitten by *S. nobilis*. Typical signs of envenomation from this species with a pale area of raised skin (red arrow) at the bite site, which can also blister, and erythema radiating from the bite site (blue arrow).

Figure 5. An immature *S. nobilis* specimen that bit a 7-year-old male and induced symptoms (Case 14). Raised to maturity in lab conditions and subsequently determined as male.

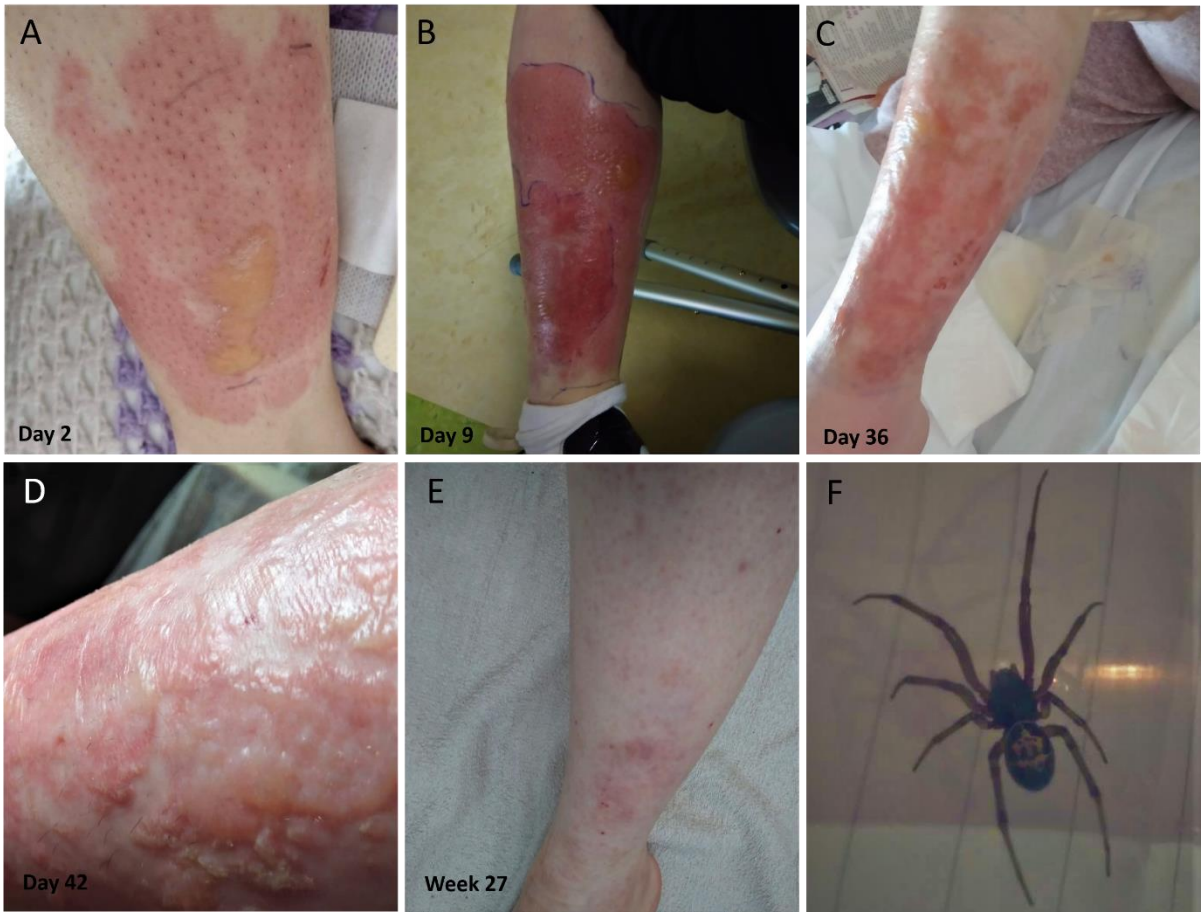

Figure 6. Severe reaction that resulted from three *S. nobilis* bites to the lower left leg (Case 15). Classic symptoms of envenomation resulting in raised skin/blistering at the bite site with radiating erythema and severe pain (A). (B-D) are representative images of cellulitis and secondary dermatitis from day 9 to day 42. (E) photo taken of leg on week 27 showing recovery. (F) the offending spider identified as an adult male *S. nobilis*.

5.2.4.3 - Genetic sequence data

Genetic sequence data was obtained from the squashed remains of three spiders. The species delimitation tree confirms the spiders involved in Case 1, 9 & 13 were all *S. nobilis* (Fig. 7). Furthermore, our phylogenetic reconstruction identifies divergence between the Californian and Irish populations of *S. nobilis* and places the offending spiders from our bite reports in the same cluster as the specimens collected from Lucan, Co Dublin used for genome sequencing. Once validated, we then deposited these genetic sequences in GenBank at NCBI to be publicly available to other researchers for rapid species confirmation through BLAST (Basic Local Alignment

Search Tool). COX1 sequences are available under the following accession numbers: genome sequence of *S. nobilis* (MT827890), case reports 1 (MT827891), 9 (MT827892) & 13 (MT827893).

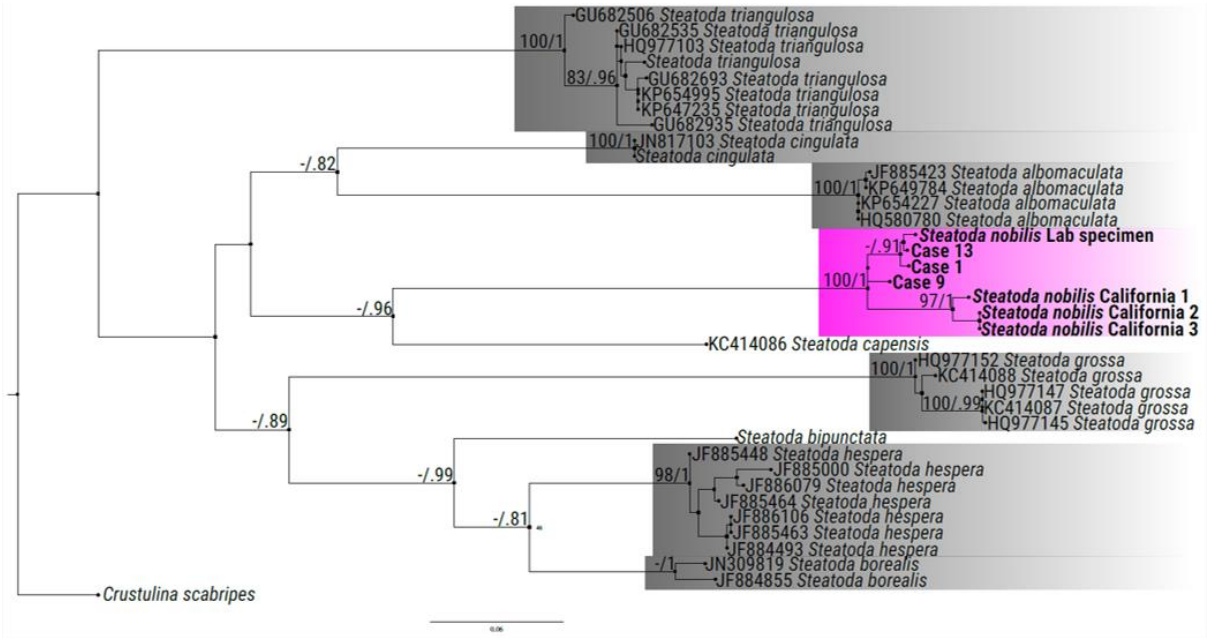

Figure 7. Phylogenetic tree of *Steatoda* species. Maximum Likelihood phylogenetic tree of *Steatoda* species. Numbers above the nodes represent bootstrap support and Bayesian posterior probabilities, respectively. Only support values > 80/0.8 are shown. Boxes indicate species delimitation based on GMYC analysis.

5.2.5 - DISCUSSION

The noble false widow spider *S. nobilis* has been present in England for 140 years and is regarded as a naturalised member of the British fauna (Snazell and Jones, 1993). However, recent shifts in rising occurrences, correlating with a global range expansion has become a cause for concern (Bauer et al., 2019). In addition to the potential negative ecological impact (Bauer et al., 2019, Dugon et al., 2017, Dunbar et al., 2018a, Hambler, 2019, Kulczycki et al., 2012), an increase in *S. nobilis* geographical range and density in urbanised habitats has resulted in an increase in human envenomations (Bauer et al., 2019, Dunbar et al., 2018d, Faúndez et al., 2020, Faúndez and Téllez, 2016, Hambler, 2019, Porras-Villamil et al., 2020, Warrell et al., 1991). Despite the earlier reports of mild to moderate symptoms from bites by *S. nobilis* (Dunbar et al., 2018d), with the increasing number of bites we are beginning to

develop a better understanding of the potential medical importance of *S. nobilis* (Dunbar et al., 2020; Dunbar et al., *In Review*)

5.2.5.1 - Variation in Venom Availability

The causes of variation in envenomation symptoms are discussed for arthropod species (Dunbar et al., 2019) and can be influenced by several factors, including: the presence of toxins with direct activity, secondary breakdown of proteins into pro-inflammatory mediators, and variation in the individual pathophysiological response of each victim to the venom (Dunbar et al., 2019). Others include seasonal variation (Junior et al., 2010), regional variation (Fry et al., 2003), and gender variation in venom composition (Valenzuela-Rojas et al., 2019). Venomous animals such as snakes, spiders, centipedes, and scorpions have all demonstrated the ability to regulate the quantity of venom delivered according to the level of the perceived threat (Dugon and Arthur, 2012, Evans et al., 2019, Wigger et al., 2002). Once venom is partially, or fully depleted, it may take several days or weeks for venom glands to fully replenish their stores, for which different venom compounds may be produced at different rates. Therefore, envenomation by different individual specimens of the same size may result in different symptom onsets depending on the level of toxin replenishment and the quantity of venom available for delivery. Ultimately, variation in symptom onsets are likely to be highly influenced by venom availability and threat perception.

We sampled a population of *S. nobilis* and found there is large variations in venom yield. We found a significant correlation linking the venom yield with the body size (Fig. 1), thus, we should also expect large variation in symptoms onsets. Although the venom yield was found to be on average higher with increasing body size, we observed variation in venom yield amongst the wild-caught spiders of the same, or similar size (Fig. 1 & 2). Furthermore, we found venom yield to be on average significantly lower in summer than in spring and autumn (Fig. 2d). To investigate further the relationship between body size and venom yield, future studies should include long term hosing without food to allow for venom glands to fully replenish. This may show a stronger correlation between body size and venom yield, however,

for the purpose of this study we wanted to capture a snapshot of quantities of available venom in wild populations. While venom yields presented here do not necessarily represent the volume of venom delivered during envenomation, the amount of venom injected undoubtedly will reflect envenomation outcomes. Overall, severity of symptoms is likely to be dose dependent and multiple bites by venomous arthropods typically induce more severe symptoms (Dunbar et al., 2019). From our dataset, we can demonstrate that some individual spiders have more than twice-fold the venom quantity available to deliver than the average spider in our dataset. This could potentially account for the fewer cases where victims present with severe symptoms that require medical attention and indicate the potential remains for more severe cases in the future.

5.2.5.2 - *Latrodectus*-like neurotoxic symptoms

Prolonged moderate to intense pain, erythema, swelling and pruritus are the most common symptoms of envenomation by *S. nobilis*. Vasodilation of capillaries and necrosis localised at the bite site were previously reported (Dunbar et al., 2018d) and are also documented here (Case 3 & 7). The wounds described in case 7 were comparable to earlier reports of necrosis (Dunbar et al., 2018d). However, these are small minor wounds and not debilitating tissue necrosis as portrayed in the media (Hambler, 2019). The main concern is limited to the exposure and susceptibility to infection (Dunbar et al., *In Review*). Other localised symptoms include radiating pain from the bite site, local sweating, a sensation of heat, tenderness, piloerection, inflammation, irritation, raised skin and paraesthesia around the bite site, muscle contractions in the affected area, reduced mobility in the affected limb, and lump/pimple, and blister formation at the bite site (Warrell et al., 1991, Dunbar et al., 2018d, Faúndez and Téllez, 2016, Faúndez et al., 2020). The venom of *S. nobilis* has the ability to induce systemic effects, such as facial flushing, feverishness, elevated and low blood pressure (Warrell et al., 1991, Dunbar et al., 2018d). The venom composition reveals that *S. nobilis* shares two-thirds of venom toxins with *Latrodectus*, including α-latrotoxins, δ-latroinsectotoxins, and α-latrocrustotoxins (Dunbar et al., 2020). In fact, while the potency of the toxin isoforms have yet to be determined from

LD_{50} models, it should be noted that α-latrotoxins, the highly potent neurotoxins that effect the nervous system of vertebrates, are more highly concentrated in *S. nobilis* venom than in *Latrodectus* (Dunbar et al., 2020). These case reports confirm that the venom is evidently highly active and can potentially induce medically important symptoms. α-latrotoxin is primarily responsible for the symptoms induced by *Latrodectus* envenomations, such as intense pain, muscle fasciculations, hypertension/hypotension, diaphoresis, neuromuscular paralysis and fatal cardiotoxic shock (Pneumatikos et al., 2003, Erdur et al., 2007, Halmo et al., 2019, Dendane et al., 2012, Vetter and Isbister, 2008, Levine et al., 2010, Sari et al., 2008). In our case series, severe debilitating pain was experienced by some victims. One victim (Case 6) felt intense pain radiating from her hip to the neck and jaw line and down to her ankle for 24 hrs. Another victim (Case 9) experienced debilitating pain requiring strong analgesics that target directly the central nervous system, and a third victim (Case 15) required weeks of opioid medication to manage the pain. More concerning is the severe debilitating pain (Cases 6, 9 & 15), tremors (Case 11), hypotension (Case 15), nausea (Case 4 & 11), muscle contractions and impaired mobility of the affected limb (Case 12), which are *Latrodectus*-like symptoms (Faúndez and Téllez, 2016, Dunbar et al., 2018d, Pneumatikos et al., 2003, Vetter and Isbister, 2008, Erdur et al., 2007). These and some of the other symptoms reported in this case series share close similarities to the neurotoxic symptoms that are consistent with latrodectism (Fig. 8) (Isbister and Fan, 2011). For this reason, even though most cases are not considered dangerous and symptoms remain mild to moderate, envenomations by *S. nobilis* should not be trivialised, especially when *Latrodectus* venom occasionally induces severe symptoms with fatal outcomes (Isbister and Fan, 2011). It is important to fully understand the full potential range of symptoms before dismissing them, especially considering the increase in *S. nobilis* bite incidents and their global range expansion. Furthermore, increasing reports of the cardiotoxic effects from *Latrodectus* venom demonstrate the complexity in which these venoms can affect biological systems (Pneumatikos et al., 2003, Erdur et al., 2007, Sari et al., 2008, Dendane et al., 2012, Levine et al., 2010). *Steatoda grossa* was first reported in Ireland in 1898 (Carpenter, 1898) and since then it has been reported in 11 counties (Dunbar et al., 2018c). The

species is also naturalised in Great Britain but have not generated the hype by the media, or concern from the public as bites are far less common in Ireland and Great Britain. However, significant effects of envenomation by *Steatoda* species are reported in Australia where *Steatoda grossa* and *Steatoda capensis* were introduced and represent all bites by *Steatoda* in Australia (Isbister and White, 2004). In some cases, the clinical syndrome was indistinguishable from *Latrodectus* (Isbister and Gray, 2003) but overall, most bites by *Steatoda* were less severe than *Latrodectus* and treatment was focussed on managing symptoms (Isbister and White, 2004). In severe cases, effective treatment was achieved using redback spider (RBS) antivenom (Isbister and Gray, 2003). In vitro studies showed that the venom from *Steatoda capensis* and *Steatoda grossa* were more potent towards insects than *Latrodectus* and less potent than *Latrodectus* towards mammals (Atakuziev et al., 2014). However, these studies have not been carried out for *S. nobilis*. Overall, case reports suggest that while most envenomations by *S. nobilis* are mostly less severe than *Latrodectus*, they significantly overlap and do have the potential for severe outcomes. The intensity in the range of symptoms were highly variable and while this can be only partly attributed to the to venom quantities injected, it is likely to be a very important part of symptom onset.

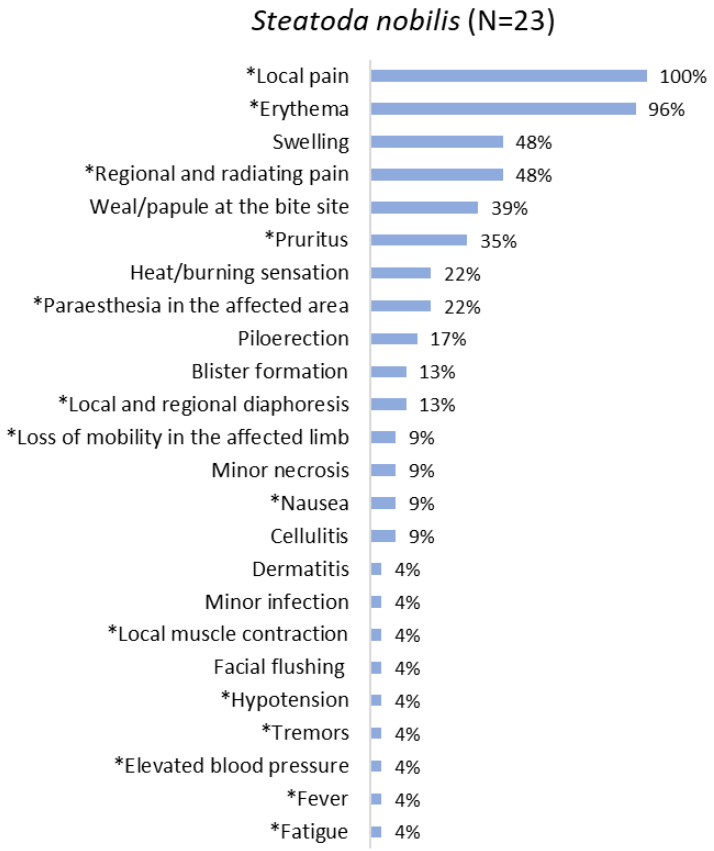

Figure 8. Most common signs, symptoms and complications resulting from *Latrodectus* and *S. nobilis* envenomations. Percent (%) shows the rate at which these symptoms occurred in reported *S. nobilis* bite cases (cases reported in this study and in previous studies (Dunbar et al., 2018d, Faúndez et al., 2020, Faúndez and Téllez, 2016, Warrell et al., 1991). Asterisk (*) marks *Latrodectus*-like symptoms.

5.2.5.3 - Bacterial Infection

In three cases (Cases 5, 14 & 15) the victims developed mild to debilitating bacterial infections including cellulitis and dermatitis. One victim (Case 15) required hospitalisation and an aggressive course of intravenous antibiotics. Bacterial infections are occasionally associated with arthropod bites (Bäckman et al., 2015, Junqueira et al., 2017), including spiders (Vetter et al., 2015a). The current consensus is that infections most likely result from skin flora, poor hygiene and rubbing of the wound. This is reinforced by the discovery of antimicrobial peptides in some spider venoms (Saez et al., 2010) leading to the erroneous claims that spider venoms are generally sterile environments (Esmaeilishirazifard et al., 2018, Ul-Hasan et al., 2019) and would neutralise bacteria during a bite (Kozlov et al., 2006). The idea that bacteria

can be commonly transmitted by virtually any insect, but that spiders' chelicerae are deemed sterile environments is simply impractical and misguided (Dunbar et al., *In Review*). It is far more likely that infections following bites will occur from antibiotic resistant strains of pathogenic bacteria present on spider fangs that penetrate the skin than it is for the infection to arise from skin commensals.

The recent characterisation of the microbiome of *S. nobilis* demonstrate that this species (and others tested) do carry opportunistic pathogenic bacteria on their body surfaces and fangs, many of which are multidrug-resistant strains (Dunbar et al., *In Review*); this is consistent with similar studies on *Latrodectus* (Ahrens and Crocker, 2011). The venom of *S. nobilis* was also shown to have no antibacterial properties and is therefore not a barrier to bacterial infection. The spiders also demonstrated they can shed bacteria while walking (Dunbar et al., *In Review*). As a result, post-bite infections are more likely to be the result of vector-borne bacterial zoonoses rather than skin flora.

5.2.5.4 - Epidemiology of *S. nobilis* bites

Habitat preference of *S. nobilis* outside of its native range is mostly synanthropic (Bauer et al., 2019, Dugon et al., 2017, Dunbar et al., 2018a, Dunbar et al., 2018b, Faúndez et al., 2018, Hambler, 2019, Kulczycki et al., 2012, Vetter et al., 2015b), and previous bites all occurred in buildings (Dunbar et al., 2018d, Faúndez et al., Faúndez and Téllez, 2016, Warrell et al., 1991). The cases previously published, and those presented here also demonstrate that female *S. nobilis* are not sedentary, and as with males, do occasionally wander inside homes (Hambler, 2019). 57% (N=13) of all confirmed *S. nobilis* bites to date were by females and 43% (N=10) by males. Regarding the circumstances of all confirmed *S. nobilis* bites, 61% (N=14) of the victims were adult females and 39% (N=9) were males (seven adults, two minors). All bites occurred in and around the home (or hospital bed (Warrell et al., 1991)). 57% (N=13) occurred when the victim was either asleep in bed, or resting on a couch, 31% (N=7) occurred when the spider was trapped in clothing, 4% (N=1) while gardening, 4% (N=1) while handling, and 4% (N=1) while performing home maintenance. Of

those cases presented here, 7% of bites occurred in Spring, 43% in Summer, 29% in Autumn and 21% in Winter.

Bites from *Latrodectus* species typically occur from females whose body length (prosoma + opisthosoma) range from 7-15 mm, which are significantly larger than males (4-7 mm) (Nentwig et al., 2019). It is thought that *Latrodectus* males are too small to penetrate human skin (Peterson and McNalley, 2013). However, unlike their 'true' widow counterparts, male (9.4-11.6 mm) *S. nobilis* grow to a similar size as females (10.5-13.7 mm) (Dugon et al., 2017), have thickened fangs (Vetter and Rust, 2012) and are evidently capable of delivering a bite mechanically strong enough to penetrate human skin and deliver venom, as with other *Steatoda* species (Isbister and White, 2004). *Steatoda paykulliana* have been noted as having a stronger mechanical bite than *Latrodectus* (Maretić et al., 1964). In almost all cases of *S. nobilis* bites, the victims were bitten by adult specimens. However, one offending spider was a juvenile specimen (Case 14), demonstrating that a 3.5 mm long specimen is capable of penetrating human skin, injecting venom, and evidently inducing symptoms. Envenomations by other juvenile *Steatoda* species have also been reported and demonstrate they are capable of causing effects as severe as adult spiders (Isbister and Gray, 2003).

5.2.5.5 - Genetic sequence data

Due to the global range expansion by some *Steatoda* species, additional studies using molecular data may shed some light on their spread and colonisation histories (Dunbar et al., 2018c). More information about genetic variation within this versatile species, allowing to track colonisation history, might allow to predict future colonisation events (Bauer et al., 2019). It is also important to maintain the rigorous standards in reporting confirmed cases by accurately identifying the offending spider (Vetter and Isbister, 2008). Advances in molecular techniques make genetic sequencing a fast and efficient method for identifying squashed specimens, especially if only fragments, such as legs, are recovered (Hambler, 2019). In this study, the identification of three squashed specimens was only made possible with genetic sequencing, demonstrating this importance. With the public availability of these

sequences in Genbank, future researchers and clinicians can now use BLAST as a tool to mitigate *S. nobilis* invasion and allow for more efficient diagnosis of bites.

5.2.6 - CONCLUSION

Envenomation can be rare, but a potentially severe, clinical syndrome caused by the noble false widow spider *S. nobilis.* Due to the limited database of *S. nobilis* envenomation syndrome we believe this manuscript is an important contribution for alerting the medical community to the potential severity of symptoms. The range of pathologies include *Latrodectus*-like symptoms and vectored bacterial infection. We speculate that the increase in bite cases over the past decade in Ireland and Great Britain are causally related to the increased *S. nobilis* population density in urbanised habitats. Given the synanthropic habitat preference and climatic suitability for *S. nobilis* population expansion in Ireland, Great Britain and globally, we further expect an increase in *S. nobilis* populations to coincide with a rise in reported bites in the future. As a result, clinicians should be on the alert for potentially severe symptoms. The rise of *S. nobilis* may result in an emerging public health issue, with potential serious outcomes for some victims. This species deserves close monitoring and research by both the scientific and medical community and updated public awareness by public health authorities.

5.3 - The kiss of (cell) death: can venom-induced immune response contribute to dermal necrosis following arthropod envenomations?

JPD contributed to the conceptualization, methodology, literature research, original draft preparation, and review and editing. This section is published in a peer reviewed journal and available at the following reference: DUNBAR, J. P., SULPICE, R. & DUGON, M. M. 2019. The kiss of (cell) death: can venom-induced immune response contribute to dermal necrosis following arthropod envenomations? *Clinical toxicology*, 1-9.

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5.3.1 - ABSTRACT

Snakes, insects, arachnids and myriapods have been linked to necrosis following envenomation. However, the pathways involved in arthropod venom-induced necrosis remain a highly controversial topic among toxinologists, clinicians and the public. On the one hand, clinicians report on alleged envenomations based on symptoms and the victims' information. On the other hand, toxinologists and zoologists argue that symptoms are incompatible with the known venom activity of target species. This review draws from the literature on arthropod envenomations, snakebite, and inflammatory processes to suggest that envenomation by a range of organisms might trigger an intense inflammatory cascade that ultimately lead to necrosis. If confirmed, these processes would have important implications for the treatment of venom-induced necrosis.

Our objectives are to describe two inflammatory pathways of regulated necrosis, tumour necrosis factor (necroptosis) and Neutrophil Extracellular Traps (NETosis);

to discuss existing knowledge about snake venom and arachnid-induced necrosis demonstrating the involvement of tumour necrosis factor and neutrophils in the development of tissue necrosis following envenomation and to contribute to the understanding of venom-induced necrosis by arthropods and provide clinicians with an insight into little known inflammatory processes, which may occur post envenomation.

ISI Web of Science databases were searched using the terms "spider bite necrosis", "arthropod envenomation necrosis", "venom necrosis", "venom immune response", "loxoscelism", "arachnidism", "necroptosis venom", "necroptosis dermatitis", "tumour necrosis factor TNF venom", "scorpionism", "scolopendrism", "centipede necrosis", "NETosis venom", "NETosis necrosis". Searches produced 1737 non-duplicate citations of which 74 were considered relevant to this manuscript. Nonpeer-reviewed sources, or absence of voucher material identifying the organism were excluded.

Necrosis is the breakdown of cell membrane integrity followed by inflowing extracellular fluid, organelle swelling, and the release of proteolytic enzymes into the cytosol. Necrosis was historically considered an unregulated process; however, recent studies demonstrate that necrosis can also be a programmed event resulting from a controlled immune response (necroptosis).

Tumour necrosis factor is a pro-inflammatory cytokine involved in regulating immune response, inflammation, and cell death/survival. The proinflammatory cytokine TNF-α participates in the development of necrosis after envenomation by vipers. Treatment with TNF-α-antibodies may significantly reduce the manifestation of necrosis.

The process by which neutrophils discharge a mesh of DNA strands in the extracellular matrix to entangle ("trap") pathogens, preventing them from disseminating. Neutrophil Extracellular Traps have been recently described as important in venom-induced necrosis. Trapped venom accumulates at the bite site, resulting in significant localized necrosis.

Insects, myriapods and arachnids can induce necrosis following envenomation. So far, the processes involved have only been investigated in two arachnids: *Loxosceles* spp. (recluse spiders) and *Hemiscorpius lepturus* (scorpion). *Loxosceles* venom contains phospholipases D, which hydrolyse sphingomyelin, resulting in lysis of muscle fibres. Subsequently liberated ceramides act as intermediaries that regulate TNF-α and recruit neutrophils. Experiments show that immune-deficient mice injected with *Loxosceles* venom experience less venom-induced inflammatory response and survive longer than control mice. Necrosis following *Hemiscorpius lepturus* stings correlates with elevated concentrations of TNF-α. These observations suggest that necrosis may be indirectly triggered or worsened by pathways of regulated necrosis in addition to necrotic venom compounds.

Envenomation often induce an intense inflammatory cascade, which under certain circumstances may produce necrotic lesions independently from direct venom activity. This could explain the inconsistent and circumstantial occurrence of necrosis following envenomation by a range of organisms. Future research should focus on identifying pathways to regulated necrosis following envenomation and determining more efficient ways to manage inflammation. We suggest that clinicians should consider the victim's immune response as an integral part of the envenomation syndrome.

5.3.2 - INTRODUCTION

Venoms have evolved to fulfil mainly foraging and defensive functions. As such, envenomation typically results in neurotoxic symptoms, which disable prey and enemies rapidly (Brodie, 2009). In some instances, however, envenomation leads to necrotic lesions in human and animal models, sometimes simultaneously with systemic organ injury (Mingomataj et al., 2014). Snakes (Katkar et al., 2016, Laing et al., 2003, Moura-da-Silva et al., 1996), cnidarians (Cegolon et al., 2013, García-Arredondo et al., 2012), bees (Mingomataj et al., 2014), wasps (Yanagawa et al., 2007), ants (Knight and Bangs, 2007), mosquitoes (Chiu et al., 2016), beetles (Srihari et al., 2017), centipedes (de Oliveira Pardal et al., 2017, Fung et al., 2011), scorpions (Chadha

and Leviav, 1979, Radmanesh, 1998) and spiders (Boissiere et al., 2016, Dunbar et al., 2018) have all been linked to the formation of necrotic wounds in patients. The potentially lytic effects of jellyfish (Cegolon et al., 2013), coral (García-Arredondo et al., 2012) and snake (Katkar et al., 2016, Laing et al., 2003, Moura-da-Silva et al., 1996) venoms are fairly well documented.

In regard to arthropods, however, and apart from the recluse spiders of the genus *Loxosceles* and the Asian scorpion *Hemiscorpius lepturus*, necrotic lesions are not consistent with the known, direct venom activity of the incriminated species. As a result, the occurrence of necrotic lesions following envenomation by arthropods remains a highly controversial topic among toxinologists, clinicians and the general public. This is particularly true in the case of spiders. While reports of alleged necrotic spider bites are on the rise, the actual spiders responsible for these lesions are rarely recovered and identified (Stuber and Nentwig, 2016) and misdiagnoses are frequent (Vetter et al., 2006) leading to a plethora of potentially misleading reports in the literature (Nentwig et al., 2017, Vetter et al., 2018, Vetter et al., 2006).

In the case of myriapods, insects, and arachnids, venom compounds have been predominantly associated with neurotoxic function, although lesser known peptides may be capable of haemolytic and cytolytic activity(Bawaskar and Bawaskar, 2012, Saez et al., 2010, Undheim et al., 2014). Large centipedes of the Scolopendridae family have been shown to occasionally produce extensive dermonecrosis, sometimes in conjunction with organ failure and acute coronary ischemia (de Oliveira Pardal et al., 2017, Fung et al., 2011, Logan and Ogden, 1985).

Africanized bees *Apis mellifera scutellata* typically induce neurotoxic symptoms but cases involving a high number of stings have led to the development of localized necrosis (Mingomataj et al., 2014). Similarly, the stings from the Asian giant hornet *Vespa mandarinia* do not typically induce necrotic symptoms, however, cases involving multiple stings have led to systemic organ injury and tissue necrosis (Yanagawa et al., 2007). The tropical fire ant *Solenopsis geminata* typically induces pain and general discomfort following envenomation. However, in rare cases involving a

large number of stings, victims might develop tissue necrosis (Knight and Bangs, 2007).

The arthropods most commonly associated with necrotic lesions belong to the class Arachnida, and more specifically scorpions and spiders. Although venom compounds and their active pathways have not been identified yet, *Hemiscorpius lepturus*, a medically important scorpion from West Asia, is currently the only known species of scorpion to possess a necrotoxic venom (Chadha and Leviav, 1979, Jalali et al., 2010, Radmanesh, 1998). In addition to inducing necrosis, other symptoms include renal failure and haemolysis (Jalali et al., 2010) and they are responsible for fatal outcomes.

A plethora of reports are linking necrotic wounds to spider bites in both the scientific and popular literatures (Vetter, 2018, Vetter et al., 2006). However, the alleged necrotic lesions produced by hobo spiders (genus *Tegenaria*) in the United States and white tail spiders (genus *Lampona*) in Australia have been systematically debunked (Gaver-Wainwright et al., 2011, White and Weinstein, 2014). Globally, confirmed spider induced dermal necrosis can be considered a rare occurrence given the taxonomic diversity of spiders (over 45,000 species described so far), with confirmed cases involving only the noble false widow spider *S. nobilis* (Dunbar et al., 2018), the yellow sac spiders of the genus *Cheiracanthium* (Vetter et al., 2006) and the recluse spiders of the genus *Loxosceles* (Boissiere et al., 2016, Cohen et al., 1999, Rubenstein et al., 2016, Swanson and Vetter, 2006).

In the case of *S. nobilis*, reports of necrotic lesions following envenomation contrast sharply with the symptoms reported from envenomation by their relatives, the true black widows (genus *Latrodectus*), which produce exclusively neurotoxic symptoms (Garb et al., 2004, Gendreau et al., 2017). Cases of venom-induced necrosis following bites from the genus *Cheiracanthium* have been reported frequently. However, an extensive review involving 59 verified bites showed that these claims were unwarranted with only a single case of minor necrosis confirmed in Europe (Vetter et al., 2006). The synanthropic spiders most commonly associated with necrotic lesions belong to the genus *Loxosceles* and are known to produce phospholipases D

(formerly referred to in the literature as sphingomyelinases D), a group of enzymes, which hydrolyse sphingomyelin [36].

With the exception of *Hemiscorpius lepturus* and *Loxosceles*, all the cases mentioned above are notable for their onset of necrotic lesions, which are inconstant with known (typically neurotoxic) venom activity. However, neurotoxicity and necrotoxicity are not mutually exclusive. Necrotic lesions could develop in at least three independent, potentially overlapping ways:

1. direct action of venom toxins on cells;

2. bacterial infection (secondary or vector borne); and

3. strong immune response to the envenomation.

The former two have been discussed previously in the literature (Ahrens and Crocker, 2011, Atakuziev et al., 2014, Esmaeilishirazifard et al., 2018, Hauke and Herzig, 2017, Vetter et al., 2015), but the latter remains a neglected topic despite earlier studies suggesting that the victim's immune response may contribute to dermonecrosis(Hogan et al., 2004, Jalali et al., 2010, Katkar et al., 2016, Laing et al., 2003, Moura-da-Silva et al., 1996, Patel et al., 1994, Ribeiro et al., 2015, Domingos et al., 2003a, Domingos et al., 2003b, Szold et al., 2003). In the light of recent studies uncovering the inflammatory pathways leading to programmed necrosis (necroptosis), we postulate that when direct toxic activity from venom toxins occur simultaneously with an innate and adaptive immune response, inflammation may be an important driving force that results in symptoms superficially inconsistent with known, specific, venom activity.

While this review aims to shed light on the role of inflammation in arthropod envenomation, some studies carried out on snake venom are also discussed as they offer an insight into the behaviour of the immune system in response to venom toxins.

The cytokine tumour necrosis factor (TNF) is involved in regulating cell survival and cell death (apoptosis) and the more recently described pathway of regulated necrosis (necroptosis).

Neutrophils are a phagocytic immune cell that typically engulf and digest foreign bodies, and once triggered by certain stimuli, they are also capable of rupturing and releasing their DNA contents, which trap foreign bodies. These are known as Neutrophil Extracellular Traps (NETs) that can lead to necrosis (NETosis). Both TNF and neutrophils have been linked to necrosis following envenomation by snakes and two medically important arthropods and will be discussed in this review.

We propose that the potential exists for necrosis to occur following arthropod envenomation via inflammatory pathways of regulated necrosis. We suggest two targetable mechanisms of inflammation, the TNF necroptosis and neutrophil NETosis pathways, as suitable candidates to study their potential involvement in the occurrence of necrotic lesions following arthropod envenomation. This hypothesis, which has been given little attention so far and has not been considered in the broader context for all arthropod envenomations, might explain the inconsistent, circumstantial occurrence of necrotic lesions following envenomation by a range of arthropods. Objectives

1. To describe two inflammatory pathways of regulated necrosis: TNF (necroptosis) and NETosis.

2. To discuss the potential involvement of TNF and neutrophils in the development of tissue necrosis following envenomation by a range of arthropods, based on the existing literature on snake and arachnid induced necrosis.

3. To contribute to the understanding of venom-induced necrotic lesions by arthropods and provide clinicians with an insight into little known inflammatory processes, which may occur post-envenomation.

5.3.3 - METHODS

ISI Web of Science databases were searched using the terms "spider bite necrosis", “arthropod envenomation necrosis”, “venom necrosis”, “venom immune response”, “loxoscelism”, “arachnidism”, “necroptosis venom”, “necroptosis dermatitis”, “tumour necrosis factor TNF venom”, “scorpionism”, “scolopendrism”, “centipede

necrosis", "NETosis venom", "NETosis necrosis". All citations (N¼2,680) were exported to EndNoteTM version X7. The citation list was inspected and all duplicate entries (N¼843) were manually removed. Searches produced a total of 1,737 nonduplicate citations, of which 74 were considered relevant to this manuscript. Because of the large volume of unsubstantiated reports on venom-induced necrotic lesions, non-peer-reviewed sources were excluded. Historical reports of suspected venom-induced necrosis published without voucher material clearly identifying the organism involved in the envenomation were also discarded.

5.3.3.1 - What is necrosis?

Necrosis occurs when cells experience organelle swelling resulting from inflowing ions and fluid from the extracellular matrix after the breakdown of cell membrane integrity. The breakdown of organelle membranes releases proteolytic enzymes into the cytosol causing further degradation of the cells and subsequent release of the cell contents into the extracellular matrix, ultimately leading to cell death (Elmore et al., 2016). Necrosis typically result from infection, envenomation, injury, or deprivation of blood supply (ischemic necrosis) (Smeeks et al., 2017). Consequences range from minor wounding causing discomfort, to extensive tissue necrosis, causing permanent disfigurement, impairment, and disability (Harrison et al., 2011).

Until the late 1980s, necrosis was considered an unregulated process, contrasting to the regulated, programmed cell death known as apoptosis (Vandenabeele et al., 2010). Apoptosis occurs in individual cells (or sometimes small clusters) that are recognized as experiencing stress by either themselves (intrinsic), or by other nearby cells (extrinsic). The cell shrinks in size, the cellular components are degraded by enzymes, but cell membrane integrity is retained until the end, which avoids triggering an inflammatory response, and then signals for the recruitment of macrophages, which engulf the cell. Apoptosis is a cell death process crucial for efficiently maintaining healthy tissue and regulating tissue growth (Elmore et al., 2016). However, the current consensus is that necrosis can also be a programmed event (necroptosis), resulting from a regulated process that can be favoured by the

immune system over apoptosis (Berghe et al., 2014, Blériot and Lecuit, 2016, Pasparakis and Vandenabeele, 2015).

5.3.3.2 - Tumour necrosis factor and the necroptosis pathway

At the cellular level, cytokines, including interleukin and tumour necrosis factor (TNF), mediate communication through signalling pathways and play important roles as mediators in inflammatory cell death. As a result, the suppression of TNF-α activity using metalloproteinase inhibitors significantly reduces TNF-α associated pathologies (McGeehan et al., 1994).

TNF is a pro-inflammatory cytokine that plays a fundamental role in regulating immune response, inflammation, and cell death/survival. It acts with other cytokines as important cell signalling molecules for recruiting immune cells from nearby blood vessels to the sites where foreign bodies occur (Rojas et al., 2017). TNF also plays an important role in determining the fate of affected cells, if it will live or die, and by which process. In recent years, studies have shed light on the processes (Figure 1(A)), by which necroptosis is the favoured outcome over apoptosis (Berghe et al., 2014, Brenner et al., 2015). Following the binding of TNF with its corresponding receptors (TNFR1, TNFR2; Table 1) located on cell surface membranes, a cascade is set in motion, which determines the life or death of the cell. Several molecules associate to form a large protein complex (complex I) (Berghe et al., 2014, Brenner et al., 2015) including TNFR1-associated death domain protein (TRADD), TNFR-associated factors (TRAF2 or TRAF5), inhibitor of apoptosis proteins (cIAP1 or cIAP2), which are linked by K63-linked ubiquitin molecule chains with linear ubiquitin chain assembly complex (LUBAC), and then with receptor-interacting serine/threonine-protein kinase (RIPK1) (Berghe et al., 2014, Brenner et al., 2015).

In living cells, survival is regulated by the transcription factor nuclear factor-kB (NF-kB) pathway. After the formation of complex I, ubiquitylation connects complex I to the IKK complex, which activates NF-kB signalling, which in turn leads to the

production of anti-apoptotic factors that prevent cell death and thus promotes cell survival. The cell death process is initiated when non-ubiquitylated RIPK1 results in switching off TNF-driven NF-kB signalling, which in turn promotes TNF-driven apoptotic signalling. When the enzymatic activity of FLIPL–pro-caspase 8 heterodimer is inhibited, it is unable to cleave RIPK1, and then, deubiquitylated RIPK1 can influence the cell death programme to favour the pathway for necroptosis over apoptosis. Non-unbiquitylated or deubiquitylated RIPK1 disassociates from complex I, which is bound to the membrane. After relocating in the cytosol, it forms an association with multiple RIPK3 complexes. These interactions trigger the formation of a new complex called the necrosome. The elevated concentrations of RIPK1/3 strongly influence necroptosis as the outcome (Berghe et al., 2014, Brenner et al., 2015).

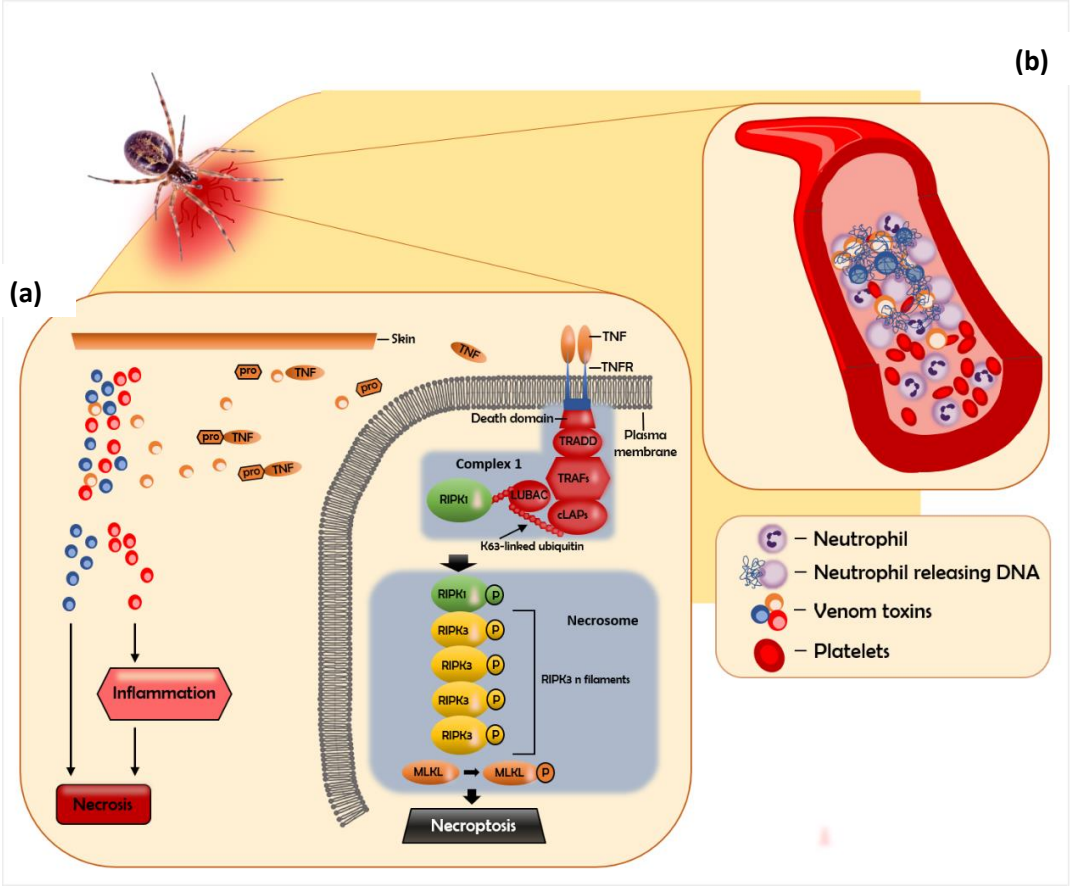

Figure 1. Potential inflammatory pathways to necrosis after envenomation. **(A)** Illustrates various ways different toxins may contribute to necrosis. Blue venom toxins represent direct lytic activity, red venom toxins represent trigger of inflammation, which leads to necrosis (regulated necroptosis), and orange venom toxin represents the convertase activity suggested

by Moura-da-Silva et al., (1996), which converts inactive TNF to its active state and further contributing to the necroptosis pathway. Illustration adapted from (Brenner et al., (2015) and Moura-da-Silva et al., (1996). **(B)** Blood vessel blockage resulting from NETs, illustrating the NET activity once triggered by certain toxins. Once NETs are activated, they trap numerous molecules including venom toxins and platelets, potentially leading to vessel blockage causing ischemic necrosis or toxin concentration that could lead to localized activity of necrotic compounds.

TNF has been shown to play a significant role in snake venom-induced pathologies. Following inoculation with venom from the European asp (*Vipera aspis*), patients can experience cardiotoxic effects. A study suggested that this pathology is mediated by the systemic circulation of TNF. Prior treatment with TNF antibodies showed a significant decrease in cardiac pathology (Szold et al., 2003).

The development of necrosis in victims of envenomation by the South American pit viper *Bothrops jararaca* and the northern East African Saw-scaled viper *Echis pyrumidum leukeyi* has been shown to be directly influenced by TNF-α. Moura-da-Silva et al., (1996) hypothesised that the pro-inflammatory cytokine TNF-α may participate to the development of tissue necrosis after envenomation by vipers. In addition to the direct potent action of venom toxins on cells, the authors (Moura-da-Silva et al., 1996) suggested that venom components such as metalloproteinases have a convertase activity that facilitates the hydrolysis of pro-TNF-α, converting it to its active state, which further intensifies the level of necrosis in the victim (Figure 1(A)). In agreement with this, the authors demonstrated that treatment with TNF-α-antibodies would significantly reduce the manifestation of necrosis.

Table 1. Nomenclature of proteins involved in necroptosis pathway

Protein	Abbreviation	Description
Transmembrane receptor molecules	TNFR1, TNFR2	Transmembrane receptor molecules that bind Tumour Necrosis Factor with cellular transduction pathways
TNFR1-associated death domain protein	TRADD	Intracellular adaptor protein that link proteins to create larger signalling complexes
TNFR-associated factors	TRAF2 or TRAF5	Adapter proteins that associate with specific tumour necrosis factor family receptors
Cellular inhibitor of apoptosis proteins	cIAP1 or cIAP3	Inhibitor of apoptosis protein that ubiquitinates RIP1
Linear ubiquitin chain assembly complex	LUBAC	Ubiquitin ligase complex that generates linear polyubiquitin chains and regulates the NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) pathway
Receptor-interacting serine/threonine-protein kinase	RIPK1, 3	Adaptor proteins whose kinase activity regulate eachothers phosphorylation through autotransphosphorylation
Inhibitor of kappa B	IKK complex	Protein that inactivates NF-κB transcription factor
FLICE-like inhibitory protein & cysteine-aspartic proteases	$FLIP_L$–pro-caspase 8 heterodimer	$FLIP_L$ is a regulatory protein of apoptosis, caspase 8 is a protease that regulates apoptosis. By forming a heterodimer, enzymatic activity is inhibited allowing for necroptosis to occur
Lysine 63-linked polyubiquitin chains	K63-linked ubiquitin molecule chains	Polyubiquitin chain linkages which are significant for linking substrates to distinct signal transduction pathways.

In concurrence with this hypothesis, a subsequent study by Laing et al., (2003) showed that, when Balb C and C57 mouse KO strains deficient in TNF receptors (TNFR1 and TNFR2) and IL-6 were injected with the venom metalloproteinase jararhagin, necrosis did not manifest. While this venom toxin typically induces haemorrhage, oedema, and necrosis in bite victims, in this study, haemorrhage and oedema continued to manifest in these KO strains at similar levels as to the WT control mice, but necrosis was consistently absent. This suggests that TNF might not be the sole target of jararhagin, and that cytokines other than TNF might be involved in the initiation of necrosis.

The inflammatory cascade and the role of important cytokines including TNF and interleukin in envenomation described by Moura-da-Silva et al., (1996) and Laing et al., (2003) tend to indicate that under certain circumstances the immune response can act independently against the host and contribute to venom-induced necrosis. In such cases, in addition to antivenom, TNF antibodies may be important in inhibiting the progression of tissue necrosis (Ferraz et al., 2015, Harrison et al., 2011).

The influence of TNF in determining the outcome of a cell death event has also been described for bacterial pathogenesis. For example, in response to bacterial proliferation within phagocytic cells, the expression levels of TNF signalling play an important role in determining the three possible outcomes for the infected phagocyte. Low expressions of TNF signalling results in unregulated necrosis, whereas moderate

expression levels of TNF result in the release of bactericidal molecules that ensure the survival of the cell. However, if the TNF expression levels are elevated, phagocytic cells can undergo another process via the RIPK1-RIPK3-mediated pathway, which ultimately leads to necroptosis (Blériot and Lecuit, 2016).

5.3.3.3 - Neutrophil extracellular traps and the NETosis pathway

Upon activation of the immune system, some of the first line responders are leukocytes, for example, macrophages and neutrophils. Because infected macrophages can relocate and ultimately redistribute pathogens, the regulated necrosis of localized cells is considered a means to control the spread of infection at the expense of these local cells (Blériot and Lecuit, 2016). In addition to phagocytosis, neutrophils can also be triggered to undergo "cellular suicide" in a process during which the chromatin becomes untightened and the nucleus swells subsequently releasing nucleoplasm into the cytoplasm. The cell dies when its membrane ruptures and discharges its DNA contents known as neutrophil extracellular traps (NETs) in the extracellular matrix. NETs prevent bacteria disseminating further by entangling them with a mesh of DNA strands (Katkar et al., 2016, Yipp and Kubes, 2013). Previously regarded as an NADPH oxidase–dependent cellular death process (Fuchs et al., 2007), recent studies indicate that NET discharge can occur independently of NADPH oxidase (NOX) and is now regarded as a non-universal pathway to cell death (Yipp and Kubes, 2013) because of its ability to be stimulated by either NOX-dependent, NOX-independent pathways, or even by both simultaneously (Katkar et al., 2016).

NETs have been shown to be involved in coagulation and identified as potentially important in deep vein thrombosis due to their potential to interact with components of the blood clotting cascade (Cimmino et al., 2017). As a result, NETs are a recognized target for treating deep vein thrombosis. In animal models, treatment with DNase 1, an enzyme that non-specifically cleaves DNA to release 5'-phosphorylated di-, tri-, and oligonucleotide products, inhibited the development of thrombus formation (Brill et al., 2012). Pathogenic bacteria can express DNase, which facilitates the denaturation of DNA strands, rendering the NETs inefficient (Fuchs et

al., 2012). It should also be noted that some snake venoms contain endonucleases (Dhananjaya and D'souza, 2010). In the event of NET initiation, the presence of snake venom endonucleases could restrict NET function and facilitate circulation of venom toxins from the bite site.

Envenomation by snakes such as *Echis* species often lead to complications such as coagulopathies, hemorrhage, and notably severe localized tissue necrosis at the bite site. Recent studies describe venom-induced NETs release, which subsequently limits the dissemination of venom toxins (Figure 1(B)) away from the bite site (Bennacef-Heffar and Laraba-Djebari, 2017, Katkar et al., 2016, Slagboom et al., 2017). As a consequence, the venom toxins accumulate at the bite site, resulting in significant localized tissue damage. Moreover, NET activity is facilitated by the absence of endonucleases, which is a common constituent of other snake venoms. NETs have only been recently described as potentially significant in snake venom induced pathologies such as localised tissue necrosis and as a target for envenomation treatment using nucleases such as DNase 1 (Katkar et al., 2016). It should also be noted that the release of extracellular traps is not limited to neutrophils and venom could potentially induce chromatin release from other cells (Brill et al., 2012).

The aforementioned studies demonstrate the potentially fundamental role of the immune system in determining the outcome of an envenomation. Within this paradigm, venom composition alone does not dictate the outcome of the bite, and intense inflammation can significantly contribute to the development of tissue necrosis.

5.3.3.4 - Arthropod venom driving necrosis

The genus *Loxosceles* comprises approximately 130 species of medium-size spiders distributed globally. The neotropical species *L. laeta*, *L. intermedia*, *L. rufescens*, *L. gaucho*, and *L. reclusa* are frequently involved in medically significant bites, which result in localised and systemic pathological symptoms, including dermonecrosis. *Loxosceles* venom possess phospholipases D, which causes hydrolysis of sphingomyelin resulting in the lysis of muscle fibres. However, while most studies

tend to focus on the direct lytic activity of *Loxosceles* venom, few have assessed the immune status of the host.

Ribeiro et al., (2015) investigated the toxicity of *Loxosceles intermedia* and the differential immune response of three mouse strains (C57, BalbC and the immune-deficient Swiss strain) to its venom. The authors demonstrated phospholipases D, hyaluronidase, metalloproteases, and serine proteases activity in the venom of *Loxosceles intermedia*. In addition to increasing venom potency, hyaluronidase facilitates the dissemination of venom from the bite site (Kemparaju and Girish, 2006). Phospholipases A2, which are thought to be the main compounds involved in the development of necrosis following snake bites (Dhananjaya and D'souza, 2010) was not recovered. However, the authors suggested this could have been due to a low abundance of Phospholipase A2 within the pooled sample, and not their absence.

Following intradermic injection of *Loxosceles intermedia* venom, C57 and BalbC mouse strains experienced a differential inflammatory response. Immune-deficient Swiss mice showed no signs of venom-induced inflammatory response but some signs of vascular congestion.

All three mouse strains developed oedema, which persisted for 16hrs in Swiss, and 24hrs in C57 and BalbC mice. Dermal necrosis was not observed during this study, but intense inflammatory infiltrate was observed in C57 and BalbC mice (Ribeiro et al., 2015). All C57 and BalbC mice died between three and six days post-injection, but Swiss mice lived up to 30 days post-injection. Although unlikely, it is possible that dermal necrosis would have manifested itself if C57 and BalbC mice had survived longer. The longer survival rate and absence of inflammatory response in the Swiss strain may be due to the fact that relevant chemoattractant cytokines are expressed at low levels in Swiss mice (Marques et al., 2011). Contrasting patterns of cell mobilization in the blood, bone marrow and spleen of C57 and BalbC mice indicated a venom induced innate and adaptive inflammatory response. It would have been interesting however, if the authors had included the Swiss mice when analysing cell mobilization. While no inflammatory reaction was observed in the histological

analysis of the skin, it may have produced an interesting comparison to C57 and BalbC in the blood, bone marrow and spleen (Ribeiro et al., 2015).

Phospholipases D from *Loxosceles laeta* venom stimulate the induction of intense inflammatory mediators, which subsequently recruit monocytes to the bite site (Rojas et al., 2017). Following the cleavage of sphingomyelin by phospholipase D, the subsequent release of ceramide 1-phosphate (C1P) can act as a pro-inflammatory mediator (Rivera et al., 2015). Patel et al., (1994) showed in vitro that the venom of *Loxosceles deserta* only leads to dermonecrosis when in the presence of infiltrating neutrophils, triggered indirectly by ceramides released from the breakdown products of sphingomyelin. The subsequent release of ceramides is significant during envenomation because they play an important role as intermediaries that regulate TNF-α and recruit neutrophils (Domingos et al., 2003b). Previous studies (Domingos et al., 2003a) suggest that *Loxosceles* venom does not produce dermonecrosis in mice because the sphingomyelin in mice structurally differs from rabbits, guinea pigs, and humans. In mice, the venom can diffuse farther, avoiding a localized build-up of venom at the bite site, but can result in a lethal effect, whereas sphingomyelin in rabbits, guinea pigs, and humans reduces the spread of venom, causing venom to accumulate thus resulting in necrosis, but prolonging the animal's life. As a result, phospholipase D directly hydrolyses sphingomyelin, ultimately leading to the release of ceramides, and the subsequent induction of inflammatory infiltrate, which leads to localized necrosis. Domingos et al., (2003a) demonstrated using BALB/c mice that venom from *Loxosceles gaucho* only induced dermonecrosis at the bite site when co-administered with sphingomyelin or ceramide phosphate plus liposome. In this study, mice injected with only venom died, whereas although the addition of sphingomyelin or ceramide phosphate resulted in inflammatory response and subsequent dermonecrosis, these mice all lived.

Hemiscorpius lepturus is a medically important scorpion from West Asia that can induce local and systemic symptoms including dermonecrosis, renal failure, and hemolysis (Jalali et al., 2010) and is responsible for fatal outcomes. *Hemiscorpius lepturus* venom possesses heminecrolysin, a phospholipase D-like enzyme

(Khodadadi et al., 2012). Studies have drawn on similarities between the molecular weights of venom compounds of *Hemiscorpius* and *Loxosceles* suggesting that symptoms including dermonecrosis may also be induced through activation of the immune system (Seyedian et al., 2010, ZARE, 2013). In a study by Jalali et al., (2011), serum collected from 36 hospital patients in the southwest of Iran after being admitted following envenomation by *Hemiscorpius lepturus* showed that concentrations of cytokines IL-1, IL-6, IL-8, and TNF-α were higher than those in healthy, non-envenomated patients. The authors found that the severity of the envenomation correlated specifically with the elevated concentrations of TNF-α and suggests that these elevated cytokine concentrations and pathology are related. In the same study, patients stung by *Mesobuthus eupeus* also showed increased concentrations of interleukins but not TNF-α. Envenomation from the latter species is considered typically mild and have never been associated with dermonecrosis.

Intense recruitment of inflammatory cells and mediators can potentially lead to disturbance in the blood (Ribeiro et al., 2015). Heavy traffic within the blood capillaries due to venom-induced inflammatory response could therefore potentially also lead to ischemic necrosis (Figure 1(B)). Necrotic lesions could therefore occur inconsistently and on an individual basis, being indirectly triggered by inflammation-inducing venom toxins rather than necrotic compounds; the outcome of envenomation would also depend on the immune status of the victim.

Variations in outcome after envenomation can also be explained by the volume of venom delivered. Snakes, spiders, centipedes, and scorpions have been shown to adapt the amount of venom delivered according to the level of the perceived threat (cf. Venom Optimisation Hypothesis) (Dugon and Arthur, 2012, Morgenstern and King, 2013, Wigger et al., 2002). In addition, venom glands may take several days or even weeks to replenish their stores and venom compounds may not be produced at the same rate. Therefore, envenomation by the same individual specimen may result in different subsequent immune response depending on the level of toxin replenishment and the quantity of venom injected (Ribeiro et al., 2015). This hypothesis is reinforced by the fact that in the case of envenomation by

hymenopteran and dipteran, necrotic lesions developed only after multiple, simultaneous envenomations (Chiu et al., 2016, Knight and Bangs, 2007, Mingomataj et al., 2014, Yanagawa et al., 2007). Regarding ischaemic necrosis, NETs could also potentially play a role in trapping material within blood capillaries and thus venom volume may have a critical role.

5.3.4 - CONCLUSIONS

Arthropod venom can induce a complex and intense inflammatory response. Although typically most arthropod venoms are not associated with direct necrotic activity, it is proposed that envenomation could in some circumstances trigger an immune cascade ultimately leading to necrosis. This would explain the inconsistent and circumstantial occurrence of necrotic manifestations following envenomation by venomous arthropods. The role of TNF and neutrophil extracellular traps in snake venom-induced pathologies is remarkable, especially from the standpoint of using TNF antibodies and DNase 1 as potential therapeutic agents to complement traditional antivenom treatment. Clinicians should consider the victim's immune response as an integral part of the envenomation syndrome. Focusing research into identifying TNF necroptosis, neutrophil NETosis, or other pathways involved in regulated necrosis following envenomation may help determine more efficient ways to manage inflammation and potentially reduce the severity of symptoms.

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5.4 - The noble false widow spider *Steatoda nobilis*: reservoir and potential vector for clinically important bacteria with diminished antibiotic susceptibility

JPD contributed to the conceptualization, methodology, data acquisition, original draft preparation, and review and editing. This section is currently submitted to a journal and is in peer review.

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5.4.1 - ABSTRACT

The false widow spider *Steatoda nobilis* is frequently associated with bites, which develop bacterial infections that are sometimes unresponsive to antibiotics. These could be secondary infections derived from opportunistic bacteria on the skin or infections directly vectored by the spider. In this study, we investigated whether it is plausible for *S. nobilis* and native European spiders to vector bacteria during a bite,

by seeking to identify bacteria with pathogenic potential on the spiders. 12 genera of bacteria were identified through 16S rRNA sequencing from the body surfaces and fangs of *S. nobilis*, and four native spiders: *Amaurobius similis, Eratigena atrica, Steatoda grossa*, and *Zygiella x-notata*. Out of 22 species isolated, 12 related to human pathogenicity, among which *Staphylococcus epidermidis*, *Kluyvera intermedia*, *Rothia mucilaginosa*, and *Pseudomonas putida* are recognized as class 2 pathogens. The isolates varied in their antibiotic susceptibility: *Pseudomonas putida, Staphylococcus capitis* (2), and *Staphylococcus edaphicus* showed the highest extent of resistance, to three antibiotics in total. All bacteria recovered from *S. nobilis* were susceptible to ciprofloxacin. Our study demonstrates that *S. nobilis* and four other native species do carry opportunistic pathogenic bacteria on their body surfaces and fangs. Therefore, some post-bite infections may be the result of vector-borne bacterial zoonoses that may be antibiotic resistant.

5.4.2 - INTRODUCTION

Bacterial infections represent a major threat to human health. For example, typhoidal *Salmonella* causes 27 million annual cases of typhoid fever resulting in 223,000 deaths (Pulford et al., 2019), and non-typhoidal *Salmonella* is responsible for over 93 million cases of gastroenteritis leading to 155,000 annual deaths (Gal-Mor et al., 2014). Bacterial infections contribute significantly to sepsis (Brandenburg et al., 2016) and in 2017, 49 million cases of sepsis resulted in 11 million deaths worldwide. Antibiotic resistance further increases the threat to human health with drug-resistant bacteria causing 700,000 annual deaths worldwide (Rappuoli et al., 2017). According to the World Health Organization's (WHO) global action plan on antimicrobial resistance, it is essential that antibiotic resistance is tackled across every contact zone between humans and the environment (Pärnänen et al., 2019). Contamination of human dwellings, and more specifically food and water storage facilities, is a major issue (Pulford et al., 2019). As such, identifying the source of contamination is crucial for reducing the spread of pathogens.

Synanthropic animals (wildlife associated with human habitats) can be major reservoirs and vectors of pathogenic bacteria. Wild, domesticated and captive

animals can be colonised by bacteria and act as reservoirs (Dunbar et al., 2015), transmitting pathogens through physical contact, including bites, stings and scratches (Cantas and Suer, 2014). For example, rats have historically caused epidemics and rat-borne zoonotic pathogens are once again increasing across Europe (Strand and Lundkvist, 2019). However, some animal groups that can potentially spread pathogenic bacteria in and around human habitats are often overlooked. Recently, venomous snakes were identified as reservoirs for *Salmonella* with potential to contribute to the health crisis through shedding contaminated faeces around homes and vectoring bacteria during bites (Pulford et al., 2019, Ul-Hasan et al., 2019). Moreover, a recent study demonstrated that bacteria can survive within the venom and venom glands of snakes and spiders (Esmaeilishirazifard et al., 2018).

Spiders are small and overlooked but occupy a varied range of synanthropic niches (Nentwig, 2015). They eat a diverse range of prey, with some capable of catching and consuming large arthropods, fish, lizards, snakes, birds, rodents (Dugon et al., 2017, Dunbar et al., 2018a, Hódar and Sánchez-Piñero, 2002, Kuhn-Nentwig et al., 2011, O'Shea and Kelly, 2017), and medically important pests, including mosquitos and house flies (Barin et al., 2010, Ndava et al., 2018, Wang et al., 2011). Some will readily feed on carrion (Barrantes and Weng, 2007, Sandidge, 2003, Vetter, 2011). Wild caught specimens of *Steatoda nobilis* were observed by us feeding on dead prey for up to eight days in laboratory conditions (unpublished data). The innate immune system of arthropods protects against pathogenic microbes (Baxter et al., 2017, Kavanagh and Reeves, 2007, Savitzky et al., 2012), however, once dead, the microbes are free to thrive and multiply. It is inevitable that spiders will encounter microbes through the environment, or through feeding, especially on carrion. The potential therefore exists for spiders to harbour virulent bacteria and they have been implicated in bite cases that subsequently led to bacterial infections (Monteiro et al., 2002).

The clinical manifestations arising from spider bites (araneism) are diverse (Dunbar et al., 2018c, Dunbar et al., 2019, Hauke and Herzig, 2017, Ribeiro et al., 2015, Vetter, 2018, Vetter et al., 2006). For example, necrotic araneism (necrosis resulting from spider bite) is most commonly documented from bites by *Loxosceles* species, but

infrequently by other species (Dunbar et al., 2019, Hogan et al., 2004, Monteiro et al., 2002, Patel et al., 1994, Ribeiro et al., 2015, Rivera et al., 2015). Bacterial infection following a spider bite could result in these prolonged and debilitating pathologies that are sometimes unresponsive to antibiotics (Ahrens and Crocker, 2011). The implication of the spider as the source of these bacterial infection is controversial. The spiders responsible usually are crushed, escape, or are captured using non-sterile methods, therefore comprehensive microbiological analysis is not possible. Infections associated with spider bites are typically caused by bacterial species commonly found in the environment and on human skin (Vetter et al., 2015a). Moreover, spider venoms are considered a rich source of antibacterial peptides (Saez et al., 2010) leading to a proposal that these are sterile environments that neutralise bacteria and therefore infections are secondary to the spider bite itself (Esmaeilishirazifard et al., 2018, Ul-Hasan et al., 2019, Vetter et al., 2015a). We therefore face a conundrum in determining if infections are caused by opportunistic bacteria already present on the skin (secondary infections), or from the fangs, and therefore directly vectored from the bite (Vector-Borne Bacterial Zoonoses).

The noble false widow spider, *S. nobilis* has expanded its range across Europe (Bauer et al., 2019), (including Ireland (Dugon et al., 2017, Dunbar et al., 2018b, Nolan, 1999) and the UK (Bauer et al., 2019)), through Western Asia (Türkeş and Mergen, 2007, Zamani et al., 2015), and the Americas (Bauer et al., 2019, Faúndez et al., 2018, Faúndez and Téllez, 2016, Taucare-Ríos et al., 2016, Vetter et al., 2015b). This species is increasingly linked to medically significant bites to humans, especially in Ireland and the UK (Dunbar et al., 2018c, Faúndez and Téllez, 2016, Warrell et al., 1991). As range expansion continues, so will the increase in bite cases (Bauer et al., 2019). Envenomation symptoms of *S. nobilis* bites include prolonged moderate to intense pain, swelling, and erythema, piloerection, diaphoresis, facial flushing, feverishness, vasodilation of blood capillaries, and minor necrosis (Dunbar et al., 2018c). At least three unpublished reports of confirmed *S. nobilis* with subsequent mild to debilitating bacterial infections including cellulitis and dermatitis with one victim requiring hospitalisation and an aggressive course of intravenous antibiotics is currently being assessed by the authors.

False widow spiders (genus *Steatoda*), like the closely related black widow spiders (genus *Latrodectus*) can occasionally subdue small vertebrates (Ahrens and Crocker, 2011, Dunbar et al., 2018a, O'Shea and Kelly, 2017, Petrov and Lazarov, 2000, Zamani, 2016) as they possess a fast-acting neurotoxic venom (Dunbar et al., 2018a, Garb and Hayashi, 2013, Gendreau et al., 2017). It is the presence of α-latrotoxin that can induce neuromuscular paralysis and death in humans following envenomation by *Latrodectus* species (Garb and Hayashi, 2013). The venom protein composition *of S. nobilis* was recently characterised and reveals approximately two-thirds of the venom is composed of *Latrodectus*-like toxins with including the most powerful toxin classes such as α-latrotoxins (11%), α-latroinsectotoxins (11%), and δ-latrocrustotoxins (11%). Also present are the enzymatic machinery that facilitate the spread of venom into the prey (metallo and serine proteases, chitinases). In high concentrations α-latrotoxin can cause localised cell death and potentially facilitated by the presence of enzymes may induce necrosis (Dunbar et al., 2020), and provide substrate that could facilitate bacterial virulence.

Previous studies on *Latrodectus hesperus* demonstrated the potential for spiders to vector bacteria during bites (Ahrens and Crocker, 2011). Fangs excised from 220 specimens recovered five pathogenic antibiotic resistant bacterial species. The microbial colonisers of *S. nobilis* fangs have never been investigated. Such a study would provide data to 1) explain why bacterial infections are increasingly associated with bites by *S. nobilis*, 2) explain why some patients are unresponsive to frontline antibiotics, and 3) determine if the etiological agent could be vectored directly from the spider's fangs, or transferred from the body surface on to the area of the bite site. This could have significant implications for advising first line medical staff who are treating bites by *S. nobilis* and help in choosing appropriate care and treatment.

The main objectives of this study were to 1) characterise the microbiome of the non-native *S. nobilis,* along with two spiders native to Ireland and the UK, *Amaurobius similis*, and *Eratigena atrica,* which are commonly found around homes and also capable of biting humans; and 2) identify bacteria species residing on the body

surface and fangs of the spiders, and test the susceptibility of these bacteria to nine broad-spectrum antibiotics.

5.4.3 - RESULTS

5.4.3.1 - Bacterial Isolation and sequence analysis of *Amaurobius similis, Eratigena atrica, Steatoda nobilis, Steatoda grossa* and *Zygiella x-notata*:

Spiders typically bite when trapped, e.g. between skin and clothing, or bed sheets, and therefore it is conceivable that bacteria may not only be transferred from the fangs, but also potentially rubbed from the spider's body onto human skin. Therefore, we investigated the presence of bacteria from fangs and from body using aseptic culture techniques. *A. similis* and *E. atrica* are commonly found around homes and are capable of biting humans. *S. grossa* is a species of false widow spider that was established in Ireland since the late 1800s and found in at least 11 Irish counties, typically inside homes under kitchen units and in garden sheds (Dunbar et al., 2018b) and is capable of biting humans (Isbister and Gray, 2003). *Z. x-notata* is commonly found in gardens and particularly around window frames in most homes across Ireland and the UK (Roberts, 1995).

In the first stage of this study, 9 different genera of bacteria were recovered from 45 samples (36 fangs (F) and 9 full body (FB)) from the 5 spider species- *A. similis* (16 F and 6 FB), *E. atrica* (10 F and 2 FB), *S. nobilis* (8 F and 1 FB) and 1 fang sample from each of *S. grossa* and *Z. x-notata*.

5 *Salmonella*, 1 *Bacillus*, 2 *Staphylococcus*, and 1 *Escherichia* species were recovered from 9 full body samples of *A. similis*, which included 3 *Salmonella* and a *Staphylococcus* spp. from bodies of euthanised *A. similis*. *Salmonella* and *Bacillus* spp. were also identified on the body of *E. atrica* and a *Staphylococcus* spp on the body of *S. nobilis*.
Of particular interest was the identification of 9 different genera on the fangs of these spiders (Table 1). *Bacillus* spp, *Raoutella* spp, *Staphylococcus* spp, were recovered from the fangs of both *A. similis* and *E. atrica*, among which *Staphylococcus* spp was predominant, occurring 7 and 9 times in *A. similis* and *E. atrica* fang samples,

respectively. Whereas *Paenibacillus* spp were predominant on the fangs of *S. nobilis* and was present in 7 out of 8 samples. The second most predominant genus found was *Bacillus*, which occurred in 4 samples from *A. similis* and *E. atrica* and in 3 samples from *S. nobilis*. *Pseudomonas* spp. were recovered from *A. similis* and *S. nobilis*. *Salmonella* and *Advenela* spp. occurred once in *A. similis*. *Yersinia* and *Listeria* spp. occurred once in *E. atrica* and *Z. x-notata*, respectively.

5.4.3.2 - Bacterial Isolation and sequence analysis of *S. nobilis*:

To test the hypothesis that spiders can carry pathogens and could play a role in infection following spider bites, bacteria were isolated from the body and fangs of *S. nobilis* and the sequence of the full-length 16S rRNA gene was determined to identify individual isolates to species level. *Streptococcus* and *Staphylococcus* were targeted by using selective CNA blood agar and Baird-Parker agar, respectively. Due to the increasing incidence of development in patients of infection associated with *S. nobilis* bites this species was an ideal candidate for this study. 20 fangs, 15 full body (5 euthanised) and 2 "spider walks" samples of *S. nobilis* were analysed.

25 different bacterial isolates were cultured, and identified through 16S rRNA sequence analysis, within the microflora on *S. nobilis*, with a total of 17 Gram-positive and 8 Gram-negative bacteria. For most sequences, the percentage identity was >99% with their respective most similar species (Table 2). Among these, 100% identity was found for 3 sequences to *Staphylococcus edaphicus*, *Staphylococcus warneri*, and *Bacillus thuringiensis*. Two isolates displayed identity of 97% and 98% for *Bacillus pumilus* and *Streptococcus anginosus*, respectively, suggesting the isolates to be closely related to these two species.

5 isolates showed haemolytic activity on blood agar plate, which includes 4 *Bacillus* sp and a *Micrococcus* sp. 12 isolates were related to human pathogenicity, among which 4 belong to *Staphylococcus* sp, 3 *Bacillus* sp, *Rothia* sp, *Streptococcus* sp, *Dietzia* sp, *Pseudomonas* sp, and *Kluyvera* sp. The association with human pathogenicity for each bacterial species was assessed using the bacterial metadatabase BacDive unless otherwise mentioned. (Table 3)

Bacteria were isolated by each of the 3 sampling methodologies: 2 species were isolated from the agar plate with spider walks, 5 from the fangs, and 18 from the full bodies (11 from dead spiders and 7 from live spiders. The 2 bacterial species from the spider walk (*K. intermedia* and *S. epidermidis*), were different from the species found on other sites. The bacteria detected on the fangs were mostly different from the bacterial community on the full body, except for *S. capitis*, which was present on both sites. Differences in bacterial flora were also observed between bodies of live and dead spiders, with a wider variety of genera isolated from dead specimens, and several *Bacillus* species from live specimens

5.4.3.3 - Anti-bacterial inactivity of *S. nobilis* venom

To investigate the hypothesis that bacteria can be transferred from the fangs into the host during the bite without being killed by the venom, *S. nobilis* venom was tested for its antibacterial property. Minimum inhibitory concentration (MIC) assays were performed by testing various concentrations of the crude venom against *E. coli* DSM1103 and DSM10973, MRSA BH1CC, and *L. monocytogenes* EGD-e. The venom did not inhibit growth of any of the pathogens at any of the tested concentrations (Fig. 2). This indicates that bacteria could survive in spider venom during transfer from the fangs to the host during a spider bite.

5.4.4 - Antibiotic Susceptibility Testing of strains isolated from *S. nobilis*:

Antibiotic susceptibility testing was performed in accordance to the CLSI standards to determine the range of antibiotic resistance properties of the bacteria residing on *S. nobilis* and to determine which antibiotics would be the most effective in treating infection caused by those pathogens following spider bite. All 27 isolates were tested against nine antibiotics of eight different classes, consisting of 8 broad spectrum antibiotics and 1 antibiotic with greater efficacy against Gram-negative bacteria (Colistin B) (Table 3)(Fig. 3). Out of 27, 10 isolates are listed in CLSI guidelines, namely – *Staphylococcus* (5), *Pseudomonas* (3), Streptococcus spp., and *Kluyvera intermedia*.

Susceptibility to antibiotics was determined by disk diffusion. For the strains listed in CLSI guidelines, resistance and susceptibility were inferred from their breakpoints for each antibiotic. For the rest, lack of, or a minimal (≤8 mm) zone of clearance around the antibiotic disk was considered as resistant. Resistance to each antibiotic was displayed by at least one isolate except for ciprofloxacin. Nearly all the isolates were susceptible to at least one antibiotic and some isolates were multi-resistant. *Pseudomonas putida*, *Staphylococcus capitis* (2) and *Staphylococcus edaphicus* were notable for resistance to 3 antibiotics. All *staphylococcus* isolates showed resistance to gentamicin (CLSI) and nalidixic acid with the exception of *Staphylococcus capitis* (1) for gentamicin and *Staphylococcus warneri* for Nalidixic acid. Furthermore, *Staphylococcus capitis* (2) and *Staphylococcus edaphicus* also showed resistance against tetracycline (CLSI) and chloramphenicol (CLSI) respectively. *Dietzia timorensis*, *Rothia amarae* and *Streptococcus* spp. showed resistance to nalidixic acid and colistin. *Serratia fonticola* (1) and (2) both showed resistance to erythromycin and amoxicillin. *Pseudomonas peli* showed resistance to erythromycin and cefoxitine. *Bacillus licheniformis*, *Bacillus thuringiensis*, *Kluyvera intermedia*, *Micrococcus aloeverae*, *P. mobilis* (1) and (2), *Pseudomonas. azotoformans*, and *Rothia mucilaginosa* showed resistance to at least one antibiotic. *Bacillus aerius, Bacillus altitudinis, Bacillus mycoides* (1), and (2), *Bacillus pumilus*, *Micrococcus endophyticus*, and *Serratia proteamaculans* showed low to high susceptibility for all the antibiotics tested

Table 1- Bacteria genera identified on fangs of *A. similis*, *S. nobilis*, *E. atrica*, *S. grossa*, and *Z. x-notata*.

Spider Species[a]	Bacterial Genus	Occurrence[b]
A. similis	*Advenella*	1
A. similis	*Bacillus*	4
A. similis	*Pseudomonas*	1
A. similis	*Raoultella*	1
A. similis	*Salmonella*	1
A. similis	*Staphylococcus*	7
E. atrica	*Bacillus*	4
E. atrica	*Raoultella*	4
E. atrica	*Staphylococcus*	9
E. atrica	*Yersinia*	1
S. nobilis	*Bacillus*	3
S. nobilis	*Paenibacillus*	7
S. grossa	*Pseudomonas*	1
Z. x-notata	*Listeria*	1

[a] 36 fangs tested from 5 different spider species: *A. similis* - 16; *E. atrica* – 10; *S. nobilis* – 8; *S. grossa* – 1; *Z. x-notata* - 1

[b] number of times the genus occurred from a spider species

Table 2- Bacteria isolated from *S. nobilis*.

Bacterial Species	Source[a]	Growth on Baird Parker	Haemolytic	Pathogenic[d]
Pseudomonas azotoformans	F	-	-	-
Pseudomonas peli	F	-	-	-
Rothia mucilaginosa	F	-	-	+
Staphylococcus capitis (2)	F	+	-	+
Streptococcus spp [b]	F	-	-	+
Bacillus aerius	FB	+	+	-
Bacillus altitudinis	FB	+	+	-
Bacillus licheniformis	FB	+	-	+
Bacillus mycoides (1)	FB	+	-	-
Bacillus mycoides (2)	FB	+	+	-
Bacillus thuringiensis	FB	+	-	+
Micrococcus endophyticus	FB	-	-	-
Bacillus spp [c]	FB-D	+	+	+
Dietzia timorensis	FB-D	+	-	+
Micrococcus aloeverae	FB-D	-	+	-
Paenibacillus mobilis	FB-D	-	-	-
Pseudomonas putida	FB-D	-	-	+
Rothia amarae	FB-D	+	-	-
Serratia fonticola (1)	FB-D	+	-	-
Serratia fonticola (2)	FB-D	+	-	-
Staphylococcus capitis (1)	FB-D	+	-	+
Staphylococcus edaphicus	FB-D	+	-	-
Staphylococcus warneri	FB-D	+	-	+
Kluyvera intermedia	SW	-	-	+
Staphylococcus epidermidis	SW	+	-	+

[a]37 samples tested from *S. nobilis*- 20 fangs, 15 full body (5 euthanised) and 2 spiders walk. F- fangs; FB- full body; D- dead; SW- spider walk

[b] 98% sequence identity to *S. anginosus*;

[c] 98% sequence identity to *B. pumilis*

(1) & (2) indicates different strains of same subspecies based on antibiotic susceptibility test (Table 3)

[d] Pathogenicity was defined based on bacterial metadatabase BacDive (https://bacdive.dsmz.de/). "+" indicates bacterial species is associated with opportunistic infections due to underlying acute or chronic health conditions. "-"indicates no known associated of bacterial species with infection.

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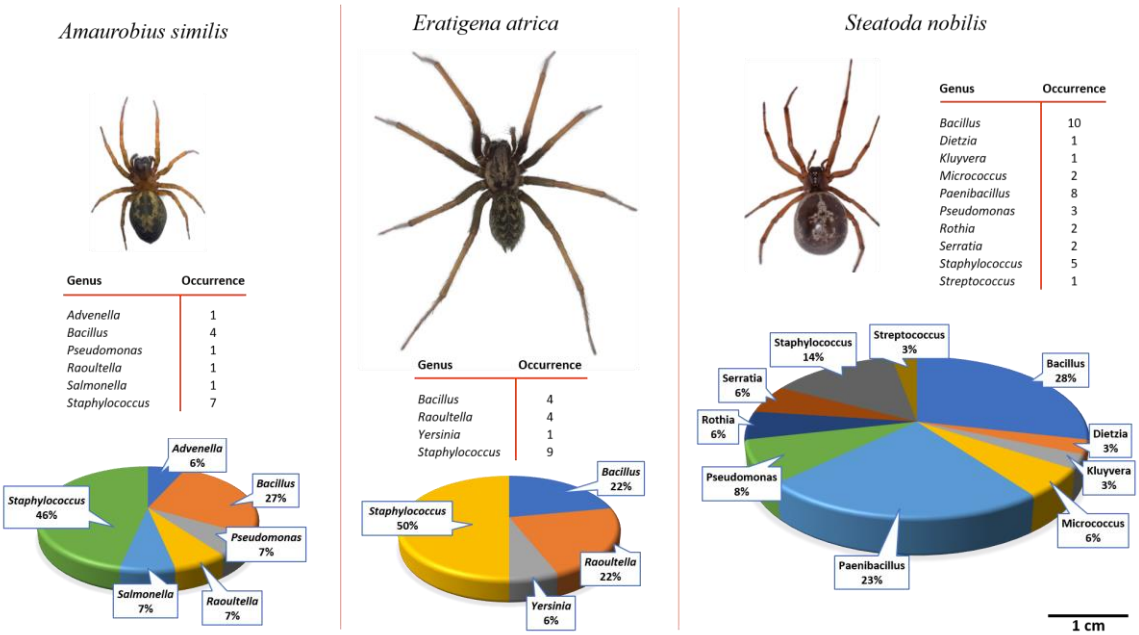

Figure 1- Comparison of the bacterial community composition and relative abundances from body and fang surfaces of *A. similis*, *E. atrica* and *S. nobilis*. Bacterial community was more diverse in *S. nobilis* (10 species) compared to *E. atrica* (4) and *A. similis* (6). *Staphylococcus* found to be most abundant on *A. similis* and *E. atrica* by appearing equal number of times as the rest of the community. Similarly, *Bacillus* was most abundant on *S. nobilis*.

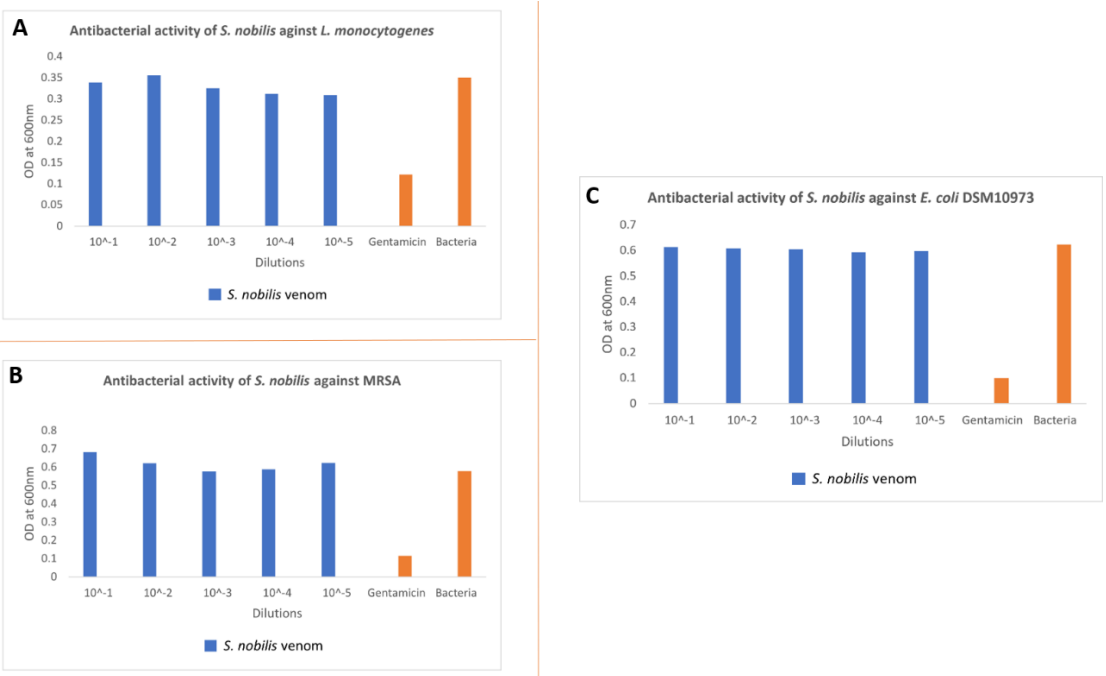

Figure 2- Non-inhibitory effects of *S. nobilis* venom against clinical isolates **A)** *L. monocytogenes*, **B)** Methicillin Resistant *Staphylococcus aureus* (MRSA), and **C)** *E. coli*. No significant effects were observed in this study.

Table 3. Antibiotic susceptibility of 27 bacterial isolates from *S. nobilis*

Bacterial species	Zone of clearance (mm)								
	5 µg	10 µg	10 µg	15 µg	30 µg	30 µg	30 µg	30 µg	50 µg
	CIP	CN	AML	E	C	TE	FOX	NA	COL
Bacillus aerius	30	20	31	26	21	27	27	19	13
Bacillus altitudinis	23	18	31	26	20	25	26	19	13
Bacillus licheniformis	31	21	13	8	16	28	31	18	11
Bacillus mycoides (1)	28	18	13	21	20	21	24	21	11
Bacillus mycoides (2)	29	23	10	23	21	25	29	22	12
Bacillus pumilus	32	20	28	27	21	25	22	19	11
Bacillus thuringiensis	17	17	8	23	23	24	15	21	11
Dietzia timorensis	36	17	20	19	26	14	10	-	-
Kluyvera intermedia	36	24	21	-[b]	27	28	24	27	22
Micrococcus aloeverae	24	20	29	21	34	32	32	-	20
Micrococcus endophyticus	32	19	40	10	16	21	39	10	16
Paenibacillus mobilis (1)	30	23	29	22	19	26	7	28	15
Paenibacillus mobilis (2)	36	25	30	22	21	30	-	28	19
Pseudomonas azotoformans	32	23	31	21	21	27	-	24	16
Pseudomonas peli	27	17	11	-	24	18	7	21	14
Pseudomonas putida	32	20	-	-	15	19	-	11	15
Rothia amarae	19	16	26	27	26	22	22	-	-
Rothia mucilaginosa	12	16	26	27	26	27	28	-	11
Serratia fonticola (1)	24	19	-	-	14	22	22	23	15
Serratia fonticola (2)	31	30	7	-	26	25	28	29	14
Serratia proteamaculans	28	19	22	13	21	19	27	20	14
Staphylococcus capitis (1)	32	24	32	23	28	27	25	-	15
Staphylococcus capitis (2)	29	19	32	24	21	12	28	-	20
Staphylococcus edaphicus	24	18	30	26	10	30	30	-	13
Staphylococcus epidermidis	27	21	38	24	19	24	31	-	9
Staphylococcus warneri	30	21	36	22	25	23	28	10	10
Streptococcus anginosus	22	16	34	33	24	28	26	-	-

This table is the result of three different experiment done in duplicate. SD ≤ ±2.

[a]Antibiotic abbreviations and classes: CIP- Ciprofloxacin (flouroquinolone), CN - Gentamicin (aminoglycoside), AML - Amoxycillin (penicillin), E - Erythromycin (macrolide), C - Chloramphenicol, TE - Tetracycline, FOX- Cefoxitin (cephalosporin), NAL - Nalidixic acid (flouroquinolone), CT-Colistin (polymyxin).

[b] -; no zone of clearance.

species listed in CLSI guidelines

Blue and red digits indicate susceptible and resistance according to CLSI guidelines; Black digits indicates antibiotic not recommended or not applicable for respective species in CLSI guidelines.

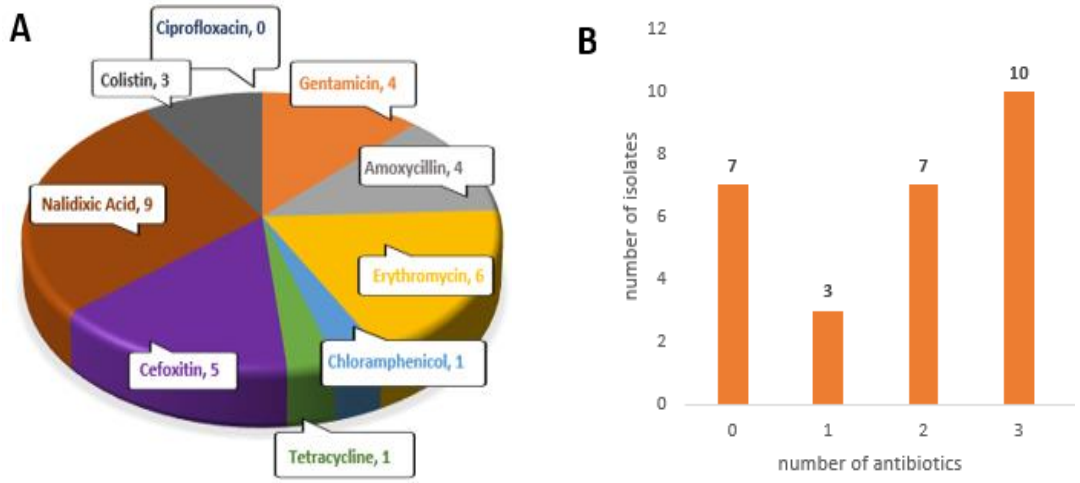

Figure 3. Antibiotic resistance profile of the bacterial community isolated from *S. nobilis*. Graph represents (A) resistance to each antibiotic shown by number of bacterial species isolated from body and fangs of *S. nobilis* and (B) number of isolates showing resistance to 0, 1, 2 and 3 different antibiotics.

5.4.5 - DISCUSSION

The role of spiders in bacterial transmission has generated much debate (Ahrens and Crocker, 2011, Vetter et al., 2015a). In recent years, increasing media reports from Ireland and the UK (Bauer et al., 2019, Dunbar et al., 2018c, Vetter, 2018) claim that victims of the noble false widow spider *S. nobilis* frequently suffer debilitating and sometimes fatal bacterial infections. While these reports are largely unsubstantiated, there have been no studies carried out to validate the true risk of bacterial infections associated with this recently established spider.

In the first part of this study, the microbiomes from *S. nobilis* (8 F, 1FB), *A. similis* (16 F, 6 FB) and *E. atrica* (10 F, 2 FB) were partially characterised, and the fangs from single specimens of *S. grossa* and *Z. x-notata* were sampled, revealing diverse bacterial compositions of eight genera (Table 1). All these bacterial genera contain some species that are associated with human pathogenicity. Since *S. nobilis* is associated with bites that lead to infections, we focused the next part of this study on the bacteria present on body and fangs from *S. nobilis* (20 F, 15 FB, spider walks N=2) (Tables 2 & 3) and identified the bacteria to species level. In this subsequent investigation, 10 genera were recovered from body and fangs of *S. nobilis*, four in common with those

found in the first part of this investigation. Testing the larger sample size of *S. nobilis* and identifying these isolates to species level allowed us to determine their potential for pathogenicity. The bacteria identified are members of normal flora of animals/ humans, and/or found in environmental settings.12 species are related to human pathogenicity (Table 2) and are recognised as opportunistic bacteria, among which *S. epidermidis*, *K. intermedia*, *R. mucilaginosa*, and *P. putida* are recognised as class 2 pathogens.

Staphylococcus spp. were recovered from *S. nobilis, A. similis*, and *E. atrica* of which four species recovered from *S. nobilis* were identified. Among them, *S. epidermidis* is a known human pathogen and responsible for severe illnesses including bacteraemia, urinary tract infections, endocarditis, septicaemia, and nosocomial sepsis originating from medical devices such as catheters and central lines (Qin et al., 2017). Other *Staphylococcus* species identified can be opportunistic human pathogens, i.e. can cause severe infection in a host with a weak immune system, an altered microbiota (such as a disrupted gut microbiota), or breached integumentary barriers, and are considered as typical components of the skin microbiome (Giordano et al., 2016, Pain et al., 2019, Premkrishnan et al., 2018).

Pseudomonads are ubiquitous in the environment and some species are associated with human infections. *Pseudomonas* spp. were recovered from *A. similis*, *S. nobilis*, and *S. grossa*, of which three species were identified, and of these one is related to human pathology. *Pseudomonas putida* can cause bacteraemia, skin, soft tissue, and urinary tract infections, localised infections, pneumonia, peritonitis, septic arthritis, meningitis, and septicaemia (Agnarsson, 2004, Ladhani and Bhutta, 1998, Mustapha et al., 2016).

Two species of Rothia were recovered from *S. nobilis*, of which one is related to human pathology. *R. mucilaginosa* is a common constituent of the oral and upper respiratory microbiota. It is commonly associated with teeth and gum disease, but is now considered an emerging opportunistic pathogen, especially in immunocompromised patients associated with endocarditis, pneumonia, arthritis, meningitis, skin and soft-tissue infections, prosthetic joint infections, and endophthalmitis. For example, it was

isolated from five cancer patients who developed bacteraemia (Horino et al., 2019, Poyer et al., 2019).

The role of bacterial araneism is controversial; however, it is accepted that it is experimentally plausible for spiders to vector bacteria, and a confirmed infection vectored directly from a spider bite is discussed in the literature (Vetter et al., 2015a). We demonstrated here that 1) a wide range of bacteria ubiquitous in the environment are carried on spider fangs and exoskeleton (Fig. 1), and 2) some are potentially pathogenic involved in a wide range of clinical manifestations. In total, 10 species of potentially pathogenic bacteria were isolated from bodies or fangs of *S. nobilis*. We believe this clearly demonstrates the potential for bacteria to be vectored during bites and that it is more likely that infections arise zoonotically than from commensal bacteria present on the skin (as is the current consensus) (Esmaeilishirazifard et al., 2018, Ul-Hasan et al., 2019, Vetter et al., 2015a).

In the case of *S. nobilis*, vectored infection may be facilitated by the venom's ability to kill localised skin cells, potentially disrupt normal immune response (Dunbar et al., 2020, Dunbar et al., 2018c, Dunbar et al., 2019), and provide substrate for bacteria to thrive. Moreover, *S. nobilis* typically bite humans when accidently trapped or squashed between the skin, and clothing/bed sheets (Dunbar et al., 2018c, Warrell et al., 1991). Therefore, the site around the bite could be contaminated by bacteria present on either the fangs or the body of the spider. Previous studies reveal spider venoms as rich sources of antibacterial peptides (Saez et al., 2010) that could neutralise bacteria in paralyzed prey (Kozlov et al., 2006, Vetter et al., 2015a). However, recent advances in venomics studies confirms that spider venoms are not sterile and should be viewed as microenvironments (Ul-Hasan et al., 2019). The results here demonstrate that *S. nobilis* venom has no inhibitory effect on bacterial growth, suggesting that the venom is unlikely to eliminate bacteria from the fangs.

Since the development of penicillin and subsequent antibiotics in the 1940s, there has been a rise in antibacterial resistant bacteria (Ventola, 2015) that kill 700,000 people each year (Rappuoli et al., 2017). Therefore, it is important to determine how antibiotic resistant bacteria move through the environment and establish contact

zones between humans and the environment (Pärnänen et al., 2019). Pathogenic bacteria recovered from the fangs of black widow spiders (Ahrens and Crocker, 2011) included multiple antibiotic resistant strains, with fluoroquinolones and aminoglycosides recommended as the most efficient antibiotics for treating infections arising from black widow bites. In one confirmed bite case from *S. nobilis* resulting in cellulitis (data unpublished), the victim was unresponsive to antibiotic treatment. We tested the susceptibility of 27 bacteria recovered from *S. nobilis* against nine antibiotics used by front line medical staff and 19 antibiotic-resistant strains were identified (Table 3). The most resistant isolates were a *P. putida*, which showed resistance to three broad range antibiotics (amoxicillin, erythromycin, and cefoxitin), *S. capitis* (2), which also showed resistance to three but completely different class of antibiotics (gentamicin, tetracycline, and nalidixic acid) and *S. edaphicus*, which showed resistance to gentamicin, chloramphenicol, and nalidixic acid. *S. capitis* and *S. edaphicus* are the only isolate in this study to show resistance against tetracycline and chloramphenicol respectively. All bacteria recovered from *S. nobilis* were susceptible to ciprofloxacin. There is a fundamental need to identify bacteria from spider bite victims. Additionally, there is a need for catalogues of the bacterial flora of spiders and cross-reference databanks with pathogenicity and antibiotic-resistance to better inform appropriate treatment for infections associated with spider bites.

5.4.6 - CONCLUSIONS

Our study demonstrates that the non-native *S. nobilis* and four native spider species carry opportunistic pathogenic bacteria on their body surfaces and fangs. Bacteria may be vectored directly from the spider, and as a result, post-bite infections may be the result of vector-borne bacterial zoonoses. Some of the bacteria carried by spiders are multidrug-resistant. Furthermore, our results showed that the venom of *S. nobilis* has no inhibitory effects against bacterial growth, indicating that it is most likely not a barrier to bacterial infection resulting from a spider bite. We believe this study provides a baseline for future research targeting synanthropic spider species to determine bacterial compositions and develop a database of bacterial species isolated from spiders, and to determine links to human disease.

5.4.7 - METHODS

5..7.1 - Spider and venom collection

Specimens of *Amaurobius similis, Eratigena atrica, Steatoda grossa, S. nobilis* and *Zygiella x-notata* were collected in Ireland, from garden walls and park railings in Lucan, Co. Dublin, Edgeworthstown, Co. Longford, Galway city, Co. Galway and Ferrybank, Co Waterford. Specimens were collected using sterile forceps, placed immediately into sterile tubes, and transported to the lab. Species identities were confirmed using identification guides specific to *S. nobilis* (Dugon et al., 2017) and Collins Field Guide for all other spiders (Roberts, 1995)

Using aseptic technique, the specimens were dispatched, and the fangs were either clipped or swabbed. For whole body cultures, spiders were either submerged in media or swabbed. For surface colonisation analysis, spiders walked directly on Brain Heart Infusion (BHI) agar. The most common method for euthanising arthropods is dispatchment. A select number of spiders were euthanised using CO_2, to determine if bacteria was recoverable.

For venom extractions, *S. nobilis* specimens were anesthetized using CO_2 for 2 min and venom was extracted by electrostimulation with repeated pulses delivered at 15-20V. Venom droplets were collected from the venom pores located on the outer subterminal part of the chelicerae using 5 µl microcapillary tubes modified with a tapered end for maximum efficiency. Venom from approximately 100 specimens was pooled and then flash-frozen in liquid nitrogen and stored at -80°C.

5.4.7.2 - Preliminary testing for microbiomes from *A. similis, E. atrica, S. nobilis, S. grossa* and *Z. x-notata* and 16S rRNA gene amplification, sequencing, and analysis:

Whole bodies or fangs from five species of spiders: *A. similis*, *E. atrica, S. grossa, S. nobilis*, and *Z. x-notata* were transferred into 750 µl (10% dilution) of Luria Bertani (LB) broth, Nutrient broth (NB), Tryptic Soy broth (TSB), MRS broth and BHI broth, and incubated at both 37°C and 10°C. Whole culture from each spider or fang were pelleted, DNA was extracted collectively from each sample using the QIAGEN

Dneasy Blood & Tissue Kit and V3-V4 region of 16S rRNA was amplified using 341F 5'-CCTACGGGAGGCAGCAG-3' (Lane, 1991), and 806R 5'-GGACTACHVGGGTWTCTAAT-3' (Caporaso et al., 2011). The amplified product was then sent to GATC Biotech for sanger sequencing. A BLAST search was carried out with the obtained sequence using the NCBI rRNA/ITS database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

5.4.7.3 - Bacterial isolation from *S. nobilis* and 16S rRNA gene amplification, sequencing and analysis:

For isolating surface bacteria, *S. nobilis* spiders were washed individually with 5 ml BHI broth for 5 min. The wash media was then incubated at 37°C overnight. For isolating bacteria from fangs, clipped fangs from each individual spider were inoculated into BHI broth and incubated at 37°C. After 24 h incubation, the cultures were diluted and plated on BHI agar and incubated 48 h to 72 h at 37°C. Selective media, Baird-Parker agar and TS-blood agar supplemented with colistin and nalidixic acid, were also inoculated with overnight cultures and incubated 48 h to 72 h at 37°C. Colonies with different morphologies were selected for further analysis.

The 16S rRNA gene was amplified using *Taq* polymerase (Bioline) and universal primers, 27F (5′-AGAGTTTGATCATGGCTCAG-3') and 1492R (5′-GGTTACCTTGTTACGACTT-3′) using Colony PCR [69]. The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced using primers, 27F 1492R (Eurofins Genomics, Germany).

A BLAST search was carried out with the obtained sequence using the NCBI rRNA/ITS database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Closest bacterial species were identified using Blast tree view produced by Blast pairwise alignment.

5.4.7.4 - Inhibitory effects of *S. nobilis* venom against pathogens

Venom extracted from *S. nobilis* was assessed for its inhibition of bacterial growth by determining the Minimum Inhibitory Concentration (MIC). Due to the limited amount of venom available, dilutions were carried out on the samples to achieve usable volumes. The antibacterial potential of the venom was assessed

against clinical isolate *E. coli* DSM1103 (aka ATCC 25922, NCIB 12210) (DSMZ, Germany) *E. coli* DSM10973, Methicillin Resistant *Staphylococcus aureus* (MRSA) BH1CC and *L. monocytogenes* EGD-e. An overnight culture was adjusted with LB broth to an inoculum density of 1×10^6 cfu ml^{-1}. 1:10 dilution of the venom was tested against *E. coli* DSM1103 and 1:100 against other pathogens in a final inoculum of 5×10^5 cfu ml^{-1}, and were incubated for 24 h at 37°C. After 24 h incubation at 37°C, absorbance at 590 nm was measured using a microplate reader (Tecan) with Magellan software.

5.4.7.5 - Antibiotic susceptibility testing

Disk diffusion assays were carried out to determine antibiotic susceptibility. Experiment was done according to the CLSI guidelines. 6 mm discs preloaded with each antibiotic (Oxoid) were placed onto Mueller-Hinton agar plate that had been spread with 100 µl overnight bacterial culture (1×10^8 cfu/ml). Plates were incubated at 37°C for 18 h and the clear zone around each disc was measured.

5.4.8 - ACKNOWLEDGEMENTS

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Chapter 6
General Discussion

Adult male *Steatoda nobilis* *Photo by Andy Wilson*

6.1 - The Invasion of the noble false widow spider *Steatoda nobilis*

Between 1850 and 2000, at least 13 alien species of Theridiidae spiders have successfully established colonies across the European continent (Kobelt and Nentwig, 2008). Species that thrive in and around human habitats have an added advantage as increasing urbanisation and encroachment into natural habitat provides synanthropic species with further room to establish and increase their range (Nentwig et al., 2017). The spread of alien species has significantly benefited from globalisation facilitated by the massive increase of imported and exported goods and services.

Since the late 1800s, four species of *Steatoda* have established colonies in Ireland. *Steatoda bipunctata* was recorded for the first time in 1929 (Pack Beresford, 1929), with its distribution restricted to the Dublin metropolitan area. We reported on a thriving and well-established colony in a commercial tractor garage in Skehana Co Galway (Dunbar et al., 2018b), the only record outside Dublin since the first Irish report almost a century ago. It should also be noted that while sampling in Maynooth Co Kildare, we found a small colony of *S. bipunctata* thriving alongside *Steatoda nobilis* on a steel outhouse situated beside the canal and adjacent to the train station (unpublished data). This suggests that *S. bipunctata* may be more widely distributed across Ireland than reports may suggest but restricted to isolated populations. It is impossible to say if these small populations have also been present here for many decades and went unnoticed, or if they only recently established here. We also added two inland county records, Roscommon and Longford for *Steatoda grossa* (Dunbar et al., 2018b). Considering both *S. bipunctata* and *S. grossa* have been present in Ireland for over 90 years, they have both remained relatively obscure and have not presented any concerns to the ecosystem, or to human health. Currently, both species are considered naturalised. *Steatoda phalerata* is originally distributed in Western mainland Europe and the UK and was recorded in Ireland from a single location in Co Down approximately 30 years ago (McFerran, 1993, Merrett, 1989). In contrast to the favourable synanthropic habitats of other *Steatoda* species in Ireland, *S. phalerata* favours undisturbed grassland habitats. While Climate Change has been discussed

as a potential determining factor for the spread of *S. nobilis* in recent years (Bauer et al., 2019), observations on the other false widow species in Ireland (Dunbar et al., 2018b) suggest that the invasion is only occurring from *S. nobilis*, which suggest factors other than rising temperatures are fundamental, for example, adaptive genetic traits may have entered the population (Bauer et al., 2019).

The first record of *Steatoda nobilis* in Ireland is comparatively recent and occurred in 1998 when a thriving colony was found in and around a residential area in Bray, Co Wicklow (Nolan, 1999). The author concluded that this species future in Ireland was secured based on the fact that it could produce large numbers of offspring and tolerate cold temperatures. Despite reaching this conclusion, no further research had been carried out on *S. nobilis* in Ireland since this initial discovery. It is highly unlikely that *S. nobilis* would have been established in Ireland and passed unnoticed long before Nolan's report as Van Helsdingen (1996) did not include *S. nobilis* in his extensive review of Irish spiders (Van Helsdingen, 1996). It would be considerably easy to find *S. nobilis* due to its conspicuous cream-coloured dorsal patterns, the bright cream crescent on the antero-lateral part of the opisthosoma, its overall body size, and its preference to rest upside down openly on its web. Rapid colonisation events by *S. nobilis* has been reported before from California, where the species became widespread within three years of the first sighting (Vetter and Rust 2012; Vetter et al. 2015). It seems plausible that *S. nobilis* may have become established in Ireland only very recently prior to Nolan's initial report.

We reported the presence of *S. nobilis* in 16 new Irish counties and suggest this species has established widespread populations across Ireland. While some county records were represented by single observations, several coastal counties, had dense populations. From our observations in the field, we can conclude that *S. nobilis* was one of the most common spiders in the Dublin urban area. Recent sightings in midland and western counties e.g Westmeath, Longford, Offaly, and Galway, where only a few individual sightings were made a few years ago, indicate that *S. nobilis* is becoming more abundant.

Despite originating from a Subtropical climate (Thorell, 1875), throughout all months of the year, regardless of ambient temperatures, specimens of *S. nobilis* can be found outdoors (Dugon et al., 2017). Our observations of tolerance to cold conditions also confirms earlier reports (Snazell and Jones, 1993). They appear to be remarkably adaptable to the cooler temperate regions but in Ireland they currently remain restricted to urbanised settings. This might suggest a dependency on the microclimate provided by artificial habitats. However, it is concerning, and conceivable that a slight change in environmental conditions could facilitate a movement of populations from an urban environment into natural habitats. In warmer climates such as Italy, *S. nobilis* occurred first in urban areas and then spread to the surrounding countryside (Kulczycki et al., 2012). This is also seen in other parts of the Mediterranean where *S. nobilis* is now found in natural habitats; in south east England, *S. nobilis* is beginning to colonise semi-natural habitats. It is also suggested that specimens collected in California were in semi natural habitats (Bauer et al., 2019). It is also possible that exponential growth and higher population density will eventually force an overflow of specimens into semi-natural habitats (Bauer et al., 2019; Hambler, 2019). It seems very likely that a similar pattern will develop in Ireland: our observations on the density of some populations in Dublin suggest that urban populations may be reaching their carrying capacity and an invasion of surrounding rural habitats may happen soon. In the UK, the spread of *S. nobilis* has accelerated from its original southern locations at a rate of 11km/year since 2010 (Bauer et al., 2019). For more than a century prior to this, there was very little movement in population size or distribution (Dugon et al., 2017). However, the northern colonies are believed to be a result of human activities such as transport of goods and services rather than a true invasion (Bauer et al., 2019). In Ireland, it is most likely the same pattern, where rapid colonisation in some counties was facilitated by similar human activities whereas the actual invasion is happening from the east and south eastern coastal counties, particularly Dublin and Wicklow.

In Ireland, *S. nobilis* appears to favour a niche that mostly overlaps with three native species: the Lacewebbed spider *Amaurobius similis*, the Giant house spider *Eratigena atrica*, and the Missing sector orb weaver *Zygiella x-notata,* all of which also thrive in

synanthropic habitats and are commonly found around gardens and homes. In some observations the webs of *S. nobilis* were often interlocking with the webs of *A. similis*, *E. atrica*, and *Z. x-notata*. In North Dublin, we observed a specimen of *S. nobilis* sitting at the centre of a web built by *Z. x-notata*. When disturbed, the specimen ran off the *Z. x-notata* web and retreated into its own hide. In another case, when an insect landed on the web of a *S. nobilis*, a neighbouring *A. similis* ran on to the web of *S. nobilis* and dragged the bug back to its own retreat. This was also observed on a few occasions with *E. atrica*. These observations provide examples of niche overlap between *S. nobilis* native spiders.

Our samples show that in Ireland, *S. nobilis* can reach a maximum body size of 13.9 mm, with an average of 9.78 mm (Class size = 10 mm). *Z. x-notata* is significantly smaller with an overall Class size of 6 mm. *A. similis* are similar to *S. nobilis* in size (Class size 9 mm) and *E. atrica* are larger (Class size 13 mm) (Jocqué et al., 2016). However, despite their size and their competitive dispositions, *A. similis* and *E. atrica* do not appear to be strong competitors against *S. nobilis*. In one observation we found a male *S. nobilis* being subdued by *A. similis*. In addition to this, we observed a cellar spider *Pholcus phalangioides* feeding on *S. nobilis*. These were the only two observations of native Irish spiders predating on *S. nobilis* over the course of this research. However, we made regular observations of *S. nobilis* feeding on the carcasses of other spiders including the garden cross spider *Araneus diadematus* and large *E. atrica*. *S. nobilis* are very efficient in tackling other spiders by using a highly effective "attack wrap" strategy that allows them to immobilize potential predators without requiring physical contact (Forster, 1995). This strategy makes them especially effective competitors and predators of other spiders. In addition to other spiders, their diet consists of a diverse range of invertebrate prey, including other venomous organisms such as the Common European wasp *Vespula vulgaris*. Perhaps the most unusual observation was the predation event we reported on a native viviparous lizard *Zootoca vivipara* (Dugon et al., 2017, Dunbar et al., 2018a), which is also the first account of any spider feeding on a vertebrate organism in Ireland. *S. nobilis* produce strong silk, and in addition to their very efficient "attack wrap" strategy, they also construct webs at any height, from ground level to several meters

above ground. This means they certainly have opportunity in some instances to encounter small vertebrates and *S. nobilis* has certainly demonstrated their ability to overwhelm animals many times larger than themselves.

S. nobilis also have a high reproductive rate (Locket, 1979, Nolan, 1999). We found that on average, eggsacs contained 94 eggs (N=50) and ranged between 34 - 208 eggs. The most prolific spider produced four eggsacs within four months (Dugon et al., 2017). Previous reports show that *S. nobilis* can still produce viable clutches 18 months following fertilisation (Locket, 1979). In comparison, *E. atrica* can produce approximately eight eggsacs over a year, each comprising between 20 - 150 eggs (Napiorkowska et al., 2018) while *A. similis* produces several eggsacs per year comprising of 80 – 100 eggs (Kim and Horel, 1998) and *Z. x-notata* has been shown to produce one to three egg sacs per year, each containing c.10–70 eggs (Wherry and Elwood, 2009).

Considering the various ecological aspects discussed above, *S. nobilis* may well have the ability to outcompete some of the most common native urban dwelling spiders in North-Western Europe. Although the impact of alien spiders on Irish ecosystems has never been investigated, *S. nobilis* appears to have an exceptional ability to compete against native Irish spiders. The prospects of a highly invasive spider disrupting native ecosystems is extremely concerning. Further field-based studies and long-term surveys will be needed to monitor the range expansion of *S. nobilis* and to assess the impact this seemingly highly invasive spider is having on our native species.

The venom of *S. nobilis* has been investigated from a proteomics perspective and will be discussed in more details further below, however, an initial conclusion indicates a predominantly neurotoxic venom. One of the limitations in this study is there are no measures made against native Irish species. Further comparative investigations into the potency of *S. nobilis* venom versus other native Irish spiders and LD_{50}'s against a range of prey would shed light on the potential of *S. nobilis* to disrupt specific niches. Predictive modelling identifying potential habitats suitable for future populations has confirmed the potential for *S. nobilis* to become one of the world's most invasive species of spiders (Bauer et al., 2019).

6.2 - Medical implications of bites from *S. nobilis*

The first recorded bite from *S. nobilis* was in 1991 when a hospital patient in Worthing, southern England, was bitten on the neck by a female specimen (Warrell et al., 1991). The victim presented with local and systemic neurotoxic symptoms resembling a moderate form of latrodectism. The next case reported in the literature was 25 years later in Chile in 2016 (Faúndez and Téllez, 2016) when an adult male was sleeping on the floor and woke to a sharp pain in the cheek. The victim experienced similar symptoms to the 1991 case (Faúndez and Téllez, 2016). Both victims recovered fully within 24 hours. In contrast, alleged reports of bites from *S. nobilis* are headlining the media throughout Ireland and the UK for over a decade. These often include alarming symptoms, such as debilitating tissue necrosis and bacterial infections, and in some cases resulting in death (Hambler, 2019).

Victims of envenomations by *Latrodectus* rarely require medical attention, and only a small percentage result in severe symptoms and death. For example, 50% of victims experience severe pain, and redness, and diaphoresis are among the common symptoms. <35% of victims experience systemic effects that include nausea, vomiting and headaches. During a period of eight years, 23,000 bites by *Latrodectus* were reported to the USA National Database. Approximately 42% experienced envenomation symptoms and 1.4% of cases developed as severe, and no fatalities were recorded (Hauke and Herzig, 2017).

The recent range expansion and high population density in urbanised habitats by *S. nobilis* has led to an increase in contact with humans resulting in envenomations. Previous cases reported in the scientific literature include one in Great Britain (Warrell et al., 1991), two in Chile (Faúndez et al., Faúndez and Téllez, 2016) and one possible case from Columbia (Porras-Villamil et al., 2020). These cases described local and systemic neurotoxic symptoms like latrodectism that include prolonged, moderate to intense pain, swelling, erythema, piloerection, diaphoresis, and facial flushing (Warrell et al., 1991, Faúndez et al., 2020, Faúndez and Téllez, 2016). We

provide 20 additional case reports bringing the total confirmed cases to 23, and we report several unreported symptoms and pathologies for this species.

Prolonged moderate to intense pain, erythema, swelling, and pruritus are the most common symptoms of envenomation by *S. nobilis* (Dunbar et al., 2018). Other symptoms include vasodilation of capillaries, and necrosis localised at the bite site, radiating pain from the bite site, local sweating, a sensation of heat, tenderness, piloerection, inflammation, irritation, raised skin, and paraesthesia around the bite site, muscle contractions in the affected area, reduced mobility in the affected limb, and lump/pimple and blister formation at the bite site, facial flushing, feverishness, elevated and low blood pressure (Dunbar et al., 2018, Faúndez et al., 2020, Faúndez and Téllez, 2016, Warrell et al., 1991). Severe debilitating pain was documented in three victims. One victim felt intense pain radiating from her hip to the neck and jaw line and down to her ankle for 24 hrs. Another victim experienced debilitating pain requiring strong analgesics that target directly the central nervous system, and a third victim required weeks of opioid medication to manage the pain. Among the more concerning symptoms is the severe debilitating pain, tremors, hypotension, nausea, muscle contractions and impaired mobility of the affected limb, which are *Latrodectus*-like symptoms (Dunbar et al., 2018, Erdur et al., 2007, Faúndez and Téllez, 2016, Pneumatikos et al., 2003, Vetter and Isbister, 2008). These symptoms share close similarities to the neurotoxic symptoms by *Latrodectus* species (Isbister and Fan, 2011). Consequently, although most cases are not considered serious with symptoms remaining mild to moderate, envenomations by *S. nobilis* have also causes severe outcomes and therefore should not be trivialised, especially when bites by *Latrodectus* have caused severe morbidity, occasionally with fatal outcomes (Isbister and Fan, 2011). It is important to fully understand the full potential range of symptoms before dismissing them, particularly since the increase in range and population density of *S. nobilis* in urbanised settings globally and bite incidents are increasing. Moreover, despite *Latrodectus* venom and envenomation outcomes are widely studied, increasing reports of the cardiotoxic effects by *Latrodectus* bites demonstrate that we still don't fully know the full extent to which the complexity of this venom can affect

biological systems (Dendane et al., 2012, Erdur et al., 2007, Levine et al., 2010, Pneumatikos et al., 2003, Sari et al., 2008).

Significant effects from other *Steatoda* species are reported in Australia. *Steatoda grossa* and *Steatoda capensis* are introduced species and solely responsible for all bites by *Steatoda* in Australia (Isbister and White, 2004). The clinical syndrome was indistinguishable from *Latrodectus* in some cases (Isbister and Gray, 2003) but overall, most were less severe than *Latrodectus*. Symptomatic treatment was predominant (Isbister and White, 2004) and severe cases were effectively resolved using redback spider (RBS) antivenom (Isbister and Gray, 2003). In vitro studies revealed the venom from *Steatoda capensis* and *Steatoda grossa* were more potent towards insects than *Latrodectus* and less potent than *Latrodectus* towards mammals (Atakuziev et al., 2014). However, these studies have not been carried out for *S. nobilis*. Overall, case reports suggest that while most envenomations by *S. nobilis* are mostly less severe than *Latrodectus*, they significantly overlap and do have the potential for severe outcomes.

Reports of tissue necrosis headlining the media has caused controversy as necrosis following spider bites are not typical except for envenomations by members of the genus *Loxosceles*. In both of our cases series, we assessed multiple occurrences of minor necroses localised at the bite. Necrosis occurred only in victims who initially experienced more intense symptoms and consistently presents as a small wound that recovers without further issue. While our findings confirm that necrosis is possible, extensive debilitating necrosis does not seem likely. It seems plausible that a larger wound could develop but the evidence currently available does not support the claim that extensive necrosis is a regular symptom of envenomations by *S. nobilis*.

Some of the most concerning media reports involve the development of bacterial infections post-envenomation, resulting in prolonged and debilitating pathologies that are sometimes unresponsive to antibiotics. In the 23 cases published to date, three of these experienced mild to debilitating bacterial infections including cellulitis and dermatitis. The implication of the spider as the source of these bacterial infection is controversial. The spiders responsible usually are crushed, escape, or are captured

using non-sterile methods, therefore comprehensive microbiological analysis is not possible. Infections associated with spider bites are typically caused by bacterial species commonly found in the environment and on human skin (Vetter et al., 2015). Moreover, spider venoms are considered a rich source of antibacterial peptides (Saez et al., 2010). It has been previously proposed that venom neutralise bacteria and therefore infections are not vectored by the spider during envenomation (Esmaeilishirazifard et al., 2018, Ul-Hasan et al., 2019, Vetter et al., 2015). So far, no study has determined if infections are caused by opportunistic bacteria either present on the skin (secondary infections), or on the chelicerae (Vector-Borne Bacterial Zoonoses) following bites from *S. nobilis*. The consequence of this knowledge gap can have profound implications for treating the victim. Knowledge on the potential species of bacteria vectored can influence the correct course of antibiotics. More importantly, if knowledge is obtained on the potential for drug resistant strains then decisions on the correct course of treatment can be made early on. We provided the first comprehensive dataset on the bacteria present on the body surfaces and fangs of *S. nobilis* and two native spiders, *Amaurobius similis* and *Eratigena atrica*, which are common house and garden spiders that are capable of biting humans. We tested the proposition that spider venom has antimicrobial properties and will neutralise bacteria present/vectored during a bite. We concluded that in the case of *S. nobilis*, the venom has no inhibitory activity. Prior to this, the consensus was that bacterial infections are unrelated to the bite and that wounds only get infected later from poor hygiene and rubbing. The social implications for this are that victims are further impacted by stigmatisation after being deemed 'unhygienic".

In the three cases presented by us, one required hospitalisation and an aggressive course of intravenous antibiotics, which took approximately 3 months to recover. The victim was hospitalized three times and treated with intravenous antibiotics and OxyContin for two weeks for pain relief. The idea that bacteria can be commonly transmitted by virtually any insect, but spiders are deemed sterile creatures is ridiculously impractical and misguided. The role of spiders in bacterial transmission should be recognised as nontrivial and infections deemed potentially as vector-borne bacterial zoonoses. Although to conclude a spider bite did occur, the spider needs to

have been seen during the bite or within close proximity shortly after, for example falling out of clothing, or in bed sheets. Given the nature of spiders, especially given the circumstances that if it was willing to bite, means it felt threatened and therefore fleeing is its priority. It is therefore reasonable to conceive that numerous infections arising from genuine spider bites must go unreported due to the absence of the spider. This could be helped by characterising bacterial communities on spiders and fangs from target species and compiling databases for clinicians to cross reference swab cultures from hospital admissions.

Further studies should include a focus on determining the LD_{50} for *S. nobilis* using mouse models and comparisons should be made towards *Latrodectus* species. Overall, there seems to be a complete absence of LD_{50} models for the genus *Steatoda*. Warrell et al., (1991) mentions that *Steatoda paykulliana* has five-fold the LD_{50} than *Latrodectus tredecimguttatus.* However, with advances in clinical techniques and our understanding of regional variation in venom composition there is a need for broader populations of *Latrodectus* and *Steatoda* species to be tested for LD_{50}'s. If *S. nobilis* individuals with adaptive genetic traits have indeed entered the population through gene flow as suggested by Bauer et al., (2019) leading to beneficial traits allowing *S. nobilis* to significantly expand its range and population density, it is reasonable to imagine that a shift in venom composition is also possible. This supports the need for comparative LD_{50}'s across populations, specifically compared to regional populations from North Africa and the Macaronesian archipelago. Other future studies should also focus on case series. While we have defined a large range of symptoms, we still need to try and establish how often people are bitten.

6.3 - The *Latrodectus-like* Venom of *S. nobilis*

Animal venoms are complex cocktails of toxic proteins that evolved as a primary means to immobilize and subdue prey. The ability of venomous animals to target the various pathways of multiple prey types is facilitated by a diverse toxin repertoire (Lyons et al., 2020, Whiteley et al., 2016). It is also suggested that venom plays a role in the pre-digestion of the prey's tissue prior the expulsion of digestive enzymes from the spider's digestive tract into the prey to facilitate the external digestion before

sucking up the liquefied meal. Consequently, as described above, *S. nobilis* are opportunistic, often faced with tackling invertebrate, and/or vertebrate prey that can be strong, fast, aggressive, and many times larger than themselves. Therefore, the most effective way to immobilize captured prey safely and efficiently is by inducing rapid paralysis.

Unsurprisingly, we report on the venom composition of *S. nobilis* being comprised mostly of neurotoxins. However, the high concentration of *Latrodectus-like* toxins (over two thirds of the overall toxin composition) was more surprising. *S. nobilis* venom contained the three most notable neurotoxins found in the venom of *Latrodectus*: α-latrotoxin, α-latrocrustotoxin, and δ-latroinsectotoxins, named after their ability to selectively target the nervous systems of prey that include insects, crustaceans, and vertebrates. Even more surprising was the higher concentration of α-latrotoxin in *S. nobilis* venom than that found in *Latrodectus* venom. Latrotoxins selectively target the neuromuscular junctions of prey. For example, α-latrotoxin binds to specific receptors called neurexons and latrophilins located on the membrane of the presynaptic nerve. α-latrotoxin is a tetramer and after binding with its corresponding receptors it inserts its base into the membrane (Orlova et al., 2000, Saibil, 2000, Ushkaryov et al., 2008). Once membrane-bound, the centre part of the protein acts as an ion channel that facilitates an influx of calcium ions into the axon terminal. Interactions with the synaptic vesicles triggers extensive exocytosis of acetylcholine, a neurotransmitter involved in muscle contraction, these are dumped into the synapse and fail to bind to receptors on the post synaptic cleft (Ushkaryov et al., 2004). The action of this toxin is predominantly responsible for the intense pain and overall neurotoxic symptoms after envenomation by *Latrodectus* and *Steatoda* (Haney et al., 2014, Ushkaryov et al., 2008). The activity is supported by a number of studies using mutant strains (Krasnoperov et al., 1999) and the development of monoclonal IgG antibodies designed to neutralise the effects of α-latrotoxin (Bugli et al., 2008). It is also likely that the actions of α-latrotoxin are key to immobilizing vertebrate prey such as small mammals and reptiles. latrocrustotoxin have a specific potency towards crustaceans, latroinsectotoxins are particularly potent towards insects and α-latrotoxins are apparently specific to targeting vertebrates. However, it

is questionable to conclude that α-latrotoxin is present to facilitate the paralysis of vertebrate prey alone and not play a primary role in subduing invertebrates.

Latrodectus and *Steatoda* are documented preying on small vertebrates including reptiles and mammals but only a handful of reports exist, studies quantifying the diet of *Latrodectus lilianae* indicate that vertebrates make up only a small part of their diet (Hódar and Sánchez-Piñero, 2002). Some studies concluded that α-latrotoxin are potent only to vertebrates and not invertebrates (Südhof, 2001). However, this is assumed by the activity of purified fractions of α-latrotoxin but little is known about the activities of associated compounds such as latrodectins. Afterall, α-latrotoxin is the most abundant toxin in the venom of *Latrodectus* and *Steatoda* and it would seem extremely counterintuitive to invest more energy to produce a toxin that targets an insignificant proportion of prey, and invest so little energy in a few toxins that target the majority of prey. Latrodectins are suspected of enhancing the potency of latrotoxins by altering ion balance near different channel types, thus regulating Ca^{2+} influx and neurotransmitter release. While they are not known to be toxic to insects or mammals in their purified form (McMahon et al., 1990) it is suggested they can synergistically increase the potency and non-specificity of α-latrotoxin towards invertebrates. This would certainly account for the high content of α-latrotoxin in the venoms of *Latrodectus* and *Steatoda*. In this context, α-latrotoxin should not be deemed a vertebrate specific neurotoxin given that these conclusions were reached solely on the activity of the toxin in its purified form without taking into consideration how other venom toxins might influence its effect when acting in synergy. In nature, α-latrotoxin may indeed be the most important toxin for inducing rapid paralysis in all prey types facilitated by the interactions of other venom components. While the latrotoxins represent the most powerful toxin classes in the venom of *S. nobilis*, other neurotoxins were detected that act simultaneously to facilitate targeting and disrupting various aspects of normal nerve function. Furthermore, enzymes including metalloproteases, serine proteases and chitinases also present in large quantities suggesting that these provide the enzymatic machinery necessary to facilitate the spread of the venom.

The array of enzymatic proteins in the venom of *S. nobilis* may also play a role in the cell death observed at the bite site. Likewise, the predominant α-latrotoxin can also kill cells in a detergent like manner (Südhof, 2001). This is associated with high concentrations of α-latrotoxin, such as the ones suggested by our data. α-latrotoxin appears to be more abundant in the venom of *S. nobilis* than in *Latrodectus*. Furthermore, these small wounds are associated in victims with the most intense symptoms. While it is currently impossible to measure the quantity of venom delivered into the victims, it seems likely that severe envenomations should correlate with a higher volume of venom delivered. The potential for necrosis to occur through inflammatory pathways of regulated necrosis is also a possibility and would also likely correlate with intense symptom onsets (Dunbar et al., 2019). This correlation is observed in a range of cases involving multiple bites/stings from venomous arthropods where necrosis forms at the bite sites after envenomations by species with neurotoxic venoms (Dunbar et al., 2019).

Future studies should focus on the venom profiling of male *S. nobilis* as other studies have demonstrated contrast between the genders, which can influence the potency. Further venom profiling should include other *Steatoda* species and regional populations, to include North Africa and the Macaronesian archipelago. Studies should also focus on selective cohorts under different diets including siblings divided and/or wild populations versus offspring fed on single diet species such as commercial crickets. This will reveal the potential for environment to play a role in influencing the venom composition.

6.4 - CONCLUSION

The objective of this study was to provide a comprehensive background of the ecology, behaviour and potential health risk of *S. nobilis* to provide researchers and the medical community with the necessary information to make decisions on where to focus targeted research and improve patient treatment and care. Data indicates that *S. nobilis* is continuing to expand its range and population density in urbanised habitats are increasing. It is extremely likely that bites by *S. nobilis* are significantly

underreported but our studies provide knowledge of the symptom spectrum, factors that contribute to pathology and variation in onsets. Overall, bites by *S. nobilis* are capable of medically important outcomes from envenoming and transmission of antibiotic-resistant strains of pathogenic bacteria and should be recognized as a species of medical importance. The rise of *S. nobilis* may result in an emerging public health issue, with potential serious outcomes for some victims. This species deserves close monitoring and research by both the scientific and medical community and updated public awareness by public health authorities. We conclude from this extensive study that steatodism is a rare, but potentially severe, clinical syndrome caused by noble false widow spider *S. nobilis*.

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