DECEPTION & MANIPULATION:
ARGYRODINAE SPIDERS AS PARASITES AND HOSTS

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Abstract

Spiders (order Araneae) are a diverse and successful taxon, present across the world in even extreme environmental conditions. Much of this success is due to their production of silk which, among other uses, forms webs with which to effectively capture prey and avoid predators. However, these resource-intensive external structures are vulnerable to exploitation by organisms utilising the silk structures and captured prey for their own benefit, termed kleptoparasites.

One such parasite is the small spider *Argyrodes argyrodes*, within the sub-family Argyrodinae. The species is commonly found in groups on the webs of other spiders across the Mediterranean, West Africa, Canary Islands and Seychelles. *A. argyrodes* has been observed scavenging, stealing food bundles, feeding alongside the host, eating host eggs and young and even attacking moulting hosts.

This thesis investigates *A. argyrodes* kleptoparasites living on the webs of colonial *Cyrtophora citricola* in southern Spain. The aggregation of *C. citricola* to form sizable, long-lasting, web structures makes them an ideal host to provide large, stable habitat patches for kleptoparasites. Research presented here shows the abundance patterns and reproductive efforts of Spanish *C. citricola* through a year, showing an unusually dominant reproductive period in spring which is closely followed by the reproductive efforts of *A. argyrodes.* High frequency of *C. citricola* egg sac infection by *Philolema palanichamyi* wasps in autumn is postulated here to skew *C. citricola* reproduction and data supports a resistance to wasps provided by aggregation into larger colonies. Larger colonies also contained lower *A. argyrodes* numbers per host, providing further selective pressures favouring colonial strategies in spiders.

Kleptoparasitic behaviour by *A. argyrodes* is described within this thesis' preliminary work and feeding alongside the host was found to be common in large kleptoparasites. *A. argyrodes* presence is then shown to reduce feeding frequency of *Cyrtophora citricola*, postulated to have a negative effect on

nutritional intake and so fitness. However, no influence was found in web repair or prey dropping behaviour of hosts.

Adaptations of *A. argyrodes* to this kleptoparasitic specialism are also considered. Firstly, it is theorised that, as the parasites produce little silk of their own, relaxation in selective pressures might lead to a higher rate of mutation retention in unused silk protein sequences,. No support for this is found, with *A. argyrodes* and other kleptoparasitic Argyrodinae showing similar variation to that of free-living Argyrodinae in their major and minor ampullate silk primary structure.

Abundance data collected as part of this thesis indicates a female-biased sex ratio in Spanish *A. argyrodes.* To this end, the microbiome of *A. argyrodes* is investigated and targeted screening explored infection frequency of manipulative endosymbiotic bacteria across populations. These studies found *Rickettsiella, Rickettsia, Cardinium* and *Spiroplasma* bacteria within *A. argyrodes* and identified strains present to be either unique to the species or to spider hosts through 16S sequence comparison. *Cardinium* and *Spiroplasma* population screening found each present in approximately one quarter of samples across populations. One population tested contained neither bacteria and this is expected to be a founder effect brought on by fluctuating and genetically similar populations.

This thesis provides an initial snapshot of a system involving colonial and parasitic spiders little explored in the literature, providing a basis with which to continue work on the ecology, behaviour, silk production and microbiome of arachnids with such extreme strategies.

Acknowledgements

While direct contributions to the results presented are listed within individual chapters, acknowledgement should be given to the huge amount of support I have received both directly in the science included but also in my journey through the doctoral process. Firstly, thanks should be given to my supervisors Sara Goodacre and Tamsin Majerus, without which none of this would exist and my time at Nottingham would have been short, sad and spider-less! I am also so very grateful to Tom Hartman for making my Nottingham University journey such an exciting and fulfilling one.

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Declaration

I, Ella Deutsch, declare that this thesis is the result of my own work, unless otherwise stated, and that it has not been presented as part of any other degree.

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Chapter 1 - Introduction: Spiders & Symbiosis

1.1 Introduction to spiders

The term ‘spider’ describes arthropods of the order Araneae, air breathing arachnids with eight legs and chelicerae including venomous fangs. The World Spider Catalogue records over 47,000 species within 117 families, showcasing a wide diversity of physiologies and ecological strategies. Diversity is demonstrated from large, hairy mygalomorphs to tiny linyphiids, able to travel on the wind with just one fine thread of silk; from the sharp eyesight of salticid jumping spiders to the eyeless huntsman *Sinopoda scurion* (Jaeger, 2012); and from the gluey, projective venom of the *Scytodes* spitting spider (Suter & Stratton, 2013) to the unusually venomless *Uloborus* (Weng *et al.,* 2006).

Spiders colonise a huge variety of environments, including arid deserts, waterlogged bogs, deep burrows and lofty tree canopies (Buchholz, 2016; Höfer *et al.,* 1994; Uchman *et al.,* 2018). They can survive and thrive in extremes of temperature, rainfall, prey availability, urbanisation and even pH (Cloudsley-Thompson, 1983; Koponen, 1992; Marco Isaia, personal communication, 2020; Magura *et al.,* 2010). The order is not only confined to terrestrial habitats, some species will dive into freshwater to hunt (Zimmermann & Spence, 1989) and others will even reside entirely underwater, using their silken sheets to trap air to breathe (Seymour and Hetz, 2011).

The diversity in spider physiology is demonstrated in Figure 1.1, along with the phylogenetic relationships between families, as presented by Garrison *et al.* (2016). Species considered within this thesis sit within the families Theridiidae (comb-footed, cobweb or tangle-web spiders) and Araneidae (orb-weavers), both of the infraorder Araneomorphae, sometimes referred to as ‘true’ spiders, which are generally short-lived and light of body compared to the Mygalomorphae.

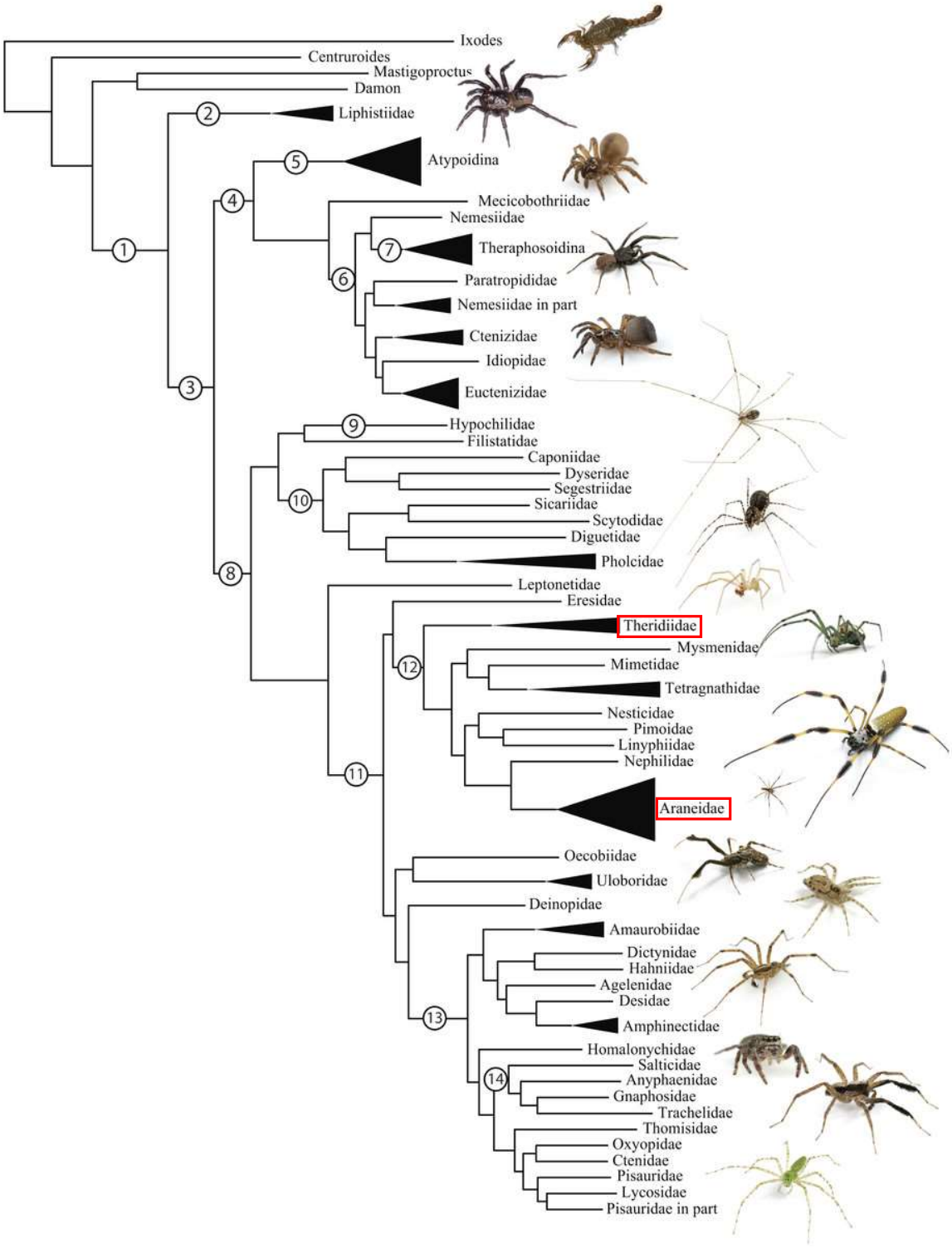

Figure 1.1. Phylogeny of spider families alongside arachnid outgroups. Positions of families considered further in this thesis, Theridiidae and Araneidae, are outlined in red. Figure modified from Garrison *et al.* (2016), under CC licence. Araneomorphae emerge from node 8. Araneoidea emerge from node 12.

1.1.1 Spiders in agriculture

Spiders are among the most common and abundant predators in terrestrial ecosystems (Nyffeler & Birkhofer, 2017) and, with their ability to travel widely and successfully colonise new and harsh environments, spiders can often be found at high abundance within agricultural ecosystems (Sunderland & Samu, 2000). They are largely generalist predators and consume an estimated 400-800 million tonnes of prey per year worldwide, over 90% insect (Nyffeler & Birkhofer, 2017), making them important natural enemies of insect pests. It is speculated that a healthy population of spiders within agricultural fields and in adjacent areas can, therefore, be considered integral to the control of crop pests especially where pesticides are discouraged due to environmental and health concerns. However, agricultural fields can present numerous threats to spider populations; including the homogenisation of plant structure, land disturbance and extensive use of pesticides. A large variety of agricultural land uses include the homogenisation of plant matter, whether through grazing by livestock reducing plant structure and diversity or the cultivation of plants of an identical species and age for harvesting (Buhk *et al.,* 2017; Salgado-Luarte *et al.,* 2019). Spiders can be very reliant on plant architecture to build webs and find shelter, so their numbers and diversity can be greatly impacted by these practices (Potapov *et al.,* 2020). Increased crop diversification as well as the provision of non-crop areas has been shown to encourage spider populations (Sunderland & Samu, 2000; Theron *et al.,* 2020).

Another direct threat is the use of agro-chemicals designed to kill insect, plant and fungal pests. Pyrethroids, a commonly used group of insecticides, have been shown to be lethal to spiderlings of the family Linyphiidae (Dinter & Poehling, 1995) and other pesticides have also shown varying lethality, up to 100%, in adult spiders (Košulič *et al.,* 2018; Pekár & Beneš, 2008). Pekár & Beneš (2008) found increasing lethality in pesticide residues over time, especially to ground dwelling spiders which might otherwise be unaffected by agricultural practices such as a reduction in plant diversity. Sub-lethal effects are also common, with pesticide presence reducing the number of eggs produced in the wolf spider *Pirata piraticus*, suggested to be due to a trade-off to increase propagule size in a stressful environment, and can also reduce the

predation rate of individual spiders even with prey at high densities (Deng *et al.,* 2008). Pesticides can also cause decreased locomotion, web building behaviour and even paralysis of legs (Baatrup & Bayley, 1993; Pekár & Beneš, 2008; Rhoades & Stoddard, 2021), impacting predation rate and survival. Other chemicals used in agricultural management can also influence spider populations, such as the production of fewer eggs, early maturation, a weakened immune system and reduced reproductive activity in spiders exposed to heavy metal pollution (Hendrickx *et al.,* 2003; Maelfait & Hendrickx, 1998; Wiśniewska *et al.,* 2022).

Even within agricultural systems managed in such a way as to encourage spiders, an obstacle to effective pest control is their generalist predation extending to other spiders, with intraguild predation becoming more common with increased density. This aggression towards conspecifics is not shared by all spider species, with some living very close with varying amounts of tolerance and cooperation. Lubin & Bilde (2007) define the use of the term ‘social’ within arachnology as spiders living in family groups, often with a shared web and cooperation in foraging and raising young. The term ‘colonial’ is used to describe spiders that also spin large, interconnected webs, but with these spiders keeping individual territories and foraging separately (Lubin & Bilde, 2007). These cooperative behaviours have evolved numerous times within different spider families (Kullmann, 1972) and are present across a wide number of environments and life history strategies, making less aggressive species potentially beneficial within agricultural systems.

Despite these potential benefits, management strategies are required to monitor colony size. Where web-living spiders live in large colonies, they provide risks to plants, including those of importance to agriculture. Colonial spiders within the genus *Cyrtophora* have been described in numerous agricultural environments as a pest, their extensive web networks covering plants and causing significant damage and death through yet unidentified means (Chander & Chauhan, 2017; García *et al.,* 2001). The dense webbing also causes reduced efficiency and safety concerns in the harvesting of crops (Chuang, 2019). This significant agricultural impact creates a requirement to

monitor and manage certain spider populations within such systems without removing beneficial spider species.

1.1.2 Silk production and use

Key to the success and diversity of the spiders is their production of silk, a biomaterial with which spiders can produce protective egg sacs, burrows, sturdy support lines, glues to form attachments and trap prey, flexible silks to form capture webs and lines to act as parachutes for dispersal (Vollrath, 1992; Herberstein & Tso, 2011). Silk can even be used to form capture nets held between four legs, underwater sheets trapping air while allowing gas exchange and even projectile glues (Coddington & Sobrevila, 1987; Correa-Garhwal *et al.,* 2019; Suter & Stratton, 2013). Diversity in spider physiology and ecology is linked to a huge diversity in both the construction behaviour and protein properties of silk creations (Hayashi & Lewis, 2000; Vollrath & Seldon, 2007).

This diversity is not only present between species but also within each individual spider. Seven different silk types have been identified and female individuals within some families, such as the orb-weaving Araneidae, produce all seven across their lifetime. These silk types and generally accepted uses are illustrated in Figure 1.2.

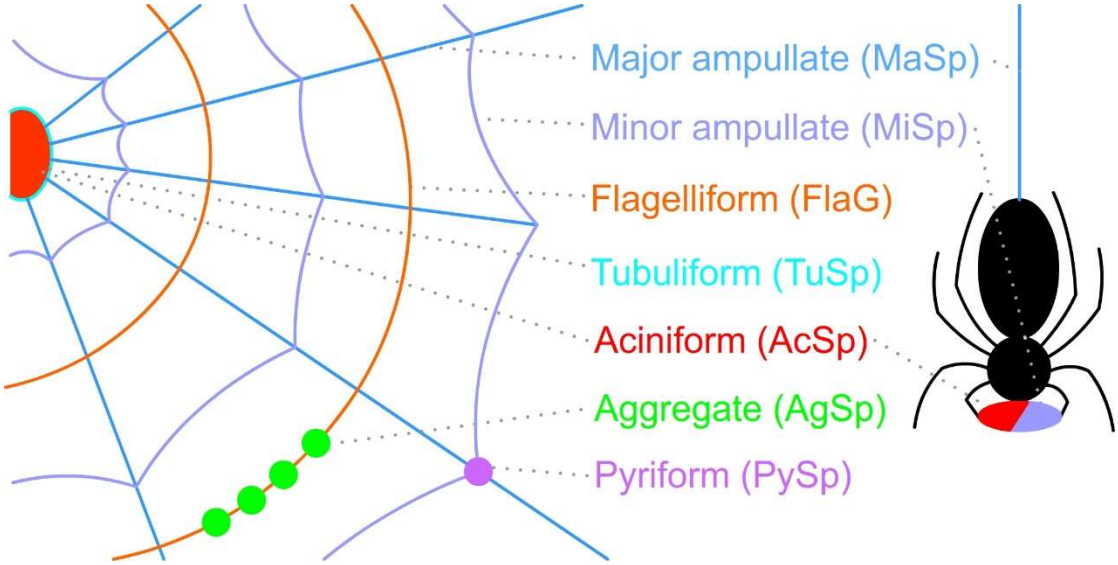

Figure 1.2. The seven silk types produced by spiders, with generally accepted uses illustrated.

Major ampullate silk (MaSp) is perhaps the most well categorised silk type, generally forming the strongest silks which are used for safety draglines and

web support scaffolds. Ancestrally, MaSp silk was utilised primarily in terrestrial webs or acted as lifelines (Blackledge *et al.,* 2009) and the innovative shift by some of the Araneomorphae towards utilising MaSp in aerial webs drove significant modification to its properties, and so encoding genes (Gatesy *et al.,* 2001). In many species, it demonstrates remarkable tensile strength, toughness and elasticity when compared to man-made materials such as Kevlar (Gosline *et al.,* 1999).

Minor ampullate silk (MiSp) is used as the auxiliary spiral in web construction, stabilising the web, providing a template for the capture spiral and sometimes in prey wrapping (Mattina *et al.,* 2008; Römer and Scheibel, 2008). While this silk shows a similar chemical structure, it is thinner, less tough and less strong than MaSp silk and is usually spun with support (Stauffer *et al.,* 1994; Papadopoulos *et al.,* 2009).

Flagelliform silk (FlaG) is used in the capture spider of an orb web and has a slightly lower tensile strength than MaSp. However, it demonstrates over a 200% extensibility, in order to absorb the force of colliding prey (Adrianos *et al.,* 2013). In some families, such as the Theridiidae, this silk is also used to wrap prey (Eberhard, 2010).

Aggregate silk (AgSp) forms an aqueous coat of sticky droplets along silk fibres, often FlaG, and acts to adhere to prey and hydrate the fibre (Vollrath *et al.,* 1990). AgSp is unusual in its composition, having a substantial nonprotein component in addition to water, and behaves as a viscoelastic material in order to best retain stuck prey (Townley & Tillinghast, 2013).

Tubuliform silk (TuSp) is used for the tough outer casing of the egg sac and, as such, is only produced by female spiders. It is the only silk that is not produced consistently during a spider's lifetime, with focused production over the reproductive period (Tokareva *et al.,* 2014). This silk shows similar properties to MiSp in strength and elasticity but demonstrates a limited capacity for bending without breaking, a glasslike or crystalline behaviour (Stauffer *et al.,* 1994).

Aciniform silk (AcSp) fibres are usually used to wrap prey and line the interior of egg sacs. It is also used in the formation of sperm webs and the decoration of webs (Foelix, 1996). AcSp is almost twice as tough as MaSp, but this is mainly due to its greater extensibility rather than the strength of the fibre (Hayashi *et al.,* 2004).

Finally, pyriform silk (PySp) is primarily involved in attachment of silks to substrates by forming attachment discs, as well as the connection of separate web fibres (Blasingame *et al.,* 2009). Attachments can differ in their adhesion strength through the spinning of different disc architectures, allowing some webs with strands designed to detach on prey capture while also ensuring strong adhesion of safety lines (Sahni *et al.,* 2012).

Each of these silk types have a conserved general structure, indicated to be necessary for the formation of the fibres. This consists of conserved N- and C-terminal domains bracketing a repetitive region which forms most (> 90%) of the protein. While the terminal domains promote fibre formation, the repetitive region is responsible for much of the diversity in silk fibre properties and shows most divergence between both spider species and silk types. For further information see Chapter 5.

With its remarkable physical properties alongside biocompatibility, utilisation of spider silks to produce novel biomaterials has long been of interest (Allmeling *et al.,* 2013). Farming spiders to produce any substantial amount of silk cost-effectively is impossible due to their cannibalistic nature, leading synthetic production to be the route forward. This involves the design of short synthetic gene sequences based on natural spider silk genes, with a focus on silks with desired properties. Protein is then produced using an expression system, with examples ranging from *Escherichia coli,* yeast and even goats (Edlund *et al.,* 2018; Decker, 2018; Fahnestock & Bedzyk, 1997; Fahnestock & Irwin, 1997). Expressed protein is in the form of a liquid ‘dope’, in the case of goats released with their milk. The resultant protein can then be purified and spun into a fibre through biomimetic processes that replicate selected important molecular mechanisms of native silk formation, such as changes in pH and temperature alongside applied forces (Andersson *et al.,* 2017; Chen *et al.,* 2006).

The production of synthetic silk, therefore, relies heavily on knowledge of the gene and protein structures underpinning exemplary silks, as well as spider silk gland physiology and spinning behaviours. This allows the targeted modification of the production process to produce materials with custom properties. To this end, increasing focus is on the sequencing of silk from diverse spider groups and understanding how this information is linked to final silk properties. Synthetic methods assist this effort through experimental modification of specific amino acid motifs in order to test the impact on final protein properties (e.g. Teulé *et al.,* 2007).

1.2 Symbiosis

Symbiosis was originally coined by Anton de Bary (1879) who defined it as 'the living together of dissimilarly named organisms', a description which encompasses parasites, commensals and mutualists (Martin & Schwab, 2012). Although there has been much debate in the subsequent years over the term, largely to better define it through extensive description, the broad definition of 'living together' can be considered in many cases.

Where a symbiont lives on (ectoparasite) or in (endoparasite) another organism and causes it harm, it can be considered a parasite. These organisms are usually much smaller than their hosts and will often not kill them, in order to receive nutrients and protection from an individual host for an extended period of time.

Not all symbiosis is negative for one party, with commensalism describing the process by which one species gains benefits with no effect to the other and mutualism describing an interaction benefiting both parties. An example of commensalism comes from the larvae of mosquitoes and midges within the neck of pitcher plants (Stephen, 1994). Each of these species feeds on decaying animal matter but they are not competitive; feeding by the mosquitoes did not affect the nutrition available to the midges and midge density increased particulate matter that was broken down sufficiently for the mosquitoes. It is often difficult within biological systems to isolate specific benefits and costs, nutritional or otherwise, that a party is receiving and so placing interactions within the banner of parasite, commensal and mutualistic can be challenging.

1.2.1 Parasitism and kleptoparasites

The symbiosis between organisms in which one benefits from the derivation of nutrients to the other's expense is termed parasitism. Windsor (1988) argues that, considering the numerous parasites present within every free-living organism, parasitism is the dominant strategy and all biological work must be underpinned by this understanding. Organisms should be explored with the parasites within as a key consideration and further work should strive to

understand host-parasite coevolution in order to best characterise biological systems.

With both parasites and hosts arising from across the tree of life, adaptations to parasitism and protective strategies by hosts are numerous and diverse. This can lead to an 'arms race', with improved strategies each organism driving the selective pressures of the other in a constant cycle of improved exploitation and defence (Dawkins & Kerbs, 1979)(e.g. Brandt *et al.,* 2005; Medel *et al.,* 2011; Soler *et al.,* 2001). With this in mind, any study on host-parasite systems is only observing a 'snapshot' in time and cannot be assumed to be stable, as with free-living organisms in fluctuating environments. This is best demonstrated in organisms with short generation times and/ or preserved samples. Studies on *Daphnia* and their microparasites extracted from sediment cores found significantly higher infectivity by parasites exposed to their contemporary hosts compared to previous or future hosts, showing rapid adaptation by both parasite and host (Decaestecker *et al*., 2007).

Kleptoparasites are often less closely associated with their host than other parasites, describing parasitism by theft of food or materials (Thompson, 1986). Kleptoparasitism can be either obligate (the sole obtainment mode of the animal) or facultative (one of a number of modes), which is more common (Iyengar, 2008). The strategy can be intraspecific, where both individuals are the same species, for example in Kelp gulls, where juveniles will commonly steal food items from others within the colony (Steele & Hockey, 1995). Intraspecific kleptoparasitism could also be considered simply competition in many cases, but specifically stealing food in these situations, especially as a common strategy in some individuals, is often termed kleptoparasitism. It can also be interspecific, where the interaction is between different species, such as dung beetles of the genus *Cleptocaccobius*, which do not roll their own faecal matter and will instead remove dung from the reserves of other beetles (Inward *et al.,* 2011).

Kleptoparasitism can involve direct interaction with hosts or a 'stealthier' approach which favours unnoticed kleptoparasites. This leads to a diversity in host behaviours and retaliatory strategies, with some hosts actively working to

protect resources and others appearing to be unaffected or unaware (Iyengar, 2008). This notice or lack thereof does not signify the extent of cost to the host and, as classification as parasites would suggest, kleptoparasites in the true sense of the word gain resources at their host's expense. Many strategies have evolved across host taxa to counteract kleptoparasitism, such as hunting and feeding in larger numbers (Caraco & Wolf, 1975), and the coevolutionary arms race demonstrated in other parasites can arise from kleptoparasitism also. It is an often overlooked interaction, described mostly in birds, and more exploration is required to develop a robust understanding of the drivers of kleptoparasitism and its impact on parasites and hosts. A model system proposed by Iyengar (2008) to provide contrast to avian examples is that of kleptoparasites within the spiders, and these are considered further in this thesis.

1.3 Kleptoparasites of spiders

The success of orb webs in catching food can prove very beneficial to their constructor but, in many cases, they can attract the wrong kind of attention. Other spiders, with the ability to traverse silk without becoming trapped, can take the opportunity to capitalise on the energy expenditure of another and can parasitise these webs, gaining nutritional benefit without the costs of web production. An example of such an interaction, spanning many species of spiders, is that of food stealing by males. Mature males can often cohabit on or around the female's web and steal prey items or feed alongside their host (Schneider & Lubin, 1998). In some spiders the males can be large and aggressive, directly taking large food items from the females. However, within many orb weavers the males' small size and behavioural adaptations allow them to take smaller prey items without aggression or detection by the large female. These small males can have little impact on her overall nutrition but, with a congregation of multiple males, she could see a reduction in food intake, especially in species where the males do not provide nuptial gifts (Schneider & Lubin, 1998).

A number of terms can be used to describe exploitative spiders such as these. These can include 'inquiline', which describes an organism living in the nest or burrow of another. 'Inquiline' generally indicates a commensal relationship, with no harm to the host, and often refers to a closely evolved host-parasite species pair. Another term used is 'kleptobiont', describing the stealing of resources by the organism, with the term sometimes being used to indicate no negative effect on the host (Hajer & Reháková, 2003). 'Kleptobiont' does not usually assume coevolution or close association between species. Finally, 'kleptoparasite' is used most widely used in reference to spiders. This term similarly describes the stealing of resources but also involves both coevolution of parasite and host as well as costs to the host. Across the literature, and so in this thesis, this term is used broadly to describe exploitative spiders, even without indication of either cost to hosts or coevolution.

The food capture resource of webs, kleptoparasitism, is also exhibited between disparate species of spider. These can include opportunistic interactions

through incidental proximity but can also regularly involve spider species with life history strategies largely directed towards the exploitation of others. Only one spider (*Curimagua bayano*) has so far been identified as an obligate kleptoparasite of the mygalomorph *Diplura* (Vollrath, 1978). This species relies on its host to capture and predigest the prey, with the parasite climbing over the host's chelicerae to share the meal. Another kleptoparasitic group of spiders is the Argyrodinae, a subfamily within the comb-footed family Theridiidae. This subfamily is known for its associations with other spiders, engaging in a range of facultative kleptoparasitic, web usurping and predatory behaviours (Su & Smith, 2014).

1.3.1 The Argyrodinae

The Argyrodinae describes a subfamily composed of six genera: *Argyrodes* (Simon, 1864), *Ariamnes* (Thorell, 1869), *Faiditus* (Keyserling, 1884), *Neospintharus* (Exline, 1950), *Rhomphaea* (L. Koch, 1872) and *Spheropistha* (Yaginuma, 1957) (Agnarsson, 2004; Su & Smith, 2014). Members of the Argyrodinae are found across the globe, inhabiting warmer climates spanning the Americas, southern Europe, central and south Asia and Oceania (Su & Smith, 2014). The foraging behaviours of this group all largely rely on other spiders though the specific strategies vary. *Ariamnes* and *Neospintharus* are largely araneophagic (predating on other spiders) while *Rhomphaea* engages in araneophagy alongside occasional kleptoparasitism. *Argyrodes* and *Faiditus* largely forage through kleptoparasitism and little is known about the strategies of *Spheropistha* (Su & Smith, 2014). While these foraging behaviours show general patterns between genera, they can vary with species and are only wide assumptions based on limited observations. Even individual spiders can show plasticity in their strategy based on situation and experience (Whitehouse, 1991).

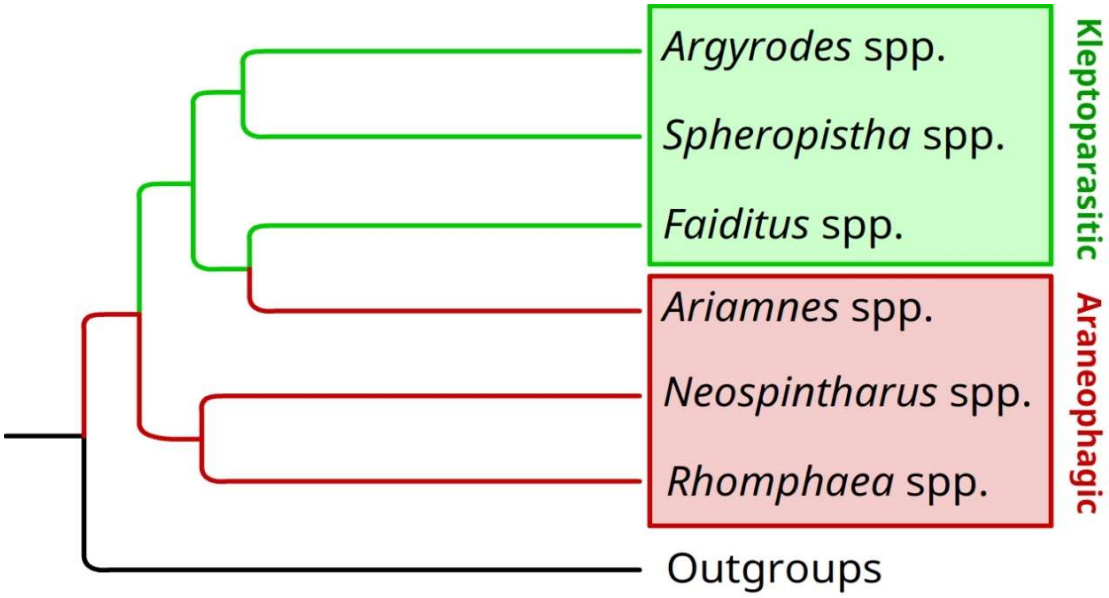

Figure 1.3. Simplified phylogeny of the genera of the Argyrodinae and feeding strategies based on that presented by Su & Smith (2014).

Araneophagy describes the consumption of spiders, and can include the consumption of eggs, juvenile and adults. It can either be entirely random, with the predator eating anything they encounter, but can also be a specific preference with adaptations to facilitate this. Spiders of the family Scytodidae often invade the webs of certain weaver spiders using venomous glue to subdue prey (Ades & Ramires, 2002) while members of the Pholcidae family are also adapted to target other spiders, showing excellent agility on certain web types (Jackson & Brassington, 1987) and using their tangled silk to effectively immobilise their eight-legged targets. This specific preference for targeting spiders extends to members of the Argyrodinae; with some catching and consuming others; often enough to develop behavioural and physiological adaptations to further their success. An example of this was studied by Whitehouse (1987) in an undescribed species of *Rhomphaea*. This spider lives on its own web independently of a host but shows a close association to other spiders in its choice of prey. It can catch spiders on its own web but will also commonly enter the webs of the prey, shaking the web in an imitation of a struggling insect to encourage its target to approach. These *Rhomphaea* prepare a silken net between their legs in order to trap the approaching prey, using the fourth pair of legs to quickly entrap the spider before wrapping it again with the minimum silk required to immobilise its legs (Whitehouse, 1987).

Where the long-term benefit of kleptoparasitism outweighs the short term consumption of another spider, or perhaps where a size difference makes the targeting of the other spider too risky, kleptoparasitic behaviour might be favoured. This can include the use of a host's web to shelter and lay eggs, the catching and consumption of small prey entrapped in the web, consumption of food parcels left over from host feeding, communal feeding with the host on large prey items or even the stealing of large prey items from the host (Whitehouse *et al.,* 2002). Host species are largely orb weavers including *Nephila*, *Cyrtophora*, *Argiope* and *Gasteracantha* but some other groups can be targeted such as individuals within the families Psechridae and Tetragnathidae (Su & Smith, 2014).

The specific challenges of performing kleptoparasitic behaviour in spiders are numerous, with escaping detection by the host being a priority. It is likely that kleptoparasitic Argyrodinae have adapted their physiology and behaviour to favour this specific lifestyle, with their reduced food capture and web building abilities making them reliant on this strategy to thrive (Whitehouse *et al.,* 2002). Vollrath (1984) compares both specialist and generalist Argyrodinae, with some targeting specific host species while others vary with availability. Specialist species have been observed using many foraging strategies to exploit their host, as they are able to avoid detection more effectively, while generalists have been recorded to use fewer techniques per host, but then showing the ability to modify these behaviours on different hosts (Cangialosi, 1997). Specialists seem to have more specific adaptations to the parasitic lifestyle, including the use of silk threads attached to the host web to provide sensory information as well as behavioural techniques in foraging. Vollrath (1984) also compares the lifestyle of two *Argyrodes* species, showing the specialists leaning towards r selection with a higher number of offspring and less maternal guarding. These specialists were hardier in their response to heat and humidity variation, suggesting that their host reliance has required them to become more resilient in other areas.

Individuals largely showing kleptoparasitic behaviour can still gain nutrients through the consumption of their hosts, in arguably less specialist ways to their araneophagic relatives. These opportunistic spiders will usually consume a

large host spider while it is moulting, and so harmless and vulnerable, or will consume the host's eggs or hatchlings, with these smaller prey items easily being scooped towards the fangs by the front legs (Whitehouse *et al.,* 2002). This behaviour could be costly for the parasite, with the removal of a host reducing their nutritional opportunities, so appears to only occur under specific conditions.

1.3.2 Kleptoparasite or commensal?

There has been great debate concerning the effects of kleptoparasites on the nutritional intake of their hosts, with the aim of determining the negative impact a kleptoparasite load can have even when removing araneophagy. Tso & Severinghaus (2000) found a significant difference in the size distributions of the prey items consumed by *Argyrodes fissifrons* and two of their *Cyrtophora* hosts in Taiwan. These parasites seemed to most commonly attack very small insects trapped by the host's web, apparently unnoticed, and were largely deterred from feeding on the host's larger food items by aggressive behaviour. This study saw no nutritional costs to the host apart from momentary distraction but personal observations on *Cyrtophora citricola* in Spain have indicated that such aggressive behaviour towards approaching parasites can commonly cause the host to drop their prey item, either into the web of another Cyrtophora or entirely out of reach. *Argyrodes* parasites on *Nephila* webs have even been observed directly stealing food items from the host (Larcher & Wise*,* 1985) and this stealing of prey was found to contribute to a slower host growth rate (Grostal & Walter, 1997).

Another cost associated with *Argyrodes* kleptoparasites, as well as other Argyrodinae, is that of web destruction (Whitehouse, 1986; Tso & Severinghaus, 1998). This behaviour can facilitate the stealing of food by the parasite but can also be taken solely to gain nutrients from the silk. The web of the host is integral to its prey capture and security and requires time and energy to produce, with any loss or damage being costly to replace. While there is a theoretical cost to web damage, Grostal & Walter (1997) found no significant difference in the juvenile growth of *Nephila plumipes* when their webs were artificially damaged.

The discussion concerning Argyrodinae and their place within the spectrum of commensal and parasitic organisms remains ongoing. Araneophagy creates a clear cost to the host but, where the smaller spiders are merely residing on the web and scavenging on tiny insects and leftovers, there seems to be fewer costs to their presence. They could even provide a benefit by cleaning away uneaten food items, or by attracting prey items due to their colouration contrasting with the night-time background, stimulating moth photoreceptors (Peng *et al.,* 2013). However, with hosts often having kleptoparasitic smaller males, the other parasites could be directly competing with these (Grostal & Walter, 1997), indirectly affecting the host's reproduction. Even with no nutritional impact at all, it has been shown that hosts with ample food availability can often still relocate when the number of parasitic spiders on their web reaches a threshold (Larcher & Wise, 1985) and such a relocation can be costly in numerous ways.

1.3.3 *Argyrodes argyrodes* and *Cyrtophora citricola*

A common example of the relationship between kleptoparasites and their hosts is that of *Argyrodes* residing on webs of colonial *Cyrtophora*. This interaction is described commonly due to the abundance of each genus across a large number of countries, with certain climates allowing their ranges to overlap, exposing *Cyrtophora* to the parasites. This interaction also encourages study due to the unusual colonial lifestyle of many *Cyrtophora* species, with clusters of individuals building conjoined webs which can span huge areas and involve hundreds of individuals of a variety of ages (Rao & Lubin, 2010). *Cyrtophora* individuals each spin an orb web which sits horizontally with a raised 'hub' in the centre as well as fixing a tangle of lines around the main web to trap flying prey (Eberhard, 1990). Each spider guards their own web structure, often acting aggressively towards conspecifics and feeding individually. However, the large, conjoined structure formed of these individual webs can benefit each individual spider by increasing their food capture efficiency, with the tangled lines forming a large and complex structure from which flying insects struggle to escape. The effectiveness of this prey capture technique, trapping insects without the need for specialist adhesive webbing, enables *Cyrtophora* to produce stronger and

more permanent webs. Living within permanent colonies can benefit the *Cyrtophora* in many ways, from prey capture efficiency to predator deterrence, but it is suggested to increase their vulnerability to kleptoparasites (Pasquet *et al.,* 1997).

Species within the genus *Argyrodes* are commonly associated with *Cyrtophora* as they are inclined to be kleptoparasites long term, remaining within the same web for extended time periods and so benefitting from the permanent tents produced by *Cyrtophora* (Tso & Severinghaus, 2000; Cangialosi, 1997). *Argyrodes* have been shown to have an increased nutritional intake on *Cyrtophora* webs compared to other hosts possibly due to the substantially larger prey they have access to (Baba *et al.,* 2007). They can also benefit from the colonial hosts through the increase in overall web space, with Agnarsson (2003) likening host webs to habitat patches and describing a more predictable number of *Argyrodes* parasites on interconnected webs due to the more stable environment and ease of movement. These parasites are largely described as having limited negative effects on *Cyrtophora*, taking food too small for the large adult females (Tso & Severinghaus, 2000). However, *Argyrodes* have been observed eating *Cyrtophora* eggs and newly emerged young (Lubin, 1974) as well as causing large prey items to be dropped through aggressive interactions (personal observation) and no recording has been made of competition or aggression between *Argyrodes* and the small *Cyrtophora* mature males.

1.4 The arthropod microbiome

In 1988, Whipps and colleagues working on the ecology of rhizosphere microorganisms provided the first definition of the term microbiome. They described the 'microbiome' as a 'characteristic microbial community' in a 'reasonably well-defined habitat which has distinct physio-chemical properties' as their 'theatre of activity' (Whipps *et al.,* 1988). Lederberg & Mccray (2001) describe the microbiome within a specifically ecological context, as 'a community of commensal, symbiotic, and pathogenic microorganisms within a body space or other environment'. The diversity of microscopic organisms within the body of others is becoming increasingly clear through the improvement of sequencing technologies and there is substantial evidence that this microbiome is intrinsically linked to an organism's life history traits. Such relationships can be fleeting, such as microorganisms introduced through ingestion, or can form much longer-term endosymbiotic relationships.

The term endosymbiont describes the intimate association of one organism residing within the body or cells of another. Arthropods are more commonly exploited by endosymbiotic bacteria than any other phyla, providing excellent model systems for host-symbiont study (Harris *et al.,* 2010). While some symbionts become obligate (or primary), with the host requiring them to survive or reproduce, often these associations can remain facultative (or secondary), with no requirement on the side of the host (Jaenike, 2012). These unseen manipulators can have huge impacts on the phenotypes of their hosts in beneficial, neutral and costly ways in order to facilitate their own transmission.

1.4.1 Manipulation by endosymbiotic bacteria

Endosymbiotic bacteria infecting arthropods are often heritable, with a point of horizontal infection within a host's lineage followed by largely vertical transmission (Russell & Moran, 2005). However, with facultative symbionts there appears to be little phylogenetic congruence between parasite and host, suggesting that these relationships are evolutionarily short-lived and horizontal transmission does happen relatively often (Werren *et al.,* 1995). With vertical transmission being key to the survival and proliferation of these symbionts, the

reproductive fitness of the host is integral to the bacteria. Where their interests diverge is in the production of male hosts, as only female offspring continue to transmit the bacteria through their gametes, and in order to increase the number of female offspring the symbiont can alter its host's phenotype in a variety of ways.

The number of female offspring can be directly increased through bacterially induced parthenogenesis and feminisation. Parthenogenesis describes reproduction from an ovum without the need for fertilisation, showing females producing other females with no need for any male input (Stouthamer *et al.,* 1993). With feminisation, fertilisation happens as normal, but a large proportion of the genetically male offspring will develop female physical characteristics, allowing them to reproduce as females and transmit the manipulative bacteria (Giorgini *et al.,* 2009). A more indirect way of increasing bacterial transmission, through the encouragement of females, is that of male killing. This describes the process by which the bacteria kill male offspring at an early stage of their development, usually before hatching. While this does not directly increase the number of females, it is hypothesised to benefit the female offspring by reducing inbreeding probability and providing the deceased males as a food resource (Hurst & Majerus, 1993). It can also serve to reduce competition for resources and so greater survival, faster growth and increased reproduction of the females (Martins *et al.,* 2010; Hurst & Majerus, 1993).

Where male offspring are produced by an infected host, they can be used by the bacteria to encourage the propagation of similarly infected females through cytoplasmic incompatibility. This process involves creation of reproductive incompatibility between an infected male and an uninfected female (unidirectional) or a female infected with a different strain of the bacterium (bidirectional). This is caused by a loss of paternal genetic material during embryogenesis (Tram *et al.,* 2003) and, in diplodiploid systems, results in embryonic death. In a haplodiploid system, this can lead to death but also can result in entirely haploid male offspring. Within a population infected by an incompatibility inducing bacterium, infected females could then find great selective advantage over those not infected due to their ability to mate with both infected and uninfected males.

For a rare horizontal transfer event to lead to long-term infection in a population or species, the newly infected host must survive and reproduce effectively. It is possible for infection rates to increase stochastically but is much more likely through reproductive manipulation by the bacteria or even beneficial effects of infection. Many beneficial effects of endosymbiotic bacteria are context dependent, retaining their facultative status with little likelihood of a completely infected host species. Benefits found within insect endosymbiont relationships include the provision of protection against natural enemies augmentation of thermal tolerance and ability to feed on specific plant species (Jaenike, 2012). Jaenike compares the process of symbiont transfer between insect species or individuals to the horizontal transfer of mobile genetic elements among bacteria, conferring traits such as antibiotic resistance and pathogenicity. As with these mobile genetic elements, benefits incurred can vary with environmental conditions and so selective forces can drive a change in the frequencies of such agents within populations.

Within one individual host it is not uncommon to find multiple different bacterial endosymbionts, including different strains of the same group or entirely different groups (e.g. Ros & Breeuwer, 2009; Zhao *et al.,* 2013). These can interact in complex ways and the presence of multiple endosymbionts can influence the phenotypic manipulations each induce (further discussion in Chapter 6).

1.4.2 *Spiroplasma*

Spiroplasma (class Mollicutes, order Entomoplasmatales, family Spiroplasmataceae) is a group of small, gram-negative bacteria often associated with a variety of plant and arthropod systems both endocellularly and extracellularly (Gasparich *et al.,* 2004). *Spiroplasma* can be commonly found within the gut of a range of arthropod species, with its movement into the host's tissues sometimes being responsible for a move to pathogenicity (Haselkorn, 2010). Pathogenicity has been described in a range of arthropods and vertebrates while also becoming damaging to plants, causing agronomically important diseases such as citrus stubborn disease (Markham *et al.,* 1974) and corn stunt disease (Davis et al., 1972). However, *Spiroplasma*

infection does not always cause such damage to its hosts, it can often be described as an endosymbiont with varied but often neutral or positive effects.

Many strains of *Spiroplasma* impact their host's reproduction through male killing; having been initially referred to as Sex Ratio Organisms before technological advances allowed them to be clearly identified (Haselkorn, 2010). This male killing phenotype (SRO) has been found in several species of ladybird beetle (Tinsley & Majerus, 2006) and butterfly (Jiggins et al., 2000) but has been largely studied in the fruit fly genus *Drosophila* (Anbutsu & Fukatsu, 2011). Within *Drosophila*, there exists both a SRO and non-male killing phenotype (NSRO), with the phenotype even switching to NSRO with a reduction of bacterial density (Yamada *et al.,* 1982).

While a *Spiroplasma* infection can severely impact a host's reproductive output by killing male offspring, levels of infection by *Spiroplasma* are high in many wild populations (Cockburn *et al.,* 2013), suggesting that the infection does not significantly lower host fitness. This could be through the addition of context dependent benefits provided by some strains of the bacterium. *Spiroplasma* has been shown to protect a number of arthropods against natural enemies; including protection against fungal parasites in pea aphids (Łukasik *et al.,* 2013) in addition to nematodes and parasitic wasps in *Drosophila* (Jaenike, 2012), with the mechanisms of this protection remaining unknown.

Transmission of *Spiroplasma*, as with many other endosymbionts, is largely vertical but with evidence of common horizontal travel. Haselkorn *et al.* (2009) describes the presence of this horizontal transmission through phylogenetic analysis, showing closely related *Drosophila* infected by highly divergent *Spiroplasma* strains as well as closely related *Spiroplasma* inhabiting distant, in genetic and physical terms, species of *Drosophila*. This common horizontal infection rate could lead to the presence of multiple strains within one individual, with then the potential for recombination. However, this possibility requires further exploration as no evidence has been found within genetic analysis for past recombination, suggesting that endosymbiotic *Spiroplasma* lack the recA gene for recombination (Haselkorn, 2010). Other *Spiroplasma* lack this gene but perform a similar function through other mechanisms (Haselkorn, 2010).

1.4.3 *Cardinium*

Six percent of arthropods tested by Zchori-Fein & Perlman (2004) contained an intracellular bacterium named *Cardinium hertigii* (class Bacteroidetes, order Bacteroidales, family Bacteroidaceae). *Cardinium* have been shown to infect nematode, insect and arachnid species (Nakamura *et al.,* 2009). One of the first reports of manipulation by this bacterium was in the parasitoid wasp genus *Encarsia*, where it was found to cause parthenogenesis as well as modifications to host selection in the females (Zchori-Fein *et al.,* 2001). Alongside parthenogenesis, a capacity for numerous reproductive manipulations has been shown in a variety of hosts.

Cytoplasmic incompatibility correlating with *Cardinium* infection has been shown by Gotoh *et al.* (2007) in the spider mite *Eotetranychus suginamensis*, where females treated with an antibacterial agent and mated with infected males showed a significantly lower hatching rate and number of F1 females. However, this study also found no such manipulation in other species tested, showing variation in the phenotype expressed by different species under a similar infection. Feminisation is also shown to occur due to *Cardinium*, with the wasp *Encarsia hispida* naturally producing entirely female clutches and antibacterially treated females producing only uninfected males, even when the bacterial numbers were only reduced and not eradicated entirely (Giorgini *et al.,* 2009).

Fitness effects of *Cardinium* infection within the spider mite *Tetranychus urticae* have been described by Xie *et al.* (2016). This included an increase in the fecundity of infected females, with a significantly higher number of offspring than individuals which were uninfected and individuals which contained *Cardinium* along with another endosymbiont, *Wolbachia*. *Cardinium* infected females within the same study also had a longer lifespan than uninfected counterparts, with no indication as to the mechanism of such a benefit.

1.4.4 *Rickettsia*

Rickettsia (class Alphaproteobacteria, order Rickettsiales, family Rickettsiaceae) are a group of intracellular pathogens responsible for a range

of diseases including typhus and rickettsialpox when transmitted to vertebrates (Carl *et al.,* 1990; Brettman *et al.,* 1981). However, they can be considered to infect invertebrates primarily, with limited movement into vertebrates, and can be vertically transmitted reproductive manipulators in the same way as other endosymbionts. Perlman *et al.* (2006) reviewed the phenotypes of this bacterial group and found both male killing and parthenogenesis to be present in separate hosts. Other effects of *Rickettsia* include negatives such as reduced weight, fecundity and suppression of positive bacteria in the pea aphid (Chen & Purcell, 1997; Sakurai *et al.,* 2005) but also positives including increased body size in adult leeches (Kikuchi & Fukatsu, 2005). As communal food sources of invertebrates, such as plants and vertebrates, can harbour *Rickettsia* infection, it is suggested that horizontal transmission through food sharing may be common (Perlman *et al.,* 2006).

1.4.5 *Rickettsiella*

Little is known about the abundance and impacts of *Rickettsiella* (class Gammaproteobacteria, order Legionellales, family Coxiellaceae), with limited literature covering its symbiotic interactions with arthropods and spiders prior to the regular use of bacterial screening using next-generation sequencing technologies. Many of the examples of *Rickettsiella* infection classify it as a pathogen, causing slow development and death (Cordaux *et al.,* 2007; Han *et al.,* 2020). However, it is also increasingly being identified as having more long-term associations as an endosymbiont and is frequently identified from the tissues of seemingly healthy arthropods (Cordaux *et al.,* 2007; Iasur-Kruh *et al.,* 2013).

Recent exploration into the phenotypic results of *Rickettsiella* endosymbiosis are striking. On full-genome sequencing of vertically transmitted *Rickettsiella* from the poultry red mite, *Dermanyssus gallinae*, B vitamin biosynthesis pathways are shown to be unexpectedly retained. This suggests a nutritional symbiosis relationship, with the bacteria providing a number of necessary nutrients not provided the host's blood diet (Price *et al.,* 2021). *Rickettsiella* has also been found in the pea aphid *Acyrthosiphon pisum*, which is naturally present in both red and green morphs. On experimental infection with

Rickettsiella, red hosts were found to produce green polycyclic quinone pigments, becoming green (Nikoh *et al.,* 2018). This can have dramatic implications for fitness, as particular predators and parasitoids have been shown to prefer specific colour morphs (Libbrecht *et al.,* 2007; Losey *et al.,* 1997).

1.4.6 Endosymbiotic bacteria in spiders

Rickettsia, *Spiroplasma, Rickettsiella* and *Cardinium* bacteria have all been shown to infect spiders, as well as the commonly characterised endosymbiont *Wolbachia* (Goodacre *et al.,* 2006; Bouchon *et al.,* 2011) and, of the spider species tested, almost all showed the presence of at least one of these manipulative bacteria, with some containing up to four (Goodacre and Martin, 2013). Some of the most common infections are that of *Wolbachia* and *Cardinium*, with 37% and 22% of species screened by Duron *et al.* (2008) containing each, respectively. There is limited understanding concerning any phenotypic manipulations of these widespread infections within spiders and its similarity to other arthropods, with detailed understanding necessary for exploration of spider ecology in any context.

Examples of feminisation, parthenogenesis and cytoplasmic incompatibility caused by symbiotic bacteria have been shown within other arachnid species such as mites. Little is, however, known about the extent of manipulation in spiders. *Rickettsiella* and *Wolbachia* has been recently recorded to induce cytoplasmic incompatibility in a linyphiid and theridiid spiders (Knight, 2018; Rosenwald *et al.,* 2020). Goodacre & Martin (2013) describe studies showing modifications by *Wolbachia* to the sex ratio of spiders within the family Linyphiidae by male killing (Vanthournout *et al.,* 2011) and manipulation of female behaviour (Gunnarsson *et al.,* 2009). The authors do, however, suggest consideration of the incomplete sex ratio balance in females cured of their infections, suggesting other additional factors.

A spider-symbiont interaction studied in detail is that of *Cardinium* and the cellar spider *Holocnemus pluchei* by Stefanini & Duron (2012). *H. pluchei* were found to be commonly infected with the bacteria and the transmission rate from mothers was very high, with 100% of offspring tested retaining the infection. No

evidence was found for any reproductive manipulation by the bacteria, including no impact on the number of offspring, sex ratio or hatch rate and with the complete success rate of wild caught uninfected females indicating no presence of cytoplasmic incompatibility. Horizontal transmission in this lineage was indicated to be very low, with the bacterial genes sequenced pointing to one infection in the lineage of all the populations studied. This contrasts with data from Perlman *et al.* (2010), who suggested that the rate of horizontal transmission was high within *Cardinium* infections of *Cybaeus* spiders.

Endosymbiotic bacteria within spiders have been observed to cause more phenotypic changes than just direct reproductive manipulation. Goodacre *et al.* (2009) describe the effect of *Rickettsia* infections on the linyphiid *Erigone atra*, small spiders which disperse by spinning silk to be caught by the wind and 'ballooning'. Individuals infected with *Rickettsia* were shown to be less likely to disperse, especially over long distance. This is hypothesised to promote the bacteria's transmission through increased matings between spiders with the same bacterial strain as well as disproportionally influencing females to remain, resulting in a bias of the sex ratio in the population. Very little is understood in the literature about both the mechanism of such manipulations as well as the diversity and prevalence of phenotypic impacts of endosymbionts across spider species.

1.5 Thesis aims

This thesis aims to explore the natural history of *Argyrodes argyrodes* on the webs of *Cyrtophora citricola* in southern Spain, as well as the impact of their unusual lifestyle on their physiology and microbiome. It will record the parasitic behaviour of these spiders, their habitat preferences, life cycle and impacts on their hosts while also exploring the impact their parasitic lifestyle may have had on silk production. Attention will also be given to the bacterial symbionts and overall microbiome contained within *A. argyrodes* and how this compares with other explored spiders and arthropods.

Chapter 2 aims to describe the study sites used and provides preliminary data which supports methods used through the following chapters. Chapter 3 follows *A. argyrodes* and *C. citricola* abundances across a whole year, aiming to provide insight into life history strategies and how they compare to other localities, as well as how the two species might see correlations in their abundances and reproductive periods. Chapter 4 aims to address the impact *A. argyrodes* might be having on their hosts, considering feeding, web repair, relocation and parasitic wasp infection as key factors based on previous literature and observations of *C. citricola* within the study sites.

Chapter 5 looks specifically at adaptations due to kleptoparasitic behaviour, aiming to identify any relaxation of selective pressures on silk proteins through lack of reliance on these fibres for survival and food capture. Chapter 6 similarly looks at an adaptation which is hypothesised to be diverged from other explored spider species due to *A. argyrodes'* kleptoparasitism and resulting patchy distribution, that of the spider's microbiome. The chapter aims to characterise the bacteria present within *A. argyrodes*, identify manipulative endosymbionts, quantify the presence of these across the population and compare the strains isolated to those of other arthropods.

This thesis is intended to be exploratory, describing *A. argyrodes* and *C. citricola* in a locality and environment rarely covered in the literature. Each chapter will focus on a topic never before studied in this system, providing data to allow further investigation into symbiosis and sociality in spiders.

Chapter 2 - The study system

2.1 Abstract

The study sites used within this thesis expand on the locations considered in previous work, with published literature on *Cyrtophora citricola* generally studying colonies outside of Europe, observations on *Argyrodes* focusing on *Nephila* hosts (Su & Smith, 2014; Whitehouse *et al.*, 2002) and studies concerning *Cyrtophora* as *Argyrodes* hosts located in East Asia (Peng *et al.*, 2013; Tso & Severinghaus, 2000). The selection of sites in Spain provides an opportunity to study both spiders in a region where their ranges overlap and where ecological conditions differ from those explored previously. This novelty, however, means that preliminary work was required to select ideal sites, understand ecological factors influencing the relationship between host and kleptoparasite within this habitat, and confirm what data could be collected accurately and safely for the main studies contained within this thesis.

This chapter establishes background information needed to study *A. argyrodes* on a new host in a new abiotic environment. It describes the study sites and identifies ecological and environmental factors which may influence the spiders studied. It covers agricultural land use, which has a significant impact on the location, shape and size of host plants. This chapter also identifies a strong correlation between web size and spider body size, which is difficult and unsafe to measure, ensuring that this important variable can be considered in the following studies.

Finally, it describes an overview of feeding interactions between *A. argyrodes* and *C. citricola,* observing feeding alongside the host in nearly one third of cases. This final section also describes these interactions in context of kleptoparasite size, with adult females shown to interact with a feeding host more than any other life stage.

These results are included to contextualise the studies covered in the following chapters (especially Chapters 3 and 4), providing support for the methods chosen as well as including information relevant to the design of future work.

2.2 Field sites

All field sites used in this thesis are located within the Province of Cádiz, Andalusia, on the southwestern coast of Spain. Most are close to the town of Rota. The main industries, and land uses, are tourism and agriculture. This leads to an area with extensive fields of cotton, olives, vineyards, fruit and livestock as well as large golf courses, beaches and apartment blocks. Across the area *Opuntia spp.* cacti are used to border high value or easily accessible crops, preventing theft. They also grow unchecked within less managed areas. This cactus has been shown by Chuang & Leppanen (2018) to be one of the preferred substrates for *Cyrtophora citricola* in the area, with the Province of Cádiz having greatest substrate availability of mainland sites surveyed in the study.

2.2.1 Species presence and dynamics

Almost all spiders observed reside on *Opuntia* cacti, with the cactus forming a frame on which *C. citricola* spiders build webs (Figure 2.1a). The *C. citricola* are colonial, with some large groups containing over 100 spiders, seemingly limited by the plant structure's size when spiders are at high densities, although most are solitary (defined here as no connecting silk architecture with another's web) or in smaller colonies (under 30 spiders). On these *C. citricola* webs live *Argyrodes argyrodes* kleptoparasites, in varying densities (see Chapter 3). The *A. argyrodes* have also occasionally been observed on the webs of *Argiope trifasciata* and *Araneus diadematus* orb weaving spiders attached to the cacti. These webs were not as permanent as those of the *C. citricola*, with none observed lasting over one week, and were not common enough to present a permanent habitat for kleptoparasites in the region.

Figure 2.1. (a) a *Cyrtophora citricola* colony of connected webs over *Opuntia* cactus plants in Spain (b) an *Argyrodes argyrodes* subadult moving on the edge of a *C. citricola* web (c) a female *A. argyrodes* approaching a feeding *C. citricola*.

Other organisms consistently reside on the cacti. The most noticeable are the paper wasps *Polistes dominula*, which form numerous nests within the *Opuntia* across the region. These wasps are present at all sites, at varying densities, but do not appear to have any interaction with the *C. citricola* or *A. argyrodes*, rarely becoming entangled in *C. citricola* webs. They are, however, occasionally trapped by marbled cellar spiders *Holocnemus pluchei*, pholcid spiders present in almost all sites. These *H. pluchei* appear to build loose, irregular webs of their own within the cacti but also reside on the outskirts of *C. citricola* webs and will often take sole control over abandoned webs. Small, aggressive interactions have been observed between *H. pluchei* and *C. citricola*, usually over food parcels. The pholcids were never seen removing food from any spider or killing any spider, although this remains a possibility.

Another common spider within *Opuntia* structures is *Agelena labyrinthica*, building funnels that sometimes sit alongside *C. citricola* webs but with no interaction observed to date. A number of species within the spider family Salticidae were observed on the cactus and would occasionally attempt to capture small *C. citricola*, although were rarely successful.

During initial visits over May 2018, no wasps were observed targeting spider egg sacs within the system. However, in October of that year small black wasps (*Philolema palanichamyi*) were observed settling on *C. citricola* egg sacs, apparently unnoticed by any spiders. Collected egg sacs in that period hatched numerous wasps, with some hatching both wasps and spiders. This prompted further investigation into both egg parasitism rates and species interactions (see Chapter 4).

Figure 2.2. A *Philolema palanichamyi* wasp on a *Cyrtophora citricola* egg sac, indicated with red arrow, with an *Argyrodes argyrodes* close by.

Few birds were observed hunting around the cacti; the spines appearing to deter them. There were, however, chickens on some agricultural sites which could remove some spiders low on the plant. Lizards of a variety of species were observed moving within the cacti and might predate spiders close to the cactus pads.

Some colonisation was observed of *Opuntia* by the scale insect, *Dactylopius opuntiae*, released in the area to control the invasive cactus (Shaw *et al.,* 2017). In a number of occasions, this had led to plant death, with very few *C. citricola* webs using the dead cactus structure as web substrate. Chuang & Leppanen

(2018) speculated that the insect was responsible for observed declines in *Opuntia* abundance observed in recent years. However, a high number of study sites included here contained both long dead and established and thriving *Opuntia* in close proximity, suggesting colonisation of a resistant cactus, be it a developed resistance of a different species to that impacted by *D. opuntiae.*

2.2.2 Climate and land use

Both biotic and abiotic factors surrounding the cacti are likely to be key to a number of considerations covered in this thesis. Temperatures in Rota, Spain (described in Figure 2.3) are lower than many tropical and desert areas used to explore colonial spider dynamics and, while temperatures are comparable or higher than East Asian localities describing *Argyrodes* and *Cyrtophora* dynamics, rainfall is dramatically lower. Temperature and rainfall are relatively constant across sites included here, since a narrow locality was used, see Figure 2.4.

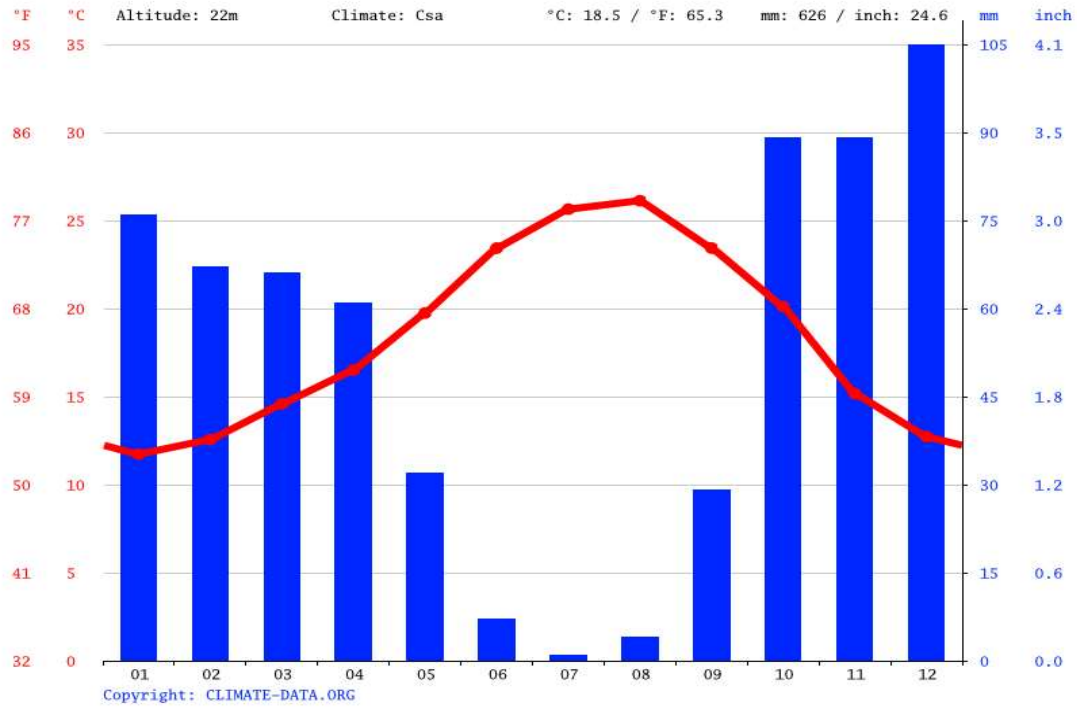

Figure 2.3. Average temperature and rainfall across each month in Rota, Spain, as recorded by Climate-Data.org (www.en.climate-data.org/europe/spain/andalusia/rota-57164, accessed July 2021).

All sites (listed in Table 2.1) were located nearby agricultural fields, usually arable, but crop types and pesticide use vary with different farmers. Cactus

patch size is also considered as an indication of habitat availability to *C. citricola.* Not all factors described in this section are considered in this thesis, with too many variables to isolate specific impacts, but are recorded for future consideration.

Figure 2.4. Location of each field site utilised in the collection of data for this thesis. Map generated using Google Maps (www.google.co.uk/maps, accessed July 2021).

Table 2.1. Summary of each field site located within the area of interest. Field sites were located by systematic searching the area surrounding Rota and also using local knowledge for areas further away. Sites were chosen for continued use due to high *Cyrtophora citricola* and *Argyrodes argyrodes* abundance as well as ease of access. 10 sites were identified, with 3 not used within this thesis but noted for future work. Included are 2018 aerial images sourced from Google Earth (earth.google.com/web) with cactus areas marked in red. Cactus area describes the approximate extent of each *Opuntia* patch within the field site within easy ambulatory travel for spiders as well as a total of these, giving an approximate available habitat size.

FF ['Friendly Farmer']	**36.651482, -6.311399**
This site was located alongside a busy main road, with a ring of *Opuntia* cactus surrounding a walled fruit orchard and bordering a cotton field. Site demolished in 2019 to make room for road expansion. Cactus area = 773 m^2.	

SN ['Snails']	**36.649485, -6.375775**
Patchy stretch of *Opuntia* along a dirt road, bordering scrubland and a cattle farm. Divided into A, B and C sections, with small (4-6 m) gaps in cactus between. Cactus area = 110 + 217 + 325 + 54 = 706 m^2.	

CM [‘Cemetario Municipal’]	36.656459, -6.372226

Opuntia patch bordering maintained cemetery (largely grassland) and dirt road. Cactus much deeper than many other sites. Cactus area = 590 + 22 = 612 m^2.

NN [‘Nursery North’]	36.663994, -6.369045

Opuntia strip between small, paved road and crop fields. Bordering an often waterlogged ditch. Cactus area = 289 + 197 = 486 m^2.

EO ['Econ Oil']	36.676486, -6.401870

Opuntia strip between small paved road and cotton field. Bordering often waterlogged ditch. Cactus area = 501 + 160 = 661 m^2.

PA ['Paradise']	36.672151, -6.391202

Opuntia strip between dirt road and wheat field. Divided into A, B and C sections, with small (4-6 m) gaps in cactus between. C section bordering an often waterlogged ditch. Cactus area = 373 + 108 + 329 + 234 = 1044 m^2.

WP [‘Water Plant’]	**36.668924, -6.389299**
Opuntia patch on unmanaged scrubland by paved road. Bordering an often waterlogged ditch. Cactus area = 133 m^2.	

Other identified sites

FL [‘Flamingoes’]: 36.612758, -6.054474
Mixed shrubbery bordering path around a lake (Laguna de Medina).

AQ [‘Aqualand’]: 36.623843, -6.194617
Extensive, patchy *Opuntia* plants in amongst smallholdings and scrubland.

BE [‘Beach’]: 36.636496, -6.376522
Opuntia strip in sandy scrubland bordering main road. Extensive construction nearby through 2017-2019.

2.3 *Cyrtophora citricola* body size estimation

The body size of an individual *C. citricola* might significantly affect its interactions with other spiders as well as response to environmental factors. Body size has been shown in spiders to impact fecundity, contest success, mating success, cannibalism, food capture, diet composition and even cooperative task allocation (Akamatsu *et al.,* 2007; Grinsted & Bilde, 2013; Koltz & Wright, 2020; Maklakov *et al.,* 2004) Therefore, it is important to collect data on *C. citricola* size. However, this is challenging to achieve in a cactus-filled environment without disturbing the spider or damaging the web.

With many spiders, web size correlates with spider size (Benforado & Kistle, 1973; Eberhard, 1988) due to both the increased physical ability of larger spiders, as well as the necessity of targeting larger prey items. These data were collected to ascertain if this is true for *C. citricola* present in this study system and if the correlation is strong enough to consider web size an acceptable alternative to direct body size measures. These data were collected for the study outlined in Grinsted *et al.* (2019) and for those contained in this thesis (Chapters 3 and 4).

2.3.1 Methods

A total of 59 *C. citricola* spiders were selected from three study sites across a full spectrum of web sizes. All selected individuals were on the edge of the cactus for easy access. The web sheet of each spider was measured to the nearest centimetre (see Figure 2.5). The spider present in the centre of the web, identified as the ‘owner’, was then collected by hand and the body length (from the tip of the cephalothorax to the end of the abdomen) was measured to nearest 0.1 mm using digital callipers.

Data was analysed using R 4.0.0 (R Core Team, 2021) with a linear model with a Gaussian error structure. All figures were created in R 4.0.0 using the package ggplot2 (Wickham, 2016).

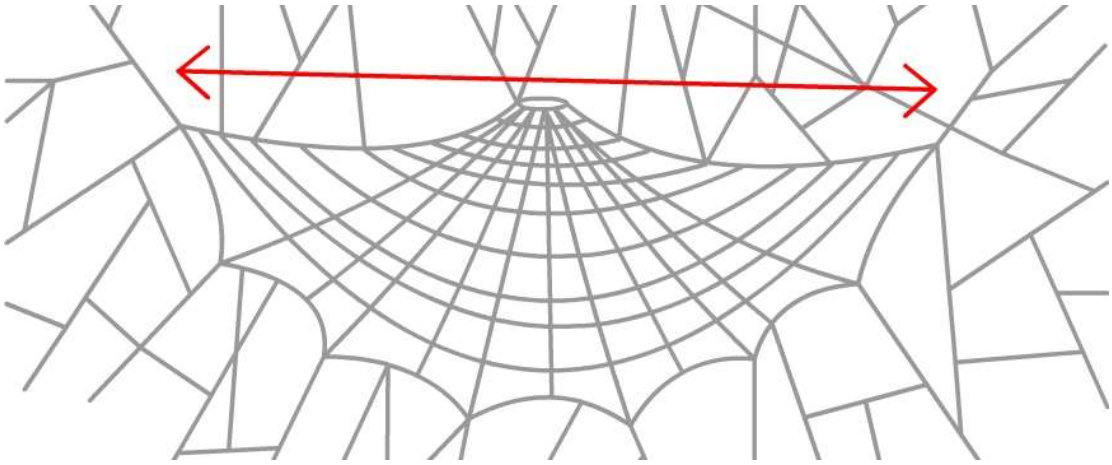

Figure 2.5. A simplified diagram showing the structure of an individual *Cyrtophora citricola* web from the side, with the red arrow indicating the points used to measure web sheet diameter in all relevant sections of this thesis.

2.3.2 Results

The linear model showed a strong positive relationship between *C. citricola* web size and spider body length (R^2 = 0.66, F(1,45) = 91.81, p < 0.001).

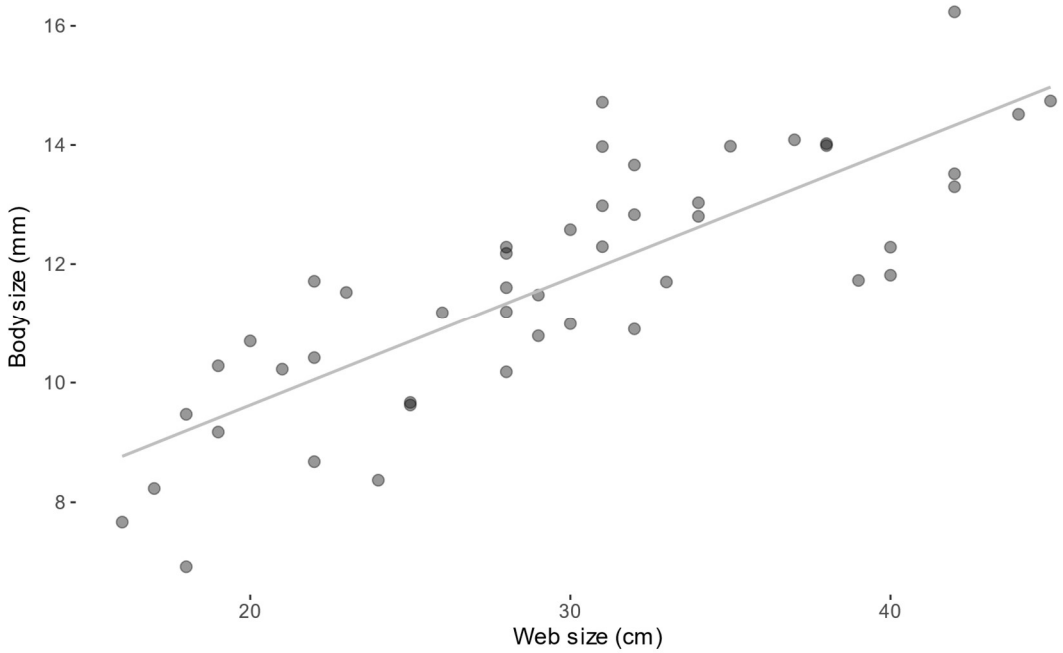

Figure 2.6. The relationship between spider body length (from the tip of the cephalothorax to the end of the abdomen) and web sheet diameter.

2.3.3 Discussion

The strong correlation found here indicates that, while web size cannot be a direct stand-in for spider size, it can be included as a close estimation. It could also be argued that many interactions showing correlations to spider size could be largely due to the larger or smaller webs spun by the spider. A larger spider might catch a higher number of food items, or larger food items, but this could be, in part, due to a bigger web structure.

It is possible that the relationship between spider body size and web size is confounded by individuals occupying webs that they themselves have not spun, with the identified 'owner' not being the creator of the web. As *Cyrtophora* webs are relatively long-lasting, they can remain after the creator has died or relocated and other *C. citricola* have been observed moving to reside in these empty webs. It is unclear how often this takes place. However, these interlopers are unlikely to stay if the web is well outside the size that they might create themselves. A small spider living on a large web is likely to be unable to complete necessary web maintenance and will find themselves unable to contain large prey caught by the less compact net. A large spider on a small web may not catch appropriately sized food items or an overall high enough prey biomass. The lack of large outliers in the data seems to support this.

2.4 Kleptoparasitic behaviour by *Argyrodes*

In order to explore and discuss the study system, it was necessary to ascertain the extent of kleptoparasitic interactions between *A. argyrodes* and their *C. citricola* hosts within the Spanish system. Exploring which *A. argyrodes* individuals interact with the host, and for how long, allows a first glimpse into what variables and behaviours could be interesting in further work and ensures studies are concentrated on the correct spiders, i.e. those most likely to interact and influence the hosts and demonstrate the most pronounced adaptations to kleptoparasitism.

2.4.1 Methods

This study was conducted in October 2018, located at field sites CM, FF, TR, NN & EO (see Table 2.1) within the province of Cadiz, southern Spain. Sites were selected for ease of access to webs for long periods of time as well as distance from other sites being monitored at the same time. Adult females were selected with webs close to the edge of the cactus. The web size was measured as well as number of *A. argyrodes* of each life stage. Life stages of *A. argyrodes* throughout this thesis have been loosely defined as ‘juvenile’ (smaller than 1 mm body length), ‘sub-adult’ (spiders between 1 mm body length and adult size, approximately 3.5 mm body length), ‘adult male’ (identified through distinctive protrusions on the cephalothorax and enlarged palps) and ‘adult female’ (over 3.5 mm body length, with reasonable confidence of maturity due to size and/ or enlarged abdomen). Some *C. citricola* individuals were found in webs where no *A. argyrodes* were observed. This allowed behaviour to be assessed in the absence of the parasite.

C. citricola were all in the centre of the web when trials began. Either a mealworm (larvae of *Tenebrio molitor*) or an ant (unidentified, collected on site), each approximately 1.5 cm long to mimic a large but common prey item, was thrown onto the main web approximately 15 cm from the spider and the *C. citricola* was allowed 5 minutes to attack (bite and/ or wrap). Where the spider did not attack, the prey item was removed and the alternate prey type was offered. The time between initial *C. citricola* movement and successful (leading to capture and feeding) contact with the prey item was noted. Spiders that

attacked were allowed one hour from contact to kill and wrap the prey item, usually bringing it to the web centre. Preliminary observations had shown almost no approach by *A. argyrodes* prior to one hour after prey capture.

After the hour had passed all of the spiders were monitored every 10 mins for a total of 6 observations. The position of nearby *A. argyrodes* was recorded, as a number of each life stage at each distance measure (5 cm from prey, 1 cm from prey, feeding on prey, scavenging from leftovers elsewhere on web, or feeding on or inspecting *C. citricola* eggs).

Data was analysed using R 4.0.0 (R Core Team, 2021). Catch time was tested as a response variable against kleptoparasite number using a linear model with a Gaussian error structure. Occurrence of kleptoparasites feeding with hosts (presence/absence) was tested against the number of kleptoparasites on the web using a generalised linear model and a second model was used to test this response against web size, both with Binomial error structures.

2.4.2 Results

A total of 42% of *A. citricola* accepted one of the prey types within the 5 minutes allowed, leaving 70 spiders to be observed out of 165 selected. Only 4 spiders dropped their prey items over the observation time (all within the first 60 minutes after capture and without noticeable kleptoparasite interaction) and were discounted, leaving a sample size of 66. Of the 62 spiders with recorded capture time, the average time between *C. citricola* moving and securing prey was 38 seconds ranging from 1 to 160 seconds. Kleptoparasite number did not predict catch time significantly (R^2 = -0.01, $F(1,60 = 0.26$, $p > 0.05$) .

Kleptoparasites did not appear to be influenced by the presence of others when approaching the host; there was no relationship between the occurrence of an *A. argyrodes* feeding with the host and the number of other kleptoparasites on the web (GLM: estimate = 0.31, z = 1.47, $p > 0.05$) or web size, and therefore the size of the host (GLM: estimate = -0.0031, z = 0.049, $p > 0.05$).

Across the 52 observed webs with kleptoparasites present, 31 adult females (across 25 webs), 46 sub-adults (32 webs), 30 juveniles (23 webs) and 1 adult male *A. argyrodes* were present. When webs were selected and all preliminary data taken, no kleptoparasites were within 5 cm of the host. During the

observation time, 33 webs had a kleptoparasite approach within 1 cm of the host, with 6 having more than one, and 23 of these had kleptoparasites within this range for more than one observation. At least 15 adult females came within 1 cm of the host (48% of total observed), 20 sub-adults (43%) and 10 juveniles (33%). The one adult male observed did not approach the host.

A total of 14 (27% of total observed) webs had at least one kleptoparasite feed alongside the host within the observation hour. 6 of these were adult female *A. argyrodes* (20% of total observed), 7 were sub-adult *A. argyrodes* (15%), 2 were a juvenile *A. argyrodes* (8%). Included in these counts is one web which had multiple kleptoparasites feeding, one of each life stage. Approximating time using observation points 10 minutes apart, the average time for the kleptoparasite to approach the host's food parcel after capture across all groups was 79 minutes, with adult females averaging 77 minutes and sub-adults 83 minutes (sample size too low and timing intervals too wide for significance testing). Two kleptoparasites reached the food parcel on the final observation, suggesting that more might approach outside of the observation time frame. No other *C. citricola* approached the host at any time over the observation window.

2.4.3 Discussion

A very low number of the spiders dropped food items, regardless of kleptoparasite presence. This may reflect the size of the prey items being 'ideal', large enough to be valuable and small enough not to pose much risk. Observation of natural dropping rate would be required to ascertain if this is a regular enough event to explore other factors which may impact dropping rate.

Capture time varied dramatically and it was unclear what factors may influence this, or even if a consistent level of skill or boldness might lead to a somewhat replicable capture time for each individual. It is suggested from this data, however, that presence of kleptoparasites does not influence this.

It is clear that kleptoparasites do commonly travel close enough to the hosts to impact them in other ways, especially to potentially impact feeding as they are shown here to approach the host in 63% of parasitised webs, feeding with the host in 27%. This is more successful than the *A. argyrodes* recorded by

Pasquet *et al.* (1997), where 47% of webs had a kleptoparasite approach captured food but only 3% had successful feeding. This could be due to the more aggressive interactions noted in that study, showing high risk high reward behaviour over the safer strategies observed here.

In this study, *A. argyrodes* feeding did not lead to dropped prey but it is unclear if this would be the case with natural prey and if there are other, more subtle, influences on feeding and dropping behaviours.

Adults, followed closely by sub-adults, were most likely to approach and feed alongside the host, indicating that larger kleptoparasites may be hypothesised to impact the host most, not only by being a bigger physical presence but also behaviourally by approaching the host more often. All but one adult included in this study were female, due to the time of year, but informal observation has indicated that males in this system do not regularly approach the host in this way. It is therefore suggested that, where impacts on hosts are being studied, larger female spiders might be the kleptoparasites to scrutinise most closely.

2.5 Statement of contribution

Sites were largely located by Lena Grinsted, with assistance by Ella Deutsch, Manuel Jimenez Tenorio, Mariano Cuadrado and members of the ‘Arañas de España y resto del Mundo’ (Spiders from Spain and the rest of the world) Facebook group. All data described in 2.2 was collected by Lena Grinsted and Alex Hadleigh, with input into study design provided by Ella Deutsch. Study design for section 2.3 was completed by Ella Deutsch with input by Alastair Gibbons and data were collected by Alastair Gibbons. All analysis of data was completed by Ella Deutsch.

Chapter 3 – Where to call home: abundance and distribution of the kleptoparasitic spider *Argyrodes argyrodes*

3.1 Abstract

The production of webs has been key to the abundance and diversity seen within spiders (order Araneae), allowing the group to effectively colonise even very extreme environments. However, the production of these large external structures opens the resource up to exploitation. This includes exploitation by spiders of the genus *Argyrodes*, small kleptoparasitic species which live on the webs of other spiders and scavenge, feed alongside the host, eat web fragments or even consume host eggs and young. This strategy has led *Argyrodes* to become reliant on these hosts for web structures and food, necessitating close associations between parasite and host. These associations have been explored in some *Argyrodes*-host systems but never in the species *Argyrodes argyrodes* residing on *Cyrtophora citricola* webs in Spain.

Data presented in this chapter show that both species largely mature and reproduce in spring, with only a small peak in autumn. This differs from other studied locations of each species, suggesting an environmental factor in Spain dampening the second reproductive period, alongside maturation speed in *C. citricola*. Patterns of abundance were not always consistent between populations, revealing local variation and aspects of boom and bust as expected in small habitat patches.

A. argyrodes abundance reduced as the number of *C. citricola* in a colony increased, showing highest kleptoparasitic load in solitary hosts but with large variation. When considering the size of the web mass, solitary hosts and those aggregating with only one other individual saw increases in *A. argyrodes* number where web size increased. Colonies did not see any significant

increase in *A. argyrodes* per spider with increasing web mass size. This indicates that web area could be a limiting factor on *A. argyrodes* abundance on solitary webs but that other factors serve to keep kleptoparasites of colonies below the carrying capacity of the resource.

3.2 Introduction

3.2.1 Kleptoparasitism in spiders

Spiders (order Araneae) have been incredibly successful at thriving in diverse and extreme environments across the world, including at limits of food and water availability and extremities of temperature and pH (Cloudsley-Thompson, 1983; Koponen, 1992; Marco Isaia, personal communication, 2020). This success is, in part, attributable to the group's use of silk to aid in the location and capture of prey, dispersal, protection of egg sacs and construction of living structures. These silk constructs are often resource-intensive but benefit the producer sufficiently to confer overall fitness success.

However, in creating such useful structures external to their bodies, spiders can become targets for exploitation by other organisms. These can include conspecifics or other species, attracted to the benefits of utilising silk without the resource and time costs of producing the structures for themselves. Exploitative behaviours can include residing on the web structure, stealing or scavenging of prey items caught in the web and consuming silk. Where such behaviour involves the stealing of resources, especially when this leads to a reduction in availability to the host, the exploiter can be termed a 'kleptobiont' (Vollrath, 1987). A more commonly used term is 'kleptoparasite', which suggests a level of co-evolution between these parasites and hosts but is often used very broadly. Examples of kleptoparasitism by other taxa targeting spiders, especially the large constructs of orb-weavers and social spiders, include hummingbirds stealing web and prey items (Young, 1971); numerous small fly (Diptera) species feeding alongside spiders and hanging from the web awaiting food (Sivinski & Sowe, 1980); scorpionflies (*Panorpa)* feeding on both trapped insects and wrapped food items, often interacting aggressively with the host (Thornhill, 1975); and the scavenging of large food items by lepidopteran caterpillars (Robinson, 1977).

Often, the kleptoparasites of spiders can be other spiders. Commonly, orb-weaving species will show extreme sexual dimorphism and the much smaller mature males will reside on the edges of female webs, scavenging food caught

in the web or left by the female (Schneider & Lubin, 1998). While some examples of kleptoparasitism by spiders occurs between conspecifics, there are also spider species which exhibit kleptoparasitism as a species-wide strategy, residing on the webs of other spiders and scavenging food items and silk from the host (Whitehouse *et al.,* 2002). As with conspecific kleptoparasitism, the degree of parasitism and strategies employed can vary with ecological and environmental factors, as well as between different host and parasite species.

The Argyrodinae form a subfamily within the spider family Theridiidae and contain a number of species that either predate on other spiders or target them as kleptoparasites. These kleptoparasitic species include those in the genera *Argyrodes*, *Ariamnes*, *Faiditus* and *Spheropistha*. Kleptoparasitic species within this subfamily have been recorded scavenging ignored or stored prey, feeding alongside the host, consuming host silk or even directly targeting host eggs or young. These strategies can vary between and within species and individuals depending on factors such as spider size, food availability and competition (Cangialosi, 1997; Whitehouse, 1991).

3.2.2 Host selection and colonial spiders

Kleptoparasitic species such as those within the Argyrodinae can see their distribution and life cycle become closely dependant on their host's due to their reliance on the resources provided by the other spider. Where a kleptoparasite cannot reside independently across its lifetime, or even for short periods of time, the host webs can be seen to function as habitat patches or 'islands' and can follow patterns of isolation explored within island biogeography (Agnarsson, 2003). This is especially important where dispersal is limited; genetic similarity has been found to be highest in group-living, kleptoparasitic *Argyrodes* residing on the same web, reducing with distance, showing low dispersal when compared to free-living, solitary related species (Su *et al.,* 2018).

Many aspects of island biogeography favour residence on larger islands, holding larger populations. These can be more stable and less vulnerable to stochastic events causing extinctions, as well as seeing generally higher genetic diversity. These same factors are also improved by shorter distances

between islands, allowing increased dispersal between patches. It would, therefore, be beneficial to kleptoparasitic spiders to target host spiders which provide larger web 'islands' which sit closely together.

Large, clustered webs can be provided by *Nephila* (and *Trichonephila*) golden orb-weavers, which produce large webs and can show aggregation in some species (Rypstra, 1985). Even more theoretically preferable hosts are colonial spiders including *Cyrtophora* tent-weaving spiders. These species produce generally smaller individual webs compared to *Nephila*, but will often spin these webs very close to conspecifics and join the structures with support lines, forming large colonies. *Cyrtophora* webs are also much more permanent than *Nephila* webs, not using fresh viscid silk to stick prey, and can last for weeks with minimal upkeep (Lubin, 1974).

It has been suggested that living in communal or colony-structured large webs can lead to increased kleptoparasite load on these large webs themselves. This has been shown in the social theriidiid *Anelosimus eximius*, where kleptoparasitic *Argyrodes ululans* are seen in higher, more predictable numbers in larger social colonies (Cangialosi, 1990a). Smith Trail (1980) also found higher numbers of multiple *Argyrodes* on communal webs of the uloborid *Philoponella oweni* and Elgar (1989) found higher numbers of *Argyrodes antipodianus* with increasing aggregation of *Trichonephila edulis*.

In some systems it appears that this pattern of increased load on larger webs is not the case. Leborgne *et al.* (1998) found no significant difference between *Argyrodes* on solitary webs of *Cyrtophora citricola* compared to colonial, with the counts being slightly higher for solitary hosts. Agnarsson (2003) also found no increase in *Argyrodes* numbers where the webs of *Trichonephila clavipes* cluster. However, numbers were more predictable, as might fit a larger habitat patch. While kleptoparasites might find colonies more often and consider them a more stable habitat, there could be protections against kleptoparasitism afforded by aggregation. Social spiders hunting cooperatively are able to monitor a larger web area, detecting kleptoparasites more often and reducing the parasite's foraging success (Cangialosi, 1990b).

3.2.3 *Cyrtophora citricola*

Cyrtophora citricola (Forsskål, 1775) is present throughout Africa, Asia, and the southern areas of Europe, around the Mediterranean sea. The species has also been introduced to both North and South America, where it has become both a common backyard nuisance as well as a growing agricultural pest (Levi, 1997; Serra *et al.,* 2003).

The species sits within the orb-weaving family Araneidae, though the genus is set apart by its long-surviving horizontal web sheets surrounded by tangled support lines, which knock prey items into a position for the spider to capture without the use of glues. Often *C. citricola* will spin these webs close to conspecifics, retaining separate web sheet territories but utilising a shared tangle structure and support lines. These colonies often include overlapping generations and can contain many hundreds of spiders at a time in favourable conditions.

Colonial *Cyrtophora* can see increased food capture and reduced variation in food capture (Grinsted *et al.*, 2019; Rypstra, 1979), greater habitat exploitation (Lubin, 1974), decreased avian predation success (Rypstra, 1979), greater web building efficiency and increased protection for eggs and young (Lubin, 1974) compared to solitary individuals. Arguments can also be made for kin selection acting on these spider colonies, with spiders in proximity likely to be closely related through large clutches and low dispersal rates (Ventura *et al.,* 2017). This could increase evolutionary success for familial lines using group living to reduce fitness variance, even with some individual costs. Costs to colonial living in spiders are not well documented but records exist of regular aggressive interactions between neighbours, increased visibility to predators (trading off with generally lower predation success once spotted) and some prey types as well as smaller individual web sizes, which could be either a cost or benefit (Leborgne *et al.,* 1998). Through such varied fitness effects, colonial web organisation is likely to be beneficial overall in some environments and not others, depending on climate, prey type and natural enemies. Therefore, we see flexible variation in *C. citricola* colony size and structure in different conditions and locations, with high but patchy food availability appearing to be

a major driver of aggregation (Mestre & Lubin, 2011; Rao *et al.,* 2010; Yip *et al.,* 2017).

It is also expected that life history traits would vary under the diverse conditions seen across the range inhabited by *C. citricola.* Females raised in laboratory conditions by Yip & Lubin (2016) lived an average of 304 days after maturation with low prey availability and 239 days with high availability. Diet restriction also led to fewer offspring, perhaps pushing wild individuals to lay eggs within periods of prey abundance, which will vary with location. Very little information exists in the literature on the longevity of *C. citricola* in the wild or when they tend to reproduce. This makes it difficult to make comment of how biotic and abiotic factors may influence this. Observation of colonies within arid environments in Israel show two reproductive periods in spring and autumn (Levy, 1997; Yip *et al.,* 2021). This involves the maturation of individuals between the two periods before laying eggs in a relatively short window of time. However, the desert is unlikely to be an environment which best reflects the life history traits common to the species as a whole, as it often exhibits extreme environmental conditions which may impact specific traits between populations of the same species. Elverici *et al.* (2012) briefly recorded *C. citricola* adults being present in coastal Turkish habitats between June and September, though made no comment on either reproduction specifically or any drop in numbers over the summer.

Cyrtophora moluccensis, a close relative which shares many key traits with *C. citricola*, has been studied in a tropical montane region of Papua New Guinea (Lubin, 1980). Females were estimated to live a total of 7.5 months, with 3.5 months as adults. Colonies contained eggs all year round, but reproduction was lowest towards the end of the dry period (July-August), along with a drop in immature spiders. Individual colonies did not follow a common pattern across the year and colony sizes fluctuated regularly, though numbers appeared to stabilise in established colonies.

The study of *C. citricola* is important for both the understanding of key biological theories and also for its increasing economic importance. Biological interest has often stemmed from its colonial nature, key to understanding the intermediate

steps to true sociality in usually solitary and aggressive species. Further exploration is needed to isolate the genetic and environmental factors leading to aggregation in the species and how this has allowed them to be so successful in areas of poor biodiversity such as deserts, agricultural fields and urban environments.

This success in colonising unwelcoming habitats has led the species to become an agricultural pest, especially outside its native range. Within South America, where the species was introduced in the late 20th century, the dense and extensive webbing of *C. citricola* within agricultural fields has led to significant plant damage through yet unidentified means (Chuang, 2019). The webbing also serves as a physical barrier to harvesting agricultural products, substantially reducing productivity. To limit the need for environmentally damaging pesticides, an understanding of the population dynamics and natural enemies of *C. citricola* is necessary to both predict and minimise economic damage. This understanding may also harness the use of the species in other areas as a pest control measure. In many localities, including southern Spain, the species resides on hardier plants located on the borders of agricultural land, where otherwise the abundance of arachnid predators of flying insects would be low. Large colonies can consume a large prey biomass and, where flying pests are a significant problem, it could be economically beneficial to support the growth and longevity of such colonies.

3.2.4 *Argyrodes argyrodes*

Argyrodes argyrodes (Walckenaer, 1983) [*sub Argyrodes gibbosus* (Lucas)] is a small spider within the family Theridiidae, subfamily Argyrodinae, found across the Mediterranean, West Africa, Canary Islands and Seychelles (Exline & Levi, 1962; Levy, 1985; Platnick, 2021). Like many of its genus, *A. argyrodes* largely lives on the webs of other spiders and forages through kleptoparasitism and sometimes predation on host eggs and young (Whitehouse *et al.,* 2002). The species can be considered a generalist, found residing on the webs of a number of the orb-weaving family Araneidae where their ranges overlap. Kleptoparasitic strategies recorded have included scavenging, stealing food

bundles, feeding alongside the host, eating host eggs and young and attacking moulting hosts (Whitehouse *et al.,* 2002).

It is common to see multiple *A. argyrodes* on one host web and so, much in the same way as *C. citricola*, it can be considered a group living species, seemingly without the cooperative behaviour and skewed reproductive strategies of truly social spiders. This aggregation is likely to be driven by patchy resource availability but also may be influenced by increased survival and foraging success where the host is confused by multiple kleptoparasites on its web. An argument could be made, as in *C. citricola*, for kin selection acting to decrease conspecific aggression; with low dispersal rates leading to high relatedness in *Argyrodes* on the same host web or colony (Su *et al.,* 2018).

Argyrodes abundance is known to be positively correlated with web size, host body size, host density, vegetation complexity and prey availability (Agnarsson, 2003; Kerr & Quenga, 2004; Spear *et al.,* 2017). Density of *A. argyrodes* can vary over time even within the same location and season. Pasquet *et al.* (1997) found an average of one kleptoparasite per web in September 1992 and three in the same month the following year. When considering *Argyrodes* abundance and reproduction, it is also important to consider the whole year rather than a snapshot of time. With such close reliance on host success as well as environmental factors, seasonal abundance would be expected to differ greatly across localities due to the adaptation of life history traits to best exploit their lifestyle.

Very little published work has been recorded on the seasonal abundance of *A. argyrodes* or its life cycle and how this might be impacted by both host species and locality. Preliminary data from Israeli populations located on a variety of vertical orb webs (Araneidae and Tetragnathidae) shows maturation of adults in early spring and late summer, with egg sacs being produced in autumn and overwintering as eggs and juveniles (Efrat Gavish-Regev, personal communication, 2021).

However, data examining other *Argyrodes* species shows significant changes with both host and the presence of competitors. *Argyrodes flavescens* was seen to mature in November where two species of *Nephila* host were present but

saw a further spring peak in adult numbers where their preferred host was removed (Miyashita, 2002). Although there were differences between hosts and localities, total *Argyrodes* numbers studied by Miyashita (2002) generally saw peaks correlating with increases in available web area and *A. flavescens* saw the highest proportion of adults when prey abundance was highest in late autumn, seemingly outcompeting *Argyrodes bonadea,* which was present on the same webs and largely matured in spring. A slightly different pattern was found by Vollrath (1987), where two cohabiting *Argyrodes* species showed slightly different peaks in total numbers while generally following the abundance of *Nephila* hosts, with peaks in adult females in spring and autumn. The two exhibited different kleptoparasitic strategies and active periods, which may explain why they don't demonstrate the completely opposite maturation seasons recorded in Miyashita (2002).

The study of *A. argyrodes* and relatives is important towards understanding the evolution of host-parasite interactions in a system where the parasite is not residing in or on the host, but is closely associated with the web, a key resource and often considered a phenotypic extension of the host. This also becomes an extreme example of island biogeography, with constant fluctuation in size and location of habitat as well as resource availability and risk. *Argyrodes* kleptoparasites are widespread in warm climates and are likely to be a key factor in the ecology of most orb weavers and other hosts in these habitats. *A. argyrodes* is widespread, associated with a number of hosts and often recorded on webs without competing *Argyrodes*, making it an excellent species to study without competition complicating the results. The species' association with *C. citricola*, interesting due to its aggregation, hardy silk and economic importance, makes understanding *A. argyrodes* a major factor in understanding influences on the life history traits of *C. citricola,* as well as considering population control measures where the species is most damaging.

3.2.5 Chapter aims

As *C. citricola* undergoes large changes in aggregation and abundance with stochastic fluctuation and changing environmental conditions, the species is expected to have very different life history strategies and abundance patterns

across different localities and seasons. This chapter aims to describe the abundance and reproductive patterns seen in Spanish *C. citricola* across one year in a number of habitat patches.

Alongside the impact of environmental conditions on *A. argyrodes,* these kleptoparasites are also closely tied to the host spiders providing their webs and food. This chapter will follow abundance and reproductive patterns of this species alongside *C. citricola* and explore how the two might be linked and how this differs from examples elsewhere. The impact of colony size will also be tested to identify if colonial spiders experience difference in kleptoparasitic load when compared to solitary individuals and how this is influenced by the number of spiders in the colony and their web size.

3.3 Methods

This study was conducted between March 2019 and January 2020. In March 2019, sites were selected based on low likelihood of disturbance and large area of *Cyrtophora citricola* habitations. A length (see Table 3.1) along *Opuntia* cactus of high habitation was selected within each site and marked using fabric tape attached to the cactus, with the focal area being 1 metre deep into the cactus to allow *Argyrodes argyrodes* to be seen. Focal area size was selected depending on the density of spiders at the site as well as natural features limiting observation over a larger area.

Sites were visited approximately every 6 weeks, with a focus on the spring and autumn (2019: 29/03, 06/05, 09/06, 19/08, 06/10, 29/11; 2020: 24/01). Visits were either brief, specifically for this study, or as part of longer trips in combination with other data collection. At each visit a number of counts were taken within the focal area, recorded separately for each web mass (colony and solitary): the total number of webs (occupied and/ or empty), the total number of *C. citricola*, the number of *C. citricola* with eggs, the total number of *C. citricola* egg sacs, the number of *A. argyrodes* at each life stage (see Chapter 2.4), the number of *A. argyrodes* egg sacs and the diameter (to the nearest cm) of each *Cyrtophora* web (see Chapter 2.3). *C. citricola* males were largely indistinguishable from juveniles at the recording distance so were not separated in total counts. The number of *Agelena labyrinthica* and *Holocnemus pluchei* within the focal area was also recorded, as these spiders were common in some sites. No observations were made in weather conditions which might cause the spiders to seek shelter including heavy wind or rain.

All figures were created in R 4.0.0 (R Core Team, 2021) using the package ggplot2 (Wickham, 2016). Population fluctuations and life stage monitoring were analysed using detailed graphs covering both total abundances across all areas and densities for each site.

Analysis of kleptoparasite load per *C. citricola* web was completed using a GLMM (Generalized Linear Mixed Model, R package lme4 (Bates *et al.,* 2015)) with a Poisson error distribution, using data from web masses (colony and

solitary) recorded across the whole year containing live *C. citricola*. Only masses completely inside the focal area were included, to ensure accurate kleptoparasite counts.

The GLMM included the total number of *A. argyrodes* in each web mass as the response, with an offset of *C. citricola* number for each colony to give a response variable of kleptoparasites per host. The number of *C. citricola* in each web mass was tested as a predictor alongside the total web area, as well as the interaction term between both variables. Random variables included site and date alongside a unique ID for each mass to reduce overdispersion. Site and date were not tested as variables for significance due to low sampling of large colonies within each. Each model was repeated with adult female kleptoparasite number in place of total kleptoparasites. Some data points may have been the same individuals recorded over time but in this analysis were treated as independent. P values for predictor variables were generated using Likelihood ratio tests.

Table 3.1. Sites used for year-long monitoring and focal area marked for observation (each 1 m deep into cactus). Total monitoring area was 137.3 m^2. For further site information see Table 2.1.

Site	**Total cactus area (m^2)**	**Focal area (m^2)**
EO	661	9.0
CM	612	38.7
NN	486	9.0
PA	1044	18.4
WP	133	23.0
SN	706	39.2

3.4 Results

A total of 1088 *Cyrtophora citricola* web masses with 1264 *C. citricola* spiders and 306 empty webs were observed over the focal area, with 1245 total observations of *Argyrodes argyrodes*, 119 of *Holocnemus pluchei* and 196 of *Agelena labyrinthica.* Across all months, 42% of web masses had at least one *A. argyrodes* present and this proportion was lowest in August and October (26 and 23%), at a medium rate in November, January and March (49, 55, and 48%) and highest in May and June (71 and 67%).

3.4.1 Population monitoring

Figure 3.1 shows the highest number of *C. citricola* webs, matching with the highest number of *C. citricola* spiders, as being in March/ April. However, the total web surface area, and so habitat availability for *A. argyrodes*, peaks later, in May. This is likely to have been influenced by the increase in web sizes in May evidenced in Figure 3.2, with a peak in all web sizes in June. Web numbers (and total *C. citricola)* peak again in October but these webs are much smaller and overall web area sees a sharp drop in June then a steady decline over the rest of the year.

The initial peak in total *C. citricola* in March/ April is closely followed by a peak in total *A. argyrodes* in May. *A. argyrodes* total numbers then remain steady throughout the year. *H. pluchei* and *A. labyrinthica* were present in very low numbers over spring and summer. No *A. labyrinthica* were visible into autumn, while *H. pluchei* saw a peak in numbers in August before disappearing over the winter.

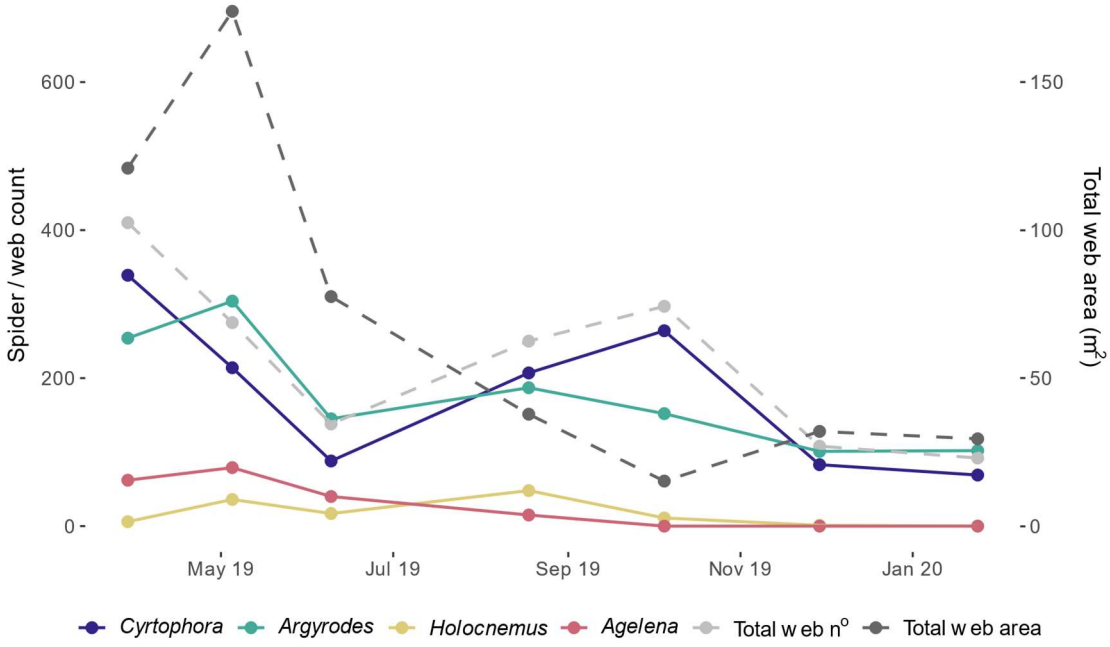

Figure 3.1. The total individual counts of *Cyrtophora citricola, Argyrodes argyrodes, Holocnemus pluchei* and *Agelena labyrinthica* within the focal areas across one year of monitoring alongside the total number of empty and occupied *C. citricola* webs and the total area of the main sheets of these webs, calculated from their diameters.

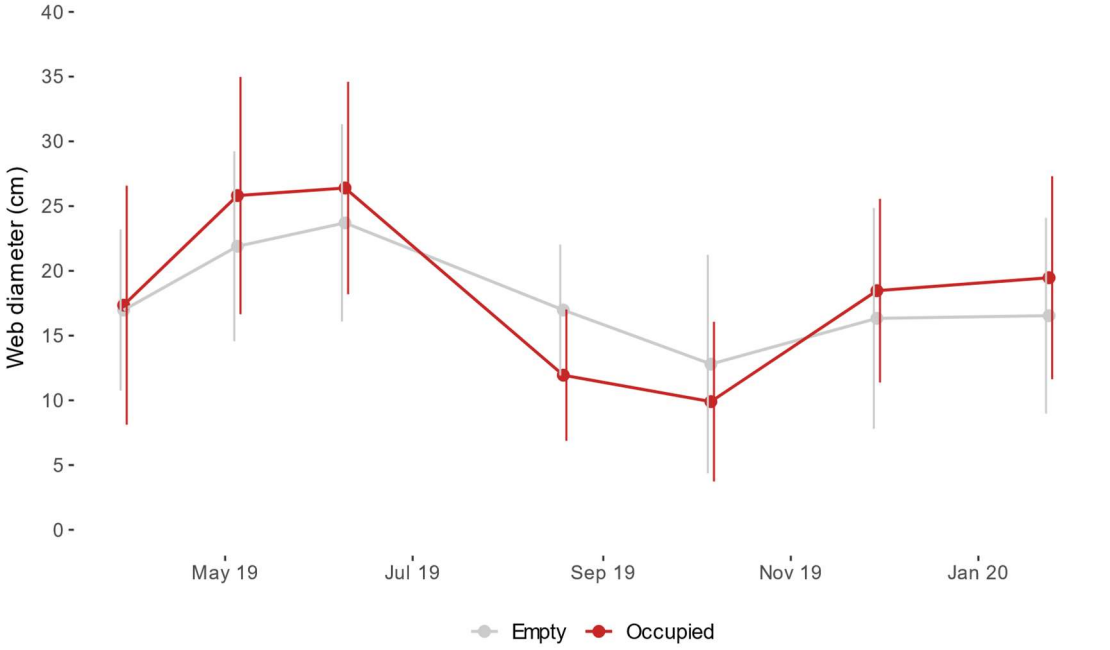

Figure 3.2. The mean web diameter of empty and occupied (by a *Cyrtophora citricola*) webs across one year with the standard deviation.

C. citricola total numbers (as described in Figure 3.3) exhibit two peaks of similar sizes, one in March/ April and one in October. Both total *C. citricola* egg numbers and number of females with eggs largely peaked in June, with a build-

up through the spring. Almost no eggs were present in the mid-summer and early winter and no eggs were present in January. *A. argyrodes* adult female numbers reported in Figure 3.4 matched with the presence of *C. citricola* egg sacs, with a large peak in June and a smaller peak in October.

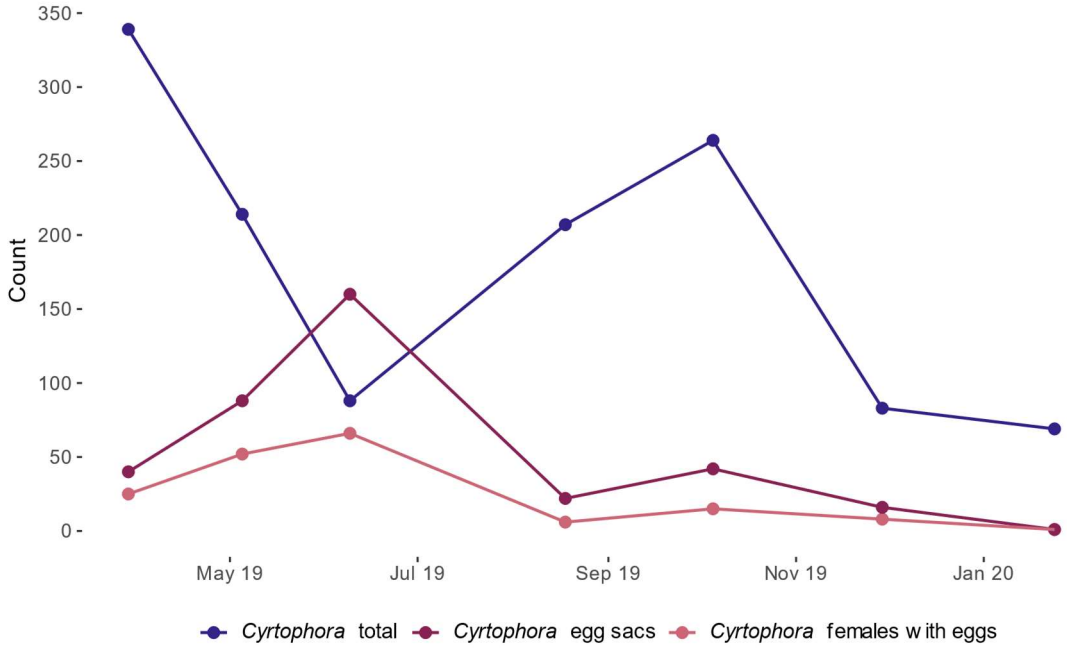

Figure 3.3. The number of *Cyrtophora citricola* counted across the focal regions in one year alongside the number of these with seemingly viable egg sacs on their webs and the total numbers of egg sacs.

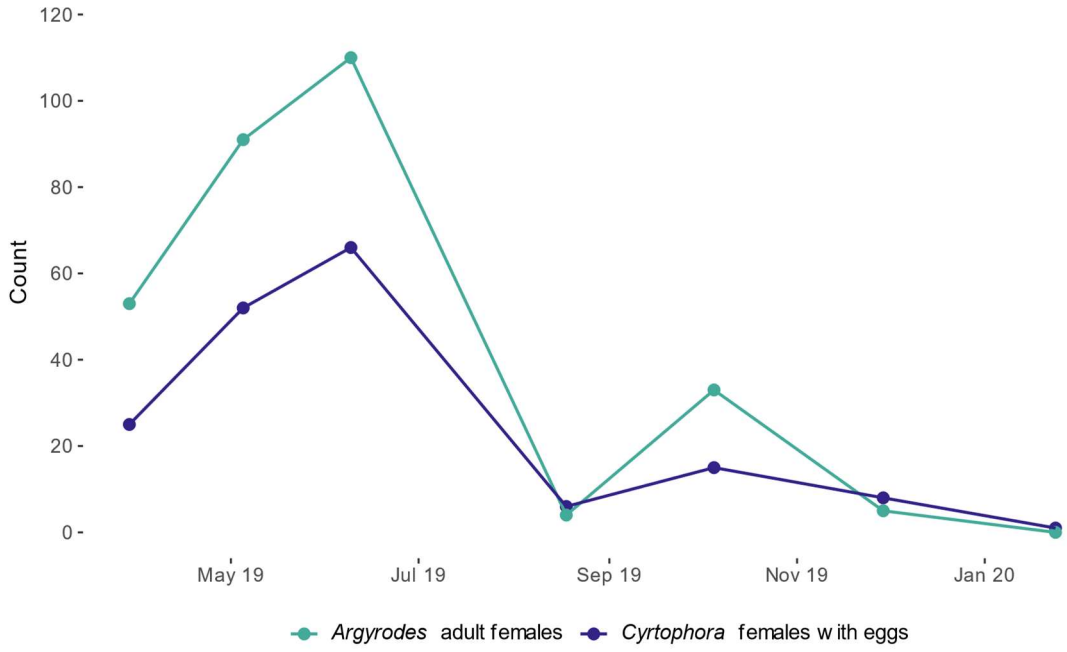

Figure 3.4. The number of *Cyrtophora citricola* across the focal regions in one year with seemingly viable egg sacs shown alongside the number of *Argyrodes argyrodes* adult females present in those same areas.

As shown in Figure 3.5, in late March approximately half of the recorded *A. argyrodes* were subadults; the other half were adults at a 50:50 sex ratio. Into May, the numbers of subadults and adult females rise while males drop. Through June, the numbers of adult females continue to rise while subadults drop dramatically and adult males continue to decrease. In August negligible numbers of adults of both sexes are observed while counts of both subadults and juveniles increase. Juveniles remain present at consistent numbers through the rest of the year while subadults drop slightly in the autumn then remain present in lower numbers. Adult female numbers see a second peak in October and drop to negligible amounts through the winter. Most *A. argyrodes* egg sacs visible in the focal area were present in May and October.

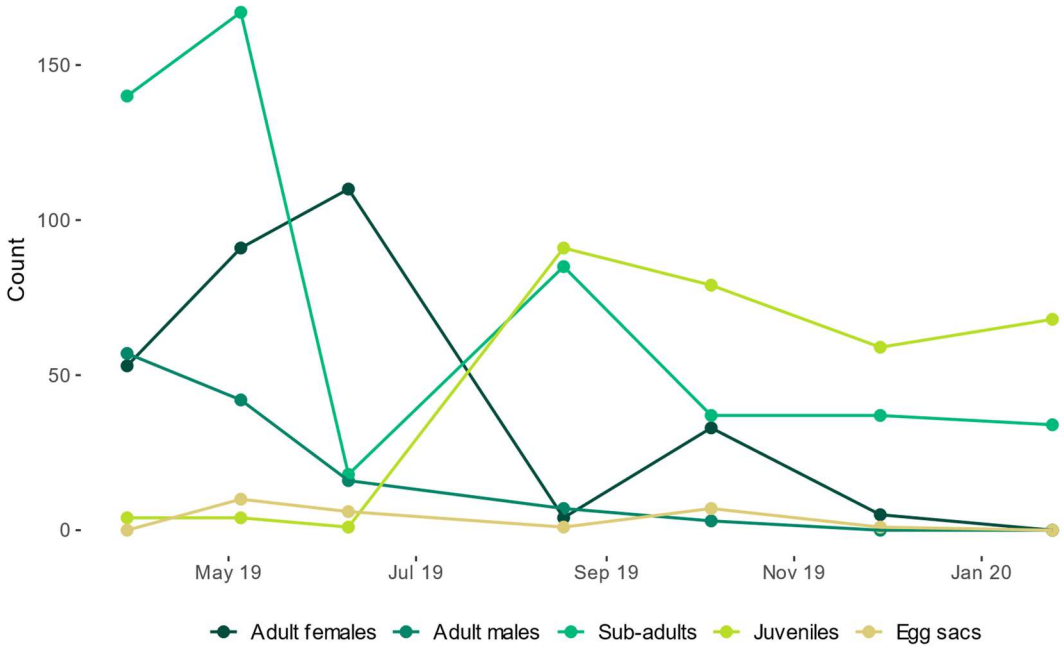

Figure 3.5. The number of *Argyrodes argyrodes* at each life stage present in the focal areas across one year along with number of egg sacs present.

When web size averages across the year are broken down by field site, they each show a consistent pattern, with generally large webs in spring and smaller webs in autumn no matter where they are recorded. CM shows above average

web size through the year but all other sites do not show consistently high or low mean web sizes.

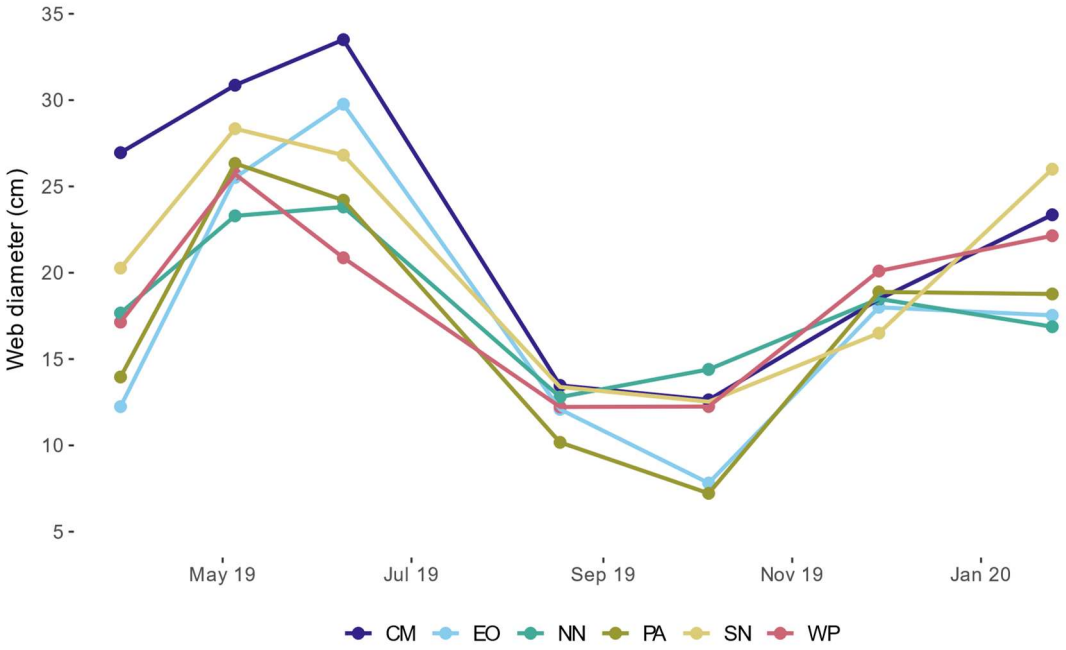

Figure 3.6. The mean web diameter of *Cyrtophora citricola* webs present across the year at each field site.

When *C. citricola* numbers are broken down by site it reveals very different overall densities as well as patterns in spider densities across the year. Compared to other sites, CM, SN and WP had consistently low *C. citricola* densities in the focal area and saw two small peaks in numbers in spring and autumn. PA saw similar low densities in spring before increasing rapidly through August and October and dropping rapidly in December. These low numbers contrast with the relatively high numbers seen in EO and NN through spring. EO then sees a steep decline into summer and a slow decline through the rest of the year. NN sees a similar drop into June but then peaks again in August and October, followed by a gradual decline into winter.

All sites see a peak in *C. citricola* egg sacs in June, apart from WP which sees an early peak in May. All sites then see a drop over the summer and, except EO and CM, a second peak in autumn which is smaller than the spring peak in all but PA. Over the spring breeding period, egg sac numbers are highest on NN and EO, with NN remaining the highest into autumn and EO dropping to below average. PA had the lowest egg sac density in spring but saw an autumn

peak which brought it in line with other sites while CM and SN dropped to the lowest towards the end of the year.

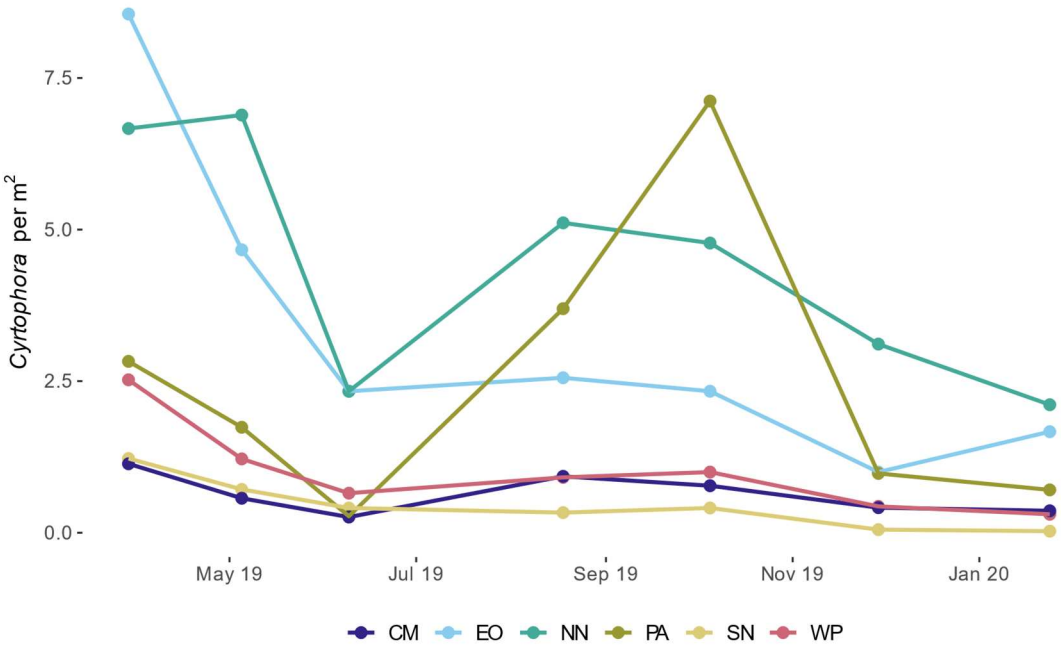

Figure 3.7. The density of *Cyrtophora citricola* for each site across one year.

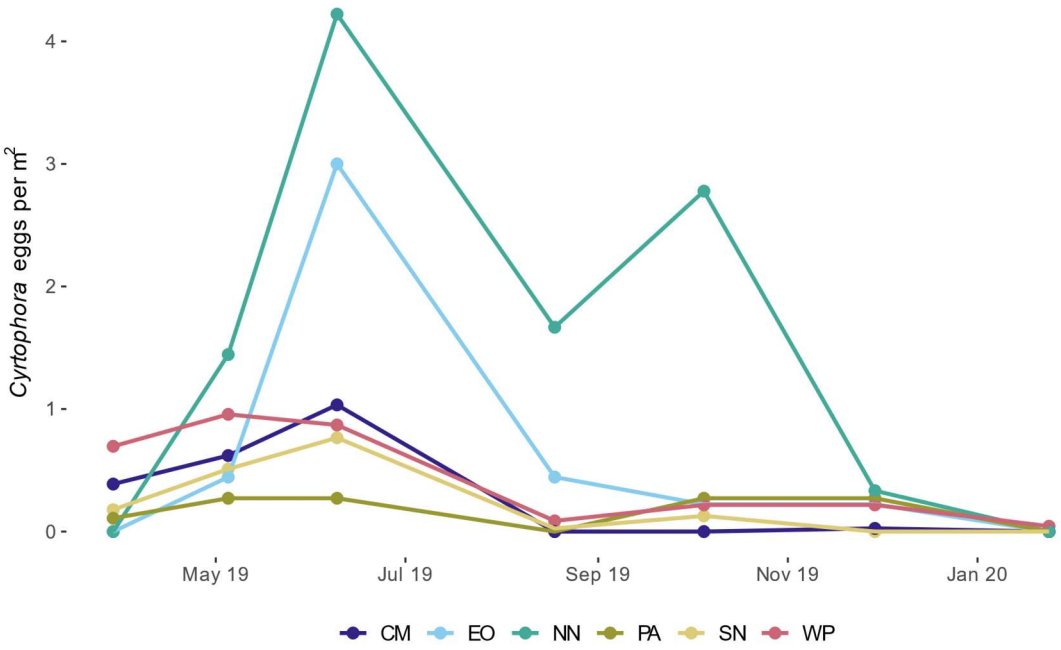

Figure 3.8. The mean density of *Cyrtophora citricola* egg sacs for each site across one year.

While the mean *A. argyrodes* density remains fairly constant through the year, with only a small spring peak, when broken down by site there is little consistency in density change across the year. NN sees a steady, large increase in numbers while EO and PA see a reduction in numbers across the

year. CM sees a small, steady drop while PA and WP remain somewhat constant with monthly fluctuations.

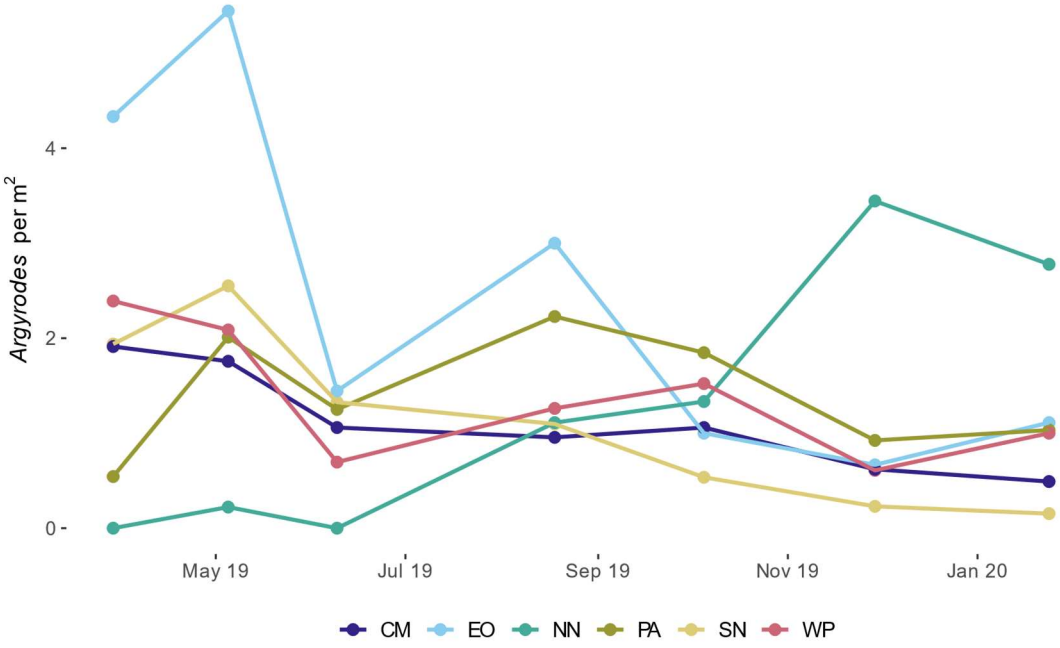

Figure 3.9. The total density of *Argyrodes argyrodes* for each site across one year

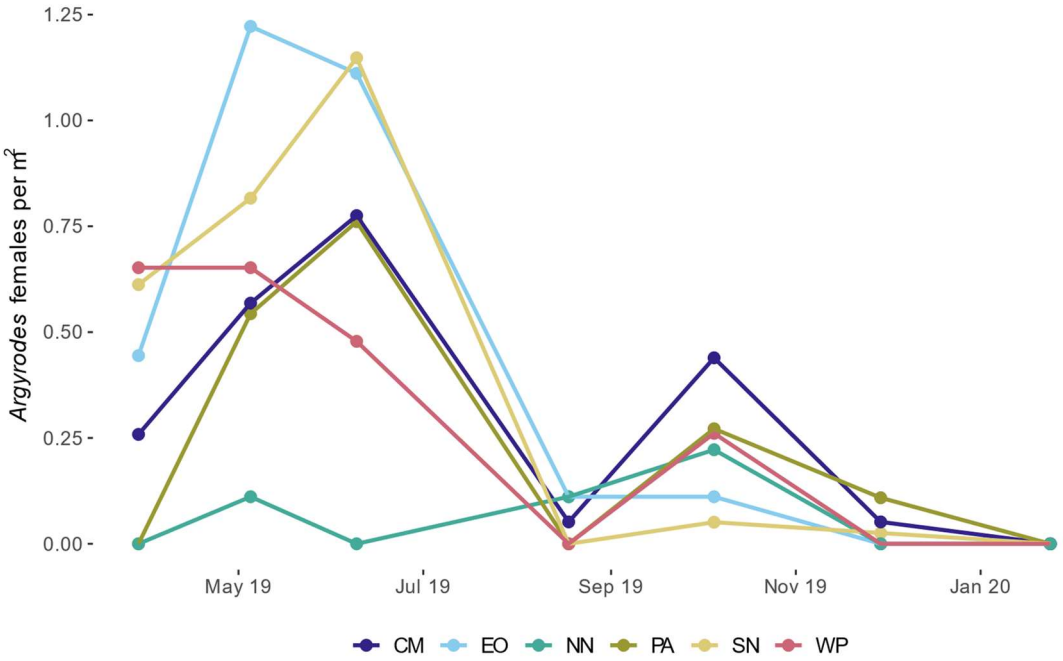

Figure 3.10. The density of *Argyrodes argyrodes* adult females for each site across one year.

When considering the densities of adult female *A. argyrodes,* a more consistent pattern emerges between sites. All sites but NN see highest adult female densities in May or June, dropping dramatically into the summer. All sites then see a smaller peak in October, except EO which saw some remaining females

in the summer and retained this number into autumn. NN sees a substantially different pattern to other sites, starting the monitoring period with almost no *A. argyrodes* and seeing an incline from spring to autumn before dropping back to starting levels over winter.

3.4.2 Kleptoparasite load

Data from 184 colonies and 671 solitary *C. citricola* were included in the analysis, with 1159 *C. citricola* and 919 *A. argyrodes.*

Overall, the number of *A. argyrodes* per host decreased with the number of *C. citricola* present in a colony (see Figure 3.11) but the number of *C. citricola* saw significant interaction with the total web area (χ^2= 33.91, df = 1, p < 0.005).

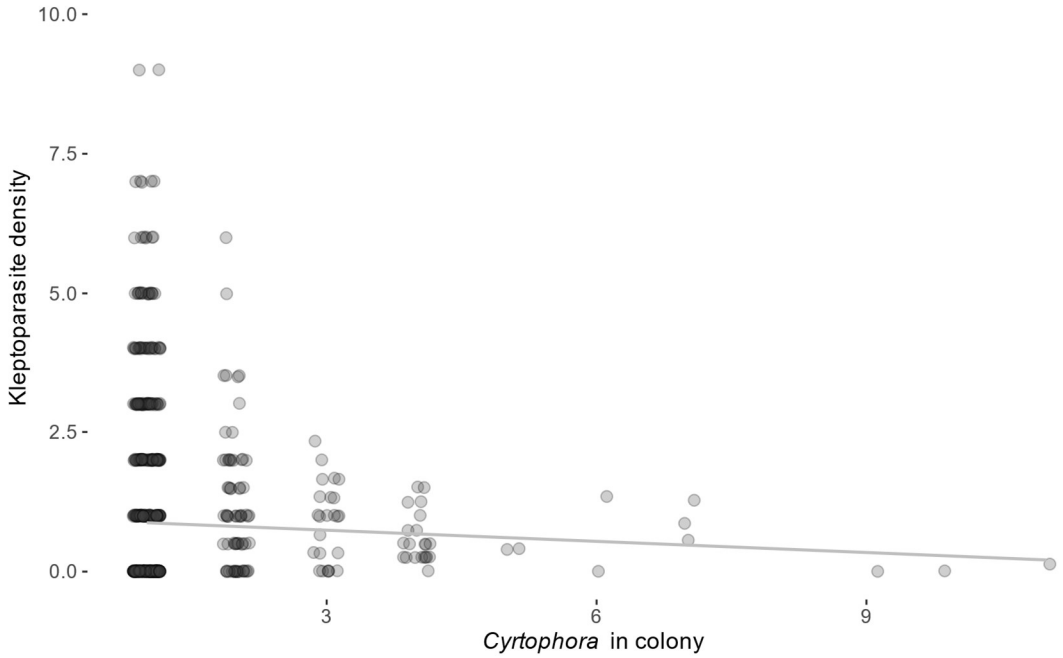

Figure 3.11. The number of *Argyrodes argyrodes* kleptoparasites present per host against the number of *Cyrtophora citricola* present in each colony.

Where the colonies are grouped by number of *C. citricola* present, as shown in Figure 3.12, a significant increase in kleptoparasites was seen with increasing web area in a sub-set of data where 1 *C. citricola* was present (X^2 = 35.18, df = 1, p < 0.005). Where 2 or more *C. citricola* were in the colony, density of *A. argyrodes* per host did not rise significantly with increasing web structure size.

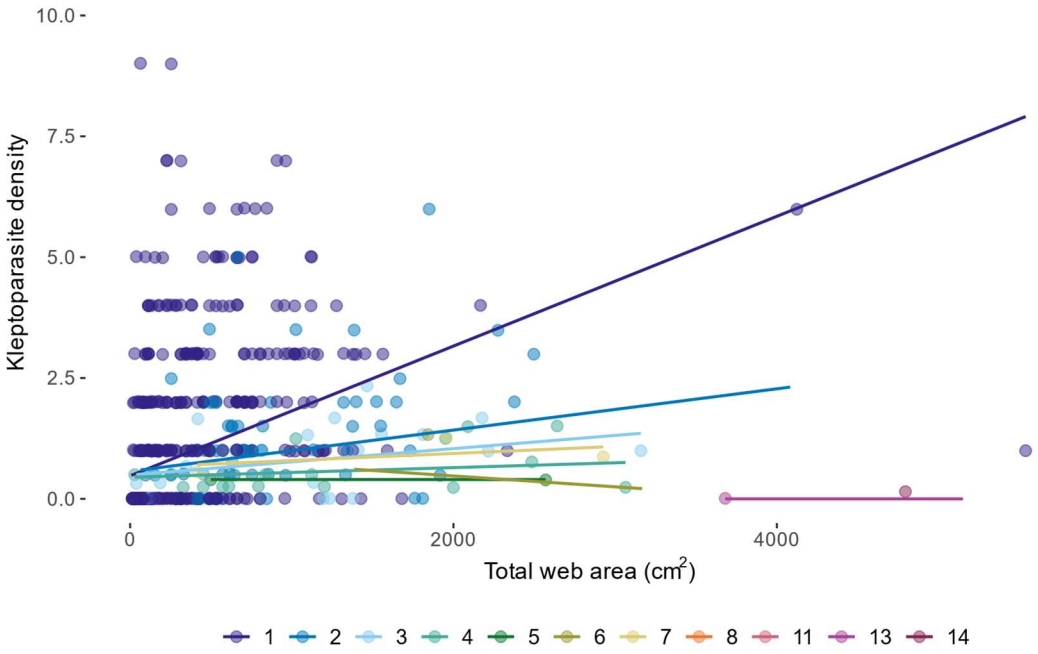

Figure 3.12. The number of *Argyrodes argyrodes* kleptoparasites present per host against the total web area of each colony. Colours separate colonies by the number of *Cyrtophora citricola* present in each, showing a significant increase in kleptoparasite density with increasing area for solitary *C. citricola* while density remains stable with area for colonies.

3.5 Discussion

Observations of *Aglena labyrinthica* and *Holocnemus pluchei* within the same areas as *Cyrtophora citricola* were sufficiently sparse, especially outside spring, to limit the expected widespread importance of these species on *C. citricola* and *Argyrodes argyrodes* ecology. Local or brief interactions such as predation, parasitism and competition, however, remain a possibility as each spider saw large differences in presence between sites. 95% of the total recorded *H. pluchei* were present within the NN site, while *A. labyrinthica* were not present at all in EO, NN or PA and at similar densities elsewhere.

While the number of *C. citricola* webs follows closely with total *C. citricola* present, the total *C. citricola* web area follows closely most months with the average web size rather than number of webs. The web size can be used as a good indicator of spider size (see Chapter 2.3), and so also loosely follows spider maturity. Therefore, the largest habitat availability exists for *A. argyrodes* when *C. citricola* are larger and older, rather than when they are at higher abundances. The total numbers of *A. argyrodes* does peak with the web area in May, dropping sharply into June but then remaining constant through the rest of the year regardless of drops in web area. This is likely to be due to the emergence of large numbers of young *A. argyrodes* in late summer and early autumn, utilising the much smaller *C. citricola* webs available in that period.

A. argyrodes were shown maturing and reproducing in line with *C. citricola* egg production, which could be in response the distraction of *C. citricola* guarding eggs, leading to higher food availability; mature *C. citricola* spinning larger and more successful webs; the availability of *C. citricola* eggs and young to prey on; or it could simply be ideal environmental conditions for both species. It has been recorded by Hajer (1995) that *A. argyrodes* spiderlings feed on the silk of the first nymphal stages of *C. citricola,* necessitating closely timed reproductive periods. This behaviour was not noted in this thesis, but activities of *A. argyrodes* at juvenile stages were not observed in any detail. The reproductive efforts shown in these data are highest in spring, May and June, while a second, much smaller peak, appears in October. While *A. argyrodes* egg sac peaks are similar for spring and autumn, the maturing of both sexes and increase in

juveniles is much larger in spring, suggesting the laying of egg sacs outside of observational view.

Both focal species recorded in Israel see similar peaks in spring and autumn but they are more even, indicating that an aspect of the Spanish environment is driving reproduction in spring over autumn. This could either be influencing both species or only *C. citricola,* with *A. argyrodes* following their host. It is unclear if the autumn *C. citricola* reproductive effort is from females born in spring, as in other localities, or if they are late maturing females born the previous year. Autumn breeding *C. citricola* females recorded in the egg sac collections described in Chapter 4, were on average smaller, with the mother web size in spring averaging 34 cm and autumn 25 cm, indicating that these may have been fast-maturing individuals. It is unlikely that many individuals mature in autumn, as the average web size is very small with a standard deviation comparable to other seasons. A possible selective pressure favouring spring reproduction is presence of *Philolema palanichamyi* wasps, discussed in Chapter 4.

A large difference between these data and those reported from Israel is sex ratio bias in *A. argyrodes*, which is not recorded from Israeli localities. Only one visit date to Spain had an equal sex ratio and numbers of males were very low the rest of the year, suggesting a mostly small window for the majority of mating behaviours and a low availability for late-maturing females. This observation is perhaps due to low visibility of males into the summer, as they shelter more from the sun away from host web centres, but this is unlikely as most web threads are visible. Another experimental error could have been mis-identification of large sub-adult males as adult females but, to test this, 15 identified females had been collected in spring 2018 and housed for a number of months with none maturing into males. Provided the accuracy of this sex ratio bias, males could also have a very short mature lifespan and narrow window of maturation early in the year. However, this would not appear to be selectively supported as any late-maturing males would be expected to see higher mating success and a higher share of paternity with the high proportion of newly matured females (Schneider & Lubin, 1998). It could be that such a low proportion of males is sufficient for the low number of newly maturing

females but, with the difference seen between Spanish and Israeli populations, it would be expected that the bias is driven by a factor only present in Spain. A final explanation is modification of the sex ratio through infection by reproductively manipulative endosymbiotic bacteria and this is explored in Chapter 6.

Abundance and reproductive patterns vary between sites for both species, indicating that local environmental factors may impact these patterns as well as the occurrence of stochastic variation due to the small populations present in each patch. Generally, sites see similar patterns of abundance and maturity with differing overall scales of abundance and extremity of fluctuation. A site of note for *C. citricola* is PA, which sees a very high autumn abundance peak. This could be a representative pattern for the site due to a low presence of whatever factor is subduing autumnal reproduction in the species, or could be a simple random 'boom' fluctuation leading to unusually high reproduction or juvenile survival on the observation year. EO is also outside the mean pattern for both *C. citricola* and *A. argyrodes* abundance, with steadily declining populations across the observation period. No explanatory environmental factor was noted but this may have been due to a stochastic population drop or unseen environmental factor such as pesticide toxicity, shown to decrease prey consumption in *C. citricola* even at below mortal levels (Mukhtar *et al.,* 2018).

When considering *A. argyrodes* abundance, the site well outside the average pattern is NN. This site had substantially lower *A. argyrodes* density than others, while having one of the highest densities of *C. citricola* hosts. This may be due to the high density of *H. pluchei* at this site, acting as a predator or competitor and therefore limiting *A. argyrodes* success. *A. argyrodes* numbers did steadily increase over the year, as *H. pluchei* numbers went down, but it is unclear if this growth was then stymied by an increase in *H. pluchei* the next spring. When comparing the prey biomass collected for each site in spring 2018, NN was found to have the highest flying insect biomass of all sites and this could be driving such high abundances of both *C. citricola* and *H. pluchei* (re-analysis of data presented in Grinsted *et al.* (2019), not shown here).

To fully consider local patterns and their replicability, similar observations must be made over a number of years. This would show both consistent patterns, such as reproductive seasons; long-term trends, such as slow population declines; as well as highlighting one-off events and patterns caused by environmental factors and stochastic fluctuation. Abundance measures would be expected to vary greatly as island patterns drive small populations up and down stochastically but are unlikely to completely die out without major damage to the habitat as *Cyrtophora* species see very high colonisation success (Rodríguez-Cabrera *et al.,* 2018). However, even with some variation, local patterns which remain, such as constantly high abundances or high reproductive rates (perhaps with high juvenile mortality) could be observed. These could then be linked to local biotic and abiotic factors such as prey availability, predator presence, agricultural practices, temperature or rainfall; but a wider sample with targeted variables would be required to identify these conclusively.

When it comes to considering kleptoparasite density on host webs, there are two measures to contemplate: kleptoparasites per host (or host web) and kleptoparasites per unit area. Both have biological reasons to be considered. A higher number of kleptoparasites per host is likely to lead to an increase in parasite-host interaction and may confuse the host, due to web vibrations from a number of sources. A higher number per host may also lead to more scavenging, web consumption and predation of eggs and young where present, leading to this measure being vital, regardless of the size of host or host web. However, the density of kleptoparasites per unit area should also factor into these considerations. A smaller host web (usually with a smaller spider and lower prey capture) with the same number of kleptoparasites as a larger web, is likely to experience any negative impact of kleptoparasites heightened and may see increased aggression between parasites over resources. The web area available, including webs no longer containing host spiders, functions as the kleptoparasites' habitat patch and therefore total area may be vital for their population stability, as described by Agnarsson (2003). While colony area functions as habitat area, the number of hosts (along with their size) could function as a measure of prey availability. These two factors are often used

interchangeably in the literature, with ‘larger colony’ being measured as either a physically larger web mass or, more often with *C. citricola*, as the number of spiders present. Kleptoparasitic load in this chapter is defined as total *A. argyrodes* per *C. citricola*, with the assumption that this best represents the parasitic load on the spiders and allows inferences into how this might impact individual fitness and evolutionary strategies. However, area is also considered when exploring the relationship between host aggregation and kleptoparasitic load and a complex interaction emerges.

When considering colony size as the number of *C. citricola* present, the number of *A. argyrodes* per spider is negatively correlated with colony size, with larger colonies having fewer parasites per host when compared to smaller colonies and solitary individuals. No comment can be made on the range of kleptoparasite loads between webs in larger colonies as the data collected in this chapter are colony-level, with no information on the variation in parasite load between webs within the colony. The reliance on colony-level data, as well as the relatively few larger colonies fully within the focal area, leads to a low sample size when considering larger colonies, so the data cannot definitively show the validity of this conclusion when considering colonies larger than 5 spiders. However, the data shown here are a strong indicator of the negative correlation across all colony sizes.

The decrease in kleptoparasites with increased colony size supports the conclusions of Leborgne *et al.* (1998), who found a decrease in *A. argyrodes* per *C. citricola* during one observation period, although this was not significant in their data. The new data show this more conclusively and suggests that aggregation in *C. citricola* could serve to reduce kleptoparasitic load and any negative impacts which might occur from a large number of *A. argyrodes* per host. This could be through increased parasite detection by colonial spiders, as seen in other group-living species (Cangialosi, 1990b), or perhaps another factor such as increased web complexity or presence of natural enemies.

When considering kleptoparasitic load per spider, there is also a relationship with the web area available. In solitary *C. citricola*, the number of *A. argyrodes* increases with web area. This supports the data available across a number of

Argyrodes hosts showing increasing load in line with web size (Agnarsson, 2003). This would be expected with both the increased area available and also may be due to higher prey capture by spiders with larger webs (see Chapter 4). It is unclear to what extent the host might be impacted by this increasing total number of parasites on their web, even with the density of parasites per web area staying somewhat constant.

C. citricola in colonies did not see any change in *A. argyrodes* per host with increasing web area. This could perhaps be due to the overall lower density of *A. argyrodes* per web in colonies, with populations not reaching the maximum possible carrying capacity of that web area and so not benefitting from increased area. There could be the impact of specific factors which appear to lead to the decreased kleptoparasites per spider in colonies, such as increased detections by hosts, which may not allow kleptoparasites to reach a higher number per spider in the larger colonies. It is important to consider that colony area is likely to be related to both the number of *C. citricola* alongside their body size, so that where two colonies have the same number of spiders but very different areas, they are likely to be recorded from different times of the year with spiders of differing ages (see the web sizes across the year in Figure 3.6). A higher sample size across all colony sizes for each date is required to replicate the same pattern seen here with time of year as a factor considered in the model.

This chapter describes a close association between the abundance and aggregation of host spiders *C. citricola* and the abundance and aggregation of their kleptoparasite *A. argyrodes* in southern Spain. As evidenced in other *Argyrodes*-host pairs, the total numbers of each spider follow each other closely across the year and *A. argyrodes* reproduction sits just slightly after that of *C. citricola*. However, evidence is shown of dramatic differences between sites, where patchily distributed populations of *C. citricola* see ‘boom and bust’ dynamics, which then dramatically impact the kleptoparasites relying on them. While larger *C. citricola* colonies are expected to provide a more reliable habitat patch, they also appear to have fewer kleptoparasites per spider, indicating perhaps increased defence against parasites with aggregation. These data show a first glimpse into the relationships present in this system and how these

differ from other reports across the distribution of *A. argyrodes* as well as those of other *Argyrodes* species and host systems.

3.6 Statement of contribution

The data contained in this chapter were all collected by Ella Deutsch following methods designed by Ella Deutsch and Lena Grinsted. All data analysis was completed by Ella Deutsch.

Chapter 4 – Nuisance neighbours: impacts of kleptoparasitic load on the colonial tent-weaver *Cyrtophora citricola*

4.1 Abstract

Kleptoparasitic spiders reside on the webs of other spiders, often scavenging food and utilising the host web for food capture, courtship behaviours and egg sac suspension. While these kleptoparasites are usually small in comparison to the hosts and go largely unnoticed by the host, studies have indicated that they impact the fitness and behaviour of the host in a number of ways. These include negative impacts, damaging webs and causing hosts to relocate, but also positive impacts, through increasing insect collisions with the web and therefore host prey capture rate. None of these studies have examined the relationship between *Cyrtophora citricola*, a colonial tent-weaving spider, and *Argyrodes argyrodes*, small kleptoparasites which reside on the webs of numerous host spiders, but which show a preference for the large and stable *Cyrtophora* webs.

This chapter describes significant impacts of *A. argyrodes* on *C. citricola* feeding behaviours while finding no influence on host relocation, web repair or parasitoid wasp infection. Feeding frequency by hosts decreased with kleptoparasite infection, suggesting a lower nutritional intake by hosts with the presence of *A. argyrodes*. It is unclear how detrimental this is to the hosts' overall fitness and, with no other factors identified to be impacted by kleptoparasite presence, it may be that the impact of parasites is not significant enough to cause *C. citricola* to resort to avoidance behaviours such as relocation and aggressive activity.

4.2 Introduction

4.2.1 Kleptoparasitism by the Argyrodinae

Kleptoparasitism describes a directly competitive behaviour involving the stealing of resources from one organism by another. This strategy is found widely across the animal kingdom and can involve conspecifics or distant taxa. Examples, among the many found across the literature, include the stealing of opened mussels from adult oystercatchers (birds of the family Haematopodidae) by juveniles of the same species (Goss-Custard *et al.*, 1998); large mammals such as bears or lions chasing smaller or less dominant mammals such as wolves or hyaenas from carcasses (Allen *et al.*, 2021; Périquet *et al.*, 2015); and dung beetles of the genus *Cleptocaccobius* taking over dung already rolled by other species (Cambefort, 2014).

In many organisms this behaviour can be opportunistic, while other taxa such as *Cleptocaccobius* have come to exploit others as a more fixed behavioural strategy. This is the case with many genera of the subfamily Argyrodinae (including *Argyrodes*, *Ariamnes*, *Faiditus* and *Spheropistha*, which lie within the family Theridiidae (Arnedo *et al.,* 2004; Su & Smith, 2014). Many of these widely distributed spiders very rarely produce their own food-capture webs and live mainly on the webs of larger spiders, usually orb weavers of the families Araneidae and Tetragnathidae (Exline & Levi, 1962).

These kleptoparasites have a number of foraging strategies, depending on factors such as host behaviour and prey availability, and will feed alongside the host on its prey, steal the prey from the host (Vollrath, 1979), scavenge leftover prey items, catch small trapped insects (Tso & Severinghaus, 2000) or feed on host eggs (Pasquet *et al.,* 1997) and spiderlings (Whitehouse, 1991). These feeding behaviours can vary with host species, kleptoparasite species, parasite experience (Whitehouse, 1991) and environmental factors (Cangialosi, 1991, 1997), leading to varied and complex interactions.

4.2.2 Parasite or commensal?

There has been great debate concerning the effects of ‘klepoparasitic’ Argyrodinae on the fitness of their hosts, with indications that they can both

benefit and cost the larger spiders. Recorded costs to hosts can include direct reductions in reproductive success caused by consumption of eggs and spiderlings. Impacts can also come through silk consumption by the parasites (Tso & Severinghaus, 1998), leading to costly repairs and a reduction in prey capture success, as well as through an increase in relocation by spiders as a result of high kleptoparasite load (Grostal & Walter, 1997). This has only been observed in *Nephila* hosts and it is as yet unclear if this effect holds true in other spiders.

While some impacts are clearly negative, there remains debate concerning more subtle impacts of kleptoparasites on their hosts. Nutrition plays an integral role in the fitness of organisms, with small changes to feeding behaviours impacting success and fecundity. Kleptoparasites most closely interact with their hosts where food is involved, and this means nutrition is an important consideration in research concerning the impacts of parasitic interaction. Some behaviours, such as directly stealing food parcels from the host, are clearly costly and can lead to slower host growth rates (Grostal and Walter, 1997; Larcher & Wise, 1985; Rypstra, 1981). However, not all kleptoparasite-host species pairs have such interactions and kleptoparasites feeding alongside the host or scavenging leftovers may have minimal impacts. Work on *Argyrodes fissifrons* on colonial *Cyrtophora* webs has even shown very little overlap in the prey preferences of each spider (Tso & Severinghaus, 2000). These parasites seemed, most commonly, to attack very small insects trapped by the host's web, apparently unnoticed, and were largely deterred from feeding on the host's larger food items by aggressive behaviour. This debate can often lead to the use of the term commensal rather than parasite, indicating a relationship benefitting the commensal species while conferring no fitness costs to the other species.

There have been indications that host spiders can even see prey capture benefits from *Argyrodes* presence. Peng *et al.* (2013) obscured the silver coloration of *Argyrodes fissifrons* and found a reduced number of moth entrapments by *Cyrtophora unicolor* webs, suggesting that the colouration of kleptoparasites is in some manner attracting or confusing the moths, increasing *C. unicolor* prey availability. It has also been suggested that, in eating small

insects trapped by webs or leftover food caught by the host, the kleptoparasites function to maintain the web, keeping it clean (Grostal & Walter, 1997). This is especially important on *Cyrtophora* webs, which are not regularly replaced so accumulate detrimental detritus over time.

Spiders of the genus *Cyrtophora* form large colonial webs within the tropical and Mediterranean regions of the world. The colonies consist of individual horizontal orb webs connected by a tangle of long draglines. These structures are often more permanent then other orb webs, with a high tensile strength and no glues used in prey capture (Blamires *et al.,* 2012). This permanence can make them vulnerable to kleptoparasites, who benefit from a high host availability, prey concentrations and prey predictability (Smith Trail, 1980; Cangialosi, 1990).

Tso and Severinghaus (2000) explored the relationship between two *Cyrtophora* species and their *Argyrodes* kleptoparasites, finding no difference in food intake by *Cyrtophora* when kleptoparasites were present. This suggested that they have no cost to the feeding behaviour or nutritional intake of the host. However, this study observed natural kleptoparasite populations, so benefits and costs could have been masked by other factors, such as more successful hosts attracting more parasites. A study artificially manipulating parasite populations is therefore required to ascertain an overall impact on host feeding and no work has been published concerning other impacts on *Cyrtophora* hosts, even with factors recorded in other spiders such as web repair and relocation.

4.2.3 Hymenopteran parasites of spider eggs

An aspect of kleptoparasitism that has been underexplored and has the potential to impact the *Cyrtophora citricola* in Spanish populations is the potential of interaction between kleptoparasitic spiders and other web residents and visitors, including other species targeting *C. citricola*. A common factor to consider is that of wasps parasitising the spider's egg sacs for their own reproductive purpose. This includes wasps of the genus *Philolema*, with some attacking insects as primary or secondary parasitoids, while many (formerly the *Iatrodecti* species group of *Eurytoma*) use spider eggs as a larval host (Noyes,

2018). Hosts are largely recorded as spiders within the ‘widow’ group of the family Theridiidae and wasps are recorded laying their eggs within the spider egg sac case, with hatching larvae predating spider eggs inside before emerging from the egg sac as adult wasps (Vetter *et al.*, 2012; Taucare-Ríos, 2018; Chuang *et al.*, 2019).

Philolema palanichamyi (Narendran, 1984) wasps were common within Spanish locations studied by Chuang *et al.* (2019). The authors found that this species, previously recorded with an Afrotropical, Neotropical, and Oriental distribution (van Noort, 2021), often infected egg sacs of Spanish *C. citricola*. This involved the laying of wasp eggs in up to two thirds of spider egg sacs, with few or no spiderlings hatching from the infected eggs. This suggests significant impacts on *C. citricola* in the region, with any impact on parasitism rate by wasps likely to impact the reproductive success and hence fitness of the spiders. No mention was made of any interactions between the wasps and any of the spiders residing on the web and no study has explored any more general factors influencing infection likelihoods of individual webs.

4.2.4 Chapter aims

With the literature describing a number of impacts on hosts from kleptoparasitic spider presence, it is to be expected that they are influencing *C. citricola* within the Spanish study system in some way. However, this interaction is likely to be complex, perhaps with both benefits and costs of varying magnitudes.

This chapter focuses on a number of possible factors impacting *C. citricola*, chosen both from literature examples in comparative species and from observations of key variables present within the Spanish study sites. The first factor tested is feeding behaviours, serving as an indication of nutritional intake. It has been widely shown that nutritional intake is key to fitness across the natural world including in spiders. Studies have indicated both positive and negative nutritional impacts of spider kleptoparasitism, which could significantly influence host survival and reproduction. This study aims to isolate any impacts of *Argyrodes argyrodes* presence on *C. citricola* feeding.

A second recorded impact of kleptoparasitism that may be important is that of reduced web repair and web abandonment after damage. As *Cyrtophora* have strong and relatively permanent webs, web repair is key to maintaining the webs for full functionality over long periods. The spiders invest more in these webs then other species and therefore abandoning these webs to move positions is more costly, requiring the construction of a new web and also loss of position within the colony structure. This study aims to discover if *A. argyrodes* presence impacts *C. citricola* web repair as well as relocation after web damage, with the previous feeding study briefly covering relocation under natural conditions.

A variable not covered by previous work on kleptoparasitism but observed to be a significant factor in the life histories of Spanish *C. citricola,* is the parasitism of egg sacs by the parasitoid wasp *Philolema palanichamyi*. This is explored in the last study of this chapter. With the wasps being so small (a mean female body size of 1.68 mm was reported by Chuang *et al.* (2019)), they appear to be easily overlooked by *C. citricola* adult females but it could be possible that they interact with *A. argyrodes*, especially adult females which sit close to the centre of the web. These kleptoparasites may act as a predator or deterrent to the wasps or perhaps they might distract the host's attention away from wasp presence. This study aims to discover if their presence has an impact on egg parasitism.

4.3 Methods

4.3.1 Feeding behaviours

This study was conducted in October 2018, located at field sites EO, WP and PA (see Table 2.1) within the province of Cadiz, southern Spain. Sites were selected with on average smaller colonies (2-5 spiders) and higher numbers of solitary webs to allow easier control and recording of kleptoparasite number per web.

To observe feeding with natural kleptoparasite loads, 61 *C. citricola* adult female webs were selected. All were within 50 cm of the cactus edge on two separate cactus patches, selected and indicated by marking the cactus. The webs' central nets were measured across the diameter with a tape measure to the nearest centimetre (see Figure 2.6) and it was noted if each web was solitary or part of a colony. Observations were made every other day for 7 total monitoring days. Feeding observations were carried out across each day (10:00-18:00) and included 8 measurements of host feeding, as a binary measure – either feeding or not feeding. Recordings were made every 30 minutes with a longer break between each batch of 5. This was performed in the daytime as that appears to be the active period for the spiders and prey within the area, perhaps with cold nights or low prey activity limiting the nocturnal feeding seen in other regions. Prior to recording feeding, each day a recording was made of the number of *Argyrodes argyrodes* on each web at each life stage (see section 2.4.1 for life stage definitions). No focal *C. citricola* died or relocated over the observation period.

For recording feeding with manipulated parasite numbers, 45 solitary *C. citricola* webs containing adult females were selected. All *A. argyrodes* were initially gently removed without damaging the web structure. Each web was disturbed lightly with the paintbrush used to transfer spiders ten times in total to ensure even treatment, with kleptoparasites removed as part of this action. The webs' central nets were measured across the diameter with a tape measure to the nearest centimetre (see Figure 2.6). They were then randomly assigned one of three treatment groups indicating the number of kleptoparasites to be added, with 15 webs in each group. The webs either had

no additions, one addition or three additions. Adult female *A. argyrodes* kleptoparasites were collected from a neighbouring cactus patch and added to each web according to the treatment category, with a very light disturbance that was replicated across webs with fewer additions. Adult females were selected as these were predicted to have the highest impact on host feeding (see section 2.4).

Over the following 6 days, the kleptoparasite numbers were recorded first thing in the morning and additions were made to ensure levels were always consistent with the treatment category. On average, 10 (22%) webs each day required additions due to kleptoparasites missing and 3 (7%) required removal of new kleptoparasites. Across the day (10:00-18:00) the feeding status of the host was noted 10 times as a binary measure, either feeding or not feeding. Recordings were made every 30 minutes with a longer break between each batch of 5.

All data analysis was performed using R 3.6.0 (R Core Team, 2017). The feeding frequency for each day was included as a proportion. Binomial GLMMs (Generalized Linear Mixed Models, R package lme4 (Bates *et al.,* 2015)) were performed with the feeding frequency as a response variable and web size and *A. argyrodes* number as predictor variables in both experiments, and with colony status added in the natural load analysis. The natural load analysis was also repeated with the numbers of *A. argyrodes* adult females substituted for the total counts. Random effects included were the day, spider ID and a generated random number to reduce overdispersion. P values for predictor variables were generated using Likelihood ratio tests. A GLM with a Gaussian error distribution was also used to test if web size was a predictor of kleptoparasite number.

4.3.2 Web repair

This study was conducted in October 2019, located at field site CM (see Table 2.1) within the province of Cadiz, southern Spain. The site was selected due to a kleptoparasite load and colony size which well represented an average for the field sites, as well as a high population size of *C. citricola*, minimising study damage to populations and allowing more extensive follow-up work to be

completed at the same site. No rain or high winds were present over the study period.

A total of 28 suitable webs (solitary or small colony individuals close enough to the cactus edge to be within reach but not in a position to be regularly damaged by the weather) were identified at 17:00 and the number of kleptoparasites present was noted. The webs' central nets were measured across the diameter with a tape measure to the nearest centimetre (see Figure 2.6). Each web was disturbed 10 times, removing the kleptoparasites without damaging the web. Webs were randomly assigned into two treatment groups, adding two adult female *A. argyrodes* (collected elsewhere on the site) to 14 webs and lightly disturbing the other 14 without any additions.

The following morning at 09:00, damage was made to the webs. Each had a slit cut into the main net, from edge to centre, parallel to the cactus line on the right (position 3 on a clock, for ease of monitoring and comparison). This cut position was chosen as a cut on the opposite side from the cactus line would damage the structural integrity of the web and access close to the cactus was poor. Three connecting lines were also cut in the same area. This destruction mimicked the observed effects of falling objects or deliberate damage by *C. citricola* in the process of prey capture. Each web was then visited at 09:00 the next morning. During this visit the repair efforts of the *C. citricola* were noted and classified as N (no new webbing to repair or compensate for damage), P (partial repair, some new webbing to either net or support lines) or R (repaired, net and support line repairs are such that function is as before).

With the low sample size, analysis of the web repair results was exploratory. All data analysis was performed using R 4.0.0 (R Core Team, 2021). A Chi-Square Test of Independence was performed comparing counts of fully repaired webs to partially repaired and unrepaired webs between treatment groups.

4.3.3 Parasitic wasp infection

Field sites NN, CM, EO, WP, PA and SN (see Table 2.1) were visited across one year (2019: 29/03, 06/05, 09/06, 19/08, 06/10, 29/11; 2020: 24/01). Where egg sacs were present in more than approximately 5% of adult webs (where

collection would be unlikely to damage the population), up to three egg sac strings were collected from webs spread across each site (2019: 29/03, 06/05, 09/06, 06/10, 29/11). The diameter of webs was noted (see Figure 2.6) as well as the number of kleptoparasites at each life stage (as defined in 2.4.1) and number of other *C. citricola* present in the colony, usually smaller *Cyrtophora* surrounding the female with eggs. A total of 121 egg sacs were collected across 78 webs.

Egg sacs were weighed, separated from others in the string, and stored within the laboratory at 25ºC in 50 ml falcon tubes bunged with foam. Trays of egg sacs were misted lightly twice per week. Egg sacs were monitored for the emergence of spiderlings and wasps and the numbers of each hatching were recorded, along with emergence date.

All data analysis was performed using R 4.0.0 (R Core Team, 2021). Data from visits with very low infection (less than 2) were excluded (2019: 29/03, 06/05), leaving the data from 66 egg sacs. A Binomial GLMM (Generalized Linear Mixed Model, R package lme4 (Bates *et al.* 2015)) was used with wasp presence or absence as a binary measure as the response variable. Predictor variables were *C. citricola* colony size, web size, total number of *A. argyrodes* and visit date. Random variables were the ID of the mother, to take into account the paired nature of two data points from egg sacs from the same web, and site. This model was repeated with adult female *A. argyrodes* counts in place of total *A. argyrodes.*

Egg sacs with wasps present in this reduced dataset (a total of 40) were included in a Poisson GLMM using the number of hatched wasps as the response and colony size, *A. argyrodes* counts, and trip dates as predictor variables. Mother ID and site were included as random variables, along with a unique egg ID to reduce overdispersion. No interaction predictor variables were tested in either model due to the low sample sizes. P values for predictor variables were generated using Likelihood ratio tests.

4.4 Results

4.4.1 Feeding behaviours

Natural kleptoparasite total numbers did not correlate with feeding frequency (see Table 4.1), and this is true also for the numbers of adult females ($\chi^2 = 0.89$, df = 1, $p > 0.05$). Web size did correlate with feeding frequency, with larger *C. citricola* webs (containing generally larger spiders, see section 2.3) feeding slightly more frequently (see Table 4.1). Web size also correlated with the total number of *A. argyrodes* (GLM: estimate = 0.42, t = 12.28, $p < 0.005$).

Table 4.1. Results from a GLMM on the feeding frequency of *Cyrtophora citricola* hosts with natural *Argyrodes argyrodes* kleptoparasite numbers.

Predictor variable	χ^2	df	p value
Web size	5.31	1	0.021
Argyrodes number	1.35	1	0.25
Colony status	0.0011	1	0.97

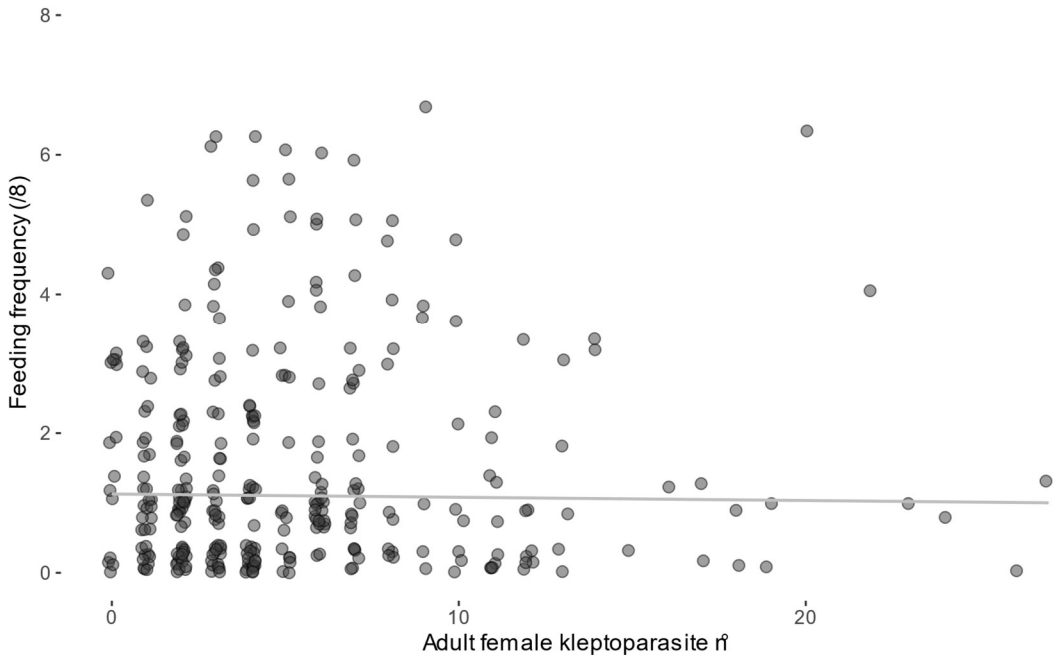

Figure 4.1. The number of *Argyrodes argyrodes* of all sizes on each web compared to the number of observations (out of 8 total across one day) where the host *Cyrtophora citricola* was feeding.

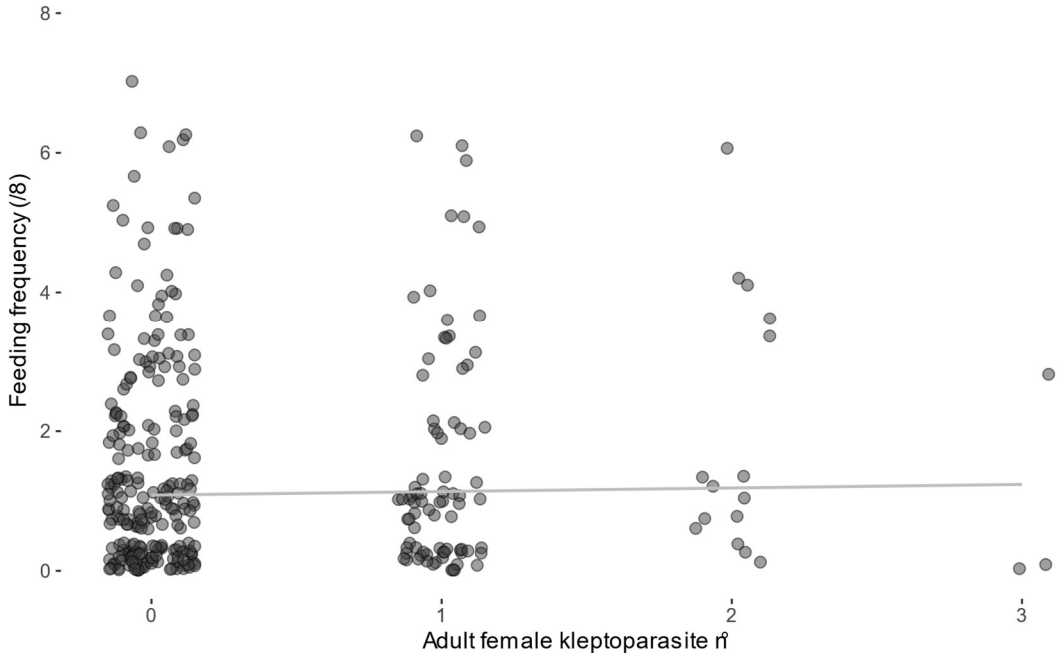

Figure 4.2. The number of *Argyrodes argyrodes* adult females on each web compared to the number of observations (out of 8 total across one day) where the host *Cyrtophora citricola* was feeding.

The manipulated number of kleptoparasites on the host web did have a significant negative impact on host feeding frequency (see Table 4.2). Web size did not have an effect on feeding frequency. A total of 5 host spiders disappeared from their webs over the course of observations, either having relocated or died. This was not significant across treatment groups (Chi-Square: $\chi^2 = 3.15$, df = 2, $p > 0.05$).

Table 4.2. Results from a GLMM on the feeding frequency of *Cyrtophora citricola* hosts with manipulated *Argyrodes argyrodes* kleptoparasite numbers (0, 1 and 3).

Predictor variable	**χ^2**	**df**	**p value**
A. argyrodes number	7.06	1	0.0079
Web size	1.79	1	0.18

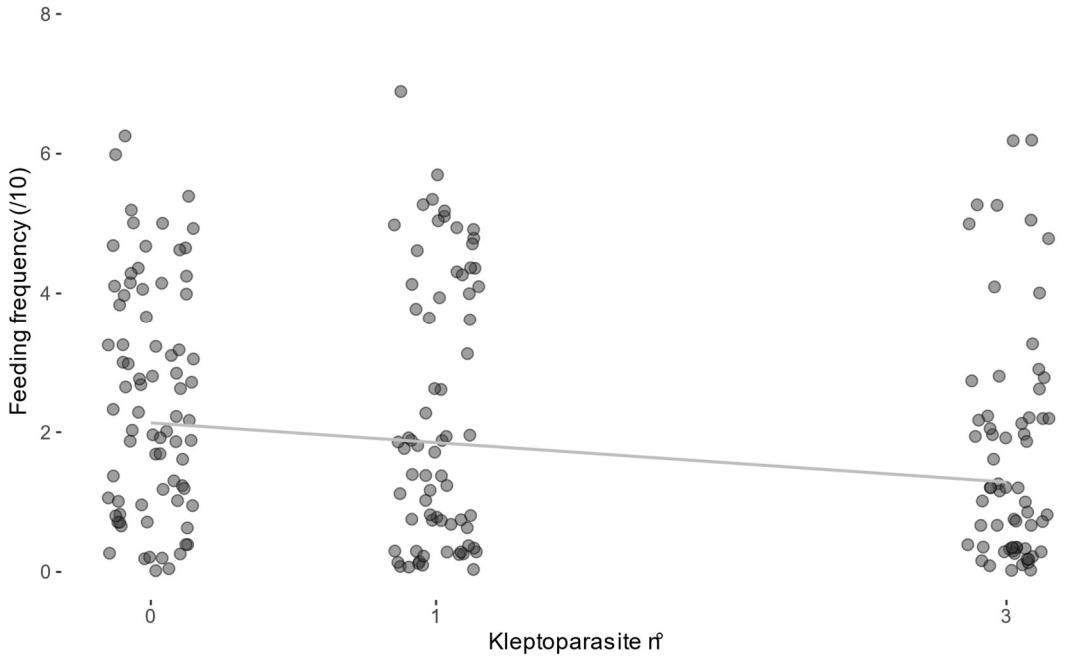

Figure 4.3. The number of *Argyrodes argyrodes* adult females placed on each web compared to the number of observations (out of 10 total each day) where the host *Cyrtophora citricola* was feeding.

4.4.2 Web repair

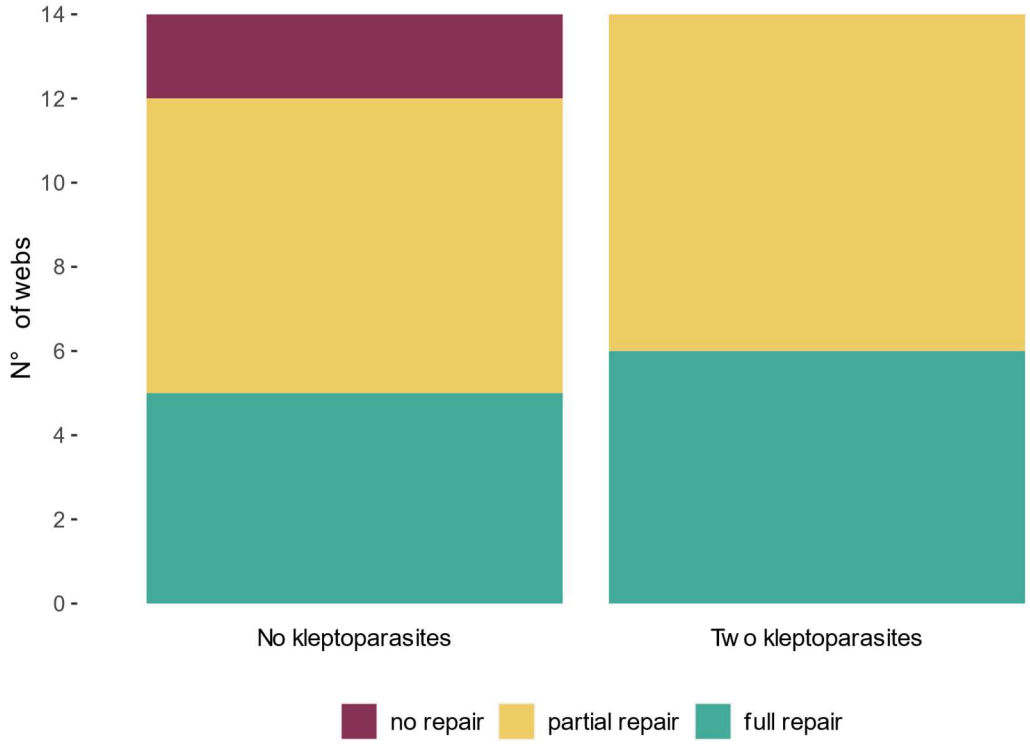

Figure 4.4. The number of *Cyrtophora citricola* webs with and without *Argyrodes argyrodes* that saw repair by the resident *C. citricola* over 24 hours.

Provided the final spiders were the same individuals as the first, no spiders, host or parasite, relocated or died over the study timeframe. A Chi-Square Test of Independence showed no significant difference in web repair between treatment groups from expected under a null hypothesis of no kleptoparasites (Chi-Square: $\chi^2 = 0$, df = 1, p = 1). This is also the case using original *A. argyrodes* number (Chi-Square: $\chi^2 = 2.07$, df = 3, p = 0.56).

4.4.3 Parasitic wasp infection

Of the 121 *C. citricola* egg sacs collected, 42% hatched spiderlings, 36% wasps and from 7% both emerged, though not usually at the same time. Wasps preceded spiders in 66% of egg sacs hatching both. 30% of egg sacs did not hatch at all within 6 weeks of collection. Hatching frequency is summarised across the year in Figure 4.5, with wasp infection being most common in June and October (eggs were rare between these visits and so not collected).

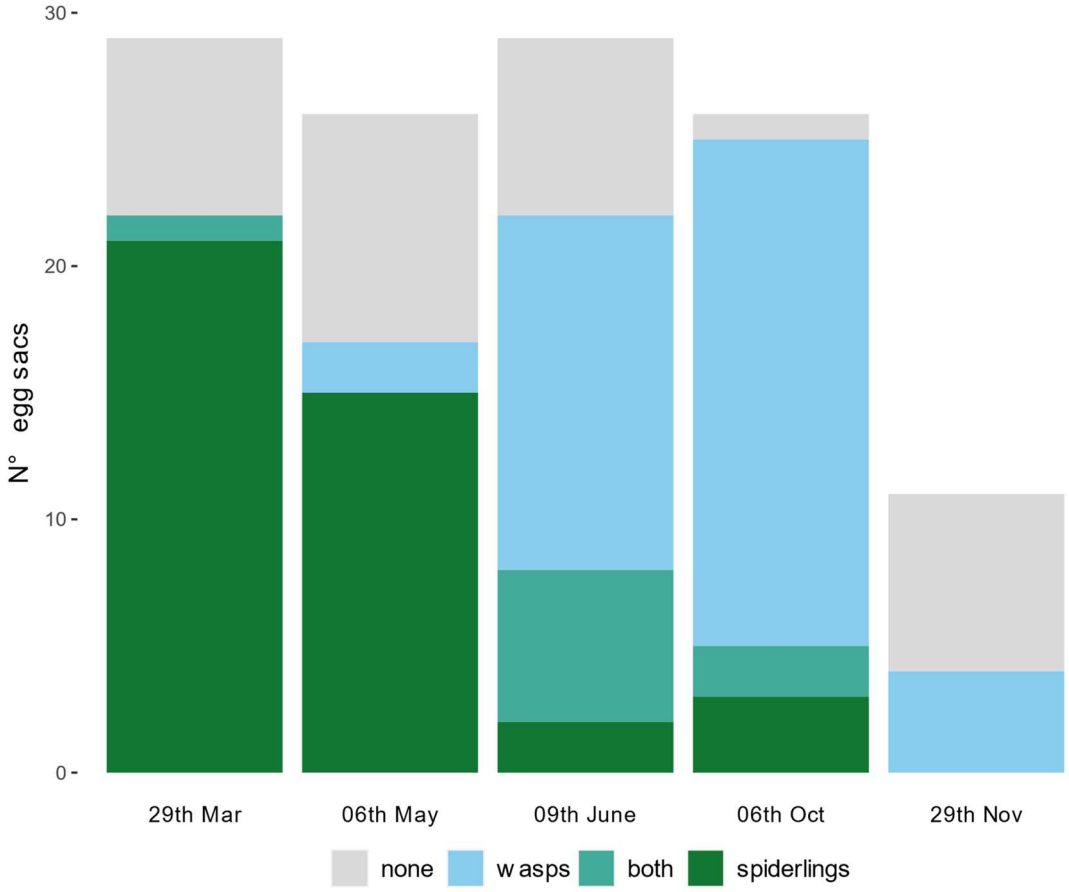

Figure 4.5. The total number of *Cyrtophora citricola* egg sacs collected on each collection date with the proportion that did not hatch, those that only hatched *C. citricola* spiderlings, those that hatched only *Philolema palanichamyi* wasps and those that hatched both spiderlings and wasps.

The infection of an egg sac by wasps did not correlate with the total number of *A. argyrodes* present on the maternal web, web size or trip date (see Table 4.3) or with the number of adult female *A. argyrodes* (χ^2 = 0.29, df = 1, p > 0.05). Infection presence did decrease with colony size (see Table 4.3).

Table 4.3. Results from a GLMM on the presence of wasps in hatching egg sacs.

Predictor variable	**X^2**	**df**	**p value**
Colony size	7.50	1	0.0061
Argyrodes number	0.42	1	0.51
Web size	0.73	1	0.39

Where wasps hatched, their total numbers, as opposed to presence or not as a binary measure, did not significantly correlate with web size (χ^2 = 3.68, df = 1, p > 0.05) colony size (χ^2 = 0.43, df = 1, p > 0.05) or *A. argyrodes* number, including total (χ^2 = 0.81, df = 1, p > 0.05) and adult female *A. argyrodes* (χ^2 = 1.24, df = 1, p > 0.05).

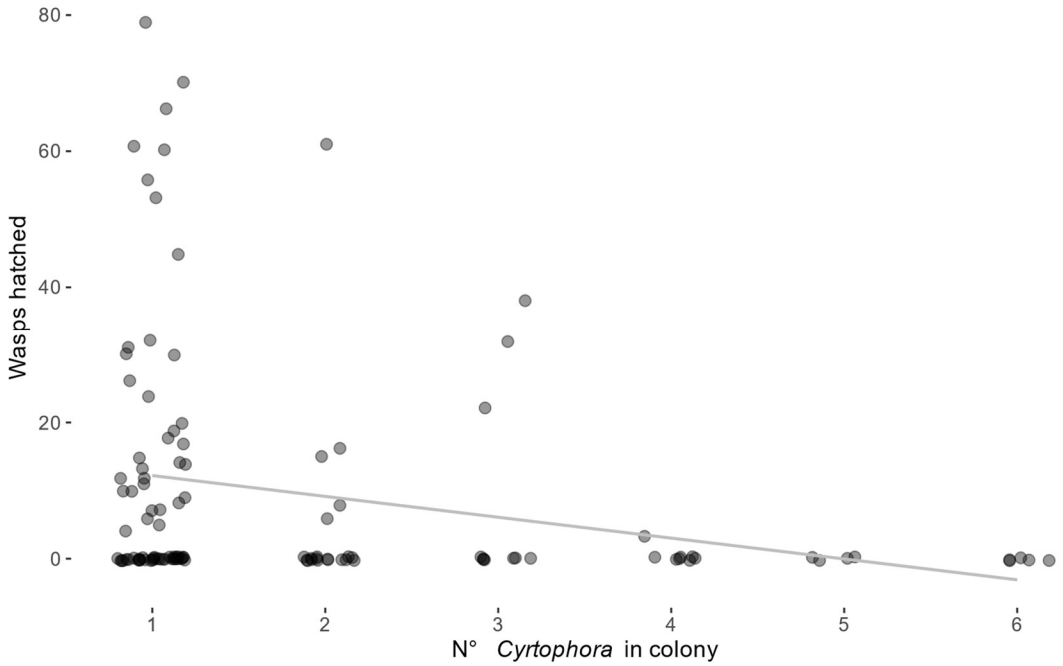

Figure 4.6. The number of *Philolema palanichamyi* wasps hatching from collected *Cyrtophora citricola* egg sacs, including those with no wasps, against the number of *C. citricola* present in the colony from which the sac was collected. While no significant correlation was found between wasp number and colony size, wasp presence as a binary measure was significantly reduced with increasing colony size.

4.5 Discussion

The lack of correlation between kleptoparasite number and feeding frequency in unmanipulated populations supports the results of Tso & Severinghaus (2000), who found no impacts on feeding by kleptoparasites. This, however, could be influenced by many factors. The relationships between web size and capture rate indicates that larger webs and spiders feed more frequently. There is then a correlation between web size and kleptoparasite load, which could indicate that successful, or merely larger, webs are more attractive to the smaller spiders. This preference for webs with a higher feeding rate could mask any impact of the *A. argyrodes* on this feeding behaviour and does not allow for clear conclusions on feeding influence.

When the numbers of kleptoparasites were manipulated and therefore did not correlate with web size, a negative relationship emerged between host feeding frequency and kleptoparasite load. There are a number of possible explanations for this pattern, as feeding frequency could be impacted by either food handling time or prey capture frequency. Food handling time could be reduced with the presence of kleptoparasites stressing the host, increasing aggressive behaviours while feeding leading to dropped prey items. This was not observed in investigations described in Chapter 2 (Section 2.4) but many factors, especially prey type, could lead to increased prey dropping under certain circumstances.

Prey capture frequency could be reduced with parasite load due to an impact on host behaviour, either distraction or stress, caused by kleptoparasite movement or a modification of web vibrations for prey location. Lower catch rates could also be due to a higher web visibility, with prey avoiding the web more successfully. Peng *et al.* (2013) indicated that *Argyrodes* were increasing web capture through prey attraction but this was in a nocturnal system, with different prey types and sensory influences and the reverse could be true with diurnal *Cyrtophora* hosts. An experiment under more controlled conditions with increased direct observation would be required to ascertain the exact change in host behaviour either in handling time or capture rate.

While it is challenging to make conclusions on the direct impact of feeding frequency on nutritional intake and consequently survival and reproduction, there is clearly a reduction in the availability of food to the host spider due to parasitism. Regardless of whether this stems from reduced web efficacy, host prey capture or handling time, nutritional availability is reduced, and this could have severe impacts on fitness under certain conditions.

The lack of correlation between web repair behaviour and kleptoparasite numbers could indicate a lack of detectable impact in this area, with any disruption caused by kleptoparasite presence having limited influence on behaviours outside of feeding. The lack of a correlation between relocation and treatment group in the feeding trials as well as the lack of any relocation during web damage, which would be expected to increase relocation chance, indicates that the increase in web abandonment by *Nephila* hosts does not hold true for *C. citricola. Cyrtophora* invest more heavily in their web and the lack of abandonment suggests that negative impacts by a higher kleptoparasite load might be less costly than avoidance measures such as relocation. The studies contained in this chapter were designed to simulate the usual observed kleptoparasite load (normally maximum two adult females per web) and web damage (not common, with time to repair before further damage) but may not simulate more extreme situations. A future study is required with more extensive kleptoparasite loads and increasing web damage to ascertain if a threshold exists which would lead to increased web abandonment by *Cyrtophora.*

No pattern appears to be present between kleptoparasite load and parasitic wasp infection. This indicates that while *A. argyrodes* are not acting positively for the hosts as a deterrent to, or predator of, wasps, they are also not significantly distracting the *C. citricola* mother in such a way as to increase wasp infection. The correlation between colony size and wasp infection suggests that colony living may serve to protect *C. citricola* females from egg parasite infection, and the decreased fecundity it causes. This indicates a selective pressure towards group living and, alongside other benefits, serves to explain why group living persists in spite of costs such as higher kleptoparasitic infection in such systems. The impacts these wasps are having on *C. citricola*

in the area are unclear, and whether the measured infection rate is a stable level, fluctuating or increasing. It is possible such infection in the area is new and *C. citricola* will dramatically reduce in numbers into the future or it could be a stable system, with *C. citricola* producing sufficient young to overcome such infection. The presence of current avoidance measures is supported by the data presented in Chapter 3 (Figure 3.3), which shows highest *C. citricola* egg sac numbers in the spring, when wasp levels are low. This could be a factor shifting Spanish *C. citricola* away from the two breeding seasons seen in the species in other regions.

Overall, these studies have provided evidence for and against theories presented in the literature. Previous work has indicated positive or no impact on feeding rates but, in artificially manipulating kleptoparasite numbers in a diurnal species of *Cyrtophora*, a negative influence on feeding rate emerges. Conversely, where in *Nephila* hosts web relocation is a cost to parasitism, this is not supported here in the more permanent colonies of *Cyrtophora*. While this study did not find that *A. argyrodes* presence influenced parasitic wasp infection, it does characterise the significant pressures faced by *C. citricola* due to *Philolema palanichamyi* infection of egg sacs, which may be contributing to selection pressure acting on the study system and lead to detectable life history changes in the future.

The new information presented here demonstrates the numerous, often subtle, influences these small spiders can have on their hosts and the unpredictability of these influences across species, habitat and other ecological factors. With a broad range of recorded host-kleptoparasite species pairs across numerous habitat types worldwide, it appears that there is a similarly broad variety of adaptations and interactions across both species.

4.6 Statement of contribution

All data were collected by Ella Deutsch except kleptoparasite counts and observations of feeding frequency under natural kleptoparasitic load (as described in 4.3.1), which was collected by Bethany Turner following methods outlined by Ella Deutsch. Study design and analysis was completed by Ella Deutsch with assistance by Lena Grinsted.

Chapter 5 – A tangled web: evolution of silk protein structure within the sub-family Argyrodinae

5.1 Abstract

Silk production can be considered key to the success of spiders (order Araneae) across the world, with great diversity in the fibre properties arising from its purpose and environment. This material can form strong support lines, protective egg sac casings, flexible prey capture strands, adhesive coatings and many more. This can include extreme examples, such as silk produced under water and in arid or low pH environments. Many species rely on specific silk properties for survival and these proteins are under constant selective pressures. However, it might be expected that species with ecological strategies less reliant on silk might see relaxation of these pressures.

This chapter considers spiders within the subfamily Argyrodinae, of which many are kleptoparasitic, living on the webs of other spiders and rarely producing their own silk for housing or food capture. They appear to exclusively produce safety lines and egg sacs, utilising a minimal number of silk types of the seven produced by their free-living relatives. This chapter considers silk generally used for support lines, MaSp, and that used for web construction and prey wrapping, MiSp, to identify relaxation of selection on the amino acid structure of MiSp proteins.

MaSp and MiSp amino acid sequences are presented from transcriptome data of 10 species within the Argyrodinae, kleptoparasitic and free-living, as well as one free-living relative. Terminal domains were analysed using gene trees and no support was identified for a greater mutation retention in primary protein structure of kleptoparasitic species. Amino acid motifs in the terminal and repetitive regions were also similarly variable across kleptoparasitic and free-living species. This finding suggests that either MiSp is used by kleptoparasites, perhaps within support lines; kleptoparasitic strategies are too recent for the

build-up of mutations; or that seemingly kleptoparasitic species are more generalist then is noted in the literature, retaining the necessary proteins with which to survive independently.

Support is found in this chapter for regions identified within the primary protein structure necessary for fibre formation, and so found to be highly conserved across species and silk types. A number of variations are noted and discussed, with many demonstrating compensatory structures elsewhere in the sequence but some seemingly without, providing insight into regions perhaps not entirely necessary for fibre formation or conversely indicating sub-standard fibre formation within individuals of the Argyrodinae.

5.2 Introduction

5.2.1 The ecological function of silk

Silk is a proteinaceous fibre produced by a number of arthropod species. Among the most well-characterised is the production of cocoons by the silkworm *Bombyx mori* due to its captive rearing for use in the textile industry, termed sericulture (Yokoyama, 1963). Silk serves a huge range of functions for its producers, forming cocoons, nests, strong attachment threads, egg clusters and protective barriers against predators. This functional diversity leads taxa to form optimised silk with interspecific, intraspecific and intraindividually diverse properties (Madsen *et al.,* 1999), with selection favouring properties to best utilise the environment and maximise fitness.

Arguably the animal with the most diverse uses for silk are spiders, forming the order Araneae. Spiders forming more basal lineages, such as the Mesothelae and Mygalomorphae with heavy bodies, two pairs of book lungs and limited ecological diversity (Coddington & Levi, 1991), demonstrate restricted silk function and diversity. These species use their silk to line burrows, lay tripwires, wrap prey and form egg sacs and sperm webs, generally with silk spun into sheets (Shultz, 1987; Starrett *et al.,* 2012). Spiders of the infraorder Araneomorphae, sometimes referred to as ‘true’ spiders, are considered to have more diverse silk production. This can include strong silk used to create large and sturdy webs, glues to form attachments and trap prey, flexible silks to form the capture spiral and nets, draglines to ‘balloon’ for dispersal, alongside a diverse range of egg casing properties across species (Vollrath, 1992; Herberstein & Tso, 2011).

Diversity between species allows taxa to become specialised to more extreme environmental conditions, for example the water spider *Argyroneta aquatica,* which is able to produce sheet webs that trap air underwater (Bell, 1852; Correa-Garhwal *et al.,* 2019). These structures are not only able to adhere to anchoring points within this aquatic environment, they are also able to allow gas exchange, permitting the spider to live and breed underwater with limited need to surface (Seymour & Hetz, 2011). Much of spiders’ success in extreme

habitats such as caves, deserts, acidic environments and even prey-limited human homes can be attributed to specialised silk use. The understanding of the structure and evolution of this silken material is key to understanding the biology of spiders as a whole.

With such diversity in silk properties, including extreme strength and flexibility, as well as antimicrobial properties and natural biocompatibility, spider silk has long been considered a desirable material for use by man. It has been traditionally used in clothes and wound dressings (Bon, 1710), but the cannibalistic nature of spiders makes harvesting material on a large scale challenging. Modern methods of synthetic production have used alternative expression organisms such as *Escherichia coli,* yeast and even goats to produce proteins based on natural silks, with varied success (Edlund *et al.,* 2018; Decker, 2018; Fahnestock & Bedzyk, 1997; Fahnestock & Irwin, 1997). Understanding the properties of natural silk and how these are informed by variations in the genetic code and protein structures produced is key to the development of effective novel materials to solve significant challenges faced in numerous industries (Lefèvre & Auger, 2016).

5.2.2 Silk use by theridiid spiders

Spiders within the family Theridiidae, commonly called cobweb spiders or tangle-web spiders, have a wide range of hunting strategies and web structures. Commonly studied species include the medically significant widow spiders of the genus *Latrodectus* and the model organism *Parasteatoda tepidariorum*, both of which produce irregular, three-dimensional tangle webs with both support and ‘gum-footed’ capture strands (Benjamin & Zschokke, 2003). These web structures are constructed incrementally and include central or peripheral retreats, where the spider will reside for extended periods and repair and expand the web where needed, in contrast to the often short-lived orb webs of the family Araneidae. Araneidae also exhibit behavioural variation within individuals in their web construction, with late-stage construction flexibility which is very uncommon in orb-weavers (Benjamin & Zschokke, 2003).

The tangle-web structure is common across the theridiid family, but some groups have modified and expanded their silk use for specialist strategies. Some produce similar structures but with viscid lines rather than the 'gum-footed' lines while other produce sheet webs with no viscid elements, relying on 'knockdown' lines above the web to capture prey (Benjamin & Zschokke, 2003). Cooperative *Anelosimus* theridiid spiders also produce sheet webs but in colonies of hundreds of spiders with meters of webbing (Nentwig & Cristenson, 1986), capturing large prey falling into the structure as a group, using both bites and sticky silk (Nentwig, 1985; Pasquet & Krafft, 1992). In contrast, spiders within the theridiid genus *Rhomphaea* specifically target spider prey. *Rhomphaea* reside on very limited webs and often travel to hunt within the prey's own web. They spin silk coated in glue between their legs, forming a net with which they entrap prey (Whitehouse, 1987).

While some theridiids have developed diverse silk and associated hunting strategies, a number of spiders within the group are kleptoparasitic, living on the webs of other spiders and feeding alongside the host or scavenging (Whitehouse *et al.,* 2002). This reduces their need to produce silk to reside on or use to capture prey, largely producing silk only for escape draglines and egg sacs. This allows these parasites to exploit a variety of environments without requiring the development of specific and costly silk properties and spinning strategies.

5.2.3 Spider silk types and protein structures

Silk fibres are largely formed of spider silk proteins, spidroins, and are formed upon the extrusion of 'dope' containing spidroins through the silk glands. An individual spider can produce up to seven different silk types, as are formed in female orb-weavers (largely family Araneidae). Diversity of silks within an individual spider allows much more effective structures, with webs formed from separate strong fibres, flexible fibres and glues that are able to capture prey more efficiently. Some spiders have all seven, such as those within the Araneidae, while other species produce fewer types and so possess fewer glands (Haupt & Kavoor, 1993; Vollrath, 1992). These silk types are: major ampullate (MaSp) for main web support structures and draglines; minor

ampullate (MiSp) for web construction scaffolds and auxiliary spirals; flagelliform (FlaG) for sticky capture threads; tubuliform (TuSp) for egg cases; aciniform (AcSp) for egg case padding, prey wrapping and sperm webs; aggregate (AgSp) for silk glues; and pyriform (PySp) for attachment bonds between threads and to substrates (for further information on silk types see Chapter 1). The above descriptions are of their generally accepted uses, but in fact some variation is seen across spider groups, for instance MiSp silk used in prey wrapping rather than web construction by the theridiid *Latrodectus hesperus* (Mattina *et al.,* 2008). Theridiids generally have slightly diverged silk uses, with MiSp being present in prey wrapping and also used alongside MaSp in some draglines (Hsia *et al.,* 2011).

Some silk fibres are formed from multiple groups of proteins of the same type which exhibit slightly different amino acid structures and final properties. This is the case with major ampullate silks; which contain MaSp1, which has a preponderance of ‘GA’ and ‘poly-A’ motifs conferring strength; MaSp2, with ‘GPGXX’ motifs promoting mobility and elasticity; and sometimes MaSp3, containing larger, more polar amino acids and as-yet undefined properties (Brooks *et al.,* 2005; Collin *et al.,* 2018; Kono *et al.,* 2019). Amino acid full names and properties can be found on Table 5.2.

Spidroin primary structures include a non-repetitive amino terminal domain (N-terminal domain, NTD) and a non-repetitive carboxyl terminal domain (C-terminal domain, CTD). The NTD is responsible for protein transport and pH dependent changes, ensuring fibre formation and protein aggregation within specific areas of the silk glands (Hagn *et al.,* 2011). The CTD controls the assembly of repetitive segments into fibres, proposed to be through the use of a pH sensitive ‘salt bridge’ (Hagn *et al.,* 2010; Strickland *et al.,* 2018). As these terminal domains are each key to the fibre formation, they show high conservation across species and silk types. Between these domains, there is a large region made up of repeating common peptide motifs combining to form repetitive structural modules (Hayashi & Lewis, 2000). Between each terminal domain and the repetitive sequence there often sits a linking region.

Figure 5.1. Broad representation of MaSp spidroin primary structure, which is generally comparable across silk types. Lengths not to scale.

The C-terminal domain is largely composed of hydrophobic and hydrophilic amino acids. These residues sit in an alternating pattern, forming alpha-helices with the hydrophilic residues exposed to promote solubility (Hagn *et al.,* 2010). The charged residue content of studied CTDs is typically less than 10% (Strickland *et al.*, 2018). One acidic and one basic amino acid are found to be almost totally conserved in CTDs, with a second of each partially conserved, postulated to be key in the formation of the salt bridge. A conserved C (cysteine) is found to form disulphide bonds, with a replacement by S (serine) in some silks still allowing fibre formation but other substitutions not leading to successful formation (Ittah *et al.,* 20017). DNA and amino acid identity are highest with the 'QALLE' motif, recognisable with few variations in all sequences analysed by Challis *et al.* (2006) and Strickland *et al.* (2018), and this region can be reliably used to identify sequences that reside within the spidroins.

N-terminal domains are longer then CTDs and usually have a higher percentage identity across species and silk types (Garb *et al.,* 2010; Motriuk-Smith *et al.,* 2005). Three conserved E (glutamic acid) residues and a single W (tryptophan) have been found to be key to the pH-dependent dimerisation of the NTD (Jiang *et al.,* 2019; Ries *et al.,* 2014). However, even where differences exist in the primary structure within the NTD across species and silk types, compensatory residues have been found to retain molecular mechanisms underlying pH-dependent dimerisation (Otikovs *et al.,* 2015).

The region between the terminal domains makes up the majority of the silk sequence (> 90%) and, while very little of this region is conserved between species and silk types, it generally consists of a pattern of smaller repeated motifs (Blackledge *et al.,* 2011). These amino acid motifs combine into larger repetitive units, sometimes called ensemble repeats or repetitive modules, that

range from < 50 to over 200 amino acids in length (Ayoub *et al.,* 2007). Such a structure makes assembly of short-read sequencing difficult, and few full-length repetitive region sequences exist (see Section 5.2.4). This region, however, is largely responsible for the final properties of the silk protein and, even where only fragments are available, inferences can be made on the final fibre from the known motifs. An example of this is in MaSp1, where 'poly-A' and 'GA' units are predicted to fold silk fibroins into the beta-sheet configuration necessary for formation of nanocrystals that interlock molecules. Also, 'GGX' motifs form a second level of crystal structure where helices can bond inter-molecularly (Xu & Lewis, 1990; Kümmerlen *et al.,* 1996; Malay *et al.,* 2017). MaSp2 is similarly structured but with the 'GGX' replaced by 'GPGXX', kinking the amino acid chains and forming a molecular nanospring, increasing the final elasticity of the fibre greatly (Hinman & Lewis, 1992; Becker *et al.,* 2003). MiSp repetitive regions analysed also contain repeats that are primarily made up of 'GGX' alongside 'poly-A' and 'GA', although these are not all predicted to form beta-sheets to the extent of MaSp, decreasing tensile strength (Liivak *et al.,* 1997; Vienneau-Hathaway *et al.,* 2017). These regions are disrupted by S and T-rich (serine and threonine) 'spacer' regions, with as yet unclear functions (Colgin & Lewis, 1998).

5.2.4 Sequencing technologies and spider silk

As the final fibrous structure and properties are intrinsically linked to features of individual spidroin primary structure, this allows RNA sequencing data to provide valuable insight into both natural and synthetic production. However, the repetitive nature of the majority of the silk gene structures proves difficult to impossible to assemble from short-read, 'second-generation' technology. Second-generation (next generation) technologies were a paradigm shift above prior methods as they allow the mass parallelisation of sequencing reactions, greatly increasing the amount of DNA that can be sequenced in any one run (Heather & Chain, 2016). However, DNA has to be fragmented and amplified in clones of between 75 base pairs and 400 base pairs, hence the term 'short-read sequencing' (Adewale, 2020). These fragments are then assembled

based on overlapping regions but, where this is completed *de novo* with no reference, errors can be common and final sequences remain short.

A number of technologies underpin 'third-generation' sequencing, but these all focus on the production of longer reads, often between 5 kilobase pairs and 30 kilobase pairs and with large overlapping regions for assembly (Adewale, 2020). Base-calling errors can be high in such technologies but are reduced by either circular consensus sequencing, where the DNA has multiple passes through the sequencer, or through the use of these long-read sequences as references for the assembly of short-read sequences. This presents the ideal solution to silk gene construction, and a number of complete silk genes have been reported using this method to date (Garb *et al.,* 2019; Zhou *et al.,* 2021). The obstacle to this, however, is that of price and computing capacity. Sequencing a sample using third-generation technologies can be over 10 times the cost of second (personal observation) and this can be prohibitive for exploratory studies and/ or those sequencing many individuals. Availability of this technology continues to increase but, in the meantime, short-read sequencing remains common in silk research.

5.2.5 Chapter aims

This chapter aims to identify the impact that kleptoparasitic specialism can have on silk protein structure. *Argyrodes argyrodes* rely on draglines as a safety line when dropping from a web and for producing single lines to sit on when given no other option. They also produce egg sacs seemingly in the same manner as relatives within the subfamily Argyrodinae. However, no other silk production was observed in either the field or laboratory environments, and this could suggest a lack of need for effective silk production of MiSp, FlaG and AgSp silk types. In fact, Kovoor & Lopez (1983) found an absence of FlaG spigots entirely in the genus. With reduced selective pressure ensuring beneficial properties to these silks, we might expect to see a higher rate of retained non-synonymous mutations, which may be removed by natural selection in other species unless beneficial.

This chapter will explore the primary structure of *Argyrodes argyrodes* spidroins and those of close relatives. *A. argyrodes* MaSp and MiSp terminal domain

sequences will be compared with others of the Argyrodinae family, both kleptoparasitic and free-living, to identify any increased variation in amino acid sequence in kleptoparasitic spiders, especially when considering underused silk types. Sequences from both terminal and repetitive regions of MaSp and MiSp silk of *A. argyrodes* will also be examined to identify variation from previously considered species and explore how this variation can be considered in context of their unique behavioural strategies.

5.3 Methods

5.3.1 Argyrodes argyrodes sequencing

For the sequencing of an *Argyrodes argyrodes* transcriptome, 10 adult female spiders from across field sites in the province of Cadiz, southern Spain (pooled for sufficient tissue mass to reach RNA concentration required) were freshly killed in ethanol, frozen using liquid nitrogen and crushed using a mortar and pestle to disrupt the tissues. RNA was extracted using GeneJET RNA purification kit (ThermoFisher) following standard protocol. Samples were then flash frozen with liquid nitrogen and stored at -80^{o}C before transferring to the sequencing facility on dry ice.

RNA sequencing was carried out by Macrogen Europe. Total RNA integrity was tested using an Agilent Technologies 2100 Bioanalyser or 2200 TapeStation. mRNA was purified using the TruSeq stranded mRNA LT sample prep kit (Illumina) and sequencing carried out using NovaSeq6000 s4 (Illumina).

5.3.2 Spidroin identification

Transcriptome processing, assembly and translation were all completed through the public server at usegalaxy.org (Afgan *et al.,* 2018). Sequencing files were trimmed using Trimmomatic (Bolger *et al.,* 2014) and Trimmomatic was also used to filter low quality reads using the standard settings: LEADING:20, TRAILING:20, SLIDINGWINDOW:4:20, MINLEN:36. *De novo* transcriptome assembly was completed using Trinity v2.2.0 (Grabherr *et al.,* 2011) and the coding region prediction method TransDecoder v.1.0.3.0 (Brian & Papanicolaou, n.d.) was used to predict protein sequences.

A database was created using all spider silk protein sequences available on the NCBI database (as of 23/06/2019). The assembled and translated transcriptome was inputted into NCBI BLAST+ blastp (on usegalaxy.org) against the custom database with an e-value threshold of 0.00004, a value identified to bring numerous but accurate results. Protein sequences were chosen for the identification of silk sequences as identity should be more highly conserved between species, to ensure conserved function, and very few

species closely related to *A. argyrodes* were present in the database. The blastp output identified possible silk sequences within the transcriptome and the amino acid sequence of each of these was extracted. MaSp and MiSp were most reliably identified due to the high number of sequences included in the database as well as high terminal domain conservation. These were therefore focused on for analysis, as one silk type expected to be conserved across species (MaSp) and one, under the hypothesis described in 5.2.4, which may be more varied in kleptoparasitic species (MiSp).

Identified sequences were aligned with silk sequences of the same silk type from the well characterised theridiids *Latrodectus hesperus* and *Latrodectus geometricus* (MaSp: AAY28935.1, QHG11083.1, ABR68855.1, ABR68855.1 (Ayoub *et al.,* 2007; Garb & Hayashi, 2005)) (MiSp: ADM14322.1, ADM14321.1 (Garb *et al.,* 2010)) to confirm similarities to silk genes and confirm which region of the gene was present.

5.3.3 Sequencing of other theridiid species

A further 10 assembled transcriptomes were provided by Kazuharu Arakawa and team at Keio University. These transcriptomes were from others of the Argyrodinae family, including both kleptoparasitic and free-living, and one theridiid outgroup, *Enoplognatha ovata* (species listed in Table 5.1 with phylogenetic relationships summarised in Figure 5.2). Each transcriptome used RNA sequences from one whole individual. RNA was extracted and sequenced and transcriptomes were processed as described in Kono *et al.* (2016), with *de novo* assembly using Bridger (Chang *et al.,* 2015).

These further sequences were then also translated using TransDecoder and possible MaSp and MiSp sequences identified in the same manner as described in 5.3.2.

Table 5.1. The species included in this study's silk gene analysis, all but *Argyrodes argyrodes* selected by Kazuharu Arakawa (Keio University) for sequencing largely due to sample availability. Each was scored as 'kleptoparasite' or 'free living' according to information available in the literature. No species had disagreement in the literature, though records were not extensive so it is possible some species may exhibit both strategies.

Species	Lifestyle	References
Argyrodes argyrodes	Kleptoparasite	Saaristo, 2000
Argyrodes bonadea	Kleptoparasite	Miyashita, 2000; Miyashita, 2002
Argyrodes flavescens	Kleptoparasite	Miyashita, 2002; Miyashita *et al.*, 2004
Argyrodes miniaceus	Kleptoparasite	Spear *et al.,* 2017; Su & Smith, 2014; Su *et al.,* 2018
Argyrodes nephilae	Kleptoparasite	Exline & Levi, 1962; Javed, 2010
Ariamnes cylindrogaster	Free living	Su & Smith, 2014
Faiditus xiphias	Kleptoparasite	Su & Smith, 2014
Rhomphaea labiata	Free living	Sekhar, 2018
Rhomphaea sagana	Kleptoparasite	Kaston, 1965; Su & Smith, 2014
Spheropistha melanosoma	Kleptoparasite	Yaginuma, 1957
Enoplognatha ovata	Free living	Stevenson & Dindal, 1982

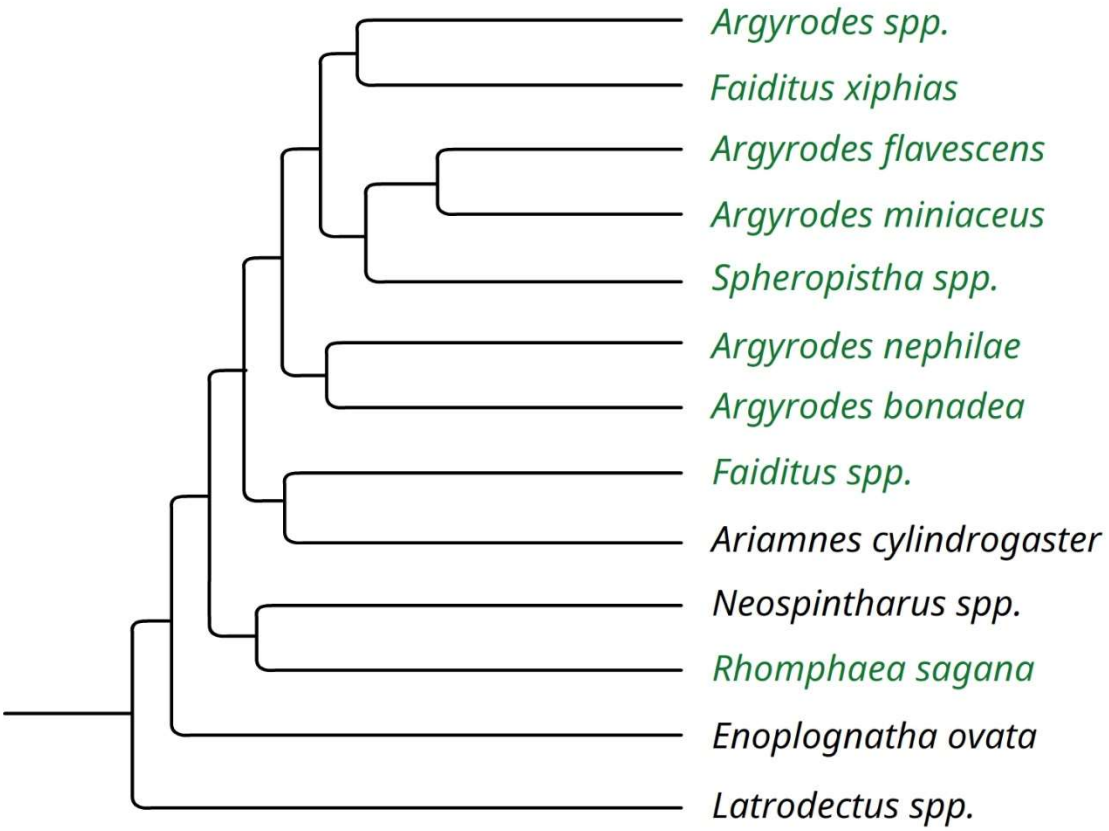

Figure 5.2. Simplified phylogeny modified from Su & Smith (2014) to isolate species considered in this chapter. Species in green are classed as kleptoparasitic.

5.3.4 Defining spidroin terminal regions

Comparison of the divergence of silk genes between the species sampled was completed using C-terminal and N-terminal regions identified for MaSp (1&2) and MiSp silk types. Terminal regions were used as their conservation allows for alignments between species, where repetitive regions are too variable to align reliably. This conservation also allows sequences to be more consistently identified as silk from whole transcriptomes, increasing the number of species that can be included as well as the higher number of unique sequences per individual. Amino acid naming conventions and properties are given in Table 5.2 to contextualise further text.

Table 5.2. Amino acids and their one letter codes, used throughout this chapter, along with key properties. Amino acids shaded in grey indicate charged amino acids.

Amino acid	Abbreviation	Properties
Alanine	A	Non-polar, hydrophobic
Arginine	R	Polar, basic
Asparagine	N	Polar, hydrophilic
Aspartic acid	D	Polar, acidic
Cysteine	C	Polar, hydrophobic
Glutamine	Q	Polar, hydrophilic
Glutamic acid	E	Polar, acidic
Glycine	G	Non-polar, Hydrophilic
Histidine	H	Polar, basic
Isoleucine	I	Non-polar, hydrophobic
Leucine	L	Non-polar, hydrophobic
Lysine	K	Polar, basic
Methionine	M	Non-polar, hydrophobic
Phenylalanine	F	Aromatic, hydrophobic
Proline	P	Non-polar, hydrophobic
Serine	S	Polar, hydrophilic
Threonine	T	Polar, hydrophilic
Tryptophan	W	Non-polar, hydrophobic, aromatic
Tyrosine	Y	Polar, hydrophobic, aromatic
Valine	V	Non-polar, hydrophobic

The NTD of the silk sequences was defined using a set of criteria based on major ampullate spidroins and is approximately 124 residues long. The start of this region was taken from the conserved first ‘PWXX’ motif (where ‘XX’ are

different amino acids, usually ‘SS’), which sits shortly after the cleavage site from the initial signal region. The NTD ends on a conserved E residue in MaSp.

Figure 5.3. N-terminal domain of *Trichonephila clavipes* MaSp1, as defined by this method. Colouring indicates polarity. Accession number MF955684.1.

The CTD of the silk sequences defined were approximately 70-72 amino acids long. The start of this region was defined using the nearest highly conserved R residue downstream from the Q in the conserved ‘QALLE’ motif (approximately 45 residues). A second R is often present further upstream but, as conservation begins to reduce towards this, only the shorter region was included. CTDs then ended 25 residues from the Q with an S or A.

Figure 5.4. C-terminal domain of *Trichonephila clavipes* MaSp1, as defined by this method. Colouring indicates polarity. Accession number MF955765.1.

5.3.4 Sequence and phylogenetic analysis

Comparisons of extracted silk sequences alongside publicly available sequences from *L. hesperus* and *L. geometricus* (defined in 5.3.2) and *Trichonephila clavipes* (MF955765.1, MF955815.1, MF955684.1, MF955710.1, MF955723.1, MF955735.1 (Collin *et al.,* 2018)) were completed using the construction of gene trees. Alignments were made of terminal regions using CLUSTAL Omega 1.2.2 (Sievers *et al.,* 2011). Trees were created in Geneious tree builder (Geneious prime v2021.0.3) using the Neighbour-Joining method without rooting in order to explore grouping of sequences rather than estimating phylogenetic relationships. Bootstrap analysis was included with 1000 replicates and nodes were retained if bootstrap values were over 50%.

SignalP v5.0 (Armenteros *et al.,* 2019) was used to predict the presence of signal peptides and Jpred4 (Drozdetskiy *et al.,* 2015) was used to predict secondary peptide structure. Sequence visualisation was completed using Geneious (Geneious prime v2021.0.3).

5.4 Results

A total of 81 unique amino acid sequences were extracted from transcriptomes and identified as containing MaSp or MiSp terminal regions for analysis. 38 included the CTD and 43 the NTD, all included a section of the repetitive region also. Sequences were identified as MaSp1 or MaSp2 using attached repetitive region motifs, with no MaSp3-like sequences identified. All sequences included appeared more than once in the assembly, reducing likelihood of sequencing error, but some assembled using Bridger contained what appeared to be assembly errors, with a region from an unrelated protein attached to the silk sequence. These regions were trimmed and only the terminal domain, clearly a spidroin sequence, used for analysis.

Table 5.3. Unique C- and N-terminal domain amino acid sequences identified from RNA sequencing. *Argyrodes argyrodes* sample was pooled from 10 whole spiders while all other species were one whole individual.

Species	**MaSp1**		**MaSp2**		**MiSp**	
	N-term	**C-term**	**N-term**	**C-term**	**N-term**	**C-term**
Argyrodes argyrodes	1	1	5	1	2	4
Argyrodes bonadea	1	0	1	0	1	1
Argyrodes flavescens	2	1	0	2	2	3
Argyrodes miniaceus	1	0	0	1	2	1
Argyrodes nephilae	1	2	2	1	1	1
Ariamnes cylindrogaster	0	2	3	3	0	1
Faiditus xiphias	1	0	1	1	1	1
Rhomphaea labiata	2	2	1	0	1	1
Rhomphaea sagana	1	0	2	1	3	0
Spheropistha melanosoma	1	3	0	1	1	1
Enoplognatha ovata	0	1	2	1	1	0

5.4.1 Terminal domain gene trees

The amino acid sequence of MaSp1 NTDs shows a clustering of *Spheropistha melanosoma* and both parasitic and free-living *Rhomphaea* with free-living outgroup species (Figure 5.5). *Argyrodes* species sit outside this cluster but do not form a monophyletic group. MaSp2 NTDs, however, see *Argyrodes argyrodes* (a-d) and *Argyrodes bonadea* form one group while *Argyrodes argyrodes* (e) and *Argyrodes nephilae* form another (Figure 5.6). From the same node branches a group of all extracted *Ariamnes cylindrogaster* sequences and another including *Faidtus xiphias* and both *Rhomphaea* species. All three free-living outgroups to the Argyrodinae sit outside this node with one *Argyrodes nephilae* species. All species form one clade separate to *Enoplognatha ovata.*

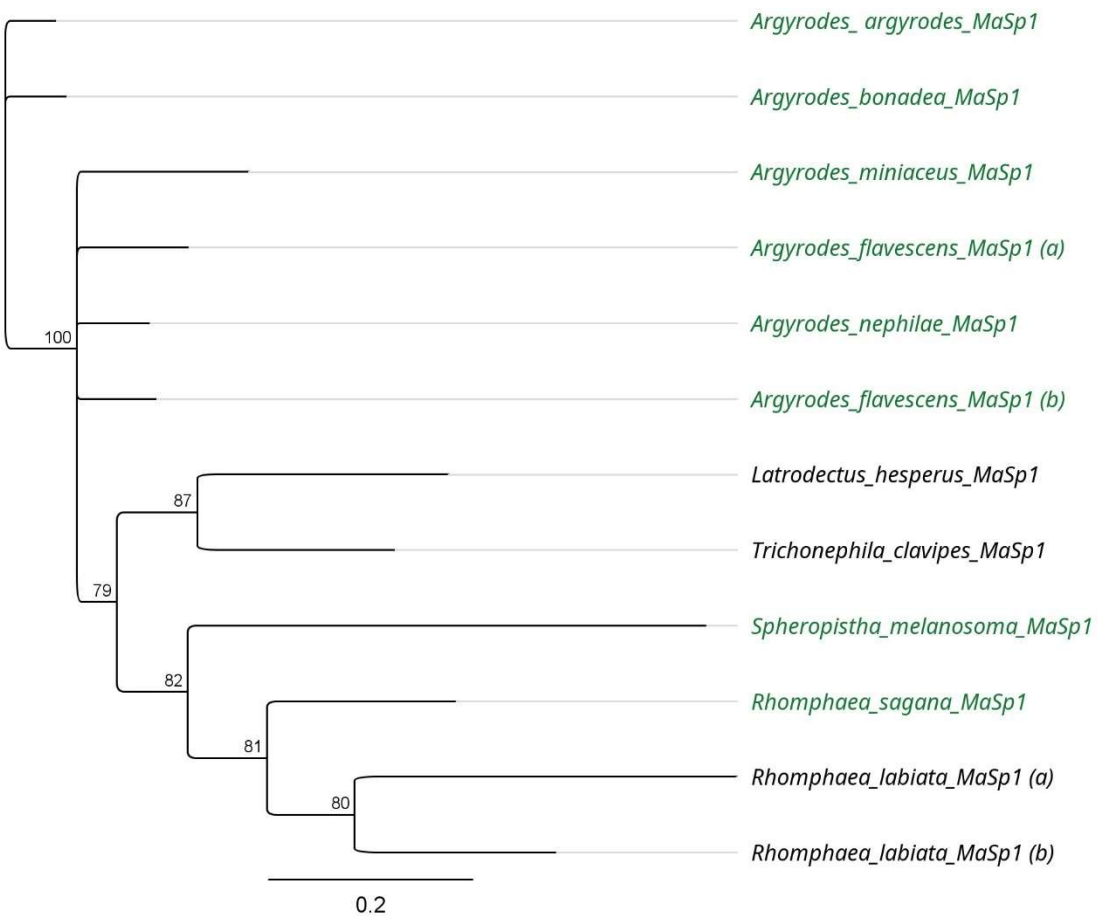

Figure 5.5. Neighbour-joining tree comparing amino acid sequences of major ampullate 1 silk protein N-terminal domains from kleptoparasitic spiders of the Argyrodinae (green) and free-living species both within and outside the subfamily (black). Bootstrap values are given at the nodes.

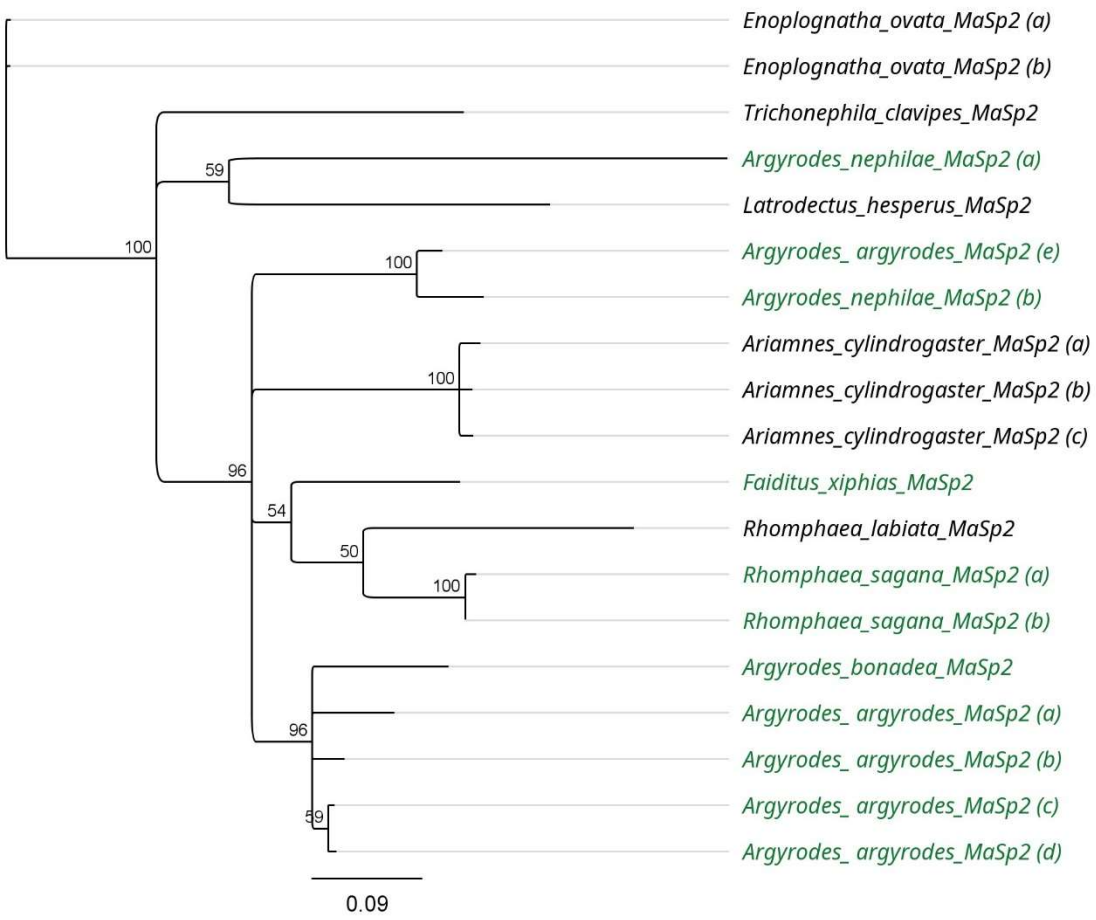

Figure 5.6. Neighbour-joining tree comparing amino acid sequences of major ampullate 2 silk protein N-terminal domains from kleptoparasitic spiders of the Argyrodinae (green) and free-living species both within and outside the subfamily (black). Bootstrap values are given at the nodes.

Neither MaSp CTD tree domain shows substantial grouping according to phylogenetic relationships or kleptoparasitism (Figures 5.7 and 5.8). Outgroups are distributed within the tree and sequences sitting outside the majority grouping are taken from *Spheropistha melanosoma* MaSp1 and *Argyrodes argyrodes* and *Argyrodes miniaceus* MaSp2.

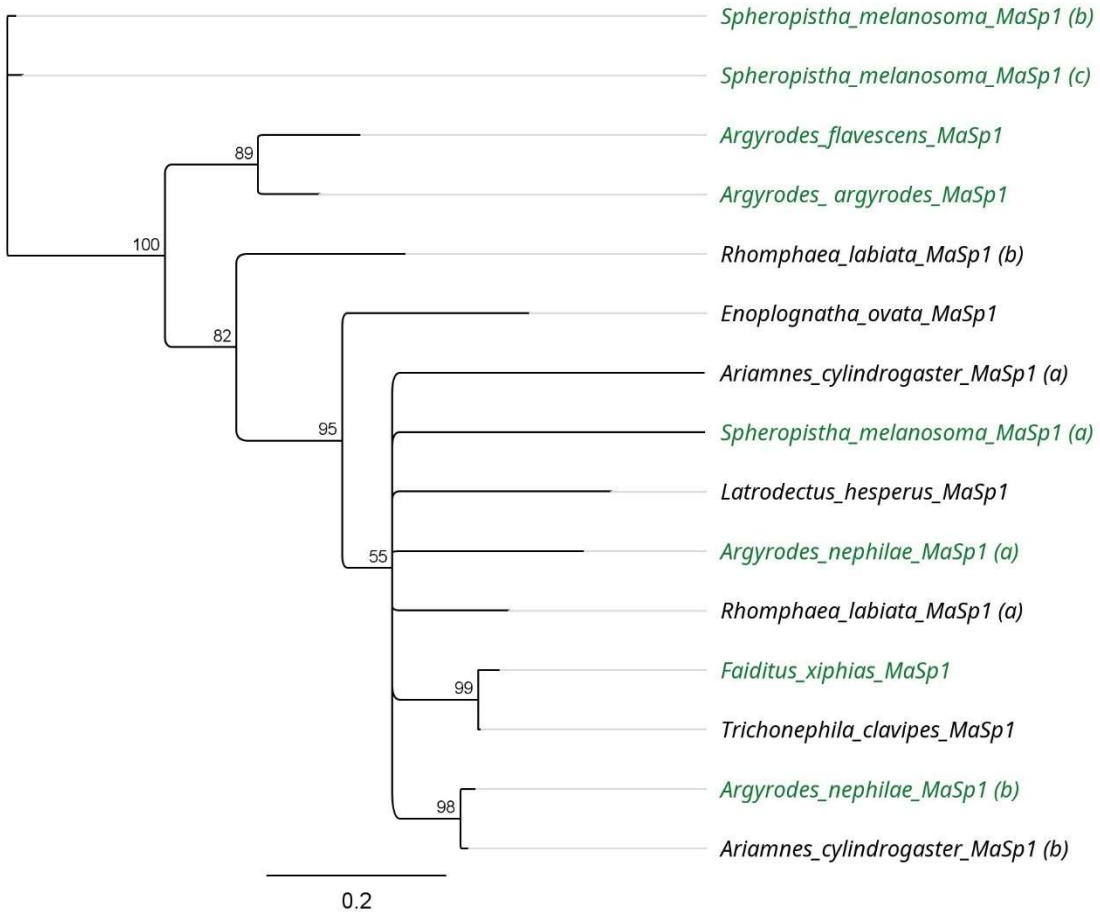

Figure 5.7. Neighbour-joining tree comparing amino acid sequences of major ampullate 1 silk protein C-terminal domains from kleptoparasitic spiders of the Argyrodinae (green) and free-living species both within and outside the subfamily (black). Bootstrap values are given at the nodes.

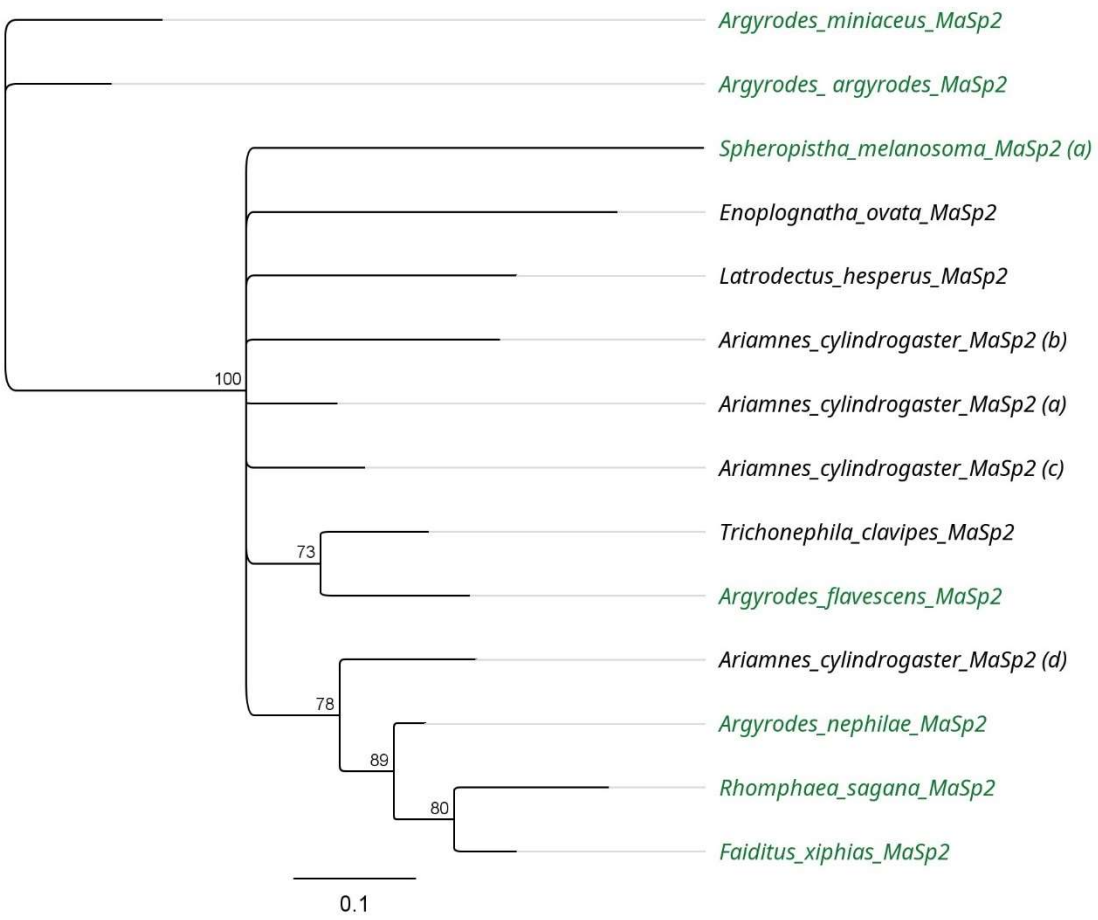

Figure 5.8. Neighbour-joining tree comparing amino acid sequences of major ampullate 2 silk protein C-terminal domains from kleptoparasitic spiders of the Argyrodinae (green) and free-living species both within and outside the subfamily (black). Bootstrap values are given at the nodes.

In comparing MiSp NTDs, *Argyrodes* do not form a single clade, and even see three sequences sitting outside the group formed by all the other species (Figure 5.9). Four *Argyrodes* sequences are, however, in this group with *Argyrodes miniaceus* (b) and *Argyrodes flavescens* (a) loosely forming one clade and *Rhomphaea labiata* and *Trichonephila clavipes* forming another. Two strongly supported clades are those grouping all three *Rhomphaea sagana* and the two theridiid outgroups, *Enoplognatha ovata* and *Latrodectus hesperus*.

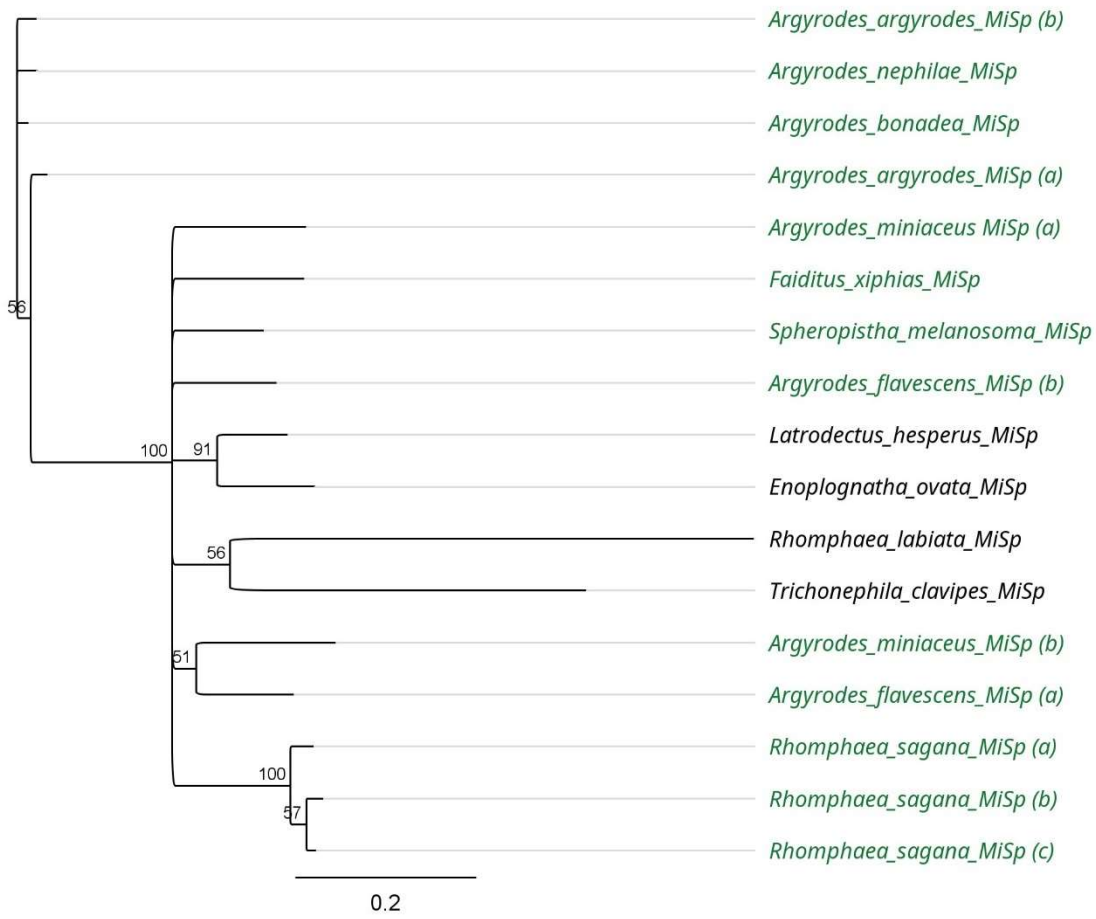

Figure 5.9. Neighbour-joining tree comparing amino acid sequences of minor ampullate silk protein N-terminal domains from kleptoparasitic spiders of the Argyrodinae (green) and free-living species both within and outside the subfamily (black). Bootstrap values are given at the nodes.

Argyrodes MiSp CTDs group into two clades, with *Spheropistha melanosoma* sitting within one and *Rhomphaea labiata* within the other (Figure 5.10). Sitting alongside these is *Ariamnes cylindrogaster*. *Latrodectus hesperus* sits outside all of these species then the two species fully outside all the clades are *Trichonephila clavipes* and *Faidtus xiphias.*

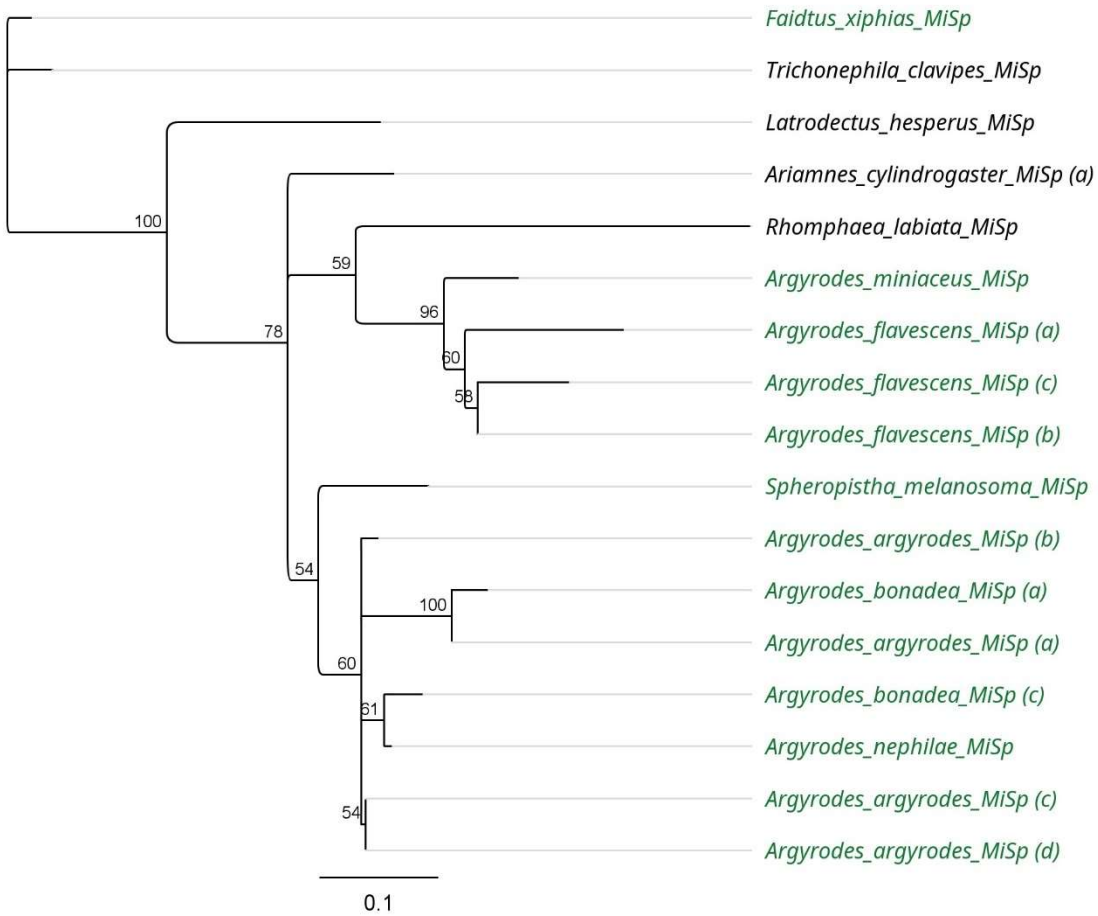

Figure 5.10. Neighbour-joining tree comparing amino acid sequences of minor ampullate silk protein C-terminal domains from kleptoparasitic spiders of the Argyrodinae (green) and free-living species both within and outside the subfamily (black). Bootstrap values are given at the nodes.

5.4.2 Amino acid composition

SignalP v5.0 (Armenteros *et al.,* 2019) analysis predicts the presence of a signal peptide in each extracted sequence prior to the defined N-terminal domain. All NTDs extracted are predicted by Jpred4 (Drozdetskiy *et al.,* 2015) to form 5 alpha helices, as indicated in Figure 5.11.

A total of 10 (8%) amino acids are identical across all MaSp and MiSp N-terminal domain sequences (see Figures 5.14 and 15, Supplementary Material for full alignment). MaSp NTD comparison shows 19 (15%) identical amino acids, 26 (21%) in MaSp1 and 33 (26%) across MaSp2. In MiSp, 34 (27%) of amino acids are identical across all sequences.

Of the amino acids specified as key to dimerisation by Ries *et al.* (2014), the W (position 2 in Figure 5.11) is almost completely conserved across extracted sequences. Exceptions come from a substitution to F in all *Ariamnes cylindrogaster* MaSp2 NTDs. The three Es (positions 69, 74 and 109) similarly show almost complete conservation. *Spheropistha melanosoma* MaSp1 shows a substitution in position 69 to a D and is missing position 74 entirely. *Rhomphaea labiata* MaSp1 (a) shows a substitution to the uncharged amino acid T at position 69 and an unusual 'EEE' across the position 74. Position 109 is conserved across all sequences extracted in this study, with the only substitution present in the MaSp2 NTD of *Latrodectus hesperus.*

The acidic D at position 32, highly conserved across species and indicated to stabilise dimer formation (Ries *et al.,* 2014), matches that found at complete conservation across silk types in multiple species (Garb *et al.,* 2010) and is proceeded here by a second acidic residue in 58% of sequences. The corresponding basic R at position 50 is present in 50% of sequences, replaced by a K in a further 42%, and K at position 55 is present in 83%, with a wider variety of substitutions.

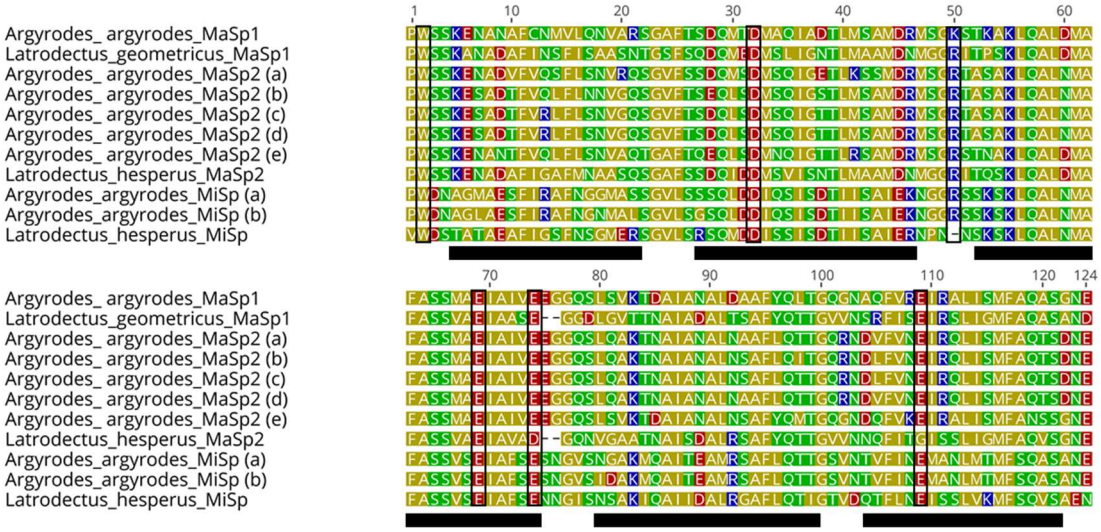

Figure 5.11. Amino acid sequences of *Argyrodes argyrodes* major and minor ampullate silk protein N-terminal domains aligned against that of *Latrodectus* species. Alignment completed using CLUSTAL Omega 1.2.2 (Sievers *et al.,* 2011). Black bars indicate predicted alpha helical secondary structures (Jnet predictions on *A. argyrodes* MaSp1). Black outlines indicate conserved areas of interest. Colouring indicates polarity.

Analysis of C-terminal domain secondary structure predictions by Jpred4 (Drozdetskiy *et al.,* 2015) shows conservation of 3 alpha helices of similar length across all CTD sequences extracted except *Argyrodes argyrodes* MaSp1, which is predicted to have a shortened or absent first helix. Approximate regions shown in Figure 5.12.

Only 3 (4%) of amino acids are entirely conserved across MaSp and MiSp C-terminal domains, with 18 (25%) across MiSp, 10 (14%) in MaSp1, 10 (14%) in MaSp2 and 7 (10%) across all MaSp (see Figures 5.16 and 5.17, Supplementary Material, for full alignment). Conservation is high in residues expected to form a salt bridge, one acidic and one basic (indicated at positions 1 and 50 in Figure 5.12). *Argyrodes flavescens* MiSp (a) is missing this conserved basic residue but contains another 9 residues upstream. The *A. argyrodes* MaSp1 sequence, however, is missing the basic residue and no alternative basic amino acid is present within 30 residues either side of the site. All sequences contain a second conserved acidic region 4 bases upstream of the ‘QALLE’, with its expected salt bridge basic pair not covered included in this analysis. Conservation of the disulphide bridge-forming C 5 positions upstream on the ‘QALLE’ is complete across MaSp and replaced by S in MiSp.

The ‘QALLE’ motif remains well conserved across MaSp sequences with two exceptions, ‘QVLLE’ replaces this motif in *A. flavescens* MaSp1 and *Enoplognatha ovata* MaSp1 contains ‘LALLE’. This *E. ovata* MaSp1 also contains a stop codon before the end of most C-terminal regions, stopping after 10 amino acids following the ‘LALLE’ motif. This motif is present in MiSp C-terminal domains as ‘QVLLE’, ‘QALLE’ and, in *Argyrodes flavescens* (c), ‘HALLE’.

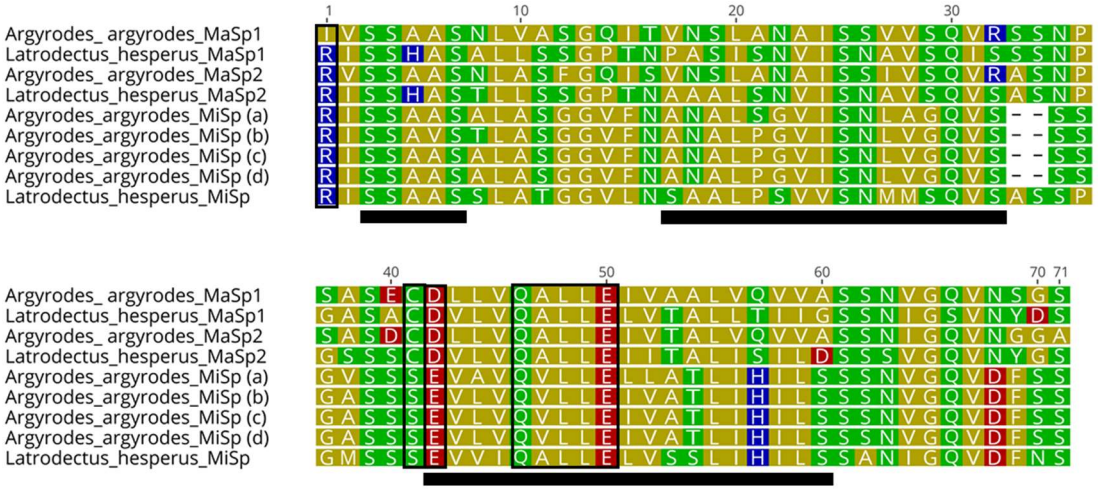

Figure 5.12. Amino acid sequences of *Argyrodes argyrodes* major and minor ampullate silk protein C-terminal domains aligned against that of *Latrodectus hesperus*. Alignment completed using CLUSTAL Omega 1.2.2 (Sievers *et al.,* 2011). Black bars indicate predicted alpha helical secondary structures (Jnet predictions on *A. argyrodes* MaSp2). Black outlines indicate conserved areas of interest. Colouring indicates polarity.

The N-terminal regions considered here show a lower pairwise identity when compared to C-terminal domains (54.5% against 57.2%). MaSp pairwise identity was higher in NTDs (63.3% against 60.3%), although identity was equal in MaSp1 (59%) and higher in MaSp2 (67.4% against 61.7%). MiSp identity was lower in NTDs (66.1% against 75.7%).

Limited repetitive regions were identified in the sequenced transcriptomes, usually as part of a sequence with a terminal domain. A repetitive region extracted from *A. argyrodes* is shown in Figure 5.13. Largely, these sequences are short, under 200 amino acids in *A. argyrodes* and 400 in all other species. Examples are present in each species and silk type. MaSp1 sequences were characterised through their lack of P and so lack the β-turn spiral motif ‘GPGXX’ across all species. They are rich in A and S and contain the helix motif ‘GGX’

(where X is usually Y, A or Q) and β-sheet motif ‘GAG’. MaSp2 sequences did contain regular ‘GPGXX’ motifs along with A and ‘GGX’ (where X is usually Y, A or Q as in MaSp1), but both at generally lower frequency. MaSp2 lacked the β-sheet ‘GAG’ motifs. S content was generally higher in MaSp2 repetitive regions compared to MaSp1. No *A. argyrodes* sequences contained the ‘QQ’ motif but some MaSp2 across other species contained ‘QQ’ at high frequency, as shown in Figure 5.14. Both MaSp types were composed largely of uncharged amino acids with occasional basic residues and much rarer acidic residues.

MiSp repetitive regions contain acidic residues alongside the basic present in MaSp, although these charged amino were at a similarly low frequency to MaSp. MiSp sequences lack ‘GPGXX’ (and ‘GPG’) motifs but did contain P in most sequences, at a much lower frequency then MaSp2. Poly-A motifs were present, but shorter than MaSp1, as well as ‘GA’ and ‘GGX’. ‘GAGAGA’ motifs were also present in MiSp but not MaSp repetitive regions.

Figure 5.13. Amino acid sequences from *Argyrodes argyrodes* (a) major ampullate 1, (b) major ampullate 2 and (c) minor ampullate silk protein repetitive regions. Other species not shown in this thesis.

5.5 Discussion

Analysis of grouping of terminal domain primary structures provides no clear evidence to support the hypothesis of an increase in non-synonymous mutations, and hence changes in amino acid sequence, due to selection relaxation on the properties of MiSp silk in kleptoparasites. Generally, terminal sequences group with phylogenetic relationships and the *Argyrodes* genus, in which all species included are kleptoparasitic, see high similarity to each other in all silk types. There is also not an observable increase in variation between sequences of the same silk type from the same individual in kleptoparasitic species, as might be the case when mutation rate is higher on the multiple copies of a silk gene present in one individual. This may be because of high conservation in the terminal domain regions, with many mutations in these regions reducing fibre assembly and functionality and so being removed from the gene pool through reduced fitness. This would suggest some production and use of MiSp silk in kleptoparasites, perhaps as a component of draglines alongside MaSp, as seen in *Latrodectus hesperus* (Hsia *et al.,* 2011). It is also possible that, with the literature providing minimal examples concerning each Argyrodinae species and the regularity of kleptoparasitism in the family, that many species considered here are more opportunistically kleptoparasitic, with independent living in some individuals or even with one individual exhibiting both lifestyles over time. This facultative parasitism would retain free-living selection pressure on the species as a whole and reduce adaptations to kleptoparasitism which disadvantage outside this strategy. More information is required on the ecological strategies exhibited by each species and the silk production demonstrated as part of these.

N- and C-terminal domains contained conservation in regions predicted to be integral to bond formation, which forms a major component of terminal domain function in the spidroin. Generally, conservation in key protein-bonding regions is not higher in free-living over kleptoparasitic species, supporting the conclusions of the gene tree analysis. Where substitutions are made, they are largely for amino acids with a similar polarity and charge and lead to retention of the same secondary structure predictions, 5 helices in NTDs and 3 in CTDs.

The only exception is that of the MaSp1 CTD for *Argyrodes argyrodes,* which has a predicted structure including a shortened or absent first alpha helix. This is likely to be caused by the unusual presence of an uncharged I where an R is usually present, caused by a single base substitution, with an absence of any compensatory basic residue nearby. With an *in silico* switch back to R, secondary structure prediction is restored. Assuming that this expressed RNA forms a functional protein, the conservation of this first helix as well as usual salt bridge formation is therefore not integral to the formation of a basic silk fibre. However, there may be negative impacts to its final properties which would indicate the importance of conservation in this position in spiders more reliant on the efficient production of silk with optimum properties. The absence of this substitution in other *Argyrodes* species suggests that this is a relatively recent, species-specific, mutation and may be lost over time if other, R containing, copies are present across the species.

N-terminal domain identity is mostly lower than C-terminal identity in sequences considered here, with MaSp just slightly higher, but MiSp much lower. This is unusual when considering previous work, where NTDs were usually more conserved. This may be suggesting a general relaxation on selection of all but integral regions of the terminal domains, with more conservation required in the CTD for very basic fibre formation. However, the lower NTD identity is also present when selecting free-living species in this study, so the pattern could be due to chance sampling bias and may also occur with random sampling of small subsets of other species. There is also a higher percentage of residues entirely conserved across the NTDs than CTDs, with perhaps a higher importance placed on these while the CTD has fewer required residues but necessary overall identity.

MaSp repetitive regions identified in this study were as expected, with all MaSp1 sequences containing extensive β-sheet forming motifs to interlock molecules and ‘GGX’ to form further bonds, forming a strong, ridged and tensile fibre. MaSp2 sequences were rich in P, forming β-spirals and creating a more elastic structure. Both of these proteins were present across the Argyrodinae family and within *A. argyrodes* but it is unclear to what extent each are produced and go on to influence the final fibre properties.

With less defined differences and short sequences, it is difficult to categorise MiSp repetitive regions against previously identified MiSp1 and MiSp2 sequences, so these were considered as a whole. No spacer regions were sequenced, probably due to the shortness of sequences and their attachment to terminal domains, meaning there was no identification of repetitive regions towards the protein sequence centre. MiSp composition was as expected, with 'GGX' and 'GA' interspersed with short strings of A. This is expected to lead to a generally strong fibre but without the especially high tensile strength of MaSp1, conferred by high A interactions, or extensibility of MaSp2, through 'GPGXX' motifs. A very low number of P residues were present in MiSp repetitive regions, and this was fairly consistent across species here, although high variation is found across other species' MiSps (Vienneau-Hathaway *et al.,* 2017). It is unclear how these may be influencing the fibre properties outside the coil motif seen in MaSp2.

As with much of the prior sequence work on spidroins, this study is limited by the use of short-read sequencing technologies. While inexpensive and not as computationally taxing as newer long-read techniques, the limited length of repetitive regions sequenced and assembled using short-read sequencing limits exploration of total silk protein structure. While the terminal domains show little variation in their structure across species due to their role in transport and binding, repetitive regions are much more variable and variation would be expected to be driven by selection on silk properties, or perhaps lack thereof. Sequencing full silk genes, where cost and computing power allows, from kleptoparasitic spiders would allow much more extensive conclusions about selective pressures arising from such extreme lifestyles and their link to loss or gain of function.

Even with the use of short-read sequencing, further analysis could involve the comparison of expression levels of silk types across species. This would require raw RNA sequencing data alongside assembled transcriptomes and one spider, of the same age and sex, in each sample. Where target sequences are known for each species and silk type of interest, qPCR could also be used to demonstrate expression levels. This would give quantitative insight into the silk

genes expressed in kleptoparasites, and which silk types are highly expressed for regular use.

With an increasing study scope, all seven silk types must be considered. With ever-expanding silk sequence diversity available to use in BLAST identification of sequences, as well as a clearer understanding of the structural features of different silk primary structures, these less-explored types are becoming easier to identify and analyse. A silk type to target in kleptoparasites may be FlaG, as these spigots have been shown to be absent in *Argyrodes* species (Kovoor & Lopez, 1983; Yoshida, 1999). Male theridiids examined by Correa-Garhwal *et al.* (2017) had no FlaG spigots and no corresponding FlaG expression. These males did, however, express AgSp and TuSp even without the corresponding spigots. AgSp may not be required by kleptoparasites so absence can also be explored for this gene's expression. PySp, on the other hand, may be required for adhesion of draglines; so production, but perhaps reduced function, may be expected in this gene and corresponding protein. Finally, the conservation of TuSp and AcSp structure across the Argyrodinae and Theridiidae could be explored, with kleptoparasites appearing to produce egg sacs of a similar structure to free-living relatives.

To ascertain reduced function in kleptoparasitic silks, further exploration is required than exclusively using RNA sequencing. Material property tests on silk produced for support and draglines would ascertain changes in properties compared to species with more extensive silk use. These were not completed in this study due to the availability of specialist equipment and silk samples but targeted tests of strength and elasticity to pair with sequencing data would be a useful addition to the consideration of silk use in kleptoparasitic spiders. Further, characterisation of proteins contained in draglines through high-magnification imaging and mass spectrometry would indicate presence or absence of MiSp in draglines. Further investigation into the spinneret morphology across the Argyrodinae would also indicate at what evolutionary stage FlaG spigots were lost and AgSp were reduced, as well as identification of morphological characteristics shared by the group and those only exhibited by kleptoparasitic species.

5.6 Statement of contribution

Argyrodes argyrodes were collected and RNA was extracted by Ella Deutsch and sequenced by Macrogen Europe. All other transcriptomes used were procured, processed and sequenced by a team led by Kazuharu Arakawa at Keio University. Study design and data analysis were completed by Ella Deutsch.

5.7 Supplementary material

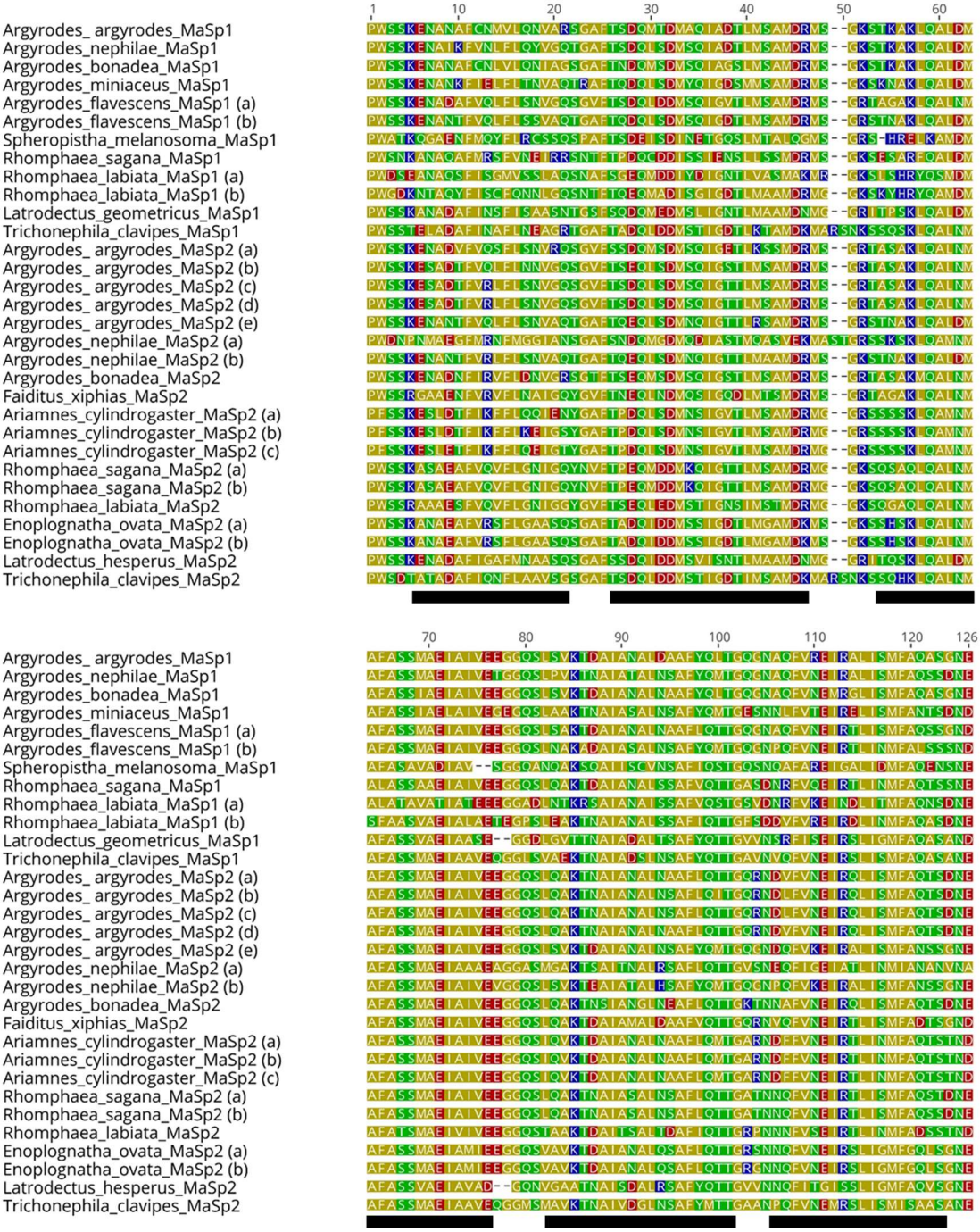

Figure 5.14. Major ampullate N-terminal domain sequences generated in this study, aligned against downloaded sequences from *Latrodectus* species and *Trichonephila clavipes* (see Section 5.3 for accession numbers). Black bars indicate predicted alpha helical secondary structures (Jnet predictions on *A. argyrodes* MaSp1). Colouring indicates polarity.

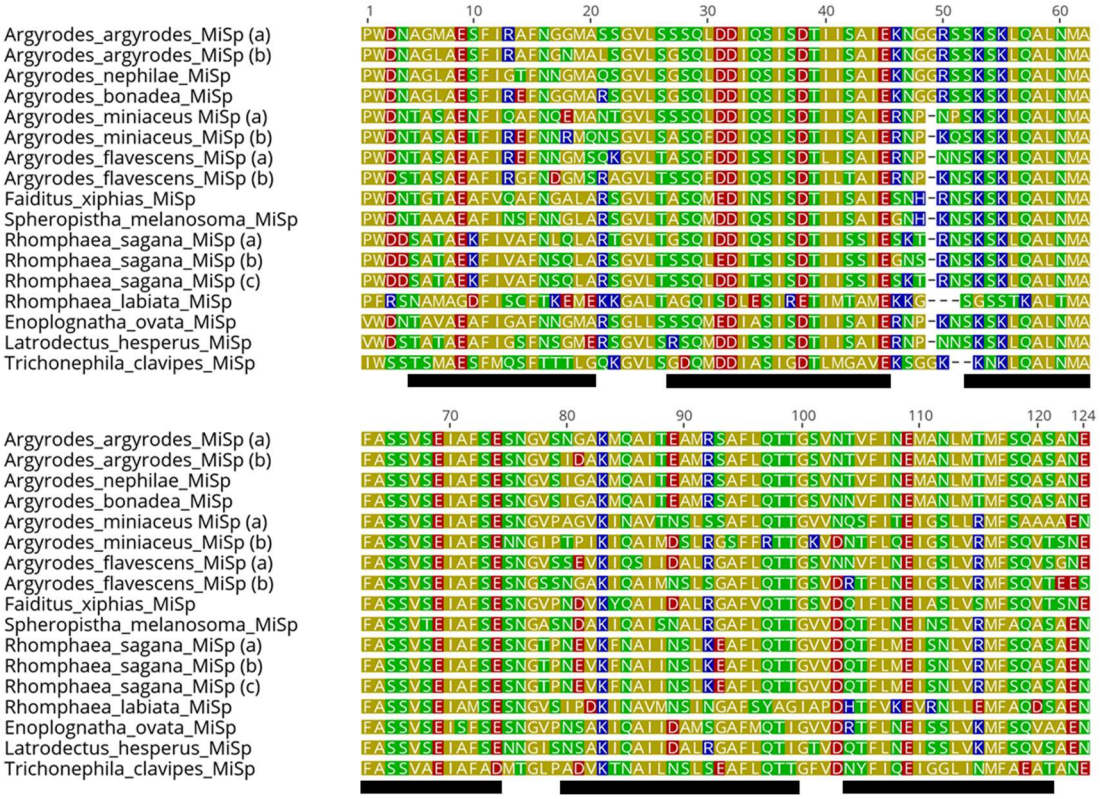

Figure 5.15. Minor ampullate N-terminal domain sequences generated in this study, aligned against downloaded sequences from *Latrodectus hesperus* and *Trichonephila clavipes* (see Section 5.3 for accession numbers). Black bars indicate predicted alpha helical secondary structures (Jnet predictions on *A. argyrodes* MiSp (a)). Colouring indicates polarity.

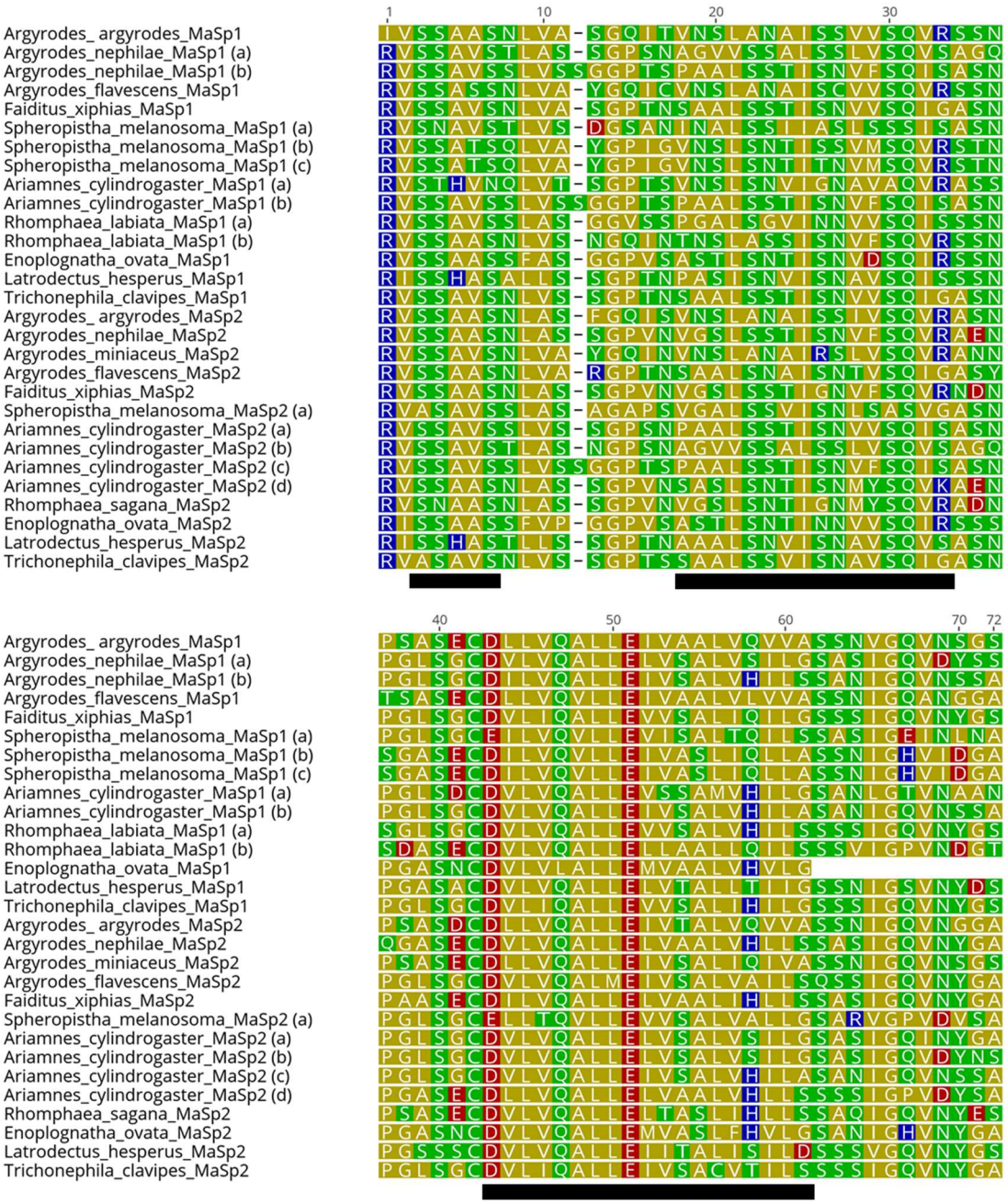

Figure 5.16. Major ampullate C-terminal domain sequences generated in this study, aligned against downloaded sequences from *Latrodectus hesperus* and *Trichonephila clavipes* (see Section 5.3 for accession numbers) Black bars indicate predicted alpha helical secondary structures (Jnet predictions on *A. argyrodes* MaSp2). Colouring indicates polarity.

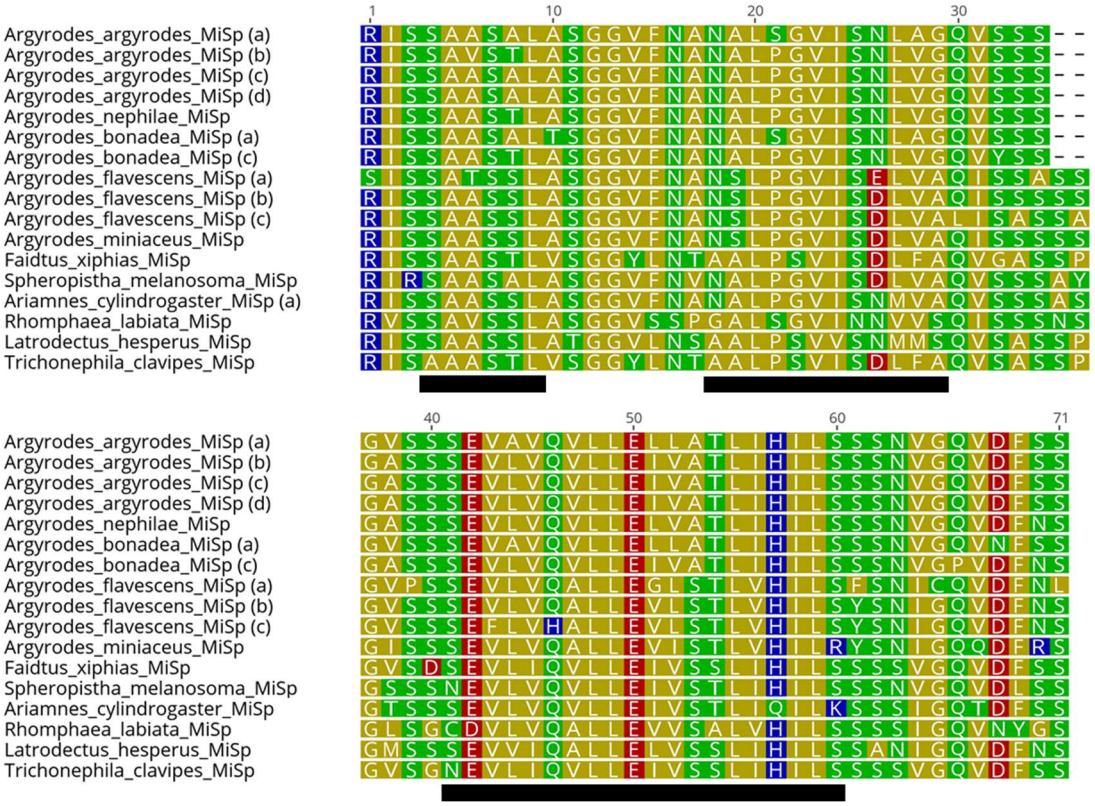

Figure 5.17. Minor ampullate C-terminal domain sequences generated in this study, aligned against downloaded sequences from *Latrodectus hesperus* and *Trichonephila clavipes* (see Section 5.3 for accession numbers) Black bars indicate predicted alpha helical secondary structures (Jnet predictions on *A. argyrodes* MiSp (a)). Colouring indicates polarity.

Chapter 6 – The Russian doll: infection of kleptoparasitic spiders by endosymbiotic bacteria

6.1 Abstract

The microbiotic community within an organism, often referred to as its microbiome, can have a substantial influence on the organism's ecology and behaviour in ways that both benefit and harm overall host fitness. Many microorganisms have long-term associations with their hosts, propagating through vertical transmission between mother and offspring, and so exhibit evolutionary strategies to manipulate the reproductive strategies of their host in favour of infected females. Understanding the modification of host phenotypes by key members of their microbiome is integral to understanding the totality of the organism as well as the impact of agricultural practices and climate change.

This chapter considers the kleptoparasitic spider *Argyrodes argyrodes*, which is expected to have an unusual microbiome through the impacts of their fluctuating and patchy habitat, apparent sex ratio bias and proximity to agricultural land. Bacterial screening identified four common manipulative endosymbionts of arthropods: *Rickettsiella, Rickettsia, Cardinium* and *Spiroplasma.* These were each identified to be closely related to strains associated with insects and other arachnids but unique to the species or within spiders. This suggests a close association with these hosts and little recent horizontal transmission, supported by the presence of identical ITS and 16S sequences extracted from the *Spiroplasma* and *Cardinium* endosymbionts present in populations of *A. argyrodes* from Spain and Israel. A close relative, *Neospintharus syriacus,* also contained identical *Spiroplasma* and *Cardinium* sequences.

Wider population screening in Spanish *A. argyrodes* showed *Cardinium* infection in 22% of individuals and *Spiroplasma* in 27%. Complete absence was found at one habitat patch recorded to have dramatically fluctuating *A.*

argyrodes populations, suggesting a founder effect biasing infection rate. Infection with both endosymbionts was identified in 5% of Spanish *A. argyrodes* and total 16S screening found infection with more than one endosymbiont in 88% of spiders and common triple infections. This has substantial implications for manipulative impacts on the spider hosts, potentially counteracting or exacerbating effects caused by individual infections.

While this study did not establish phenotypes arising from endosymbiont infection, it did find substantial infections in male *A. argyrodes*, with all four identified endosymbionts found at a similar rate to that within females. This indicates that the bacteria are not causing complete or even majority feminisation or killing of infected male offspring. More subtle effects must be explored to ascertain the impact of these endosymbionts and whether they are responsible for the observed sex-ratio bias recorded in Spanish *A. argyrodes*.

6.2 Introduction

6.2.1 The invertebrate microbiome

The term ‘microbiome’ is defined by Whipps *et al.* (1988) as ‘a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties.’ Research on the topic builds upon centuries of interest in microbial ecology and the ways in which microscopic organisms may influence the visible world. Traditionally, scientists relied upon microscopy and physical characteristics, but the advent of new sequencing technologies has opened the field to extensive possibilities across any number of fields including health, agriculture, mathematics and more.

Research concerning the microbiome of animals has been of particular interest, especially when considering the implications for human health. More than £1.2 billion has been spent in the last decade on the exploration of the human microbiome, challenging medicine’s view of microorganisms solely as disease agents and inspiring new therapies through the targeted manipulation of microscopic communities (Proctor, 2019).

A lesser considered but growing field is that of invertebrate microbiomes. The microbial communities living within these ubiquitous taxa are key to conservation biology, agricultural pest management, disease vector control and understanding the inherent inter-connectedness of the natural world (Bahrndorff *et al.*, 2016; Hoffmann *et al.,* 2011; Peterson & Osvatic, 2018; van Lenteren *et al.*, 2018). Microbiotic communities in arthropods have been shown to influence mating preference (Sharon *et al.,* 2010), reproductive efforts (Lopez-Madrigal & Duarte, 2019), pathogen transmission (Abraham *et al.,* 2017; Douglas, 2015), digestion (Jing *et al.,* 2020) and pesticide resistance (Broderick *et al.,* 2006; Gibbons, 2020).

6.2.2 Manipulative endosymbiotic bacteria in arthropods

‘Symbiosis’ describes the ‘living together’ of organisms and ‘endosymbiosis’ describes the living of one organism inside another’s body or even cells (De Bary, 1879). Symbiosis can include a range of relationships from parasitism to

mutualism and usually includes a level of reliance and co-evolution, whether in collusion or conflict. Many microorganisms within the internal microbiome can be described as endosymbionts, forming long-lasting associations and influencing the host in a number of ways. Endosymbiotic bacteria have been found to exploit arthropods more than any other taxon (Harris *et al.,* 2010), making arthropods an ideal group to explore when considering the co-evolution and manipulation involved in such relationships.

A number of commonly identified endosymbionts of arthropods are vertically transmitted bacteria, passed from mother to offspring through gametes. Horizontal transmission is also possible through shared substrates or ingestion but this is not common in most species described as endosymbiotic (Goodacre & Martin, 2013). Due to the prevalence of vertical transmission, the reproductive efforts of the host and especially the production of daughters, which can further pass the symbiont to offspring, are closely tied with the propagation success of the symbiont.

As a result of this close association and reliance on the production of host daughters, these endosymbiotic bacteria have developed a number of manipulative strategies to promote their own fitness (Werren & O'Niell, 1997). This includes the direct increase of female offspring through feminisation and parthenogenesis. Feminisation describes the process of male offspring developing physically and reproducing as females while parthenogenesis involves reproduction from an ovum with no need for fertilisation, generally with no need for the production of males (Stouthamer *et al.,* 1993). Where both male and female embryos are produced, females can be promoted by the bacteria through male-killing (Hurst & Majerus, 1993). This reduces competition for resources, increasing female fitness. A final common strategy by endosymbiotic bacteria, where infected males are produced, is that of cytoplasmic incompatibility. This process involves reproductive incompatibility between infected males and uninfected females, or females infected with a different strain of the bacterium, leading to reproductive advantages for infected females (Hurst & Majerus, 1993).

From the limited breadth of information available, it is suggested that spiders (order Araneae) have a higher frequency of infection and diversity of endosymbionts than other arthropod taxa, with endosymbionts forming the vast majority of their microbiomes (Vanthournout & Hendrickx, 2015; White *et al.,* 2020; Zhang *et al.,* 2018). The most common bacterial endosymbionts identified in spiders are *Cardinium, Spiroplasma, Rickettsia* and *Wolbachia*, with *Rickettsiella* also being increasingly identified in spiders following the introduction of 16S sequencing methods (Zhang *et al.,* 2020).

Rickettsia (class Alphaproteobacteria, order Rickettsiales, family Rickettsiaceae) species have been mostly explored due to their association with blood-feeding arthropods, with the group being passed to vertebrates as an agent of diseases such as typhus and Rocky Mountain spotted fever. The group is increasingly being identified, however, as a widespread intracellular endosymbiont of arthropods involved in a range of manipulative behaviours such as male-killing, parthenogenesis, and effects on fertility (Perlman *et al.,* 2006). *Rickettsia* have been shown to have a high rate of horizontal transmission compared to some other arthropod endosymbionts, on an 'ecological' time scale rather than 'evolutionary time' (Caspi-Fluger *et al.,* 2012; Perlman *et al.,* 2006). When considering spiders, *Rickettsia* have been recorded from spiders across divergent groups (Goodacre *et al.,* 2006). *Rickettsia* have been shown to contribute to feminisation in the linyphiid *Mermessus fradeorum* (Curry *et al.,* 2015) in combination with other endosymbionts. Further, Goodacre *et al.* (2009) found decreased dispersal behaviour in infected individuals of the linyphiid *Erigone atra*, which opens possible manipulation of hosts far wider than solely reproductive phenotypes. However, very little is known about the impacts of *Rickettsia* on spider hosts and the implications at population and species levels.

Spiroplasma (class Mollicutes, order Entomoplasmatales, family Spiroplasmataceae) is another endosymbiont regularly observed within arthropods, both intra- and extracellularly, along with being responsible for a number of arthropod, plant and human diseases. Extensive work on pleiotropic male-killing *Spiroplasma* in *Drosophila* has led this bacterium to be recommended as a model system for exploring endosymbiont-host dynamics,

with the molecular mechanisms more clearly understood than in other systems. Other arthropod host impacts described have included defence against natural enemies, colour variation and reproductive timing (Ballinger & Perlman, 2019; Haselkorn, 2010; Jiggins *et al.,* 2000). While *Spiroplasma* has regularly been documented across spider groups, no definitive manipulations have been recorded.

Cardinium endosymbionts (class Bacteroidetes, order Bacteroidales, family Bacteroidaceae) infect 6-10% of arthropods intracellularly. Phenotypic effects of infections across taxa have included feminisation, parthenogenesis, cytoplasmic incompatibility, oviposition behaviour and enhanced fecundity, showcasing diverse and subtle impacts on hosts (Giorgini *et al.,* 2009; Gotoh *et al.*, 2007; Kenyon & Hunter, 2007; Xie *et al.,* 2016; Zchori-Fein *et al.,* 2001). *Cardinium* infection has been strongly linked to temperature, with presence being higher in warmer habitats (mean > 25°C) but substantially reduced in laboratory conditions over 34°C, possibly due to reduced transmission efficiency (Charlesworth *et al.,* 2019; Jeyaprakash & Hoy, 2010). Phenotypic manipulation by *Cardinium* can also be influenced by temperature (Doremus *et al.,* 2019). This group is very prevalent in spiders, found in circa 20% of screened individuals (Duron *et al.,* 2008), with no clear manipulations observed in the few reports exploring the subject.

Little is known about the abundance and impacts of *Rickettsiella* (class Gammaproteobacteria, order Legionellales, family Coxiellaceae), with limited literature covering its symbiotic interactions with arthropods and spiders. Many of the examples of *Rickettsiella* infection classify it as a pathogen, causing slow development and death (Cordaux *et al.,* 2007). However, it is also increasingly being identified as having more long-term associations as an endosymbiont and is frequently identified from the tissues of seemingly healthy arthropods (Cordaux *et al.,* 2007; Iasur-Kruh *et al.,* 2013). A key study by Rosenwald *et al.* (2020) found *Rickettsiella* to be present in all tested individuals of the linyphiid spider *Mermessus fradeorum*. Further exploration found that infection caused cytoplasmic incompatibility between infected males and uninfected females, the first record of such in any Gammaproteobacteria.

Many arthropods, including spiders, have been found to regularly harbour more than one group of endosymbiotic bacteria within the same individual, with implications for the overall manipulation of the host (White *et al.,* 2020). A commonly reported infection overlap is that of *Wolbachia* and *Cardinium*. This was shown by Ros and Breeuwer (2009) to be integral to the study of cytoplasmic incompatibility in a spider mite, with the phenotype expressed by *Cardinium* infected females but not present in doubly infected females. Spider mites with this double infection have also shown an increase in fecundity (Zhao *et al*., 2013) with no difference in longevity, indicating a selective advantage for this infection combination. *Spiroplasma* has also been found alongside *Wolbachia* in *Drosophila* flies, with quantities of *Wolbachia* in the host being greatly reduced by *Spiroplasma*, which showed no reduction in numbers within co-infected hosts (Goto *et al.,* 2006). All the current knowledge of co-infection indicates a vast difference in phenotype even with bacteria which usually manipulate in the same ways; it also indicates competition and exclusion from certain tissues and so highlights infections by multiple bacteria as an important factor within work exploring bacterial endosymbiosis.

6.2.3 *Argyrodes argyrodes* kleptoparasitic spiders

Argyrodes argyrodes (Walckenaer, 1983) are small kleptoparasitic spiders within the family Theridiidae (subfamily Argyrodinae). This species does not produce its own webs and is largely seen residing in groups on orb webs made by much larger host spiders, usually of the family Araneidae, and scavenging from these hosts in a number of ways. The species has an extensive range across the Mediterranean, West Africa, Canary Islands and Seychelles (Exline & Levi, 1962; Levy, 1985; Platnick, 2021), exhibiting an especially patchy distribution in most areas due to their reliance on host webs as habitat ‘islands’ with minimal dispersal (Su *et al.,* 2018).

Targeted PCR testing of the close relative *Argyrodes antipodianus* did not find presence of the endosymbiont *Wolbachia* (Rowley *et al.,* 2004) but no other research has been published on the microbial community present in *A. argyrodes,* any close relative or any other kleptoparasitic spider. The most comprehensive study on the full microbiota found within theridiid spiders

covered *Latrodectus*, *Steatoda* and *Parasteatoda* species (Dunaj *et al.,* 2020). This analysis found *Wolbachia, Spiroplasma, Rhabdochlamydia* and *Gilliamella* to have the highest abundance. While within the same family as *A. argyrodes*, these included species have vastly different ecological strategies and physiologies and cannot be expected to represent the family as a whole.

A. argyrodes can be expected to contain an unusual microbiome, even when compared against other spiders, for a number of reasons. The first is their patchy distribution and low dispersal leading to exacerbated founder effects, where the microbiome present in the few individuals first colonising the site becomes widespread as their offspring dominate. Also, habitat specificity, where the differences between localities such as available prey types and environmental conditions could lead to diverged microbial communities. *A. argyrodes* on the webs of *Cyrtophora citricola* are also often found in proximity to agricultural fields (personal observation, see Chapter 2), where the diversity of endosymbiotic bacteria has been shown to be both high and protective against pesticides (Gibbons, 2020; White *et al.,* 2020). A combination of these factors might be expected to lead to microbiomes which show little diversity between individuals located in the same area but high diversity between areas. As presented in Chapter 3, *A. argyrodes* in Spain appear to show a marked sex ratio bias even at peak breeding season, a pattern not observed in Israeli populations. This may be due to high mortality in males but could also arise through manipulation by endosymbiotic bacteria.

6.2.4 Chapter aims

This chapter aims to explore the natural microbiome of Spanish *A. argyrodes*, with a focus on manipulative endosymbionts, which would be expected to influence the behaviour and reproduction of the spiders. With such unusual life history strategies and spatial distribution, we might expect these kleptoparasitic spiders to attract and retain microorganisms with similarly uncommon strategies. This chapter will explore whether this is true, leading to a diverged microbiome when compared to other spiders, and will discuss how any differences in manipulative bacterial endosymbionts might drive divergence of the hosts' ecological strategies.

6.3 Methods

Six adult female *Argyrodes argyrodes* were collected from site PA in March 2020 and a further 10 in June 2020, with 4 adult females from SN, 4 from PA and a further 2 adult males from PA. For further site information see Table 2.1. Spiders were killed after a 3 day starvation period to minimise gut contents then stored in 95% ethanol before being transferred to DNA/RNA Shield (Zymo Research) for transport. DNA extraction and 16S sequencing were completed using the ZymoBIOMICS® Targeted Sequencing Service (Zymo Research).

The ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research) was used to extract DNA using an automated platform. Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S NGS Library Prep Kit (Zymo Research), with primers amplifying the V3-V4 region of the 16S rRNA gene. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean & Concentrator (Zymo Research), then quantified with TapeStation (Agilent Technologies) and Qubit (Thermo Fisher Scientific). The final library was sequenced on Illumina® MiSeq with a v3 reagent kit (600 cycles) and 10% PhiX spike-in.

Unique amplicon sequence variants were inferred by Zymo Research from raw reads and errors and chimeric sequences were removed using the DADA2 pipeline (Callahan *et al.,* 2016). Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 (Caporaso *et al.,* 2010) with the Zymo Research Database. With data returned from Zymo Research, graphs were created in R 4.0.0 (R Core Team, 2021) using the package ggplot2 (Wickham, 2016).

See Tables 6.1 - 6.4 (supplementary material) for full list of 16S sequences sourced from public databases used for phylogenetic analysis. Sequences were aligned using CLUSTAL Omega 1.2.2 (Sievers *et al.,* 2011). Trees were created in Geneious tree builder (Geneious prime v2021.0.3) using the Neighbour-Joining method, rooted using outgroups. Outgroups were selected based on available sequences from closely related bacterial species. Bootstrap

analysis was included with 1000 replicates and nodes were retained if bootstrap values were over 50%.

6.3.2 PCR isolation of endosymbiont specific markers

To confirm the presence of *Spiroplasma* spp. and *Cardinium* spp. endosymbionts in the haemolymph of *A. argyrodes* and survey infection frequency in the wider population of *A. argyrodes*, PCR amplification using common spider endosymbiont primers was completed. 82 spiders (45 adult females, 28 adult males and 9 subadults) were collected across sites SN, NN, PA, CM and WP in April and May 2018. Samples were killed after a starvation period of 3 days to minimise gut contents then stored in 95% ethanol until use. In order to compare bacterial endosymbionts of Spanish populations with others, 18 *A. argyrodes* (15 adult female and 3 adult male) samples were also collected from Ma'agan Mikha'el Cemetery in Israel (sourced from Efrat Gavish-Regev, Hebrew University of Jerusalem) in August 2018 and immediately stored in 95% ethanol. This population was indicated to be distinct from the Spanish through CO1 sequencing (data not shown). A further 8 samples of adult female *Neospintharus syriacus*, a free-living spider also within the sub-family Argyrodinae, were taken at the same date, in the same Israeli locality.

Only legs (removed at the second segment, where no gut is present) were used in endosymbiont screening to further minimise gut contamination. Tissue was disrupted manually using a small pestle then DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich). Samples were eluted in 200 µl of distilled water and stored at -20°C. Successful DNA extraction was confirmed by a PCR reaction using the primers hedin-O and hedin-C (Hedin & Maddison, 2001), amplifying the D2–D3 region of the spider 28S nuclear ribosomal RNA gene.

Infection from the *Spiroplasma* spp. was detected by amplifying the intergenic ribosomal spacer with the primers Spits-J04 5′-GCCAGAAGTCAGTGTCCTAACCG-3′ and Spits-N55 5′-ATTCCAAGGCATCCACCATACG-3′ (Majerus *et al.,* 1999) alongside the 16S amplifying primers 23F 5’-CTCAGGATGAACGCTGGCGGCAT-3’ and TKSS 5’-TAGCCGTGGCTTTCTGGTAA-3’ (Haselkorn *et al.,* 2009). *Cardinium* spp.

infection was identified by the amplification of the 16S ribosomal DNA (rDNA) gene with the primers Ch-F 5′-TACTGTAAGAATAAGCACCGGC-3′ and Ch-R 5′-GTGGATCACTTAACGCTTTCG-3′ (Zchori-Fein & Perlman, 2004).

The total volume (10 µl) of PCR reactions contained; Thermo Scientific Reddymix PCR Master Mix 1x (1.5 mM MgCl2 concentration), 0.5 µl each of forward and reverse primers (10 µM concentration) and 1 µl of DNA. An initial denaturation at 94°C for one minute was followed by 35 cycles of 94°C for 30 seconds, 55°C for 20 seconds and 72°C for 30 seconds. Negative controls with no template DNA were included in each reaction as well as positive with known bacterial presence. PCR products were separated by gel electrophoresis (1.5% agarose gel in TBE) and visualised using the NuGenious imaging system (Syngene).

This reaction was then repeated with a 30 µl volume and the product was purified and sequenced by Macrogen Europe using a 3730xl DNA Analyzer (Applied Biosystems). See Table 6.5 (supplementary material) for full list of *Spiroplasma* spp. ITS sequences sourced from public databases used for phylogenetic analysis. Sequences were aligned using CLUSTAL Omega 1.2.2 (Sievers *et al.,* 2011). Trees were created in Geneious tree builder (Geneious prime v2021.0.3) using the Neighbour-Joining method, rooted using outgroups. Bootstrap analysis was included with 1000 replicates and nodes were retained if bootstrap values were over 50%.

6.4 Results

Rickettsiella, Rickettsia, Cardinium and *Spiroplasma* were all found to be present within the body of at least one *Argyrodes argyrodes* screened using 16S sequencing (see Figure 6.1 for read proportions). *Rickettsiella* was present in 75% (12) of the samples, *Rickettsia* in 13% (2), *Cardinium* in 63% (10) and *Spiroplasma* in 70% (11). All bacteria were present across both collection dates and sexes except *Rickettsia*, which was not present in 16S samples from SN.

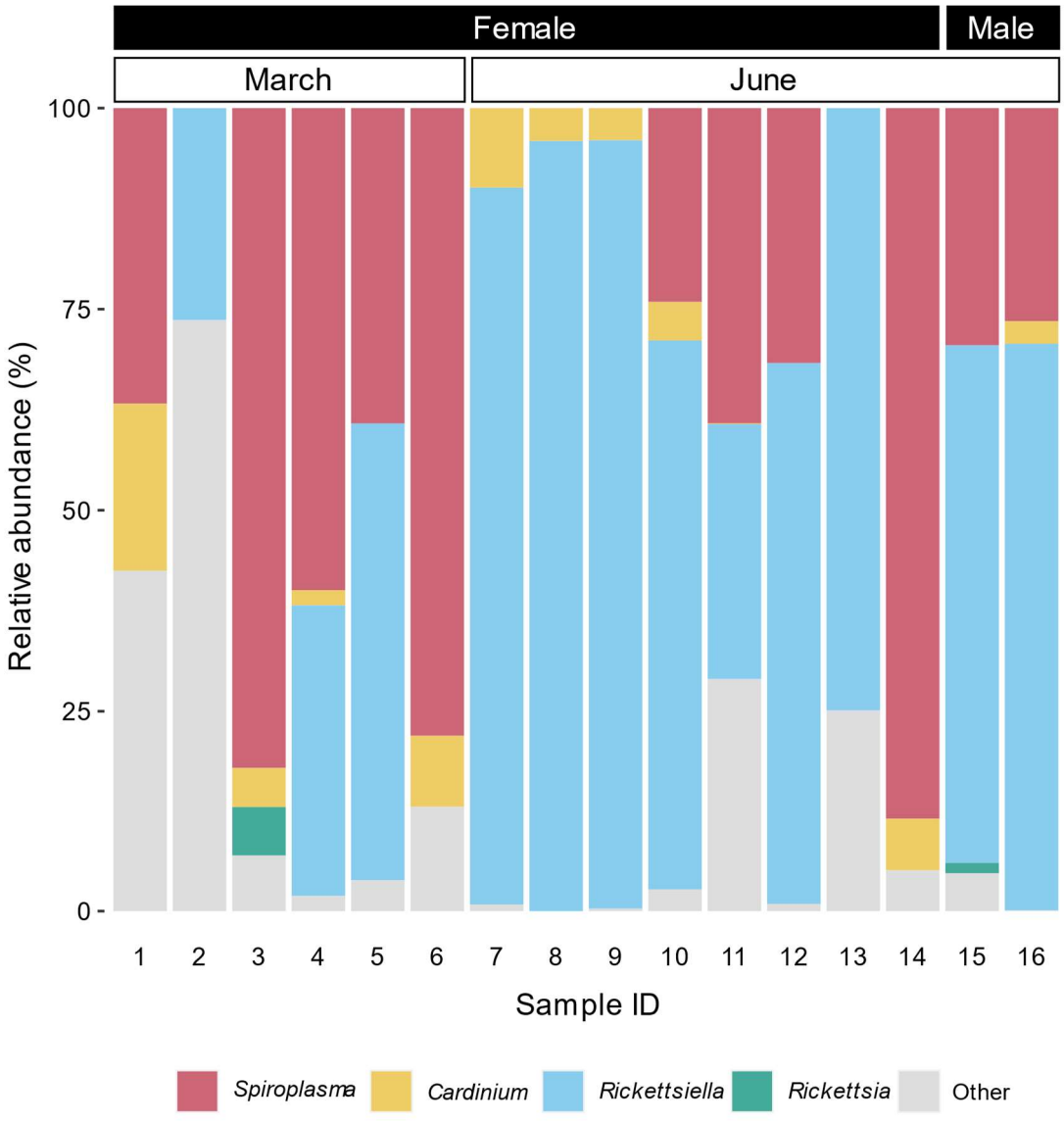

Figure 6.1. Microbial composition of 16 adult *Argyrodes argyrodes* based on 16S sequencing. Samples vary in locality, date collected and sex.

While the presence of endosymbionts in 16S screening data is similar between the samples collected in March and June, there is some difference in the read

abundance. In samples collected in March, *Spiroplasma* are the most abundant bacteria in all but one sample. Conversely, spiders collected in June show dominance of *Rickettsiella* in all but two samples.

Other bacterial sequences identified were typically under 1% of reads. Notable exceptions were *Turicibacter sanguinis*, present in samples 1 (5%) and 2 (3%), *Staphylococcus succinus* in sample 2 (8%) and sample 6 (4%) and *Romboutsia ilealis* in sample 2 (4%).

Four *Rickettsiella* 16S sequences were identified from the 16S screening, with '*Rickettsia* a' being present in all samples with *Rickettsiella* and in majority in all. The other 3 'b-d' were only present in 1 or 2 samples and all at less than 1% of sequences. In aligning these sequences with others across arthropods (see Figure 6.2) the sequences extracted from *A. argyrodes* in this study cluster strongly and show most similarity to *Rickettsiella* extracted from the hemipteran insect *Ommatissus lybicus*.

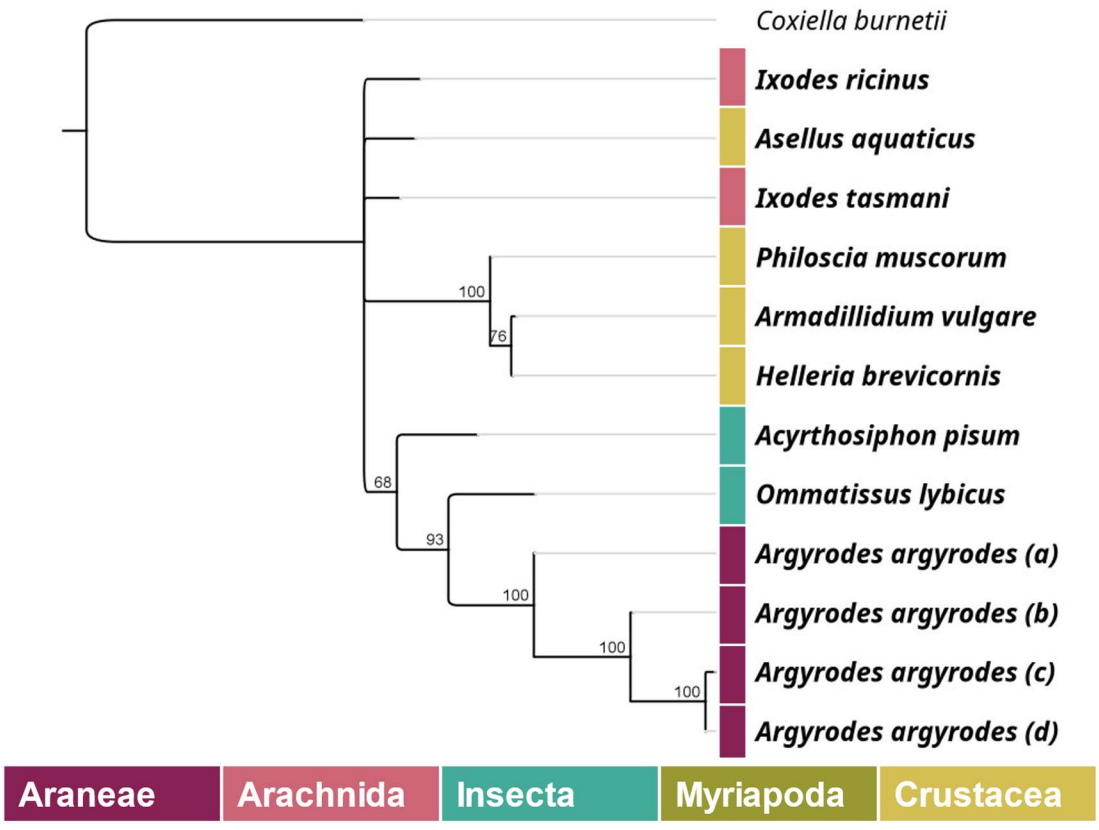

Figure 6.2. Phylogenetic tree of *Rickettsiella* spp. endosymbionts of arthropods based on V3-V4 16S rRNA gene sequences. Endosymbionts indicated in bold, with host names as branch labels. Bootstrap values are given at the nodes. For further information on included sequences see Table 6.1.

All *Rickettsia* 16S sequences identified were identical. When considering their relationship with other arthropods, as shown in Figure 6.3, the 16S extracted from *A. argyrodes* group into one clade with *Rickettsia* from all included insect hosts except coleopterans, as well as those from other spider species.

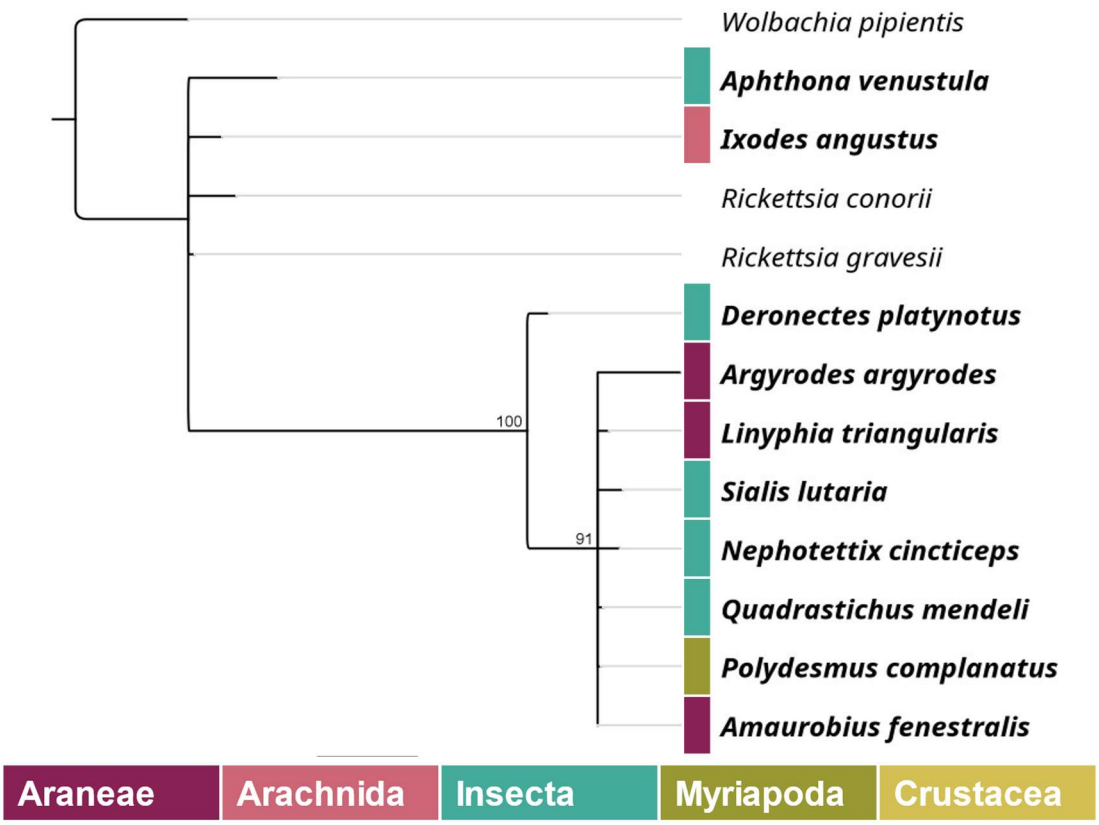

Figure 6.3. Phylogenetic tree of *Rickettsia* spp. endosymbionts of arthropods based on V3-V4 16S rRNA gene sequences. Endosymbionts indicated in bold, with host names as branch labels. Bootstrap values are given at the nodes. For further information on included sequences see Table 6.2.

All *Cardinium* 16S screening sequences from *A. argyrodes* were identical. The sequence showed greatest similarity to *Cardinium* extracted from the mite *Microzetorchestes emeryi,* grouping with that from another mite host, *Brevipalpus phoenicis* and the spider *Cybaeus sanbruno,* as shown in Figure 6.4.

PCR screening of leg tissue found *Cardinium* infection in 22% of Spanish *A. argyrodes* and 50% of Israeli *A. argyrodes*, with the difference being non-significant (Fisher's Exact Test: $p > 0.05$). 25% of *Neospintharus syriacus* were infected with *Cardinium.* It is of note that none of the 9 samples collected from

Spanish site NN (9) or PA (6) contained *Cardinium*, although neither of these rates were significant compared to all other sites (Fisher's Exact Test: $p > 0.05$; Fisher's Exact Test: $p > 0.05$). Sequencing of PCR products within the 16S region showed identical nucleotides across both species and all populations. These sequences also overlapped (217 base pairs) with the V3-V4 sequencing of the screening data and showed 100% similarity, with a lower similarity to other *Cardinium* included in Figure 6.4.

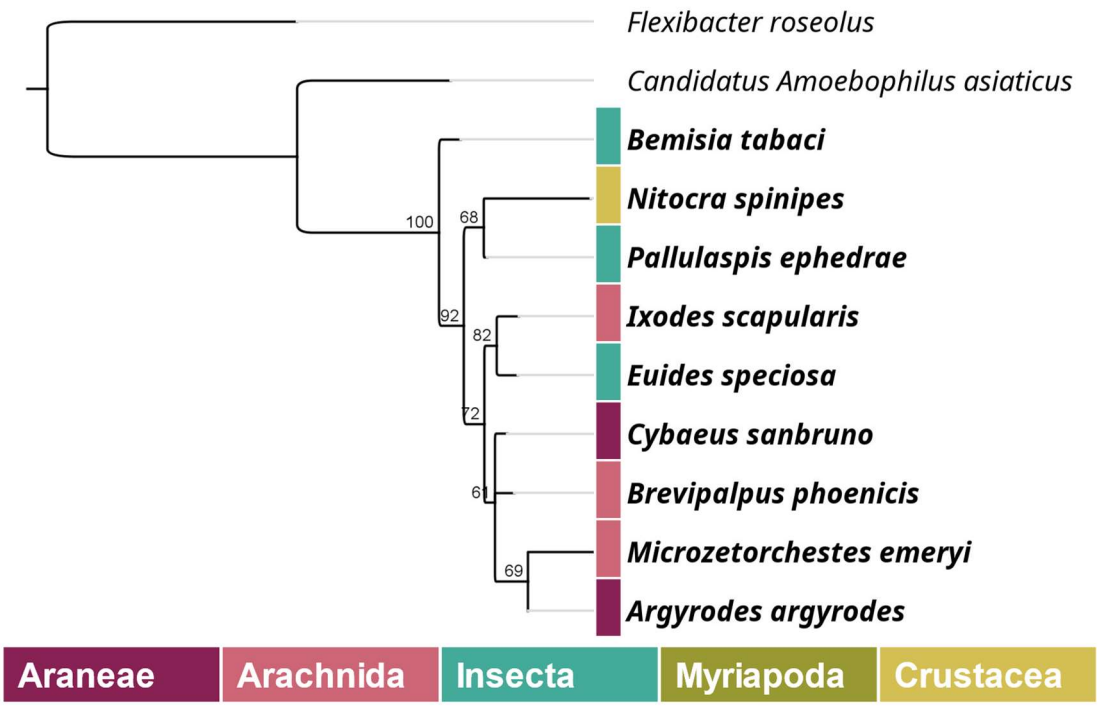

Figure 6.4. Phylogenetic tree of *Cardinium* spp. endosymbionts of arthropods based on V3-V4 16S rRNA gene sequences. Endosymbionts indicated in bold, with host names as branch labels. Bootstrap values are given at the nodes. For further information on included sequences see Table 6.3.

Three *Spiroplasma* 16S sequences were identified from screening of *A. argyrodes*. '*Spiroplasma* c' is present alongside '*Spiroplasma* a' in all infected samples collected in March but is at approximately 10% of the abundance of 'a' in all. 'c' is then not present in any samples collected in June, where 'a' is the sole strain in all but sample 15, which was entirely infected with strain 'b'. 'a' and 'c' sequence similarity is high, with only one synonymous base pair substitution, and these strains cluster with the *Spiroplasma* endosymbiont of

the spider *Larinioides cornutus* in Figure 6.5. 'b' shows only a 98% identity with 'a' and clusters alongside the *Spiroplasma* of the weevil *Curculio sikkimensis*.

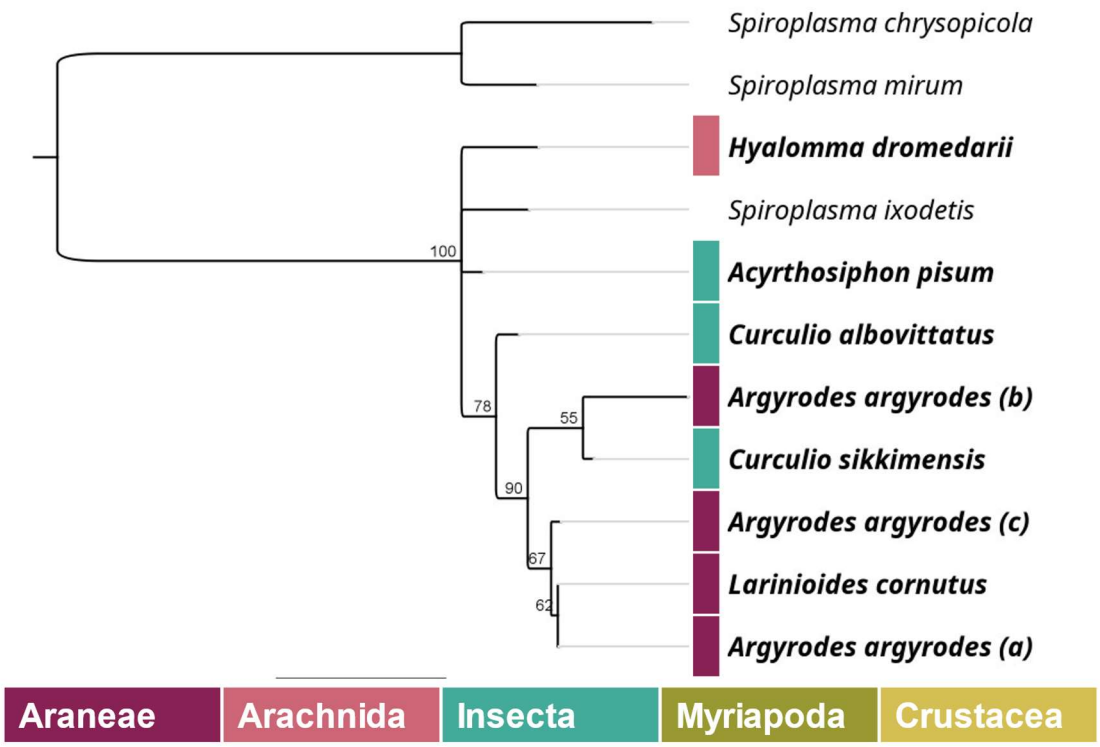

Figure 6.5. Phylogenetic tree of *Spiroplasma* spp. endosymbionts of arthropods based on V3-V4 16S rRNA gene sequences. Endosymbionts indicated in bold, with host names as branch labels. Bootstrap values are given at the nodes. For further information on included sequences see Table 6.4.

PCR screening of *A. argyrodes* leg tissue found a 27% *Spiroplasma* infection rate in Spanish populations, a 28% rate in Israeli papulations and 50% in *N. syriacus.* 31% of Spanish adult females were infected against 21% of adult males and this was not a significant difference (Fisher's Exact Test: $p > 0.05$). It is of note that none of the 9 samples collected from site NN contained *Spiroplasma* and this is significant when compared to infection rates across all other sites (Fisher's Exact Test: $p < 0.05$).

Sequences derived from PCR screening of the ITS region were all identical across populations and species. Phylogenetic comparison of ITS sequences, shown in Figure 6.6, showed *A. argyrodes* and *N. syriacus Spirplasma* clustering with that from all the spider species included except *Tetragnatha montana*, which clustered in a sister clade alongside the samples extracted from insects and cultured *Spiroplasma ixodetis.* Sanger sequencing of the 16S

region using 23F and TKSS primers showed a 128 base pair overlap with 16S screening sequences and showed 100% identity, though also showing this identity to all the endosymbiotic *Spiroplasma* sequences included in Figure 6.5.

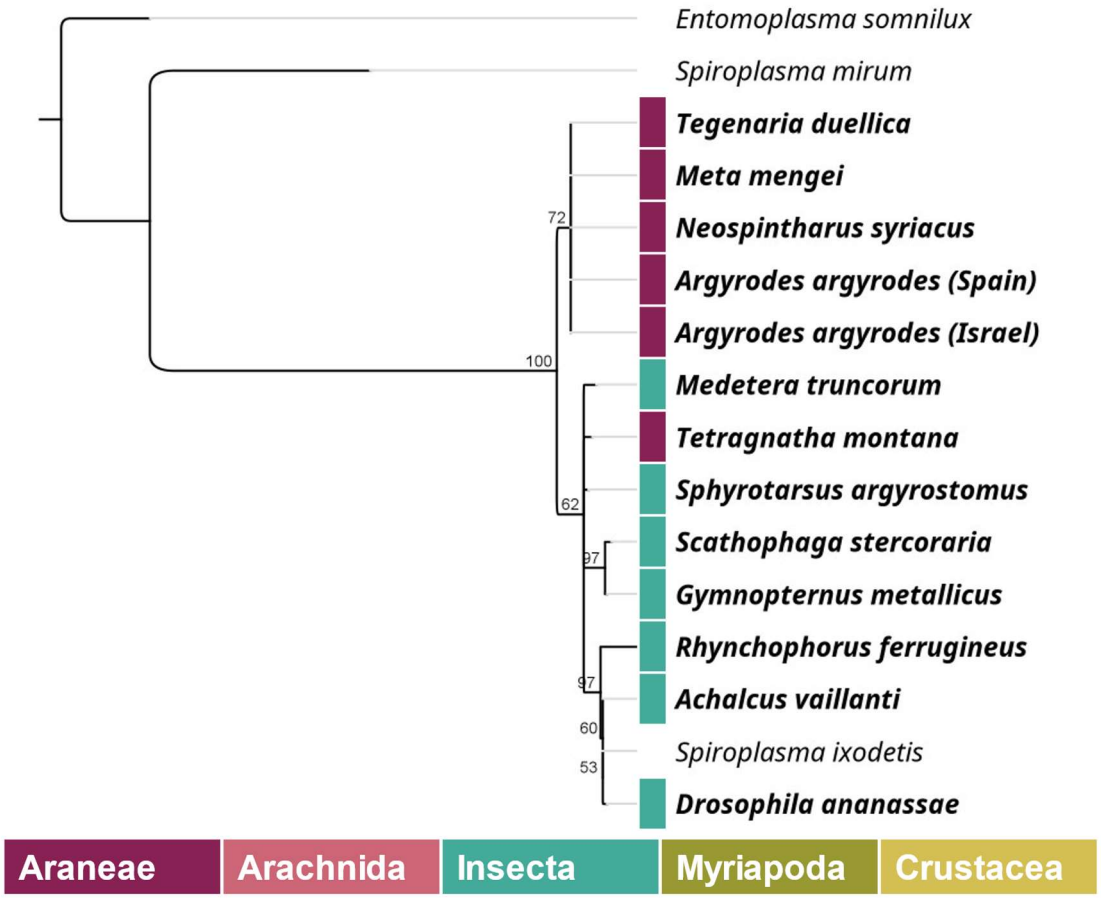

Figure 6.6. Phylogenetic tree of *Spiroplasma* spp. endosymbionts of arthropods based on ITS sequences. Endosymbionts indicated in bold, with host names as branch labels. Bootstrap values are given at the nodes. For further information on included sequences see Table 6.5.

PCR screening showed a number of *A. argyrodes* infected with both *Cardinium* and *Spiroplasma*. 5% of spiders tested from Spain showed a double infection while 12% of Israeli samples showed the same.

6.5 Discussion

The dominance of *Rickettsiella, Cardinium* and *Spiroplasma* can be expected from current knowledge of spider microbial communities, with each being found across a number of spider species screened in previous work. The combination of all three, alongside *Rickettsia* in some samples, is uncommon in previous screening and appears to be widespread in *Argyrodes argyrodes*. This is especially true of theridiid screening, where only *Rickettsiella* was present in both *A. argyrodes* and the other theridiids.

Sequencing of 16S and ITS regions placed most *A. argyrodes* (and *Neospintharus syriacus*) endosymbionts phylogenetically close to those of other spiders and mites and loosely with examples from insects. This is consistent with a level of co-evolution with associated taxa arising from a long period of vertical transmission, diverging the bacterial strains from those of other hosts. Horizontal transmission is also possible in all cases, as some clustering with insects is present. Further host examples from both arachnids and insects as well as additional regions of genetic material are required for a more complete phylogenetic analysis. The grouping of a large diversity of *Rickettsia* might be expected based on relatively high rates of horizontal transmission reported by Perlman *et al.* (2006) and Hundertmark (2020), seeing less isolation and host specialisation. All four strains of *Rickettsiella* identified in *A. argyrodes* cluster away from those in other hosts, indicating a level of host specificity with mutations forming new strains, which are vertically transmitted, rather than being explained by horizontal transmission introducing new strains or contamination by gut contents. One strain of *Spiroplasma*, however, formed a clade alongside an insect example and away from both other sequences. This might suggest either an insect-derived strain or presence from gut contents, present from infected insect prey.

Other relatively abundant species found in 16S screening, *Turicibacter sanguinis, Staphylococcus* and *Romboutsia ilealis,* might be expected in samples through their presence in animal guts and external surfaces. Their low frequency across samples and low read counts within samples does not indicate significant association with *A. argyrodes,* but further screening would

be required to ascertain a high enough occurrence rate in the species or all local arthropods, to be worth exploring for impacts of infection on individuals or populations, or perhaps interaction with common endosymbionts.

There was a lack of clear difference between the two sites screened for 16S diversity, indicating that habitat factors and patchy distribution may not strongly influence the occurrence of each endosymbiont, but sample size was low. However, PCR screening for *Spiroplasma* found no infection in NN and no infection of *Cardinium* was found in NN or PA. This divergence by NN could be predicted as the site sees uncommonly low and fluctuating levels of *A. argyrodes* (Chapter 3). This could lead to founder effects by uninfected individuals homogenising infection, supported by findings indicating a higher dispersal tendency in uninfected spiders (Goodacre *et al.,* 2009). This similarity in infection status in some sites supports a higher level of relatedness between nearby individuals surveyed, as presented by Su *et al.* (2018), although more data on web-level infection would be needed to support this in all sites.

There are marked differences between the relative abundance of endosymbionts across the March and June collection periods in 16S screening. This could be through infection of differing tissues, with these changing in relative mass over this period, where most *A. argyrodes* mate and begin laying eggs. This is challenged by the two males collected in June, which follow the pattern of *Rickettsiella* dominance, even though they would be expected to have very different physiological changes over the period. Another explanation could be differing environmental conditions such as rainfall or temperature, impacting the success of one endosymbiont over the other. While all screened individuals were judged to be adults, the individuals in June also may be older than the other group and this shift could be a natural community shift over time. Finally, individuals with an abundance of one endosymbiont over another could be manipulated to reproduce earlier or later than others, with the individuals maturing in March being the offspring of early reproducing mothers and the spiders maturing in June being a higher proportion of late reproducing mothers. Regardless of cause, there is not a consistency in dominance between *Spiroplasma* and *Rickettsiella,* although both are consistently more abundant then *Cardinium* and *Rickettsia*. This may be expected to have implications for

the final phenotype exhibited by the hosts, with variation possible between individuals, across the year or over a longer period of time as one endosymbiont begins to succeed over others.

It is unclear what impact these bacteria are having on the host but, with such high infection rates by four manipulative endosymbionts, it is to be expected that a number of phenotypes may be associated with infection status. The presence of *Rickettsiella, Rickettsia, Cardinium* and *Spiroplasma* in the two males screened using 16S sequencing, alongside similar infection rates in PCR screening of *Spiroplasma* and *Cardinium,* suggests that no endosymbionts are entirely removing males from the population through complete feminisation, male killing or other method. This is expected as, unlike some insects, sexual reproduction utilising males is a requirement for reproduction in most spiders and relying on uninfected males being present may not be successful in the long term. However, incomplete sex ratio influence by an endosymbiont is common (Ebbert, 1993) and may be key to understanding the bias observed in Chapter 3. A further study following reproduction in a laboratory would be required to observe the sex ratio of offspring arising from mothers with differing infection statuses. Cytoplasmic incompatibility could also be tested within a more controlled environment, but this may not be expected to be of import to *A. argyrodes* due to their high level of genetic relatedness with those nearby (Su *et al.,* 2018), leading spiders of related maternal lines, and so with similar infection status and strain type, to be most likely to reproduce.

When considering the microbiome of kleptoparasitic spiders, it may be important to also consider that of their hosts. Where horizontal transmission does occur, the sharing of prey items or consuming of hosts could lead the kleptoparasites to exhibit similar microbiological profiles. A lack of such similarity might suggest a minimal level of horizontal transfer. *C. citricola* themselves have a patchy distribution and likely high relatedness in groups so patterns of endosymbiont infection may show similarities to *A. argyrodes* even with no transmission between. Due to the difficulty in identifying *C. citricola* mature males within the studies contained in this thesis, it is unclear if these host spiders see patterns of sex ratio bias unexpected for the species or wider family.

6.6 Statement of contribution

Argyrodes argyrodes for 16s sequencing were collected by Ella Deutsch and processed, sequenced and identified by Zymo Research. Endosymbiont screening was completed by Ella Deutsch with assistance by Alastair Gibbons and sequencing by Macrogen Europe. All study design and further analysis was completed by Ella Deutsch.

6.7 Supplementary material

Table 6.1. 16S sequences of *Rickettsiella* and outgroups retrieved from NCBI GenBank (Sayers *et al.,* 2019) included in phylogenetic tree (Figure 6.2) alongside sequences extracted in this study.

Accession	Bacteria	Host species Host class: order		Reference
U97547.1	*Rickettsiella grylli*	Various	Various	Roux *et al.,* 1997
AY447040.1	*Rickettsiella* sp.	*Asellus aquaticus*	Malacostraca: Isopoda	Wang *et al.,* 2007
AM490939.1	*Rickettsiella* sp.	*Philoscia muscorum*	Malacostraca: Isopoda	Cordaux *et al.,* 2007
AM490938.1	*Rickettsiella* sp.	*Helleria brevicornis*	Malacostraca: Isopoda	Cordaux *et al.,* 2007
AM490937.1	*Rickettsiella* sp.	*Armadillidium vulgare*	Malacostraca: Isopoda	Cordaux *et al.,* 2007
AB522697.1	*Rickettsiella* sp.	*Acyrthosiphon pisum*	Insecta: Hemiptera	Tsuchida *et al.,* 2010
KX790338.1	*Rickettsiella* sp.	*Ommatissus lybicus*	Insecta: Hemiptera	Karimi *et al.,* 2019
KP994858.1	*Rickettsiella* sp.	*Ixodes tasmani*	Arachnida: Ixodida	Duron *et al.,* 2015
KP994857.1	*Rickettsiella* sp.	*Ixodes ricinus*	Arachnida: Ixodida	Duron *et al.,* 2015

Table 6.2. 16S sequences of *Rickettsia* and outgroups retrieved from NCBI GenBank (Sayers *et al.,* 2019) included in phylogenetic tree (Figure 6.3) alongside sequences extracted in this study.

Accession	Bacteria	Host species Host class: order		Reference
AJ429500.1	*Wolbachia pipientis*	*Liposcelis bostrychophila*	Insecta: Psocoptera	Yusuf & Turner, 2004
NR_157982.1	*Rickettsia gravesii*	*Amblyomma triguttatum*	Arachnida: Ixodida	Abdad *et al.,* 2017
L36107.1	*Rickettsia conorii*	Various	Various	Roux & Raoult, 1995
MH618375.1	*Rickettsia* sp.	*Polydesmus complanatus*	Diplopoda: Polydesmida	Li & Zhang, 2018
KX592503.1	*Rickettsia* sp.	*Quadrastichus mendeli*	Insecta: Hymenoptera	Gualtieri *et al.,* 2017
KU586121.1	*Rickettsia* sp.	*Nephotettix cincticeps*	Insecta: Hemiptera	Guo *et al.,* 2016
FM177876.1	*Rickettsia* sp.	*Deronectes platynotus*	Insecta: Coleoptera	Küchler *et al.,* 2009
MK078309.1	*Rickettsia* sp.	*Aphthona venustula*	Insecta: Coleoptera	Kolasa *et al.,* 2018

MF156636.1	*Rickettsia* sp.	*Sialis lutaria*	Insecta: Megaloptera	Gerth *et al.,* 2017
HF935069.1	*Rickettsia* sp.	*Ixodes angustus*	Arachnida: Ixodida	Anstead & Chilton, 2013
LR899445.1	*Rickettsia* sp.	*Amaurobius fenestralis*	Arachnida: Araneae	Pilgrim, 2020
LR812273.1	*Rickettsia* sp.	*Linyphia triangularis*	Arachnida: Araneae	Pilgrim, 2020

Table 6.3. 16S sequences of *Cardinium* and outgroups retrieved from NCBI GenBank (Sayers *et al.,* 2019) included in phylogenetic tree (Figure 6.4) alongside sequences extracted in this study.

Accession	**Bacteria**	**Host species** **Host class: order**		**Reference**
AB681070.1	Flexibacter roseolus	-	-	Nakagawa *et al.,* 2011
HM159369.1	C. Amoebophilus asiaticus	*Acanthamoeba* spp.	Discosea: Centramoebida	Schmitz-Esser *et al.,* 2010
JQ364959.1	C. *Cardinium* sp.	*Nitocra spinipes*	Maxillopoda: Harpacticoida	Edlund *et al.,* 2012
GQ455429.1	C. *Cardinium* sp.	*Pallulaspis ephedrae*	Insecta: Hemiptera	Gruwell *et al.,* 2009
AB506775.1	C. *Cardinium* sp.	*Euides speciosa*	Insecta: Hemiptera	Nakamura *et al.,* 2009
HG421084.1	C. *Cardinium* sp.	*Bemisia tabaci*	Insecta: Hemiptera	Santos-Garcia *et al.,* 2014
KJ675560.1	C. *Cardinium* sp.	*Brevipalpus phoenicis*	Arachnida: Trombidiformes	Novelli *et al.,* 2014
MG889459.1	C. *Cardinium* sp.	*Microzetorchestes emeryi*	Arachnida: Sarcoptiformes	Konecka & Olszanowski, 2019
AB001518.1	C. *Cardinium* sp.	*Ixodes scapularis*	Arachnida: Ixodida	Kurtti *et al.,* 1996
GQ480753.1	C. *Cardinium* sp.	*Cybaeus sanbruno*	Arachnida: Araneae	Perlman *et al.,* 2010

Table 6.4. 16S sequences of *Spiroplasma* and outgroups retrieved from NCBI GenBank (Sayers *et al.,* 2019) included in phylogenetic tree (Figure 6.5) alongside sequences extracted in this study.

Accession	**Bacteria**	**Host species** **Host class: order**		**Reference**
NR_121794.2	*Spiroplasma mirum*	-	-	Wang & Zheng, 2010
MN166762.1	*Spiroplasma ixodetis*	Various	Various	Matet *et al.,* 2020

NR_103945.1	*Spiroplasma chrysopicola*	*Chrysops* sp.	Insecta: Diptera	Ku *et al.,* 2013
AB559934.1	*Spiroplasma* sp.	*Curculio sikkimensis*	Insecta: Coleoptera	Toju & Fukatsu, 2011
AB604655.1	*Spiroplasma* sp.	*Curculio albovittatus*	Insecta: Coleoptera	Toju *et al.,* 2013
MT302369.1	*Spiroplasma* sp.	*Acyrthosiphon pisum*	Insecta: Hemiptera	Romanov *et al.,* 2020
MG564240.1	*Spiroplasma* sp.	*Hyalomma dromedarii*	Arachnida: Ixodida	Senbill *et al.,* 2017
KX559380.1	*Spiroplasma* sp.	*Larinioides cornutus*	Arachnida: Araneae	Yun & Ren, 2016

Table 6.5. ITS sequences of *Spiroplasma* and outgroups retrieved from NCBI GenBank (Sayers *et al.,* 2019) included in phylogenetic tree (Figure 6.6) alongside sequences extracted in this study.

Accession	**Bacteria**	**Host species** **Host class: order**		**Reference**
DQ439664.1	*Entomoplasma somnilux*	Various	Various	Volokhov *et al.,* 2006
DQ917756.1	*Spiroplasma mirum*	Various	Various	Bi *et al.,* 2008
DQ004912.1	*Spiroplasma ixodetis*	Various	Various	Volokhov *et al.,* 2006
JQ925448.1	*Spiroplasma* sp.	*Medetera truncorum*	Insecta: Diptera	Martin *et al.,* 2013
JQ925385.1	*Spiroplasma* sp.	*Gymnopternus metallicus*	Insecta: Diptera	Martin *et al.,* 2013
FJ657372.1	*Spiroplasma* sp.	*Drosophila ananassae*	Insecta: Diptera	Haselkorn *et al.,* 2009
JQ925485.1	*Spiroplasma* sp.	*Sphyrotarsus argyrostomus*	Insecta: Diptera	Martin *et al.,* 2013
JN601189.1	*Spiroplasma* sp.	*Scathophaga stercoraria*	Insecta: Diptera	Martin *et al.,* 2012
MH745004.1	*Spiroplasma* sp.	*Rhynchophorus ferrugineus*	Insecta: Coleoptera	Awad *et al.,* 2021
DQ231494.1	*Spiroplasma* sp.	*Tegenaria duellica*	Arachnida: Araneae	Goodacre *et al.,* 2006
DQ231496.1	*Spiroplasma* sp.	*Meta mengei*	Arachnida: Araneae	Goodacre *et al.,* 2006
DQ231498.1	*Spiroplasma* sp.	*Tetragnatha montana*	Arachnida: Araneae	Goodacre *et al.,* 2006

Chapter 7 – Thesis discussion

7.1 Study system

This thesis introduces a study system that provides an ideal opportunity to examine the association between kleptoparasitic spiders and colonial hosts, providing insight into the evolution of both parasitic and social strategies. Low plant diversity and increasing urbanisation have led *Cyrtophora citricola* to be the most abundant web building spider in all areas the species was observed, providing the only kleptoparasitic Argyrodinae in the area, *Argyrodes argyrodes,* a single host to associate with. *C. citricola* colonies present are of variable size, with many solitary individuals also.

A key component of these study sites is the constant presence of agricultural land, usually arable and orchard. Many crop pests of concern in the area, such as the cotton bollworm (*Helicoverpa armigera*) and olive fly (*Bactroceraoleae* spp.), are flying insects and fall well within possible *C. citricola* prey types. With *C. citricola* dominance, understanding its impact on these pests and the factors which might encourage abundance and predation in agricultural areas may be key to a reduction in pesticide reliance in the area.

7.2 Sociality in spiders

Both species considered in detail in this thesis, *Cyrtophora citricola* and *Argyrodes argyrodes*, live in groups of conspecifics. This is an unusual tactic within the normally aggressive and cannibalistic spiders and has long been studied to give insight into what fitness benefits might be driving and influencing this strategy.

When considering *A. argyrodes*, data presented here supports previous work finding an increase in kleptoparasites with increasing web size in solitary host spiders. This indicates a carrying capacity of the web, either driven by area alone or by the amount of food caught by the host, which is suggested in Chapter 4 to be higher with increasing web area. This increase in group size with increasing web area does not hold true for colonies of host *C. citricola* (of

more than 2 individuals). This indicates there are factors limiting *A. argyrodes* density where hosts aggregate. The data presented does not, however, describe the distribution of kleptoparasites within the colony and it could be the case that, while the overall load is low, they are distributed unequally. For example, a *C. citricola* with attractive features to kleptoparasites, such as a large web, high food capture rate, ideal colony position or spiderlings, might experience a higher *A. argyrodes* density than if they were solitary. This web-level kleptoparasite data would also support previous work on the predictability of kleptoparasite numbers in colonies, driven by larger and more stable web 'islands'. Most interactions between nearby kleptoparasites are outside of the scope of this thesis but Chapter 2 does show that direct kleptoparasitic behaviour, feeding alongside the host, is not influenced by the number of kleptoparasites on the web. This suggests that parasite group living does not distract the host sufficiently for increased *A. argyrodes* feeding or decrease feeding through competitive interactions. More subtle impacts, such as reduced or increased risk of host detection through increased web movement, may be present. However, *A. argyrodes* have clear evolutionary drivers towards reduced conspecific aggression and group living. Kleptoparasitic living would become next to impossible where they fiercely guarded territories and access to hosts, with aggressive interactions attracting the notice of the host and such low densities in small, fluctuating habitat patches regularly driving population numbers to dangerously low levels.

A more complex drive for colony living is that of *C. citricola,* which for the most part do have the space and resource to remain solitary. Suggested fitness benefits of the strategy are described in Chapters 1 and 3 and additional data presented in this thesis provide additional insight. The decreasing kleptoparasite load in larger *C. citricola* colonies indicates a potential driver of group living where kleptoparasites are present and negatively impacting the hosts. Protection against natural enemies is also shown by a reduced infection of egg sacs by *Philolema palanichamyi* as colony size increases, presented in Chapter 4. With the predation by the young wasps on spiderlings within the egg sacs, even a small reduction in infection chance could dramatically benefit the reproductive output of colony females. These wasps, only described recently in

Spain (see Chapter 4), are shown in this thesis to infect a large proportion of *C. citricola* egg sacs across the summer and autumn and may be the reason for the low *C. citricola* egg sac production into those months, as presented in Chapter 3. However, all considerations of colony size impact on *C. citricola* presented in this thesis contain a low sample size of larger colonies. Further sampling of colonies above 5 spiders is needed to expand conclusions, to verify that patterns remain and to suggest comparative relationships in other regions of the world with generally much larger colonies.

7.3 A true kleptoparasite?

In considering the reduction of kleptoparasite load in larger colonies a positive outcome for *Cyrtophora citricola* fitness, it is assumed that the *Argyrodes argyrodes* have a negative impact on host survival and reproduction. This assumption has had mixed support in published literature. Presented in Chapter 4, this thesis finds a negative correlation between *C. citricola* feeding frequency and kleptoparasite load, with even one adult female *A. argyrodes* reducing feeding. Feeding frequency cannot be a direct measure of nutritional intake and it is unclear how this might impact survival and reproductive success. However, this is a compelling indication of impacts on *C. citricola* behaviour which should be explored further. This exploration would be most effective in a laboratory environment, observing prey capture efficiency and feeding duration to ascertain cause.

Contrary to observations in other host species, no impact on *C. citricola* web repair behaviour and relocation was seen with kleptoparasite presence. This may be expected in *Cyrtophora* species, due to their more permanent and energetically costly web. In addition, no impact was found on host infection by *P. palanichamyi* wasps with *A. argyrodes* presence. Ascertaining overall *A. argyrodes* impact on *C. citricola* fitness will require a number of further, targeted studies, considering factors which are most vital to host survival and reproduction. These could include reproductive success and survival in a captive environment, removing many confounding variables from wild studies. Consideration must also be made for the survival of *C. citricola* males and their access to females with *A. argyrodes* presence.

7.4 Adaptations due to kleptoparasitism

While kleptoparasites may have impacts on their hosts, they also reveal their own adaptations both to best exploit hosts and also, more indirectly, as a result of their unusual distribution and behaviours. Life cycle influences may be demonstrated in the close relationship between the reproductive periods of each spider, with *Argyrodes argyrodes* females maturing as *Cyrtophora citricola* lay eggs, laying their own eggs closely thereafter. Such a correlation might arise through convergent evolution driven by abiotic selection pressures but, in spiders so different in behaviour and physiology as well as differences seen between populations in Spain and Israel, this seems less likely.

An immediate observation on *A. argyrodes* is their drastically reduced requirement for silk production, limited to draglines and egg cases. However, sequencing data presented in Chapter 5 of this thesis suggests little or no relaxation on selective pressures, arising from kleptoparasitism, acting on conserved regions of silk proteins MaSp and MiSp structure. Repetitive regions also appear to show expected motifs, leading to generally conserved properties. Expansion of this sequence analysis to include other silk types as well as long-read sequencing of complete silk sequences is required to ascertain what novel variations this specific lifestyle may have shaped.

An under-considered factor influencing all organisms in their behaviour and physiology is the microbiome, and especially endosymbiotic bacteria developing close, often manipulative, host associations. Chapter 6 of this thesis presents the first microbiome analysis of *A. argyrodes* and identifies abundant infections of four common endosymbionts of arthropods. Each of these bacterial groups has been previously found to exhibit manipulative impacts on their host in order to promote their own transmission and it is expected that they would be influencing the behaviour of *A. argyrodes* in some way. Chapter 3 describes a female-biased sex-ratio observed in *A. argyrodes* and it is suggested that this could be influenced by one of more of the identified endosymbionts. 16S and ITS sequences of bacterial strains were largely novel and some specificity for spider or arachnid hosts was found, suggesting vertical transmission over a long period of time with minimal gene flow between

arthropod groups. Analysis is made challenging by the low sampling diversity in most bacterial groups, with *Rickettsiella* and *Rickettsia* less commonly screened for in arachnids, especially within the 16S regions used here. Further sequencing is required across arthropods to ascertain possible transmission routes and further targeted regions of the genome will add strength to those conclusions.

Captive breeding and behavioural work on endosymbiont-infected spiders is required to identify how these bacteria are influencing *A. argyrodes* and whether these manipulative effects are impacting kleptoparasitic strategies. Endosymbiont benefits to survival may also influence the success of *C. citricola* and *A. argyrodes* in agricultural areas, where the use of pesticides is expected to be extensive. These bacteria have been shown to provide some resilience under such pressures and understanding this may impact on the management of spiders as natural predators of pests.

7.5 Concluding remarks

This thesis explores a number of life history traits and behaviours associated with *Argyrodes argyrodes* and *Cyrtophora citricola* as well as introducing methods of exploring adaptation novel to the exploitative Argyrodinae. In finding close associations between *A. argyrodes* and its host, as well as costs to *C. citricola* of *A. argyrodes* presence, this thesis supports the use of the term 'kleptoparasite' in all its assumptions. Results presented highlight the complexity inherent both in all natural systems, but also specifically this system, where such a large number of organisms directly rely on the others for appropriate habitat and nutritional provision. This work will provide a first step to utilising *C. citricola* colonies in temperate agricultural land to explore extreme and exploitative evolutionary strategies.

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Appendices

Appendix A - Covid-19 project impact

From March 2020 onwards there was a number of disruptions to the initial project plans due to the Covid-19 pandemic. The largest impact was the inability to travel to Spanish (and potentially Middle Eastern) field sites in 2020 and 2021 to record data and collect spiders for behavioural and molecular work in the laboratory. Another impact was the severely reduced access to laboratory and other university facilities across 2020 and somewhat in 2021, reducing some time-intensive molecular work and removing access to specialist facilities such as scanning electron microscopes. This led to a reduced volume of outputs associated with the project, much of this is described in Chapter 7. Future Work.

Appendix B - PIPS placement reflection

From the 1st February to the 1st April 2020 I completed a placement with the University of Nottingham School of Life Sciences Zoology Collection, focused on the development of public engagement resources and curation of the museum specimens. This included activities outside this time frame due to the inflexibility of externally organised public engagement events, delays in funding allocated to curation activities, and the premature finish of the placement period due to Covid-19 restrictions. The placement was initially arranged as 3 months, with a 2 week placement at the National Museum of Scotland, but due to facility closures and social distancing policies this additional time was cancelled.

Activities & Outcomes

During the placement, my aims were to:

1. Learn about and aid the curation of specimens including fluid preserved, taxidermy and skeletal remains. Curation included the maintenance of specimen preservation and the catalogue and organisation of samples.

2. Develop the public engagement resources for both the museum collection and associated outreach activities carried out within the school, including those by the SpiderLab.
3. Deliver outreach events using the museum collection (and some associated live animals where relevant).
4. Secure funding for the collection to procure both curation equipment and specialist services.

Due to Covid-19, in-person curation was scaled back, with some completed outside of the placement time frame, and in-person public engagement was also reduced. Resources were created with both digital outreach and in-person future outreach in mind and included extensive photographs and illustrations, taxonomic games and informational signs and labels. Funding was secured from the UoN Cascade fund in February 2020, based on an application written by me, but purchasing was mainly completed well after the placement due to university spending freezes. I was involved in a large amount of spending decisions and university purchasing logistics.

Personal & Professional Development

My goals for development across the placement were mainly focussed on the enhancement of my project management and administrative skills, building on experiences I had gathered throughout my PhD but in a more focussed and professional setting. I aimed to manage my own time and deadlines to maximise return, coordinating effectively with stakeholders while working in a self-driven, independent way. I feel I achieved this, with self-motivation and the ability to organise my own time improving across the time I worked on the project. I achieved most of my aims to time and effectively managed Covid-19 related delays and changes in an organised and strategized way. I had some opportunity to manage student volunteers and, while the pressure of being in this leadership role was significant, I leaned into my organisation skills to ensure they were useful and also saw development the students' own personal and professional confidence and skills.

Another benefit to both personal and professional skills was experience communicating with a number of people with a range of roles in my project. This

included my direct supervisor, other department academics, external academics and experts, student volunteers and the wider public. Modifying my communication style has been something I have excelled at previously and I further enhanced that here due to my leadership in the project and the wide range of stakeholders. I feel my outcomes were in large part thanks to my communication both delivering information and also effectively understanding feedback. This has aided my teamwork skills, which are largely based on organisation and communication.

Lastly, I have developed my writing style for a professional organisation, with a number of written outputs including a grant proposal, public engagement strategy plans and outreach resources with the aim to entertain and inform. Writing with such a range of tones and styles was challenging and not something I had extensive experience with. I feel I was successful in my production of these documents, utilising the expert help available as well as online resources to ensure professional and clear communication.

The placement was not directly within my career aspirations, which lie within Professional Services roles at research organisations. However, many of the skills and experiences are very relatable. Experience with administration in a university environment, especially procurement, was enlightening and I now have a much clearer understanding of the management structure within the University system. All of the developed communication and management skills will be beneficial in my future roles, and I value the chance to enhance these outside of my PhD project.

Appendix C - Publications

Book chapter: **Deutsch, E. K.** & Gibbons, A. (2020). Nutritional symbiosis in arachnids. In: Microbiomes of Soils, Plants and Animals: An Integrated Approach.

Journal article: Grinsted, L., **Deutsch, E. K.**, Jimenez-Tenorio, M., & Lubin, Y. (2019). Evolutionary drivers of group foraging: A new framework for investigating variance in food intake and reproduction. *Evolution*, 73, 2106-2121.