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**The Mating Systems and Introgression of New Zealand Fishing Spiders  
(*Dolomedes*)**

A thesis

submitted in fulfilment

of the requirements for the degree

of

**Doctor of Philosophy in Biological Sciences**

at

**The University of Waikato**

by

**Simon J. Connolly**



THE UNIVERSITY OF  
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*Te Whare Wānanga o Waikato*

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*Dolomedes minor* female – Photographed by Helen Macky

*“There you will find her clinging to the web, having crept out from her daytime hideout to take up her protective stand for the evening hours. She is a magnificent creature whose body seems to be clothed with the finest velvet.”* – Forster and Forster 1973

# Abstract

Mating systems describe the number of mates different sexes in a species have in their lifetime. Mating systems are key to sexual selection and the evolution of species, as a mating system sets the rules for future evolution and sexual conflict. Spiders show a wide variety of mating systems and extreme adaptations and behaviours are associated with the evolution of monogynous (males mate once in their life) mating systems, including males breaking their genitalia and sacrificing themselves to be eaten by their mate.

Another process that is highly influential on evolution is introgression, the movement of genes from one species to another. This generally occurs through species mating to produce hybrids which then facilitate gene transfer. Despite requiring two different species to interbreed, introgression is often not investigated through the lens of mating behaviours and how these can facilitate or limit introgression.

*Dolomedes aquaticus* and *D. minor* are fishing spiders endemic to New Zealand. Their mating systems and behaviour are undescribed, but members of this genus in other parts of the world are known for their extreme mating behaviour including obligate monogyny, spontaneous male death and sexual cannibalism. Previous genomic work on *D. aquaticus* and *D. minor* revealed a one-way and geographically limited introgression occurring between the two species. That is, genes are being transferred between the two species, but only via *D. aquaticus* females mating with *D. minor* males; and only in the extreme south of the two species' shared range.

In this thesis I investigated the mating systems and introgression of *D. aquaticus* and *D. minor*, using laboratory experiments, field surveys, and 3D morphometrics. I found *D. aquaticus* females will generally only mate once, followed by a syndrome of aggression after mating, whilst males will mate multiply. *D. minor* mating is typified by low probability of mating for males and females, but potential for multiple mating in both sexes, rapid escape behaviour by males following copulation, and genital damage. I also found that the two species are able to meet outside of the Introgression Zone, making it unlikely that habitat plays a key role in the introgression. Instead, my results suggest behaviour plays a significant role in limiting the introgression, with male-choice controlling the geographic element and

female-choice the one-way element. Using micro-computed tomography and 3D morphometrics I analysed the potential role of genital shape in this introgression. Whilst the genitalia of the two species were quantifiably different, there was little evidence that genital shape limits the introgression.

The results of this thesis show that closely related species can have drastically different mating systems, showing the strong impact sexual selection can have on the behaviour of species. They also highlight the importance of behaviour to promoting and limiting introgression between species. Future work on these species should include investigation of sperm dynamics, the rapid copulation behaviour of *D. minor*, and testing the effectiveness of genital plugging to reduce sperm competition in *D. minor*.

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## Chapter 1: General Introduction



*D. minor* female with egg sac – Helen Macky

## 1.1 Mating Systems

Few topics in evolutionary biology are as seemingly simple, yet as deceptively complex, as mating systems. A mating system is a description of how many mates each sex will have in its lifetime (Kokko, Klug, and Jennions 2014). For example, a monogamous mating system is one where both sexes have only one partner, while a monoandrous and polygynous system is one where females will only mate with one male, but males will mate with multiple females. Kokko et al. (2014) describe mating systems as the “cause and consequence of the sexually selected adaptations”. Species’ mating systems are formed by evolution, especially via sexual selection, but simultaneously sets the rules for future evolution and sexual conflict. This makes mating systems of extreme importance to the understanding of the behaviour and evolution of any sexually reproductive animal.

There are several aspects of spider biology that make their mating systems particularly fascinating and informative for studies on evolution. These are: complex communication through vibration and pheromones; intromittent genitalia, where the males carry sperm in secondary structures not connected to their true genitalia (called pedipalps, these structures are also often paired, meaning males in some species can only mate a maximum of twice and need to allocate their matings accordingly (W. G. Eberhard 2004)); the females’ ability to store sperm; and the potential for a male to be cannibalised (either before or after copulation) by a female (Schneider and Andrade 2011). Sexual cannibalism, in particular, attracts research attention as it is a relatively rare behaviour in animals, places strong selection on males and their courtship; and allows for excellent experiments on female choice under extreme circumstances (Schneider and Andrade 2011). Spiders are also known for their widespread use of mating plugs to limit further female mating after copulation (Uhl, Nessler, and Schneider 2010).

Spiders exhibit variety in their mating systems, with polygyny, polyandry, monogyny and monoandry in various combinations. For example, in *Stegodyphus lineatus* (Eresidae) a highly female-biased sex ratio renders females monoandrous (mating with one male) and males polygynous (mating with multiple females) (Berger-Tal and Lubin 2011). In *Agelenopsis aperta* (Agelenidae), males are limited in their matings by the extreme distances between females, and females are only receptive to one mating, rendering the species

monogamous, that is both monogynous and monoandrous (although in this case the authors noted some individuals would take multiple mates in the wild and were capable of mating multiply under laboratory conditions) (Singer and Riechert 1995). Additionally, given their paired genitalia, *Argiope bruennichi* (Araneidae) males are conditionally bigynous (using one genital with each of two females), depending on the body condition of females (Cory and Schneider 2020; 2018). However, spiders are most often studied and perhaps most infamous for monogyny.

Monogyny describes the reproductive system, whereby males will only mate with a single female in their lifetime. Classical sexual selection theory argues that male gametes (sperm) require little investment, so a male maximises his reproductive (and evolutionary) success by producing as many gametes as possible and mating with as many females as possible, thereby ensuring the maximal spread of his genes (Bateman 1948). Whilst sperm are not as low-cost in some species as previously thought (Godwin et al. 2017), polygyny is still widely seen throughout the animal kingdom. Monogyny is less numerically common, but is still observed in a many varied taxa, including spiders, anglerfish, ants and honeybees ((Michener and Michener 1974; Monnin and Peeters 1998; Peeters 1990; Vollrath 1998; Wilhelm et al. 2011), leading to questions about its evolutionary origin and maintenance (Schneider and Fromhage 2010).

Monogyny in spiders has several independent evolutionary origins (a minimum of five in araneoids alone (Miller 2007)) and is typically associated with a set of life history and behavioural traits: sexual cannibalism, genital damage, male-biased sex ratios, protandry (early maturation of males) and female-biased sexual size dimorphism (Miller 2007; Schneider and Fromhage 2010). Sexual cannibalism and genital damage are thought to be evolutionarily correlated, as is the evolution of male-biased sex ratios, protandry and female-biased sexual size dimorphism (Miller 2007; Schneider and Fromhage 2010). However, all five mating system elements are strongly correlated with the evolution of monogyny (Miller 2007) (Fig. 1.1). This type of interconnected evolutionary relationship is what makes mating systems “cause and consequence of the sexually selected adaptations”, to quote Kokko et al. (2014) once again. Sexual selection creates mating systems like spider monogyny and defines the elements of said system, but simultaneously, sexual selection (and therefore reproductive success) within that species is defined by the mating system.

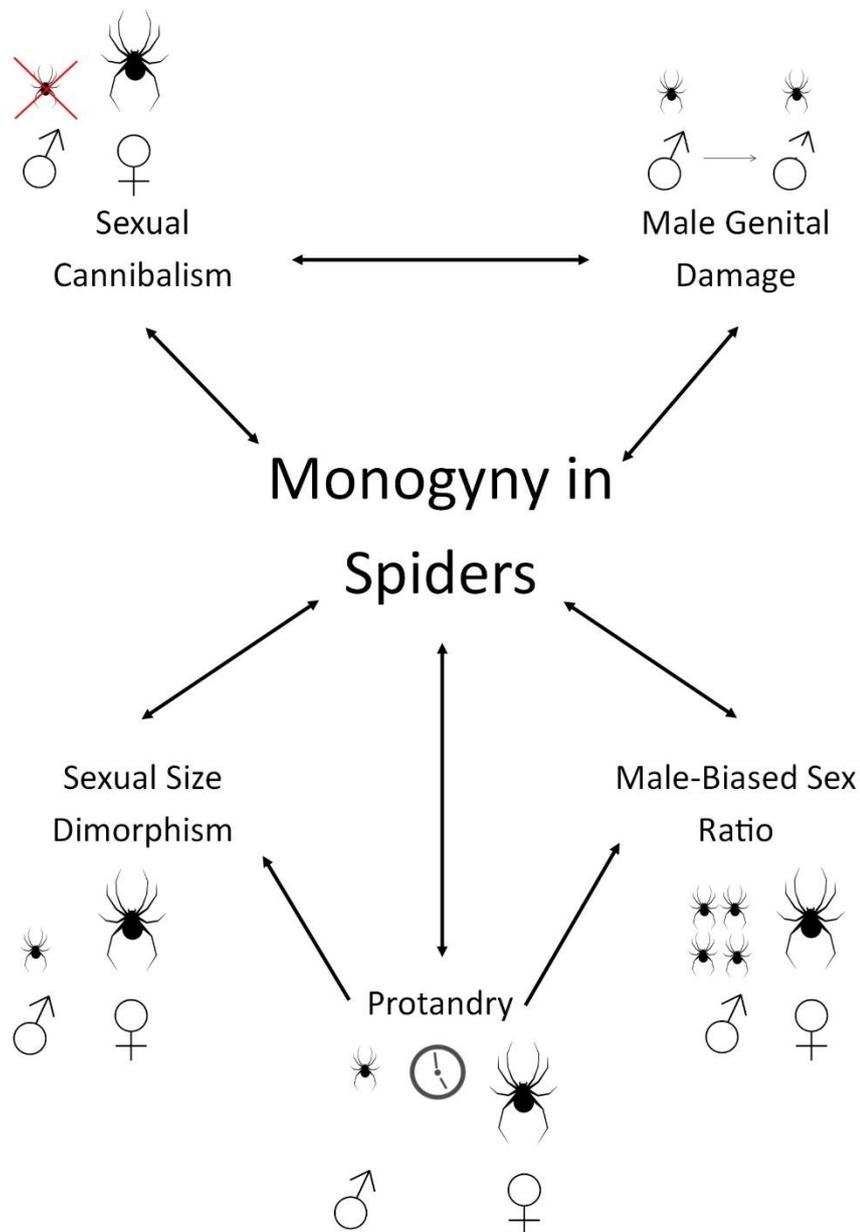


Figure 1.1. Conceptual diagram of mating system elements (each represented by a sketch) and the evolution of monogyny in spiders, based on hypotheses outlined in Miller (2007). Arrows represent hypothesised causal links, with double arrows representing situations where causality could be in either direction.

A highly male-biased sex ratio is commonly associated with monogyny in spiders. Fromhage et al. (2005) used mathematical modelling to explain the evolution of monogyny, with the assumption that mating with fewer females increases the number of offspring a male will

father with that female, relative to other males. Their simulations showed that both monogyny can only be evolutionarily persistent in highly male-biased species. This conclusion is supported by other simulations that state that sex ratio is one of the most important predictors of a mating system (Harts and Kokko 2013). Under a male-biased sex ratio, a male should invest his resources into protecting his sperm investment into one female, rather than allowing the plentiful other males to mate with her (Harts and Kokko 2013). This finding agrees with field data from several monogynous spider taxa, which show males can be up to ten times more abundant than females of the same species (Schneider and Fromhage 2010). There are also field observations of males aggregating on webs of lone females, whilst the female is maturing, in order to secure matings as soon as the female matures, thus demonstrating an adaptive response to male-biased sex ratio (Fromhage, McNamara, and Houston 2008).

The aggregation of males on webs is related to another factor that is often associated with spider monogyny: male search mortality. In most spider taxa, even in non-web building taxa, the females remain immobile and are sought out by wandering males, (Johnson 2005a; Schneider and Fromhage 2010). The high risk of mortality during this search (D.J. Hosken et al. 2009) could incentivise males to prioritise a single female, rather than risk death in the search for subsequent mates. The risk to males searching for multiple mates is only increased when female aggression is considered, as an unsuccessful courtship can lead to death for the male (Johnson 2005b). Indeed, mathematical modelling supports this assertion, showing that monogyny (in this case simulated via self-sacrifice) should evolve if it has a strong effect on female fecundity and a male is at high risk of mortality whilst searching for another mate (Buskirk, Frohlich, and Ross 1984). Empirical research supports these modelling predictions. For example, in white widow spiders (*Latrodectus pallidus* Theridiidae), females often mate multiple times, but only 20% of males may find an opportunity to mate whilst searching (D.J. Hosken et al. 2009). However, this risk would be lessened for non-web building species who are able to hunt during their search for females, a situation which is less well studied (Schneider and Andrade 2011). Whatever the relative risk for each species, the above example of widow spiders demonstrates intense male-male competition, a high-risk search, and a heavily male-biased sex ratio (given that females may mate multiple times despite the high mortality in males). Under such circumstances, a male

should try to maximise his investment through a paternity assurance mechanism (e.g. genital plugging) and sacrificing himself to raise the fecundity of his mate.

Male-biased sex ratios are correlated with often extreme female-biased sexual size dimorphism (Fig. 1.2), whereby males in many species mature earlier (protandry) and are therefore smaller and occur in greater numbers than females (Fig. 1.1) (Miller 2007; Schneider and Fromhage 2010). Early male maturation would be strongly selected for in taxa where there is first male paternity advantage due to mate plugging (Miller 2007; Schneider and Fromhage 2010). This selection pressure would further lead to selection for males to mature at a smaller size, reducing the growth and therefore time necessary to reach maturity (Schneider and Fromhage 2010). Maturing at a smaller size can also reduce male mortality before mating opportunities arise, due to a decreased risk of predation (Gunnarsson 1998; D.J. Hosken et al. 2009; Schneider and Fromhage 2010), and thus potentially decrease male mortality when searching for females. However, female-biased size dimorphism may also be evidence of a positive effect of size on female fecundity (Schneider and Fromhage 2010), and in many taxa it is thought that dimorphism has arisen due to an increase in female size for fecundity benefits, rather than a decrease in male size (Miller 2007). Whatever the cause, with selection pressures for males to be small and females to be large, there is an abundance of evidence for how sexual size dimorphism could evolve in relation to monogynous systems.



Figure 1.2: Sexual size dimorphism in Saint Andrews Cross Spider (*Argiope keyserlingi*). Photographed by Graham Winterflood. Accessed under Creative Commons.

Sexual cannibalism, where a female consumes the male as part of the mating process, is common in spiders and is associated with many unusual adaptations. In some taxa, the males will attempt to resist being cannibalised, either behaviourally or morphologically (Anderson and Hebets 2017; Schneider and Fromhage 2010). For example, *Trichonephila fenestrata* males will deliberately excise their own legs to provide food for and pacify cannibalistic females (Neumann and Schneider 2020). However, in other taxa, the male is voluntarily consumed. For example, in *Latrodectus hasseltii* males somersault into the jaws of the waiting female following copulation (Snow and Andrade 2004). For the females, the male may represent a simple meal, but the evolutionary benefit of being consumed is less apparent for the male.

Evolutionary theory dictates there must be significant benefit to the males in being consumed, which must outweigh (or at least balance) any evolutionary fitness a male would gain from future matings. This benefit could be an investment in the male's future offspring, either through quality of offspring or assurance of paternity. Evidence from *Dolomedes tenebrosus* found that sexual cannibalism increases the size, number and survival of

produced offspring (Schwartz, Wagner, and Hebets 2016). In *L. hasseltii*, males that copulate for longer are able to discharge more sperm, increasing their odds of producing offspring with the female, and allowing themselves to be cannibalised extends this duration even further (D.J. Hosken et al. 2009). Whilst operating through different mechanisms, both of these examples show a strong selection pressure for the male to be cannibalised.

Male genital damage is correlated with monogyny in many spider species (Fig. 1.1) (Miller 2007). Male genital damage comes in many forms including breaking of the embolus (the tip of the male genitalia that inserts into the female) (Foelix 1996; Miller 2007), the genital bulb being unable to deflate after copulation, permanently causing the genital to be unusable (Schwartz, Wagner, and Hebets 2013), or the male excising his entire pedipalp from his body (Li et al. 2012). The evolution of genital damage could be regarded as logical when considered alongside sexual cannibalism: the re-usability of the pedipalps is irrelevant if males are eaten after their first copulation. Equally, the structure of the pedipalp could explain the development of sexual cannibalism: a male should sacrifice himself to provide a meal for a female, as without functioning pedipalps he cannot produce any further offspring regardless. Accordingly, Schneider & Fromhage (2010) demonstrated that whilst these factors are inter-related (Miller 2007), they cannot explain their evolution or the evolution of monogyny alone. However, genital damage in some spider species is also related to sperm competition.

## 1.2 Sperm Competition and Genital Plugging

Male competition for reproductive success often does not end at copulation. Since Parker's (1970) seminal work, sperm competition has been recognised as an extremely important factor in shaping the mating systems of animals. It refers to competition between male's after copulating, to fertilise the limited female gametes (Simmons 2014). The relative importance of sperm competition in a species is decided by that species' mating system, as, if females are monoandrous, then there is no sperm competition, and if females are polyandrous males will typically either engage in or avoid sperm competition (Schneider and Andrade 2011; Simmons 2014). This is another example of mating systems defining how sexual selection will alter a species' evolution.

Sperm competition and cryptic female choice are of particular importance in spiders, given the female's ability to store sperm, potentially even being able to choose between the sperm of multiple males (Schneider and Andrade 2011). This is achieved via paired sperm storage organs, called spermathecae. Mate guarding is a common practice in males of many species to prevent other males from their mate, and therefore avoid sperm competition, and has been noted in multiple spider species (Schneider and Andrade 2011). It has also been speculated that male spiders are able to alter the behaviour and signalling of females during mating, making them less receptive to future males (Estramil and Costa 2007; González and Costa 2008; Norton and Uetz 2005; Perampaladas, Stoltz, and Andrade 2008). However, the most infamous measure to avoid sperm competition in spiders is genital plugging.

Genital plugging is common in entelegyne spiders, which are a large group of spiders, defined by the double-ended female genitalia. This means males can plug a female to prevent future copulations, without risking the female's ability to lay eggs (Uhl, Nessler, and Schneider 2010). Some spiders form their plugs from substances produced during mating, however others use the broken portions of their damaged genitals (Uhl, Nessler, and Schneider 2010), hence why genital damage is linked to preventing sperm competition in spiders. The effectiveness of these plugs is debated, with some females and competing males being able to remove them, but their ability to limit sperm competition has been demonstrated across multiple spider taxa (Masumoto 1993; Sentenská, Pekár, and Uhl 2018).

### 1.3 Species Concepts and Introgression

The notion of species is simultaneously intuitive and highly complex. At the most basic level, a species defines individuals of a type of organism, distinct in some way from other types of organism. A species was recognised by both Darwin and Linnaeus as the basic unit for their work on natural selection and taxonomy, respectively. Mayr (1942) defined a species as a group of individuals who are able to breed with each other, but not with individuals from other groups (Biological Species Concept). Whilst simple, this concept is flawed in that it cannot be applied to asexual organisms and is impractical when applied to organisms separated by geographic barriers, or where the ability of organisms to interbreed is unknown or difficult to test. In the years since, a multitude of species concepts have

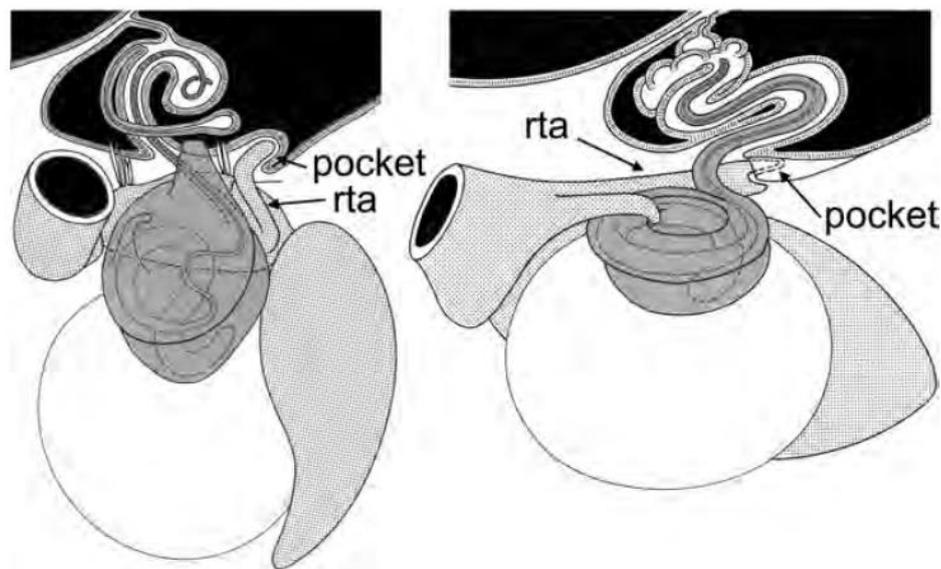
emerged, based on morphology, ecology, phylogenetics and other delimitable traits (Aldhebiani 2018). This culminated in calls for pluralism in species concepts, and that researchers should use whichever concept is most useful for their work (Mishler and Donoghue 1982; Sluys and Hazevoet 1999).

In this pluralist world, biologists are left to deal with the reality of species interbreeding with each other, and introgression is the flow of genes between species (Croucher, Oxford, and Searle 2004; Ohshima and Yoshizawa 2010; Wall 1970). Introgression has wide ranging effects on plants, animals, fungi, and other taxa and is a strongly influential force in evolution, as it can alter species at a much greater rate than natural selection (Chang, Song, and Zhou 2007; Leduc-Robert and Maddison 2018; Wall 1970). In animals, it is also strongly influenced by mating systems and behaviours, as it requires the formation of hybrid specimens via mating between the two species (Anderson 1949; Lattimore et al. 2011; Sweigart and Willis 2003). However, the impact of mating systems and other behaviour in animals is often ignored in studies of introgression (Domènech et al. 2020; Ortiz et al. 2021). As always, the mating systems of the species involved would set the rules for such interactions and determine the evolutionary success or failure of interbreeding species.

## 1.4 Genital Shape

Animal genitalia are highly diverse, despite their seemingly simple function of transferring gametes. The reasons for this diversity have been a source of debate for decades, with various mechanisms of sexual selection being among the most prominent explanations (W. G. Eberhard 2010; W. G. Eberhard and Huber 2010; Masly 2011; Shapiro and Porter 1989). The lock-and-key mechanism is a classic theory, which claims that genitalia are diverse to prevent maladaptive interbreeding between different species (Dufour 1844). Whilst this explanation is controversial, and evidence from different taxa are mixed (Masly 2011; Shapiro and Porter 1989), many researchers still argue it has a strong role in determining genital shape (Alichi 2022; Anderson and Langerhans 2015; Langerhans, Anderson, and Heinen-Kay 2016; Sloan and Simmons 2019). Therefore, genital shape can be highly relevant to investigations of introgression between animal species.

The Retrolateral Tibial Apophysis (RTA) is a structure on male spider genitalia that hooks the male genitals onto the female (Fig. 1.3). This aligns the male genitalia with the female genitals, and the male to insert his delicate embolus into the female reproductive tract, and transfer sperm. It is therefore a structure that is key to reproduction in these species, and its shape is formed by sexual selection, leading to a wide diversity of forms between species (Huber 1995; Langerhans, Anderson, and Heinen-Kay 2016).



*Figure 1.3: Diagram of male RTA hooking into the female genitalia to allow locking of the genitalia and sperm transfer. Pocket refers to the structure on the female genitalia to accommodate the RTA. Figure from (W. G. Eberhard and Huber 2010).*

X-ray computed microtomography (micro-CT scanning) can be used to construct a series of digital-cross sections or a 3D model of the structure, using data from x-rays focused into a subject from multiple angles. This method has several advantages over traditional dissection. The first advantage is that of resolution, with micro-CT scanning able to analyse the complex structure of the male pedipalp, including the RTA (Michalik et al. 2013), a structure key to understanding spider mating behaviour. A second advantage is that those scans can be converted to 3D models for geometric morphometric analysis.

Geometric morphometrics quantifies and compares variation in trait shape across individuals or populations (Adams, Rohlf, and Slice 2004; Mitteroecker and Schaefer 2022).

This allows for objective description of the shape variation in structures and has been used for: demonstrating genital diversity and selection pressure in millipedes (Sloan, Kennington, and Simmons 2023), taxonomic descriptions of spiders (Costa-Schmidt and de Araújo 2010; Michalik et al. 2013) and investigating evolution of amphibian cranial shape (Sherratt et al. 2014), and much more. There are three main kinds of geometric morphometric techniques: landmarking, where landmarks are manually placed by researchers on analogous points on different samples; semi-landmarking, where some landmarks are placed manually and others are placed automatically based on a specific algorithm; and landmark-free techniques, where all landmarks are placed automatically (Mitteroecker and Schaefer 2022). Whilst each technique has its advantages and disadvantages, landmark free techniques are highly useful in cases where smooth or rounded surfaces need to be analysed, or there is difficulty placing homologous landmarks across samples (Mitteroecker and Schaefer 2022). The latter situation is relevant when analysing spider RTAs with variable numbers of teeth, precluding the placement of homologous landmarks.

### *1.5 Dolomedes* fishing spiders

*Dolomedes* (Pisauridae) spiders comprise over 100 species found around the globe (NMBE - World Spider Catalog 2024). Instead of building webs to catch prey, most species are known for waiting on the edge of water and striking out at prey that moves past, hence their common name of fishing spiders. For this reason, they are adapted to quickly sprint across the water, reaching speeds of up to 50 cm/s (Pullar and Paulin 2018). They are opportunistic hunters, usually of small invertebrates, but are also known to predate on fish (Nyffeler and Pusey 2014; Williams 1979) and frogs (Cabrera-Guzmán, Crossland, and Shine 2015; Moore and Townsend 1998). They will take advantage of available prey on land (Williams 1979), and some species have evolved to hunt independently of large bodies of water (Forster and Forster 1973). Their other common name, nurseryweb spiders, comes from the tightly woven silk structure that females of some species create so that their young can be guarded by the mother until they disperse (Forster and Forster 1973; Vink and Dupérré 2010).

*Dolomedes* have been the subject of several studies into mating rates and mating behaviour. In the wild, male courtship is triggered by female draglines (lines of silk that bear pheromones to lead the male to the waiting female) (Arnqvist 1992; Johnson 2005a). This

courtship then involves vibratory, visual, and chemical cues, with the forelegs being waved in the air and tapped against the female's body (Arnqvist 1992). It seems the purpose of this communication is for the male to avoid being rejected by the female, either passively or via pre-copulatory sexual cannibalism. Some studies have noted high aggression from females when courted by males (Arnqvist 1992; Arnqvist and Henriksson 1997; Forster and Forster 1973; Johnson 2001), and field studies have shown that males can make up a significant portion of females' diets in the wild (Greenwood, McIntosh, and Harding 2010; Johnson 2001). Across the genus *Dolomedes*, there is a wide variety of mating rates and behaviours, from monogyny through spontaneous male death in *D. tenebrosus* (Schwartz, Wagner, and Hebets 2013) to polygyny and monoandry in *D. scriptus* (Schoenberg, Hebets, and Sullivan-Beckers 2022).

## 1.6 New Zealand *Dolomedes*

In Aotearoa New Zealand, there are four species of native *Dolomedes* (Fig. 1.4): *D. minor* (which is found throughout the mainland in swamps, scrubland and modified habitats such as parks; and unlike most *Dolomedes* is not obligate to hunt on water (Forster and Forster 1973; Williams 1979)), *D. aquaticus* (which is found on the rocky margins of braided rivers south of Taranaki), *D. dondalei* (which is found throughout the North and South Islands on streams and rivers in native forest), and *D. schauinslandi* (which is found on the Chatham Islands and is classified as At Risk: Relict due to its restricted location on several mammalian-predator free islands) (Vink and Dupérré 2010).



Figure 1.4: Top Left: *D. aquaticus*, sourced from Wikimedia Commons, Top Right: *D. dondalei* from Vink and Dupérré (2010), Bottom left: *D. minor* by Helen Macky, Bottom Right: *D. schauinslandi* from Wikimedia Commons

A relatively small amount of work has been done on these species, mostly focusing on their ecology and links with the riparian environment (Collier, Bury, and Gibbs 2002; Greenwood, McIntosh, and Harding 2010; Greenwood and McIntosh 2008; 2010; 2011), as well as analysis of their locomotion (Pullar and Paulin 2018) and feeding behaviour (Williams 1979). The mating behaviours of *D. minor* and *D. aquaticus* have each received some anecdotal investigation. Forster & Forster (1973), as part of their much wider study of New Zealand spiders, noted extreme levels of aggression from *D. aquaticus* females to courting males, with only one male in the study observed successfully copulating with a female. The Forsters assumed that this cannot be representative of the species' behaviour at all times, as this level of aggression would be maladaptive, and speculated that the females give priority to the first male to mate with them and are aggressive thereafter. For *D. minor*, Vink & Dupérré

(2010) found evidence of genital damage in preserved specimens, which they assumed to be accidental on behalf of the male. Specifically, broken emboli were found in the epigynal folds (outer genitalia) of females. No observations of courtship behaviour or copulation have been undertaken for *D. minor* to date, and it is therefore unknown how the mating systems of the New Zealand species compare to the more well-known species in other parts of the world. All New Zealand *Dolomedes* are known to be sexually dimorphic, with large females relative to the males (though they do not show extreme sexual size dimorphism shown in many monogynous spider species) (Vink and Dupérré 2010), but the presence or absence of other mating system traits and behaviours has yet to be investigated. This investigation is the goal of Chapter Two.

A taxonomic and genetic study of New Zealand *Dolomedes* found *D. aquaticus* and *D. minor* to be sister species, with introgression occurring between the two (Vink and Dupérré 2010). It is a one-way introgression between *D. aquaticus* and *D. minor*, with *D. minor* males mating with *D. aquaticus* females to produce hybrids, evidenced by *D. aquaticus* mitochondrial DNA being found in specimens of *D. minor*, but no such transfer taking place from *D. minor* to *D. aquaticus* (Vink and Dupérré 2010). As well as being one-way, the introgression is also geographically restricted to parts of the lower South Island (hereafter referred to as the Introgression Zone), despite the two species co-occurring throughout the range of *D. aquaticus* (Lattimore et al. 2011).

Investigation of *D. minor* and *D. aquaticus* introgression has been limited to describing the genetic transfer. However, Vink & Dupérré (2010) and Lattimore et al. (2011) provide several hypotheses as to why introgression is only one-way and geographically restricted. The first (habitat overlap hypothesis) is that flooding within the introgression zone causes habitat overlap between the two species, which is not the case outside of the introgression zone. This could force *D. aquaticus* further from their river habitats into the habitats of *D. minor*. Another potential hypothesis (reproductive timing hypothesis) considers the timing of reproductive maturity, which may significantly differ between the species and sexes and across their range, if their mating systems have diverged between monogyny and polygyny, limiting the direction of introgression (Johannesen and Veith 2001).

I also propose hypotheses in relation to divergences between the species' mating behaviour. A third hypothesis (male courtship hypothesis) is that variation in courtship initiation between males of the two species and regions limits introgression. For example, some males are willing and able to court females, but others are not. This could be due to differences in signalling or other behaviour between species. Female choice differences between the two species could also limit the introgression to one-way, or there may be a behavioural cline (a difference in the species along its range) explaining the geography of the introgression zone. These females would not be receptive to male courtship (female receptiveness hypothesis). The above hypotheses are tested in Chapter 3.

A final hypothesis (genital morphology hypothesis) is that differences in genital morphology of the species limit the geographical range of the introgression and restrict it to one-way. Variation in the Retrolateral Tibial Apophysis (RTA) of *D. minor* males could explain the geographical restrictions. The RTA of *D. minor* can vary, with specimens from Southland being anecdotally closer to that of *D. aquaticus* (Vink and Dupérré 2010), but it is unclear if this variation is actually consistent with geography. This hypothesis is tested in Chapter 4.

## 1.7 Thesis Outline

This thesis uses a combined approach including laboratory experiments, field observations, micro-CT, and geometric morphometrics to examine the mating systems and introgression in *D. minor* and *D. aquaticus*, thereby providing new information on these species, as well as expanding our insight into these evolutionary forces. Chapter Two describes the mating rates of these two species for the first time and use them as a case study to examine the evolution of genital plugging via damage and related mating system traits, such as protandry and sexual cannibalism in spiders. Chapters Three and Four examine the unique introgression between *D. minor* and *D. aquaticus* and attempt to explain its one-way and geographic limitations. Chapter Three uses field observations and behavioural experiments to systematically examine each step that could limit the introgression, from the likelihood of the two species meeting in the wild to their ability to court and attempt copulation. This chapter is under review at the *Journal of Evolutionary Biology* following resubmission. Chapter Four uses micro-CT and 3D geometric morphometrics to examine the variability of

the male genital structure in the two species both within and outside of the introgression zone to test if this is a barrier to one or more aspects of the introgression.

## Chapter 2: Mating rate predicts evolution of genital mutilation and sexual cannibalism in New Zealand fishing spiders



*D. minor* female on nurseryweb – Helen Macky

## 2.1 Abstract

The mating rate of a species defines the parameters for success in sexual selection. Polyandry, the system where females mate with multiple males, can lead to sexual conflict as males seek to maximise their own paternity by preventing female remating. One measure found in many species is genital plugging, where males block the female genitals after mating to prevent future males from mating with her. This is a common tactic in entelegyne spiders and is associated with male genital damage, sexual cannibalism, and low male mating rates. We investigated and compared mating rates and associated mating system elements in two New Zealand *Dolomedes* fishing spiders: *D. aquaticus* and *D. minor*. Despite being sister species, these two spiders have drastically different mating systems. Our laboratory experiments found *D. aquaticus* males to mate multiply (polygynous), while females mostly mated once (monoandrous). In contrast, *D. minor* males and females typically had low mating rates, though the possibility of multiple mating exists for both sexes. Consequently, *D. minor* showed genital damage, specialized escape behaviour for males to avoid sexual cannibalism and extreme choosiness in mates, potentially by both sexes. *Dolomedes aquaticus* however showed no such patterns, as males are assured that females are unlikely to remate with subsequent males, removing any post-mating competition. These results are evidence of the strong effect of mating rates and sexual selection on the evolution of species' behaviour.

## 2.2 Introduction

Mating rates of species are highly influential on sexual selection and evolution, as they define the structures and behaviour necessary for reproductive success. Polyandry, where females mate with multiple males, can be beneficial to a female's total reproductive output (Pizzari and Wedell 2013; Simmons 2014; Taylor, Price, and Wedell 2014). Simultaneously, however, females having multiple male partners can lead to intense post-mating competition among males to fertilise a female's gametes via sperm competition engagement or tactics to avoid sperm competition (Firman and Simmons 2008; Pardo et al. 2016; Parker 1970; Simmons 2005). Avoidance measures can constitute sexual conflict, as males attempt to control females' mating opportunities, when females would otherwise benefit from mating with multiple partners (Elias, Andrade, and Kasumovic 2011; Perampaladas, Stoltz, and

Andrade 2008; Schneider 2014). Strategies males can use to influence their partner's mating behaviour include: mate guarding, where males guard females from subsequent males (Leivers, Rhodes, and Simmons 2014); altering female behaviour to avoid future matings (Civetta, Rosing, and Fisher 2008); and genital plugging.

Genital plugging is blocking of the female genitalia, usually done by males to prevent female remating (Uhl, Nessler, and Schneider 2010), though can be performed by the female to signal unavailability to males (Kuntner et al. 2016). Genital plugs are often made from specialised structures or produced substances, but one form of genital plugging is genital damage, where a male breaks off a piece of his own genitalia to leave the females reproductive tract (Herberstein et al. 2012; Smith 2020; Uhl, Nessler, and Schneider 2010).

Male genital damage and copulatory plugging of females has been investigated in many diverse taxa, such as butterflies (Klein and De Araújo 2010), stingless bees (Smith 2020), and nematodes (Timmermeyer et al. 2010). Of particular focus are the entelegyne spiders, known for the double-ended layout of the female genital tract. This layout allows for males to plug females without impeding the females' ability to lay eggs (Uhl, Nessler, and Schneider 2010). Many spiders also have paired genitalia, with the males having two sets of genitalia, and the females having paired genital openings. This adds an extra consideration to the sexual conflict, as a male breaking his genitalia to plug a female would need to copulate twice to effectively plug the female (Uhl, Nessler, and Schneider 2010). Female spiders can also store sperm, making the structure of the female's spermathecae (sperm storage organ) an important contributing factor to mating system evolution (W. G. Eberhard, Guzmàn-Gòmez, and Catley 1993). Whether the spermatheca favour first or last male sperm precedence (Austad 1982; W. G. Eberhard, Guzmàn-Gòmez, and Catley 1993; West and Toft 1999) and whether a male or female benefits from filling both spermathecae during mating (Cory and Schneider 2020) are important evolutionary factors altering the adaptive significance of genital damage and plugging. These factors make spiders a compelling group for the study of sexual conflict.

Genital damage in spiders is often linked with low male mating rates, including monogyny – a scenario in which a male acquires only one mate throughout its life (Schneider and Fromhage 2010). Furthermore, genital damage tends to be associated with several other

traits such as sexual cannibalism, male-biased sex ratios, protandry (early maturation of males) and female-biased sexual size dimorphism (Miller 2007; Schneider and Fromhage 2010). In particular, there is a strong phylogenetic link between genital damage and sexual cannibalism (Miller 2007), with cannibalism either appearing as a female counter measure to being plugged by the male or facilitated by the male to maximise his investment in his mate (Uhl, Nessler, and Schneider 2010). The behaviour of females post-mating is also a key factor in the evolution of genital damage. If a female spider will remate after her first mate, then this can drive the evolution of genital plugging and genital damage (Neumann and Schneider 2011; Uhl et al. 2014), or other strategies such as mate guarding (Kralj-Fišer et al. 2011). However, if females are unlikely to remate, then a male is free to place his investment into securing more mates and has no need to compromise his genitalia (Norton and Uetz 2005; West and Toft 1999). Given their paired copulatory organs, spiders can also have bigynous mating, where males expend one set of genitalia with one female and the second with another, often because aggressive female enforcement prevents males from copulating and plugging a female's second genital opening (Uhl, Nessler, and Schneider 2010). However, bigynous systems are often only discussed as an intermediate form in relation to monogynous systems (Cory and Schneider 2020; Schneider and Fromhage 2010). Whilst the diversity of spider mating offers many opportunities for comparison, the precise causal relationships in the evolution of genital damage and related factors such as sexual cannibalism are unknown (Schneider and Fromhage 2010).

In spiders, studies investigating mating rates and the adaptive significance of post-mating adaptations, such as genital mutilation, have almost exclusively focused on web-building species, especially those in the Therididae, Nephilidae and Arenidae families (Andrade 2019) (although see Izquierdo and Rubio (2011) for a description of genital damage in the litter-dwelling spider *Unicorn catleyi*, Oonopidae). Much less focus has been placed on non-web building hunters. One group that fits this gap and has received some attention are the *Dolomedes* fishing spiders.

*Dolomedes* spiders across the world have diverse male and female mating rates and behaviour. Some studies have noted high aggression from females, leading to sexual cannibalism of mating partners (Arnqvist 1992; Arnqvist and Henriksson 1997; Johnson 2001; Kralj-Fišer et al. 2016; Schwartz, Wagner, and Hebets 2016). Sexual cannibalism in

*Dolomedes* has been studied in the context of behavioral syndromes (Johnson and Sih 2005; 2007), but more recent research points to an important role of the size difference of the mating pair (Kralj-Fišer et al. 2016; Schoenberg, Hebets, and Sullivan-Beckers 2022, Šramel et al. 2024). Furthermore, in North American *D. triton* and *D. tenebrosus*, cannibalism of males has been shown to have a positive impact on the fecundity of the mating pair (Johnson and Sih 2005; Schwartz, Wagner, and Hebets 2016). In *D. tenebrosus*, the fecundity benefit of being eaten has led to an extreme mating system where males mutilate their genitals and spontaneously die during their first copulation (Schwartz, Wagner, and Hebets 2013). In this mode of genital mutilation, the male palpal bulb does not deflate after mating; essentially the genitalia are unable to 'reset' for a second mating (Schwartz, Wagner, and Hebets 2013). The obligate male death makes this species monogynous. *D. tenebrosus* also shows extreme female-biased sexual size dimorphism and a male-biased sex ratio (Schwartz, Wagner, and Hebets 2013), key evolutionary factors of monogynous mating systems (Schneider and Fromhage 2010). Whilst it was speculated that the male's death in *D. tenebrosus* allows him to serve as a mating plug (with his whole body being lodged inside the female), the evidence instead suggests that spontaneous death functions to facilitate cannibalism, as females can go on to mate with subsequent males, unimpeded by previous matings (Schwartz, Wagner, and Hebets 2014; 2016). Conversely, another North American *Dolomedes*, *D. scriptus*, shows a system of polygynous males and monoandrous females (Schoenberg, Hebets, and Sullivan-Beckers 2022). *Dolomedes scriptus* females also perform sexual cannibalism, however, it is thought that this is a mechanism of mate rejection by females as pre-copulatory sexual cannibalism is more likely than post-copulatory sexual cannibalism (Schoenberg, Hebets, and Sullivan-Beckers 2022). In yet another species, *D. triton*, female aggression and cannibalism led to females only accepting one copulation per male, producing a potentially bigynous system (Johnson 2001). *Dolomedes triton* also show a male-biased sex ratio in the wild (Zimmermann and Spence 1992). Given the variety of mating rates and behaviours in *Dolomedes*, they seem an excellent group to test for coevolution of different mating system elements, including sexual cannibalism and low male mating rates.

New Zealand *Dolomedes* have received little study on their mating behaviours, though *D. aquaticus* have been noted for high levels of female aggression and cannibalism (Forster and

Forster 1973; Greenwood, McIntosh, and Harding 2010). Genital damage has been observed in *D. minor*, through examination of museum specimens, with females having broken tips of male emboli around the outer folds of their genitalia (Vink and Dupérré 2010). However, it is not known to what extent genital breakage in this species occurs during mating. *Dolomedes aquaticus* and *D. minor* are sister species endemic to New Zealand, capable of interbreeding in a small area of their range (Lattimore et al. 2011; Vink and Dupérré 2010). In this study, we used field observations and laboratory experiments to compare the mating rates, associated behaviours, courtship dynamics and population dynamics of *D. aquaticus* and *D. minor* to elucidate their mating system and mating dynamics.

## 2.3 Methods

### 2.3.1 Spider Collection & Rearing

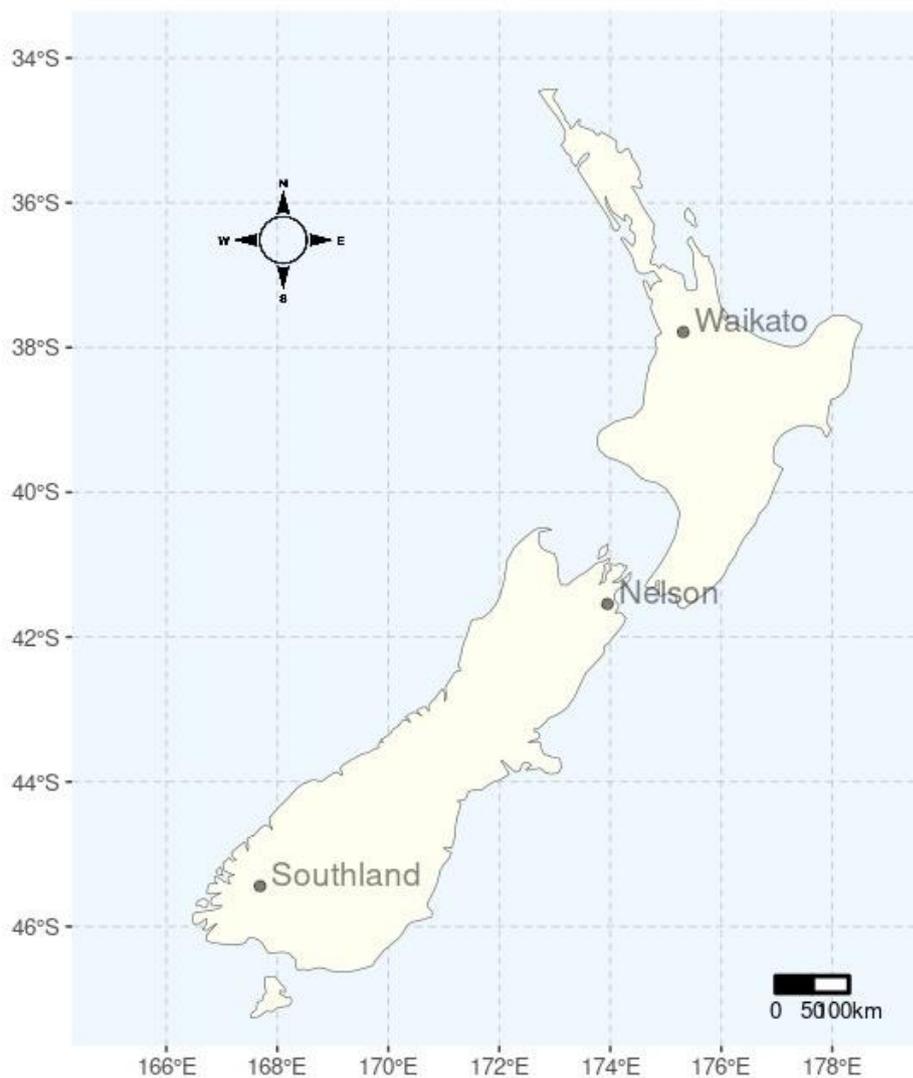


Figure 2.1. Map of New Zealand showing collection locations of spiders for mating trials. Spiders were collected from Waikato (*D. minor*), Nelson (*D. aquaticus*) and Southland (*D. aquaticus*).

We collected spiders in New Zealand in 2020 and 2021. *D. aquaticus* were mainly collected in the Nelson region of the South Island, with a small subgroup being collected in Southland (South Island), which is inside the Introgression Zone (see Chapter 3). *D. aquaticus* were collected from stony riverbanks. *D. minor* were collected in the Waikato (North Island), from shrubs in parkland (Fig. 2.1; See Supplementary Table 1 for a full list of collection locations). Spiders were hand-collected at night, using eyeshine as outlined by Benson and Suter (2013). All spiders were juveniles or sub-adults when collected to ensure that they were unmated. Collection was permitted by local councils (Waikato Regional Council, Marlborough District Council, Southland District Council).

Spiders collected in the field were returned to the Invertebrate Behavioural Research Facility at the University of Waikato, where they were raised to adulthood in individual containers (150 mm x 95 mm x 60 mm). Each container had mesh holes to allow air flow, as well as a corked hole for deposition of food. Spiders had access to water ad libitum, via a dental cotton bud in the bottom of the container that led to a vial of tap water, and shelter within their containers with a piece of egg carton. We cleaned the containers as required by transferring the spiders to fresh containers, and hand washing the used containers. Spiders were fed at least twice weekly, with a mix of live field crickets (bred in the lab and sourced from iNZect Direct New Zealand), and woodlice, earwigs and earthworms that were caught locally. Food detritus, dead spiders and spider moults were removed from the containers daily, with deaths and we recorded moults to monitor the status of the lab population.

We also measured the spiders' cephalothorax width to the nearest 0.01 mm, as a proxy for size, using Fuller digital callipers.

### 2.3.2 Laboratory Experiments

On reaching adulthood, spiders were used in experiments in the laboratory as soon as was practical. Trials for *D. minor* took place from March 2021 – August 2021 and trials for *D. aquaticus* took place from October 2021 – December 2021. Spiders from different regions of the country were not intermixed during experimentation.

To describe mating rates for both sexes, we structured trials to provide opportunities for males and females to remate with new, unmated spiders after their initial matings. After a pair mated, and assuming both individuals survived the experiment, the male and female were placed in at least two subsequent experiments with unmated spiders of the opposite sex. Each of these spiders (herein 'Focal Spiders') had the opportunity to mate with at least three unmated new partners, again assuming they survived each subsequent pairing (Fig. 2.2). However, in *D. minor* we increased the number of retrials to account for a low willingness of individuals to mate during our trials. No specific number was set for these retrials, and instead it was based on performing the maximum practical number with the spiders available in the lab population.

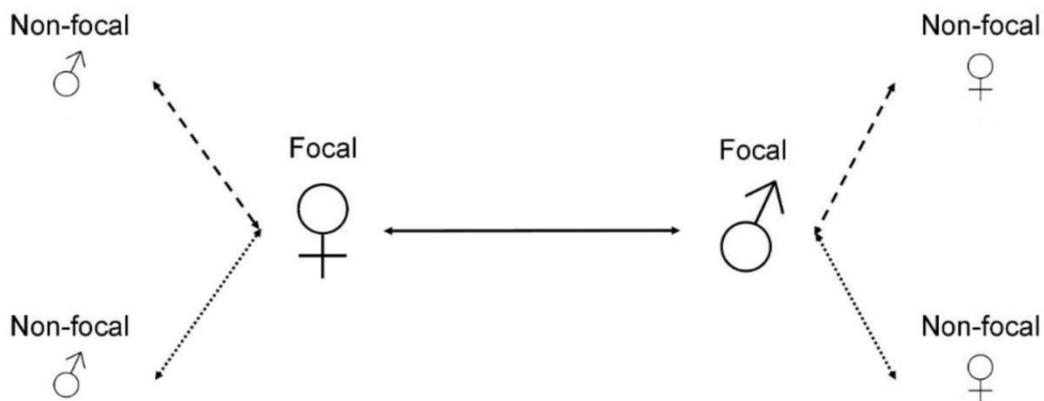


Figure 2.2 Experimental design of mating trials. Solid arrows represent first matings, dashed arrows represent second, and dotted arrows third.

Our aim was for each spider to be used only once, however, uneven population sizes and difficulties in rearing made this impractical. Hence, some spiders were used more than once to increase replicates. However, spiders were only reused as non-focal individuals (i.e spiders used in focal trials were always previously unmated), and every spider was given a new

partner for each experiment and given rest days to recover between trials (minimum time between trials was 48 hours).

We fed females the night before an experiment to control for hunger and introduced her to the arena to be left overnight to release pheromones and webbing in the arena. A small number of trials were performed with females only being left in the arena for at least ten minutes before the males were introduced. However, this was altered early on in an attempt to improve the perceived low rates of mating in *D. minor*. Arenas were 265 mm x 235 mm x 120 mm plastic containers, with air holes like the rearing containers. The arenas featured a climbable mesh wall and a mesh ceiling. Females were released onto the container floor but were then free to move throughout the arena. Males were added to the arena far from the female to avoid the female prematurely killing the male. Upon the introduction of the male, spiders were video recorded using Sony FDRAX53 4K digital cameras. Trials lasted for four hours, unless cut short by female aggression or cannibalisation of male. For pre-copulatory aggression, males would be removed from the arena if it was clear the female would not accept attempts at courtship and there was obvious risk to the life of the male. Trials were occasionally extended if a long courtship or mount was still underway at the four-hour mark.

Behaviours were coded using BORIS software (Friard and Gamba 2016). For each trial I recorded: latency for males to begin courtship, latency for males to mount females, latency for pairs to copulate for the first time, whether pairs mated, number of insertions per mating (maximum of two, one per pedipalp), copulation duration (measured as contact between the genitalia), whether a male was killed pre or post copulation, number of female attacks on the male, and number of pseudocopulations/flubs (any instance of the male reaching with his palps for the female epigyne but not making an insertion).

Pedipalp insertion (copulation) was determined by the male embolus being broken after contact with the female epigyne; or extended contact between the male pedipalp and the female epigyne and other behavioural cues (such as the male raising his setae indicating sperm transfer). Male genitalia were checked under a dissecting microscope for signs of damage after any experiment with males either attempting to copulate or pseudocopulating with the female.

### 2.3.3 Field Observations

To measure temporal variation in sex ratio and the proportion of juvenile to adults in the wild, we undertook regular field observations of *D. minor*. We chose Hammond Park (a public park with a mix of vegetation types) in Waikato to observe spiders from October 2020 to March 2023 (with a gap in the winter of 2021 due to COVID19 pandemic lockdowns and park closures). These were done by walking the same transect approximately every fortnight (the specific day of the fortnight was changed to coincide with fine weather when reasonable) for two hours, and recording all spiders encountered for their sex and maturity. *Dolomedes aquaticus* was unfortunately unable to be studied in a similar long-term fashion, due to the species not being found in the Waikato region. However, we did undertake a similar survey with a smaller timeframe and sample size in Chapter Three.

### 2.3.4 Statistical Analysis

We used binomial general linear models (GLMs) to compare the likelihood of focal spiders mating when unmated, after one mate, after two mates and so on. A similar analysis was performed based on the number of pedipalp insertions observed, to test whether any differences in probability to mate were the result of the number of mates a spider had, or the number of genitalia/genital openings used. Two-sided binomial tests were used to determine if each species was more likely to use one or both pedipalps during a mating. Differences in duration of copulation between the two species were tested with a t-test. Effects of male and female cephalothorax width on the probability of mating were also tested in previously unmated pairs, using t-tests.

When spiders copulated, the difference in likelihood of post-copulatory cannibalism between species was calculated using a binomial GLM. Two separate analyses were performed to test for differences in female aggression for mated and unmated females, comparing between the two species. Each analysis involved a binomial general linear model to test the difference in the probability of a female attacking between the two species, and an ANOVA testing differences between the two species in the number of attacks per hour (standardised to account for variable trial length).

Differences in latency to court, mount and copulate were tested between the two species using t-tests. In unmated pairs, the difference in probability between the two species of male's initiating courtship was tested with binomial GLMs, and the difference of the mean duration of courtship events was tested with ANOVA.

To account for different mating rates between the two species, the number of pseudocopulations was compared to the number of pedipalp insertions within each species, rather than directly comparing the number of pseudocopulations between species. This was done via t-test.

Two-sided binomial tests were used to test the male:female ratio for each night of spider field observations. All statistical analyses were performed in R 4.2.2 (R Core Team 2022).

## 2.4 Results

Mating was common between unmated *D. aquaticus* pairs, with 15 trials out of 20 resulting in successful mating (75%). Precopulatory sexual cannibalism was rare between unmated *D. aquaticus* with only one trial resulting in cannibalism, and another being cut short to avoid the loss of the male after female attack (10% of trials total). Males were likely to remate in a subsequent trial (18/24 trials, 75%), while females remated less frequently (5/28 trials, 18%).

*Dolomedes minor* showed a low likelihood of mating in first encounters between unmated individuals (17/87 trials, 20%). Precopulatory sexual cannibalism was rare between unmated individuals with four trials out of 87 resulting in pre-copulatory kills and a further four ended due to female aggression to the male (total 10%). I found that a few individuals of each sex remated: males (4/39, 10%), and females (8/50 16%).

For unmated *D. aquaticus*, there was a significant effect of male cephalothorax width on the chance of mating, where large males were more likely to mate than small males ( $t = 2.25$ ,  $df = 14.4$ ,  $p\text{-value} = 0.04$ ) (Fig. 2.3). There was no effect of size for *D. aquaticus* females ( $t = -1.42$ ,  $df = 8.2$ ,  $p\text{-value} = 0.191$ ), *D. minor* males ( $t = 0.375$ ,  $df = 19.1$ ,  $p\text{-value} = 0.711$ ) or *D. minor* females ( $t = -0.196$ ,  $df = 19.7$ ,  $p\text{-value} = 0.846$ ).

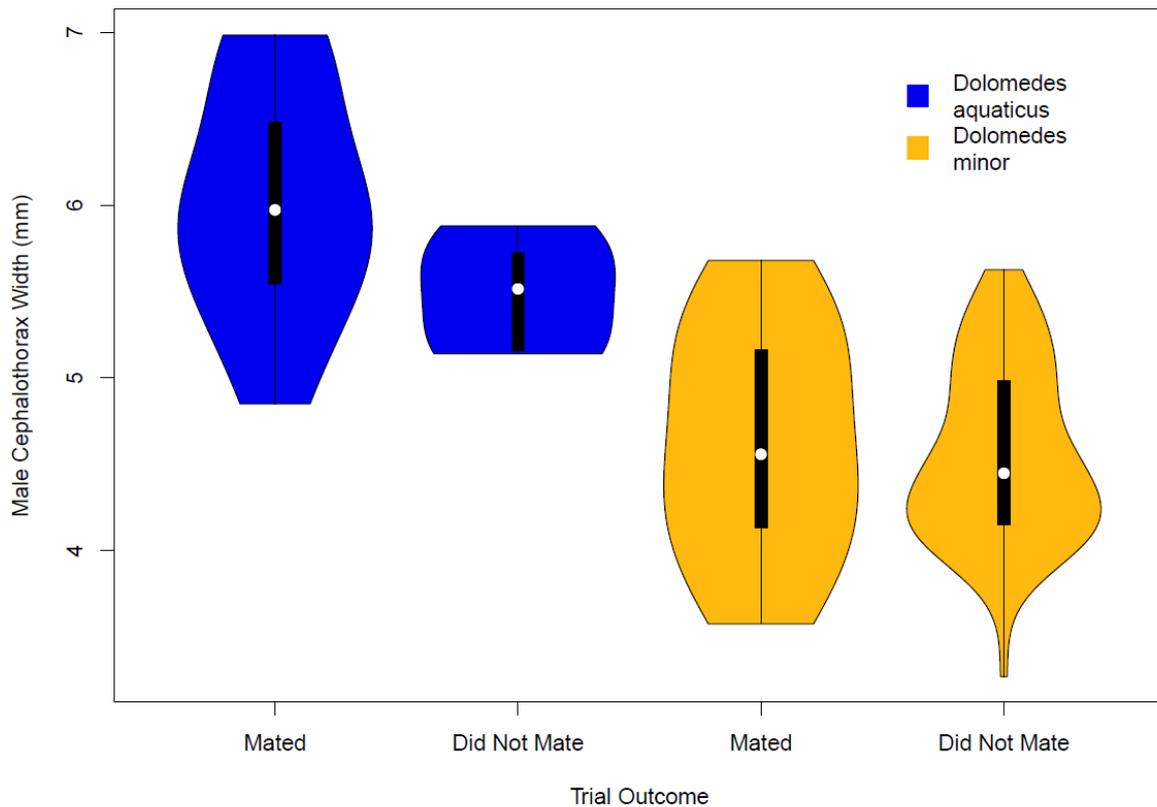


Figure 2.3: The relationship between body size and mating success in *Dolomedes* spiders. Larger *D. aquaticus* males are more likely to mate than smaller ones, when courting unmated females. *D. minor* shows no difference. Boxes show interquartile range, with the central point indicating the median. The curved lines represent the full spread of the data.

### 2.4.1 Remating Rates

*Dolomedes aquaticus* males showed no significant differences in their probability of copulating, regardless of mating status ( $z = 0$ ,  $p = 1$ ,  $df = 43$ ). In other words, male *D. aquaticus* were just as likely to copulate whether or not they had mated before (Fig. 2.4). *Dolomedes aquaticus* females, however, showed significant differences after prior matings. Females with one ( $z = 3.521$ ,  $p < 0.001$ ,  $df = 47$ ) and two ( $z = 2.081$ ,  $p = 0.044$ ,  $df = 47$ ) prior matings were significantly less likely to mate than unmated females were (Fig. 2.4).

No *D. minor* males mated more than twice, however, low overall probability of mating made statistical comparison difficult for this species (Fig. 2.4). We found no significant change in the probability of mating of *Dolomedes minor* males after a mating ( $z = 0.946$ ,  $p = 0.344$ ,  $df =$

125). *Dolomedes minor* females can mate up to three times (Fig. 2.4). Unmated females showed no significant difference in the probability of mating, compared to females who had mated once ( $z = 0.273$ ,  $p = 0.785$ ,  $df = 130.19$ ), twice ( $z = 0.607$ ,  $p = 0.544$ ,  $df = 130.19$ ), or thrice ( $z = 0.010$ ,  $p = 0.992$ ,  $df = 130.19$ ).

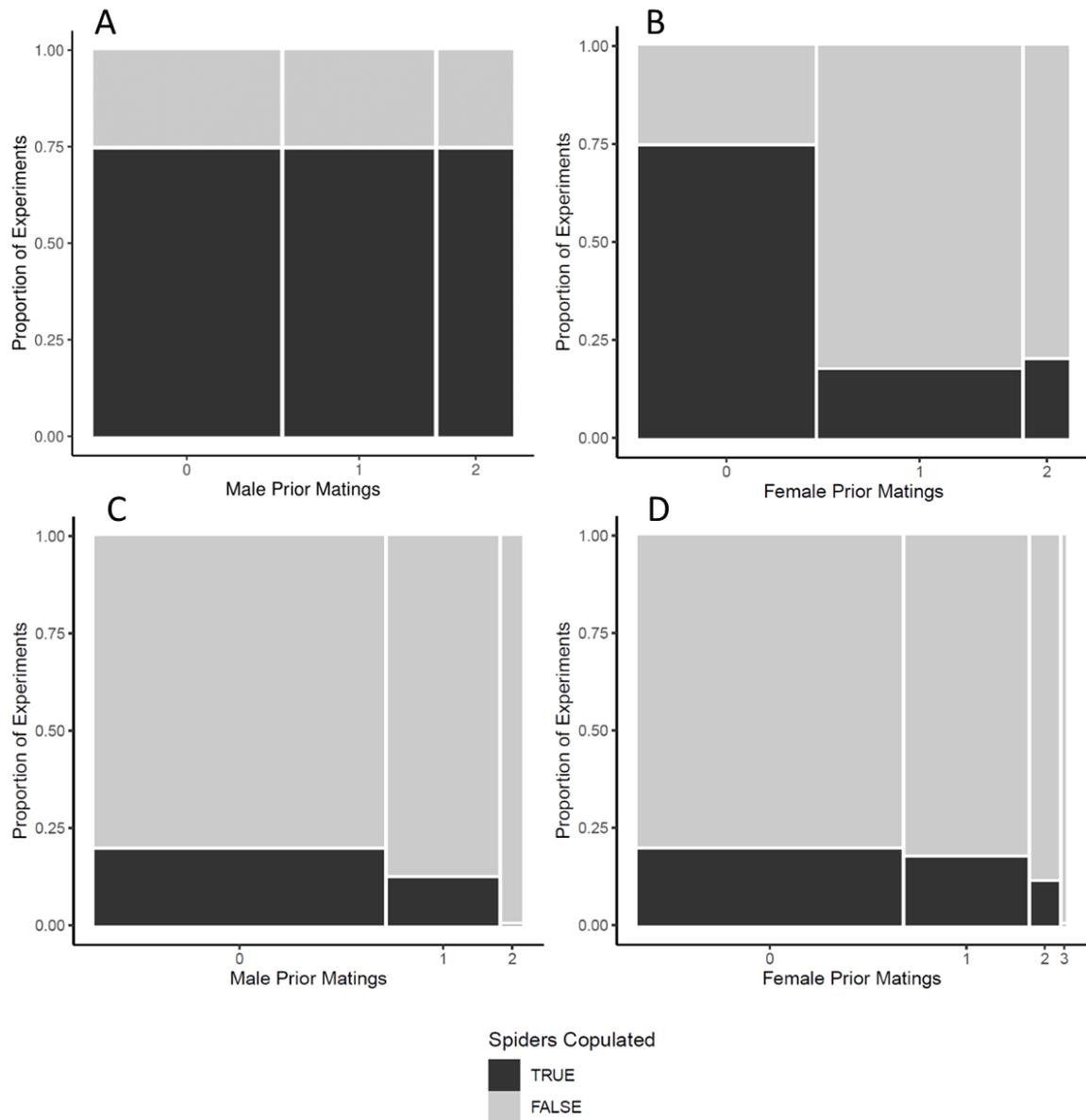


Figure 2.4: Probability of mating in *Dolomedes* species and sexes in relation to previous mating history. A: *Dolomedes aquaticus* males had high rates of mating success with virgin females, regardless of whether males have mated previously, IE a male's mating success was independent of his mating history. B: *Dolomedes aquaticus* females had high rates of mating success with virgin males when the females are also unmated. Following this first mating, there was a significant reduction in the likelihood of female re-mating, meaning a female's likelihood of mating was dependent on her mating history. C: *Dolomedes minor* males had low rates of mating success with unmated females. D: *Dolomedes minor* females had low rates of mating success with unmated males. 'True' represents experiments where spiders mated, and 'False' represents experiments where they did not successfully mate. Prior matings indicates how many times the male or female had mated in

its life before the beginning of the given experiment. The width of the bars indicates the relative sample size for each category.

#### 2.4.2 Number of Pedipalp Insertions

*Dolomedes aquaticus* males were more likely to use both pedipalps (28 matings, 74% of all matings) when mating with a female, rather than one (10 matings, 26%) (p-value = 0.005) (Fig. 2.5). *Dolomedes aquaticus* females were therefore likely to receive two insertions with their first mate, and not mate with other future males (though this was still a possibility) (Fig. 2.5). Maximum lifetime insertions observed for *D. aquaticus* males and females was six.

In contrast to *D. aquaticus*, *D. minor* males were more likely to use a single pedipalp (23 matings, 79% matings) when mating with a female, rather than both (6 matings, 21%) (p-value = 0.002) (Fig. 2.5). Maximum lifetime insertions observed for a *D. minor* males and females was four, and this was from the only male observed copulating with already damaged genitals.

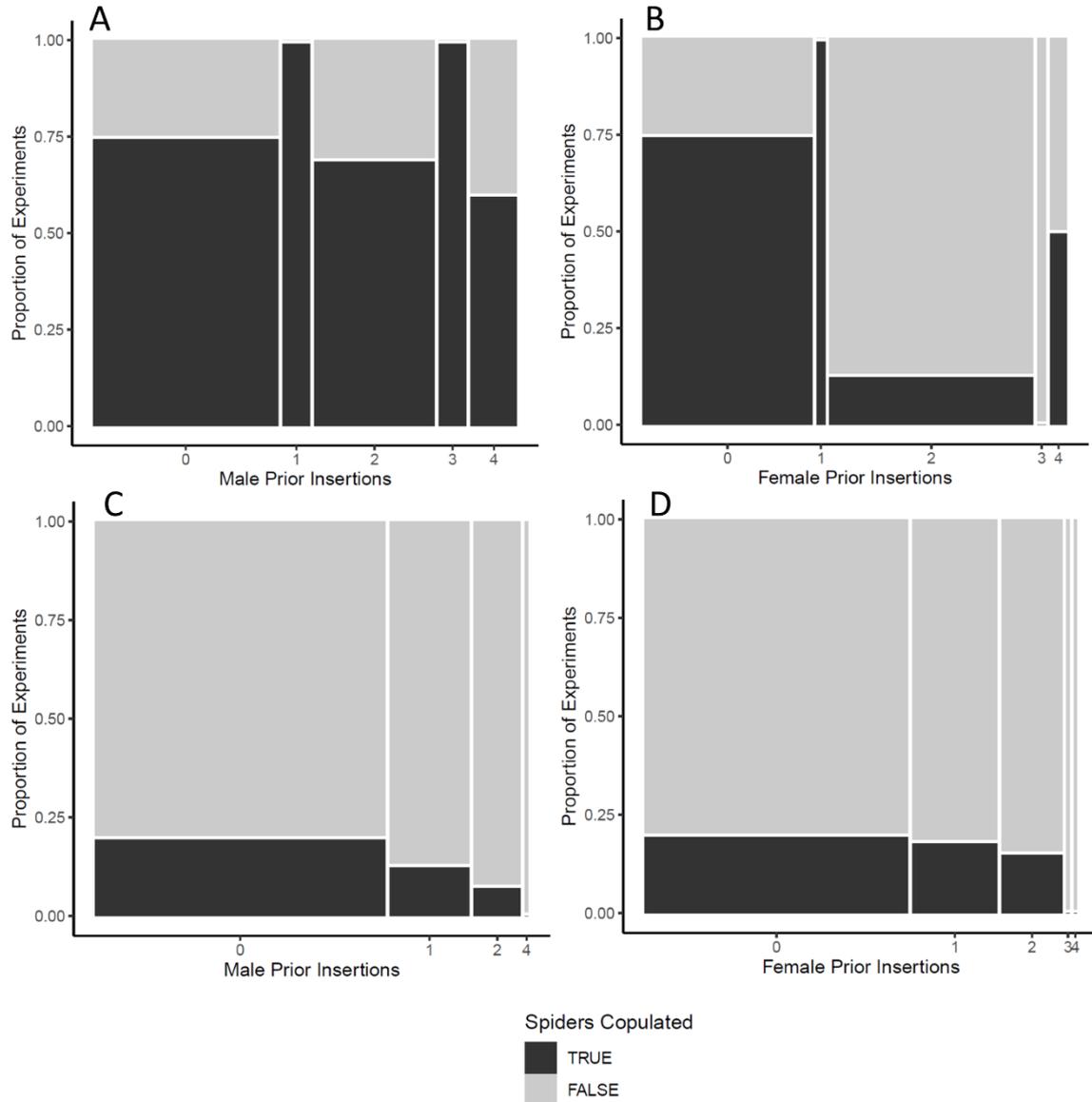


Figure 2.5: Probability of mating in *Dolomedes* species and sexes in relation to previous pedipalp insertion history. A: *Dolomedes aquaticus* males were most likely to use both pedipalps with a single female, leading to very few males who have odd numbers of insertions. B: *Dolomedes aquaticus* females were most likely to receive two insertions from a male using both pedipalps, leading to very few females who had odd numbers of copulations. Following these insertions, females were unlikely to mate again. C: *Dolomedes minor* males were most likely to use one pedipalp with a female. D: *Dolomedes minor* females were most likely to receive one insertions from a male. 'True' represents experiments where spiders copulated, and 'False' represents experiments where they did not successfully copulate. Prior insertions indicates how many times the male or female had used its genitals in its life before the beginning of the given experiment. The width of the bars indicates the relative sample size for each category.

### 2.4.3 Copulation Duration and Genital Damage

*Dolomedes aquaticus* copulated on average for significantly longer than *D. minor* ( $t = 8.81$ ,  $df = 98.67$ ,  $p < 0.001$ ), with most copulations in *D. minor* taking less than a second (Fig. 2.6).

Genital damage was observed in both species, with the tip of the embolus breaking just before the conductor (Fig. 2.7). However, of the 66 *D. aquaticus* insertions we observed, only one resulted in genital damage. We observed 35 *D. minor* insertions, of which 19 resulted in genital damage. Two insertions were performed by a male whose genitalia were already damaged.

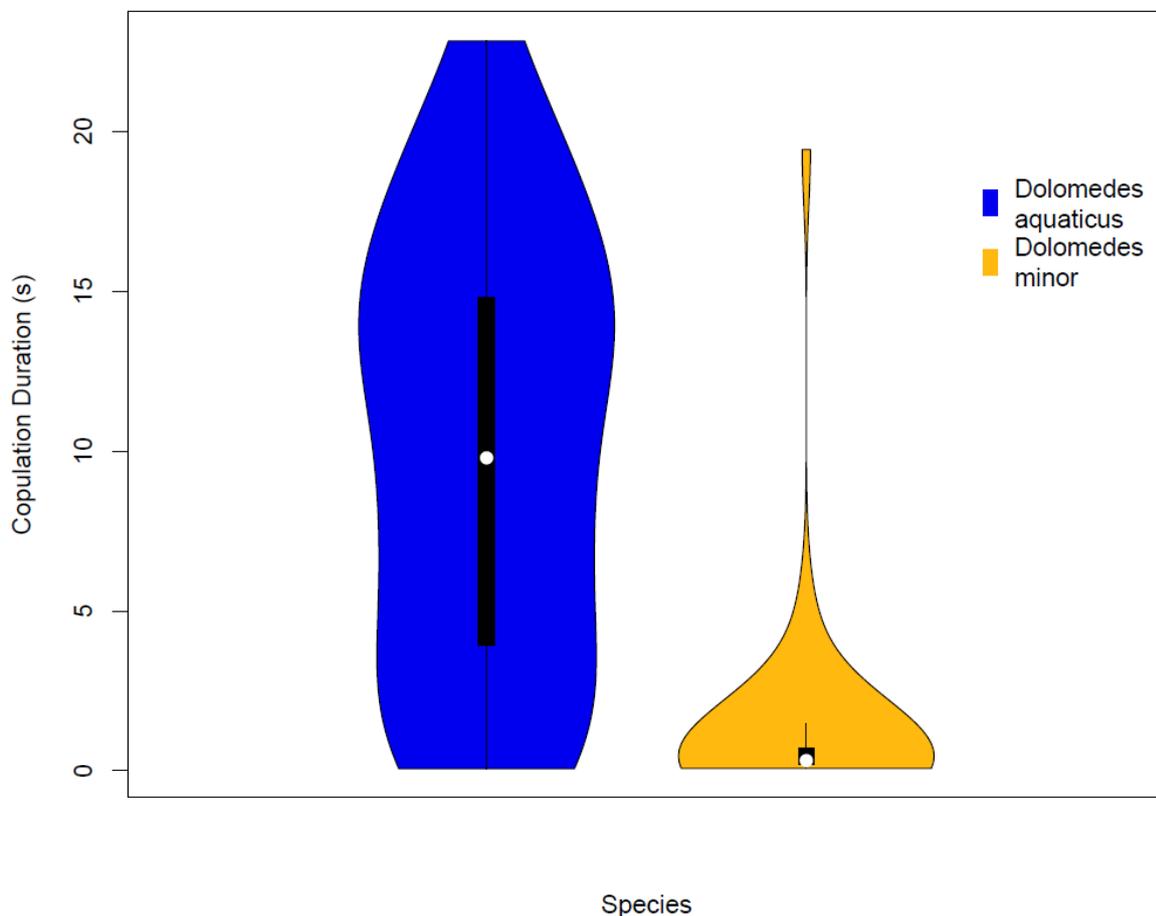


Figure 2.6: Copulation durations were significantly different between two species of *Dolomedes* spider. *Dolomedes aquaticus* had wide variation in the duration of copulations from less than a second to over 22 seconds. *Dolomedes minor* had dramatically shorter copulations than *D. aquaticus*, with most being less than a second in duration. Boxes show interquartile range, with the central point indicating the median. The curved lines represent the full spread of the data.

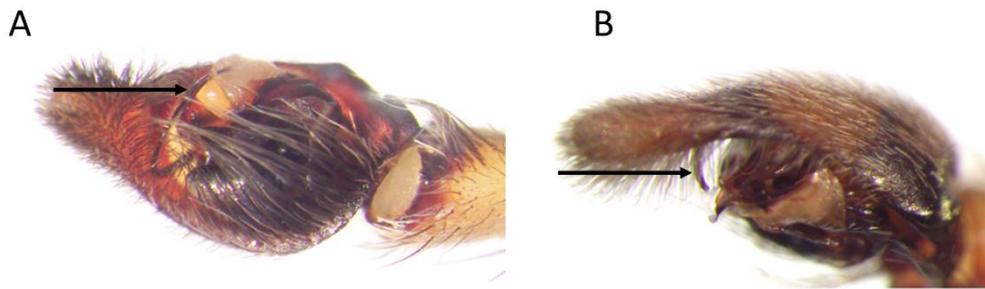


Figure 2.7. Pedipalps of *D. minor*. A: Front view of an intact embolus. B: Side view of a broken embolus. Arrows indicate embolus.

#### 2.4.4 Rates of sexual cannibalism and female aggression

Of the 38 observed matings in *D. aquaticus*, nine resulted in post-copulatory cannibalism (24%), of which two were made by mated females and 7 by unmated females. Of the 29 observed matings in *D. minor*, seven resulted in post-copulatory cannibalism (24%), of which two were made by mated females and 5 by unmated females. There was no significant difference between species for the likelihood of a mating resulting in post-copulatory cannibalism ( $df = 65$ ,  $z = 0.043$ ,  $p = 0.966$ ). When females were unmated, *D. aquaticus* females were more likely to attack the male than *D. minor* females ( $df = 168$ ,  $t = -2.138$ ,  $p = 0.033$ ) (Fig. 2.8). However, in experiments where females did attack, there was no significant difference in the per hour rate of attacks between the species ( $df = 71$ ,  $f = 0.666$ ,  $p = 0.417$ ).

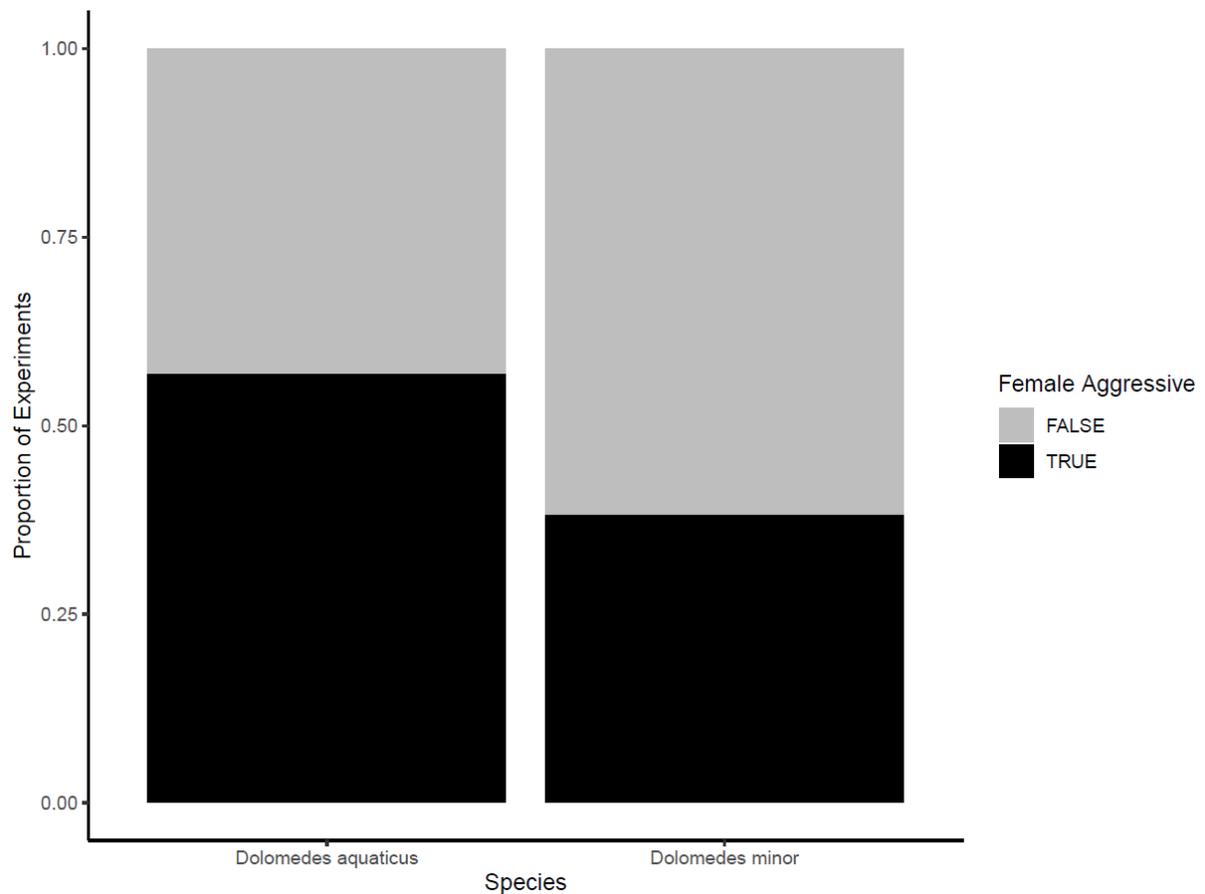


Figure 2.8: Probability of attack in unmated females by species. Unmated *D. aquaticus* females were more likely to attack than unmated *D. minor* females. The y axis describes the Proportion of experiments where females attacked a male. 'True' represents experiments where any attack behaviour was observed from females, while 'False' represents experiments where no attack behaviour was observed.

When females had already mated previously, there was no significant difference between species in female's likelihood to attack (df = 70, Z = 1.241, p = 0.215). However, the per hour rate in *D. aquaticus* was significantly higher than *D. minor* (df = 43, F = 26.483, p < 0.001) (Fig. 9).

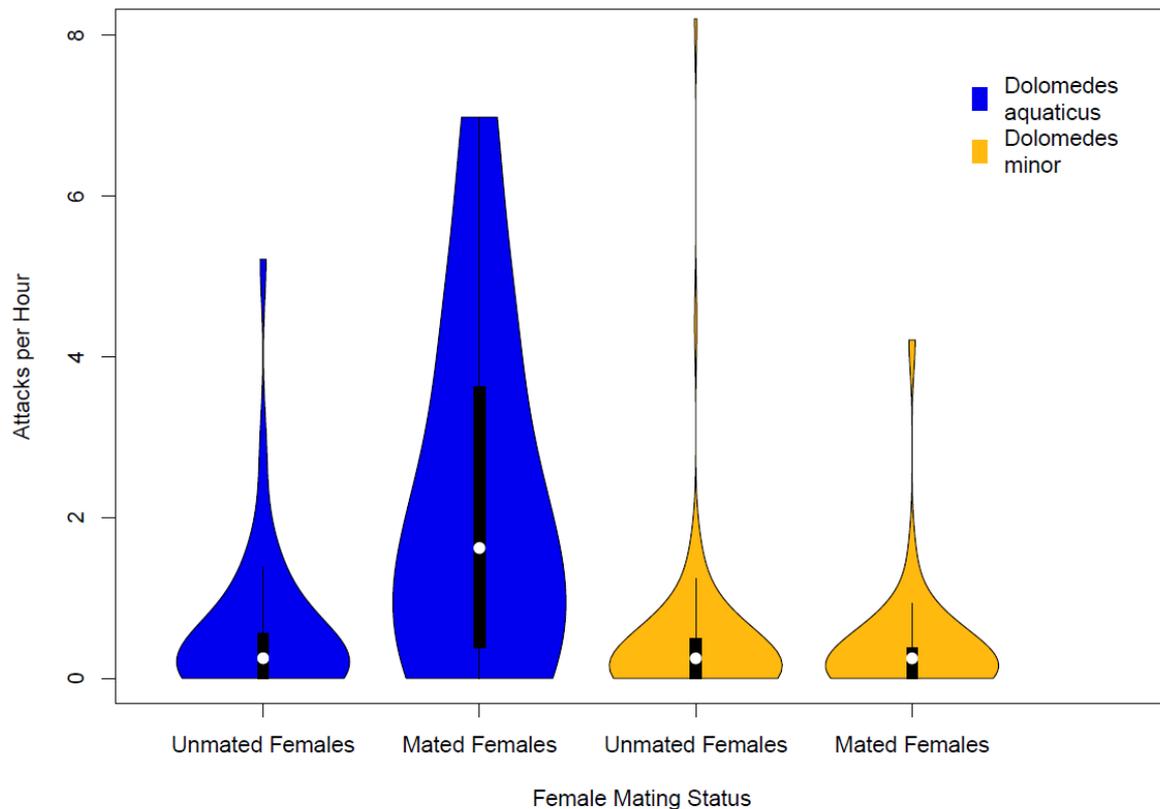


Figure 2.9: Attack rates by female *Dolomedes* spiders on male partners in relation to female mating status. *D. aquaticus* females showed a significant increase in aggression after their first copulation, while *D. minor* females showed no change in the per hour rate of attack regardless of their mating status. Boxes show the interquartile range, with the central point indicating the median. The curved lines represent the full spread of the data. There are several outliers for the *D. aquaticus* mated females subset which are not plotted for the sake of readability (however, they were included in the statistical analysis): 19.946, 19.867 and 60.

#### 2.4.5 Courtship Behaviour

Latency to court ( $t = -6.069$ ,  $df = 160.94$ ,  $p < 0.001$ ), latency to mount ( $t = -4.891$ ,  $df = 92.759$ ,  $p < 0.001$ ) and latency to copulate ( $t = -5.629$ ,  $df = 61.467$ ,  $p\text{-value} < 0.001$ ) were all significantly longer in *D. minor* than in *D. aquaticus* (Fig. 2.10). However, there was no significant difference between the likelihood of males of the two species to court females when the spiders were unmated ( $df = 106$ ,  $z = -1.642$ ,  $p = 0.101$ ), or a significant difference in mean courtship duration per trial (*D. aquaticus* mean = 2534.3 seconds, *D. aquaticus* SD =

3089.9, *D. minor* mean = 1820.1 seconds, *D. minor* SD = 1388.6, df = 72, F = 1.845, p = 0.179).

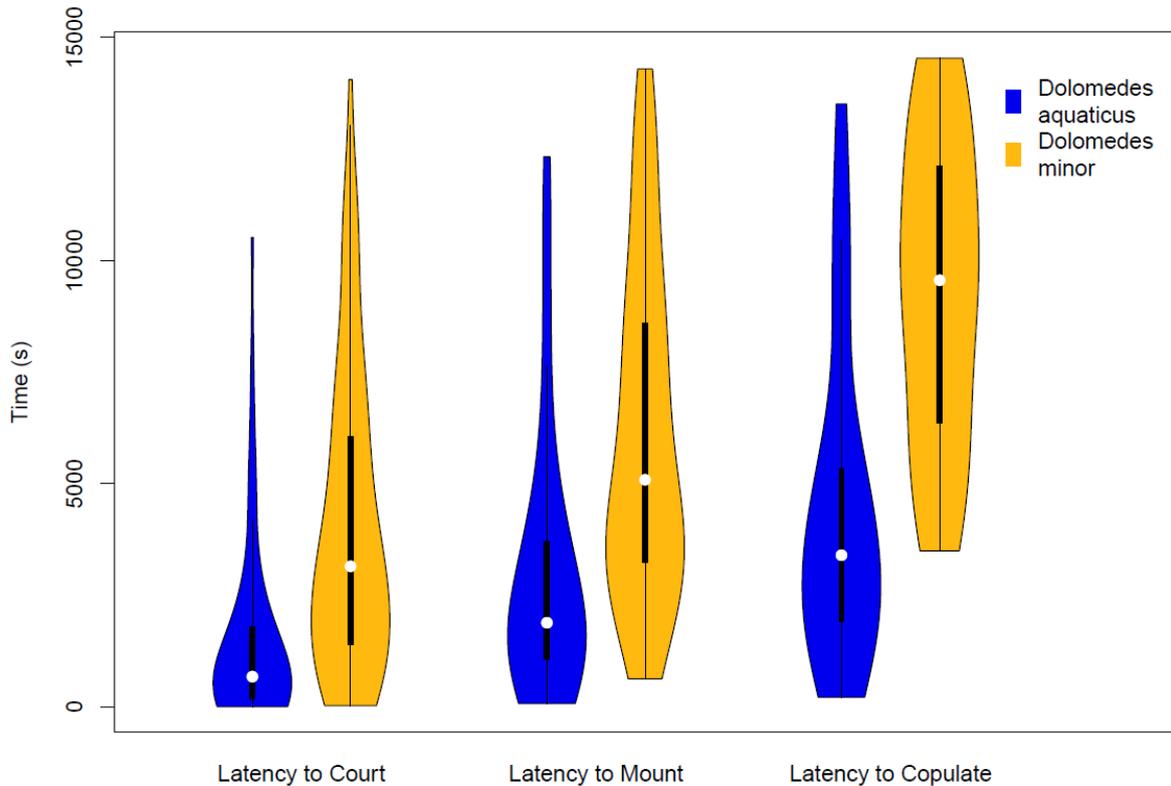


Figure 2.10: Latency periods for stages of mating by species. *Dolomedes aquaticus* has shorter latency periods for all stages of courtship than *D. minor*. Boxes show interquartile range, with the central point indicating the median. The curved lines represent the full spread of the data.

In *D. aquaticus*, there was no significant difference between the number of pseudo copulations and the number of copulations during a trial ( $t = -1.588$ ,  $df = 81.347$ ,  $p$ -value = 0.116). However, for *D. minor*, the number of pseudo copulations was significantly higher than actual copulations ( $t = -5.0294$ ,  $df = 183.12$ ,  $p$ -value =  $1.168e-06$ ) (Fig. 2.11).

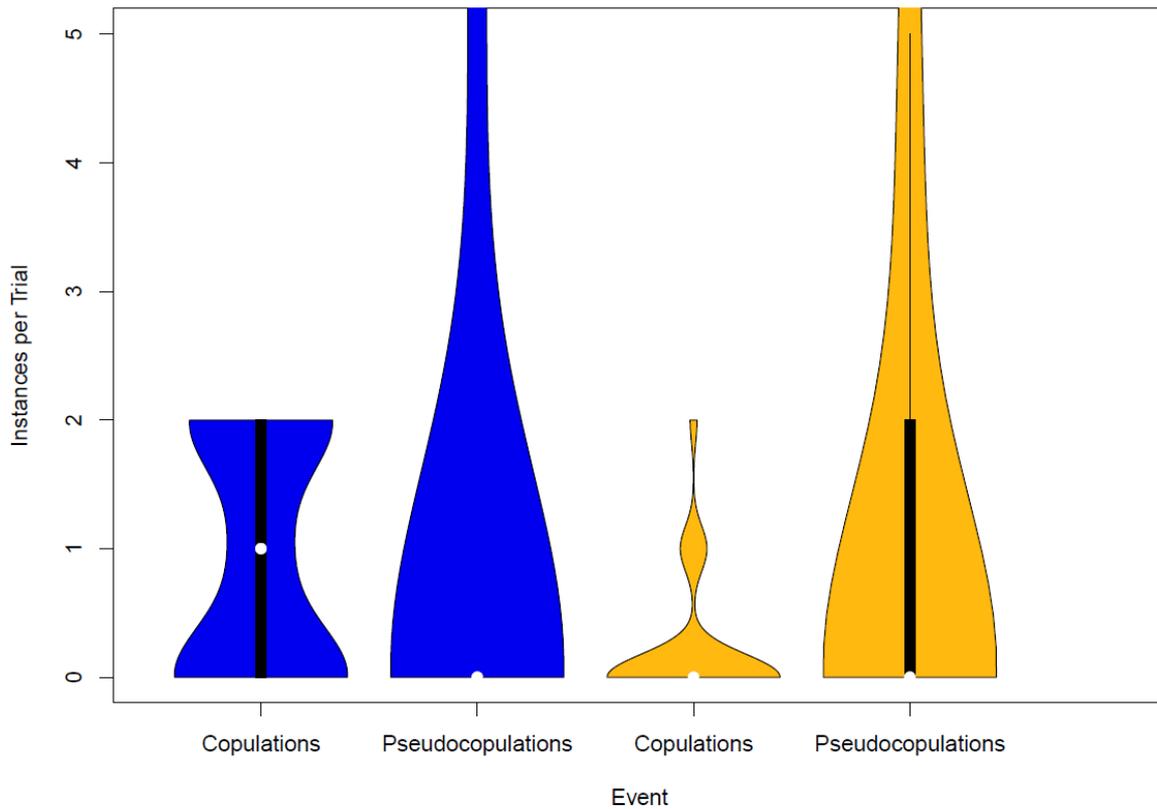


Figure 2.11: Rate of pseudocopulations and copulation by species. Pseudocopulations in both species had a much wider range than copulations per trial (which had a maximum of two). Boxes show interquartile range, with the central point indicating the median. The curved lines represent the full spread of the data.

#### 2.4.6 Population Dynamics

At no point during field observations of *D. minor* was there a statistically significant male biased sex ratio, except for a single observation in November 2022 (Supplementary Table 2). The pattern of sex ratio over time can be seen in figure 2.12. Proportion of adult and penultimate *D. minor* in the population is generally lower near the beginning of the year, with a steady increase beginning around March. Lower levels are also found in the latter half of the year, with the exception of November-December. However, for almost all sampling, juveniles made up the majority of the population (Fig. 2.13).

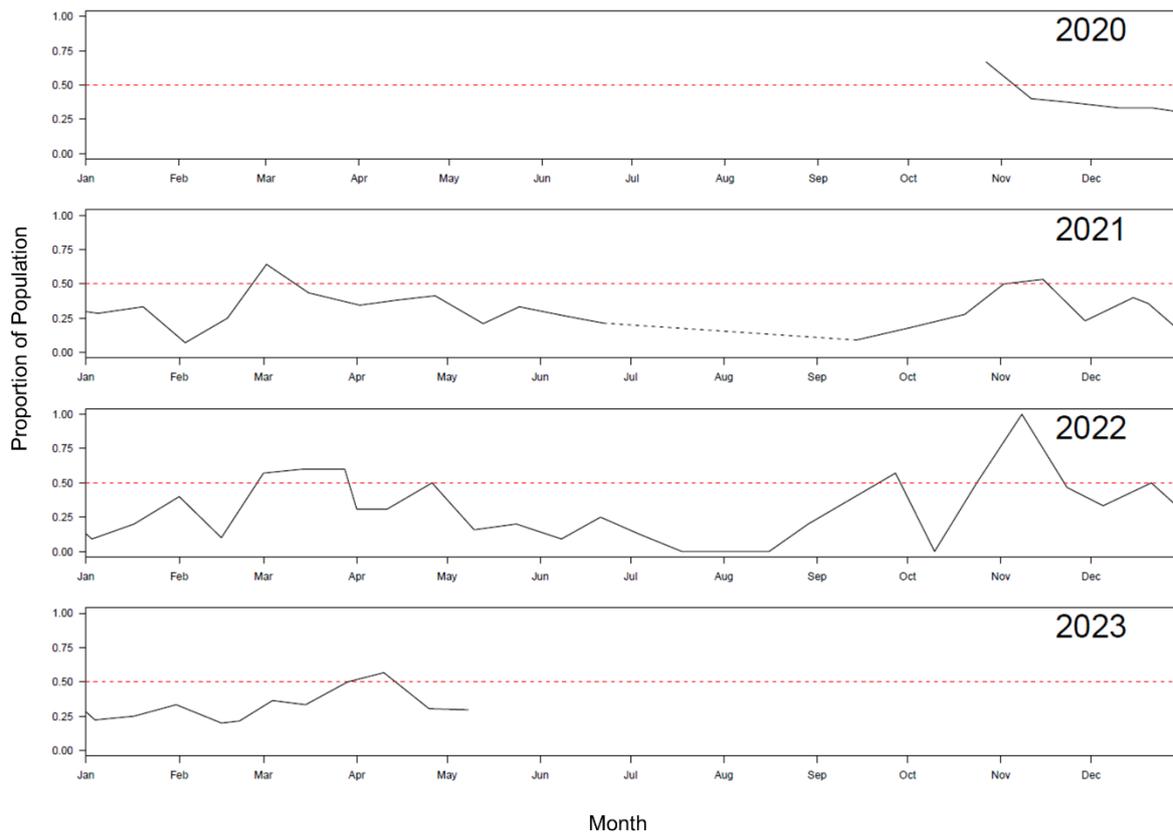


Figure 2.12: Field observations of the proportion of male *D. minor* over time. Male population tended to be low in *D. minor* in the middle of the year, with an increase in mature males in October, November, and March. However, at no point was the population significantly male biased. The red dotted line indicates an even male:female ratio. The black dotted line indicates a gap in observations.

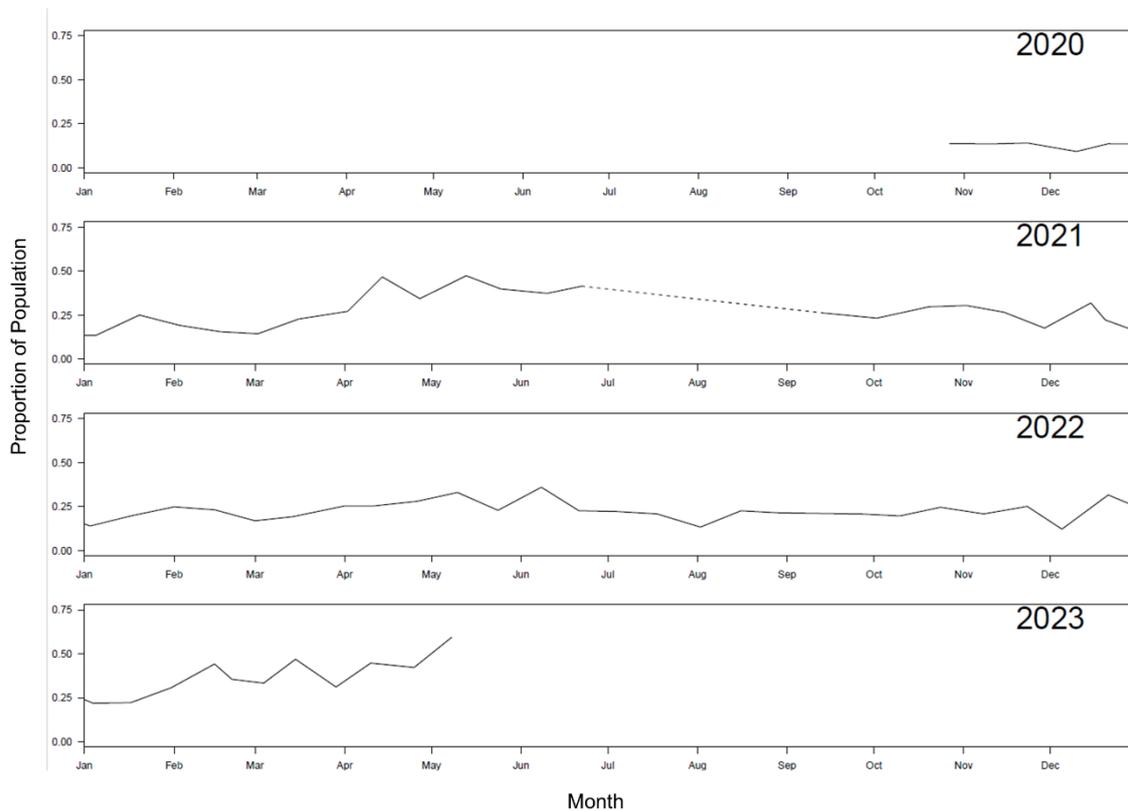


Figure 2.13: The proportion of adult and penultimate *D. minor*, relative to the proportion of juveniles, over time. Peaks in spider maturity are present at the end of year and around March. Ratio of adult and penultimate *D. minor* to juveniles over time. The black dotted line indicates a gap in observations.

## 2.5 Discussion

Despite being closely related sister species, *D. aquaticus* and *D. minor* have radically different mating systems. Our mating experiments found that *D. aquaticus* are mostly polygynous, with a male able to copulate with both pedipalps repeatedly; and monoandrous, with females becoming aggressive after their first mating. Genital damage is extremely rare in *D. aquaticus*, and males will most often copulate twice with a female in a single encounter. The behaviour of *D. aquaticus* is supportive of our hypotheses where female mating rates preclude the need for genital plugging. In contrast to *D. aquaticus*, we found that most *D. minor* mate up to twice and males usually expend one palp per female. However, we found that females will attempt to mate with more than two partners if given the opportunity, and males can attempt to use each palp multiply. Females also lack a syndrome of aggression

post mating, and it is therefore possible that females mate multiple with multiple males. This introduces a sperm competition risk, which could explain the presence of genital damage in this species. In further contrast to *D. aquaticus*, we found genital damage by breaking of the embolus in male *D. minor* in a large proportion of pairings, which is a rare finding outside of web-building spiders. In addition, *D. minor* mating was also typified by extremely short copulation durations, low rates of copulation and one copulation per mating. However, despite multiple female mating and the presence of genital damage in *D. minor*, we did not detect other traits that tend to be correlated with these mating behaviours, including an absence of monogyny (although male mating rates are lower than in *D. aquaticus*), protandry and extreme female-biased sexual size dimorphism (see Chapter Three). Sexual cannibalism in *D. minor* does not seem to be facilitated by male self-sacrifice (predicted by Miller 2007) but is instead associated with a specialised male escape behaviour. Table 2.1 summarises the key aspects of each species' mating behaviour. These spiders are evidence that even in closely related species with a similar ecology, mating systems and behaviours can evolve in drastically different ways.

Table 2.1: Summary of mating characteristics for each species. Note that these are only trends of the data. For a full analysis, see the Results section.

Characteristic	<i>Dolomedes aquaticus</i>	<i>Dolomedes minor</i>
Male Mating Rate	Polygynous (mating with multiple females)	Low mating rates, but potential for multiple mating
Female Mating Rate	Monoandrous (Mating with one male)	Low mating rates, but potential for multiple mating
Male Pedipalp Usage	Two per female	One per female
Sexual Cannibalism	Relatively Uncommon	Relatively uncommon, despite male escape behaviour
Female Aggression	Extreme after mating	Not extreme at any mating status stage
Male Self Sacrifice	None	None
Genital Damage	Very rare	Common
Sexual Size Dimorphism	Females ~2x larger than males	Females ~3x larger than males (See Chapter Three)
Protandry	Possibly, based on small sample size (see Chapter Three)	None
Courtship latency	Relatively short lag periods	Long lag periods
Copulation Duration	Relatively long	Extremely short

*Dolomedes minor* show low mating rates, and a low probability of successful mating in any given encounter. Some males are monogynous, as a failed escape from sexual cannibalism curtails their future mating opportunities. They also show genital damage, however, other typical correlates of a monogynous mating system are absent (Schneider and Fromhage 2010). Male-biased sex ratios and protandry (early maturation of males) were not shown in the field data. Additionally, female-biased sexual size dimorphism is only slight in this species, compared to the extreme examples shown in other taxa (Kuntner et al. 2009; Schneider and Fromhage 2010) and even in other *Dolomedes* (Schwartz, Wagner, and Hebets 2013).

With no female syndrome of aggression post-mating, and one male using his palps multiply, it is possible that both sexes of *D. minor* can mate multiply. However, in practice males are likely to be limited by sexual cannibalism and search mortality (whilst the risk of search mortality may be lowered in non-web building taxa (Chapter 1), it likely still exists). Females on the other hand are at much less risk during the mating process. Whilst one night of observations did show a significant male bias, this was due to low sample size on this night leading to no detections of mature females (error rate in these data were increased by the relatively high number of juveniles, leading to small sample size when analysing adults alone). However, observations made during spider collection and monitoring showed several instances of females being courted by multiple males at once. Therefore, whilst the population ratio of *D. minor* does not show strong evidence of significant male bias, it is likely that highly successful females would be able to mate multiple times.

*Dolomedes minor's* genital damage is very different from that described in *D. tenebrosus* (Schwartz, Wagner, and Hebets 2013). In that species, the palp bulb did not deflate after mating. In *D. minor*, the tip of the male embolus breaks away, making this a distinct type of genital damage, not currently known in any other *Dolomedes*. Whilst this study did not aim to test the effectiveness of genital plugging in *D. minor*, it likely has or previously had adaptive significance, unlike in *D. tenebrosus* where the male plugging the female is to allow himself to be cannibalised (Schwartz, Wagner, and Hebets 2014; 2016). Females in *D. minor* do not become aggressive after they mated, unlike *D. aquaticus* females. Hence there would be an evolutionary incentive for males to plug their mates because there is a possibility that the female will go on to remate with an additional male, introducing sperm competition risk.

There are two spider groups with comparable genital damage that are illustrative here. In *Latrodectus* spiders (Theridiidae), plugs made of the tip of the male genitalia are common, and with proper positioning these plugs are highly effective at preventing future sperm transfer (Uhl, Nessler, and Schneider 2010). If this is the case in *D. minor*, this would provide an incentive for the female to terminate the copulation as quickly as possible via cannibalism, to reduce the male's ability to place the plug correctly; a classic manifestation of sexual conflict. With this context, genital plugging could also explain the extremely rapid copulation durations seen in *D. minor*. The speed with which *D. minor* males copulate appears to facilitate a quick escape from the females, with males launching themselves away from the female after copulation, which has been described in other taxa (Zhang, Liu, et al. 2022) and other *Dolomedes* (Arnqvist 1992). Males who failed in this escape were cannibalised by the female. This makes the similarity in post-copulatory sexual cannibalism rates between the *D. aquaticus* and *D. minor* notable, as even with this adaptation, *D. minor* males only achieve the same escape rate as *D. aquaticus*. Without the short copulations, the rate of cannibalisations would likely be much higher, and *D. minor* males would likely be limited to one copulation. The other illustrative group are nephilid spiders, where the effectiveness of a male plug varies between species (Kuntner et al. 2009). In *Nephila pilipes* (Nephilidae), the broken genitalia lodge in the female's genitalia, but do not block future copulation. Females have even been found with multiple male genitalia lodged in their epigyne (Kuntner et al. 2009). Kuntner et al. (2009) speculate that genital damage was ancestrally successful as a plugging method in nephilid spiders, but males in *N. pilipes* have lost the evolutionary arms race with females for their plugs to become ineffective. Such a situation is also possible in *D. minor*, with plugs becoming ineffective in recent evolutionary time, potentially due to cannibalism and the resulting necessary escape behaviour. In either case, the genital damage in *D. minor* implies sperm competition has played a role in the species' evolution (Uhl, Nessler, and Schneider 2010; Uhl et al. 2014). Given the high speed of the *D. minor* copulations, it is also arguable that the damage observed to male genitalia is accidental and that a male will break off his genitalia when withdrawing at speed to avoid cannibalisation. However, such a conclusion would require more investigation of the genital mechanics involved, especially given the evolutionary association between sexual cannibalism and genital damage (Schneider 2014).

The tendency of *D. minor* to copulate once with each female, rather than copulate twice is intriguing. It could be that this is a system similar to the one described by Cory & Schneider, (2020) in *Argiope bruennichi* (Araneidae), where male mating strategy is conditional on female quality and availability. This kind of flexible system allows males to make the choice between minimising sperm competition (by plugging both genital openings of a female) and mating with multiple females (Cory and Schneider 2020). It could also be an adaptation by females to prevent being plugged by a single male, which would be a classic example of sexual conflict (Elias, Andrade, and Kasumovic 2011; Perampaladas, Stoltz, and Andrade 2008; Schneider 2014; Uhl, Nessler, and Schneider 2010). Female aggression has previously been shown in *D. triton* to limit male copulation to one instance per female (Johnson 2001).

The potential for cannibalism could also explain the long latency for male *D. minor* to begin courtship, as males are at a high risk of death when copulating with a female and should therefore be choosy (Schneider 2014). The choosiness also provides one possible explanation for the high rate of pseudocopulations, where males may abandon copulation attempts to avoid cannibalisation. However, pseudocopulations may also be used to assess female mating status (with males potentially even searching for broken male emboli to indicate a female is already mated), alternatively or in addition to avoiding female aggression. Altogether, this could also explain the overall low mating probability, with *D. minor* males being extremely choosy in their mates and circumstances of copulation (Schneider 2014). If a male only has two optimal copulations, he should use them in situations where he mates with an ideal female, with a clear escape route to avoid cannibalisation. We observed one *D. minor* male copulating with broken emboli. Whilst the effectiveness in sperm delivery is unknown, it has been shown before in *Latrodectus* that males with such damage can continue to mate unimpeded (Uhl, Nessler, and Schneider 2010). However, any future matings would potentially be suboptimal for a male as the male would be unable to plug the female and therefore subject to sperm competition.

The speed of *D. minor*'s matings is consistent with our observations in the field where we saw a male attempting an extremely brief copulation with a female and subsequently performing the characteristic escape behaviour. This escape behaviour is also similar to the "catapult behaviour" described by Zhang et al (2022a) in *Philoponella prominens* (Uloboridae). We also observed another copulation in the wild where a male was attempting

copulation with a female emerging from her final moult. This would be an excellent way for males to avoid cannibalisation and has been observed before in other spider species (Schneider and Andrade 2011; Uhl et al. 2015). Whilst this behaviour has shown to be extremely effective in avoiding cannibalism, males lacking the cues from mature females have been shown to have difficulty encountering a moulting female (Uhl et al. 2015). The prevalence of this behaviour, its effectiveness, and the prevalence of related behaviours (such as males guarding sub-adult females) would be excellent avenues for future studies in *D. minor*.

For *D. aquaticus*, the absence of male genital damage makes sense considering the absence of female remating. With little to no sperm competition, *D. aquaticus* males have no reason to damage their genitals or plug females, and instead maximise their fitness by searching for more mates (Norton and Uetz 2005; Uhl, Nessler, and Schneider 2010; West and Toft 1999). A similar system has been shown in wolf spiders (Norton and Uetz 2005) and other *Dolomedes* (Schoenberg, Hebets, and Sullivan-Beckers 2022). This scramble for mates would seem to provide an evolutionary advantage for those males who mature earlier and find more mates before females become aggressive and unavailable (Herberstein, Painting, and Holwell 2017). A male with access to multiple unmated females would likely only be limited by how quickly he can refill his pedipalps with sperm, assuming that these males are capable of doing so effectively. Note that the maximum mating of six copulations was set by our experimental design, and the true number may be even higher.

Whilst population studies on *D. aquaticus* were limited (See Chapter Three), the lack of extreme female-biased sexual size dimorphism supports the absence of protandry. Protandrous species usually have tiny males who mature quickly, leading to strong female-biased sexual size dimorphism (Schneider and Fromhage 2010; Schwartz, Wagner, and Hebets 2013). Given there is an advantage to large males to achieve mating, it makes sense that large male size has been preserved in this species. Large size in males could serve to resist cannibalisation from mated females (Kuntner and Coddington 2020). Indeed, in the wild, male *D. aquaticus* make up a significant proportion of a females' diet (Greenwood, McIntosh, and Harding 2010). Therefore, it would be advantageous for a male to be able to resist cannibalisation to find more mates. Investing in the ability to search for more mates has been shown to be advantageous in other spider species (Berger-Tal and Lubin 2011).

The adaptive function of female aggression after mating in *D. aquaticus* is unknown, though this has been described in spiders before (Herberstein, Schneider, and Elgar 2002; Wilder and Rypstra 2008). It may simply be that males are able to fill a female's spermatheca during the relatively long copulations, and therefore new mates have little to offer, other than their potential as a meal (Aisenberg and Costa 2005). This explanation would also explain *D. minor* female's receptiveness to further mates, assuming they are receiving relatively little sperm from their mates. Other spider species are known to become sexually receptive a long time after mating (Perampaladas, Stoltz, and Andrade 2008). Given *D. aquaticus* females likely live for several years (Greenwood and McIntosh 2011), it is possible they will remate in subsequent years. This would make them serially monoandrous, rather than strictly monoandrous. Male spiders have also been speculated to influence female behaviour and receptivity to future mates via products in their sperm (Estramil and Costa 2007; Gonzalez et al. 2019; Norton and Uetz 2005). This could be an adaptation of *D. aquaticus*, in that males are increasing the aggression of females post-mating to avoid sperm competition. This could be seen as an evolutionary alternative, where *D. minor* reduce sperm competition via genital plugging, while *D. aquaticus* instead employ an alteration of female behaviour.

A notable finding of this study is the divergence in mating systems, rates, and behaviours between two sister species. The relationship between these two species is so close they can interbreed in part of their range (Lattimore et al. 2011). In fact, the only *D. aquaticus* male to break his embolus during mating came from this region where introgression occurs. Whilst speculation should be limited based on the data, this could be evidence of an intermingling of the mating systems in this region and is a prime avenue for further exploration.

The extremely rapid duration of *D. minor* matings and high rate of pseudocopulations presented a significant challenge and limitation to this study. Identifying copulations was difficult, and biased sampling to those copulations where contact between the genitalia was longer, or the embolus broke confirming copulation. Future studies could overcome this by examining sperm deposition or allowing females to produce egg sacs and offspring for paternity analysis. This would also allow study on the effectiveness of these genital pieces as mating plugs and their adaptive significance.

Our study found that sister species *D. aquaticus* and *D. minor* have radically different mating systems; with *D. aquaticus* being monoandrous and polygynous, and *D. minor* having generally low mating rates for both sexes, but with the possibility of multiple mating. Whilst *D. minor* show genital damage, they are notable in the absence of other mating system elements generally associated with genital damage, such as monogyny, protandry and extreme sexual size dimorphism. Whilst these observations were challenged by difficulty in assessing copulations in *D. minor*, these findings are indicative that more work is needed to understand the diversity of spider mating systems, in relation to genital plugging and damage.

### Chapter 3: Mating Behaviour Influences the Direction and Geographic Extent of Introgression in New Zealand Fishing Spiders (*Dolomedes*)



*D. minor* female – Helen Macky

### 3.1 Abstract

Introgression is a highly influential process in evolution, where genes flow between species that are not fully reproductively isolated. Studies on introgression often focus on describing gene transfer and environmental changes that facilitate the meeting of species. However, the impact of mating systems and behaviour that facilitate gene transfer is less well-known. *Dolomedes aquaticus* and *D. minor* are sister species of fishing spiders undergoing one-way, geographically limited mitochondrial introgression, making them an excellent case study for the factors that promote and limit introgression. We used a combination of field observations and crossing experiments to systematically investigate potential pre-fertilisation barriers that could limit introgression and explain the one-way and geographic barriers to introgression in these species. We found that habitat overlap and timing of reproductive maturity were not likely to be important limiting factors to introgression. However, behaviour was an important factor, with male mate choice being implicated in the geographic limitation, and female mate choice being implicated in the one-way limitation. Our results show the importance of using behavioural approaches in the investigation of introgression.

### 3.2 Introduction

Introgression is the flow of genes between species that are capable of interbreeding (Croucher, Oxford, and Searle 2004; Ohshima and Yoshizawa 2010; Wall 1970), but would generally be considered separate species by any other definition (e.g. morphological analysis (Vink and Dupérré 2010)). Understanding introgression is highly informative for studies on speciation and phylogenetics (Croucher, Oxford, and Searle 2004; Leduc-Robert and Maddison 2018) as it can reduce lineage diversity, sometimes causing genetic extinction of a species (Lattimore et al. 2011; Rhymer and Simberloff 1996). Conversely, introgression can also increase the rate of evolution (Chang, Song, and Zhou 2007; Leduc-Robert and Maddison 2018; Wall 1970) as it is an efficient method of introducing new genetic material into a population, compared with random mutation (Hedrick 2013).

The precise causes of introgression are sometimes complex (Wall 1970), but it typically occurs via the backcrossing of hybrid individuals with one or both of their parent species (Anderson 1949; Lattimore et al. 2011; Sweigart and Willis 2003). The mating systems and reproductive morphologies of a species can strongly affect the circumstances and direction of introgression, sometimes limiting it to one-way where the genes flow from one species to the other, but not in reverse. For example, Sweigart & Willis (2003) found that the mating systems of different *Mimulus* plants affected the direction of introgression, with DNA from a self-fertilising species flowing into non-self fertilising species (i.e. the species with plants taking on pollen are more likely to take on genetic material that is not their own). Relationships between mating systems and the direction of introgression have also been demonstrated in animals (Stein and Uy 2006; While et al. 2015) and fungi (Sun et al. 2012), as well as through mathematical modelling (Hartman et al. 2012). Animal systems of note include red-backed fairy-wrens and swordtail fish (Baldassarre and Webster 2013; Rosenthal et al. 2003; Schumer et al. 2017). In the wrens, introgression occurs asymmetrically between lineages with different male plumage colours. This is due to extra pair mating behaviours found specifically in males with red plumage (Baldassarre and Webster 2013). In swordtails, introgression is maintained through frequent interbreeding between hybrids, with precopulatory barriers making matings between the parent species rare (Culumber, Ochoa, and Rosenthal 2014; Rosenthal et al. 2003). Differing environmental conditions across species' ranges can also alter species' mating preference, as mating experiments with spadefoot toads have demonstrated, with females preferring to hybridise for faster maturation of offspring when waters are shallow (Chen and Pfennig 2020; Schmidt and Pfennig 2016). However, the link between introgression and mating systems is often absent in studies, with most work identifying introgression through genome sequencing, with analysis focusing on environmental changes that facilitate meetings between species (Aguillon et al. 2022).

Spider mating behaviour often involves intricate signalling between males and females (Elias, Andrade, and Kasumovic 2011; Herberstein et al. 2014), as well as the interaction of complex genital and chemical structures (Croucher, Oxford, and Searle 2004). With so many potential barriers to interbreeding, spiders are a fascinating group to study cases where introgression does occur, and how this is facilitated. Spiders are extensively studied for their unusual

mating behaviours associated with their high rates of monogyny, female aggression and sexual conflict (Herberstein et al. 2011; Schneider and Fromhage 2010). This means that that males courting females of different species risk loss of life and all future mating opportunities if they are unsuccessful in courtship or copulation.

Numerous techniques are used to investigate introgression in spiders including molecular and phylogenetic approaches, crossing and behavioural experiments (De Busschere, Van Belleghem, and Hendrickx 2015; Hedin, Foldi, and Rajah-Boyer 2020; Oxford and Croucher 2014). For example, genetic sequencing and crossing experiments in *Tegenaria* spiders revealed genital morphology to be the key factor limiting the direction of introgression, whose shape favoured crosses in one direction, but not the reverse (Croucher, Oxford, and Searle 2004). Another example comes from a review of *Habronattus* (jumping spiders), which found that the preference of female jumping spiders for foreign courtship displays (Hebets and Maddison 2005) has led to several separate introgressions within the genus, significantly complicating their phylogeny (Leduc-Robert and Maddison 2018). *Habronattus* have in fact been shown to have species with deeply connected evolutionary history through frequent introgression events (Bougie, Brelsford, and Hedin 2021), mediated by habitat corridors and contact zones (Hedin, Foldi, and Rajah-Boyer 2020; Bougie, Brelsford, and Hedin 2024). However, morphological, and behavioural mechanisms facilitating introgression in spiders are rarely investigated, even though introgression can make it more difficult to distinguish species using solely genetic methods (Domènech et al. 2020; Ortiz et al. 2021). A pair of introgressing spider species, where the morphology and behaviour facilitating introgression can be investigated, could be informative for what factors allow or limit gene transfer between species, and therefore what traits are influential more broadly in broader spider evolution.

Fishing spiders (*Dolomedes* spp.) demonstrate diverse mating behaviours, including complex courtship, monogyny, and sexual cannibalism. Courtship involves vibratory, visual, and chemical signals, used in part for the male to avoid being cannibalised by the female before he can copulate (Arnqvist 1992; Arnqvist and Henriksson 1997). The females of several *Dolomedes* species are highly aggressive when courted by males (Arnqvist 1992; Arnqvist and Henriksson 1997; Johnson 2001), males can make up a significant portion of females' diets in the wild (Greenwood, McIntosh, and Harding 2010; Johnson 2001), and sexual

cannibalism is common (Johnson 2005b; 2005a; Schwartz, Wagner, and Hebets 2014; 2016). *Dolomedes* mating systems are also diverse, including at least one monogynous species, where males spontaneously die during copulation, leaving their bodies hanging from the female's genitalia (Schwartz, Wagner, and Hebets 2014), while others are polygynous (Schoenberg, Hebets, and Sullivan-Beckers 2022). Size is also an important factor in *Dolomedes* mating. Whilst many *Dolomedes* species do not show the extreme female biased sexual size dimorphisms common in other spider taxa, females are still larger than males. For example, in *D. scriptus* females are larger than males, but pairs that are more similar in size are more likely to mate (Schoenberg, Hebets, and Sullivan-Beckers 2022). When species with different sizes of males and females interact, the relative sizes of the males and females of each species could be important in determining which sex/species combinations lead to mating, and therefore determine the direction of introgression.

Mitochondrial introgression has been identified between two *Dolomedes* sister species endemic to New Zealand: *D. aquaticus* Goyen, 1888 and *D. minor* Koch, 1876 (Lattimore et al. 2011; Vink and Dupérré 2010) (Fig. 3.1). It is a one-way introgression, with *D. minor* males mating with *D. aquaticus* females to produce hybrids, evidenced by *D. aquaticus* mitochondrial DNA being found in specimens of *D. minor*, but no known transfer taking place from *D. minor* to *D. aquaticus* (Vink and Dupérré 2010). As well as being one-way, the introgression is also geographically restricted to a region in the lower South Island of New Zealand, at the extreme southern end of the range of both species (hereafter referred to as the Introgression Zone), despite the two species co-occurring throughout the range of *D. aquaticus* (Lattimore et al. 2011) (Fig. 3.2). However, the mechanisms facilitating and limiting introgression between these species is currently unknown.

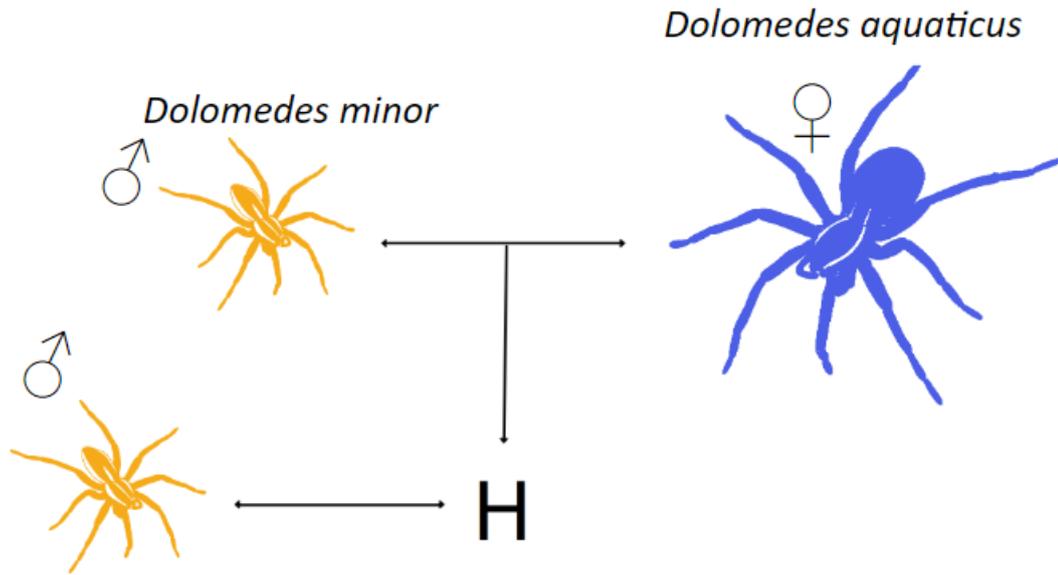


Figure 3.1. Diagram of introgression in *Dolomedes* species. *Dolomedes minor* males mate with *D. aquaticus* females, producing a hybrid specimen, represented by the letter H. These hybrids can then backcross with *D. minor*, allowing the flow of *D. aquaticus* mitochondrial DNA into *D. minor*.

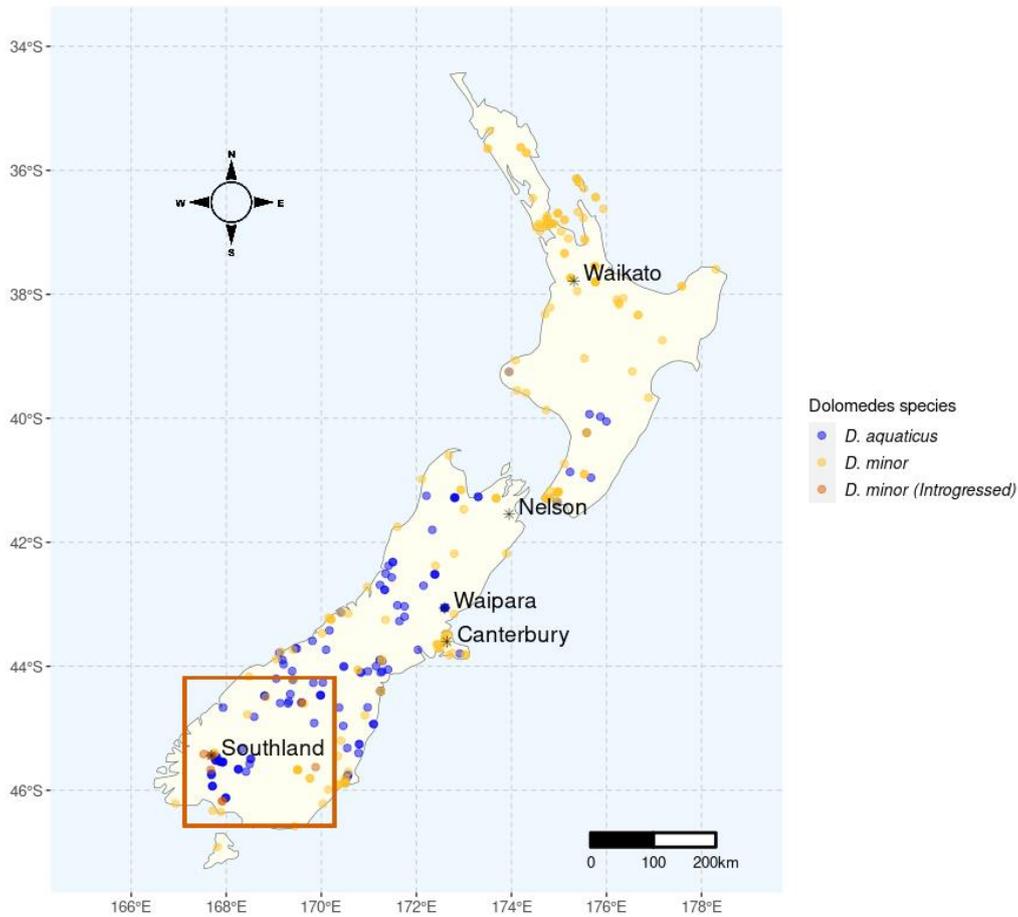


Figure 3.2. Map of New Zealand showing locations of museum specimens of *D. aquaticus* and *D. minor* based on data from Vink & Dupérré 2010 and Lattimore et al. 2011. All specimens with introgressed mitochondrial DNA are found in the south of the country, highlighted with an orange box (i.e. the Introgression Zone). Also shown are locations of collection and fieldwork for this study.

Several limiting factors could be controlling the gene flow between these species, which can be broadly categorised into pre- and post-fertilisation barriers. Pre-fertilisation barriers to hybridisation include habitat overlap, timing of reproductive maturity (i.e., do the two species overlap in the wild when they are sexually receptive), and differences in courtship behaviour. Reproduction in *Dolomedes* relies on male courtship in response to female chemical cues or other stimuli and represents another factor that could vary across species or environmental gradients. Female receptiveness to male courtship is also key for introgression to occur, including female ability to recognize male courtship, female-choice and pre-mating aggressive behaviour, which could all prevent interbreeding in ways that could vary throughout the species' range. Complex interactions of male and female signals and behaviours have been shown to explain asymmetric mating preferences across a

species' range (McLean et al. 2020). It is also possible that the various species/sex combinations could be capable of responding to courtship, but not of reaching attempted copulation; a male may be willing to attempt courtship, but aborts before making genital contact, or a female cannibalises the male before he gets the opportunity to attempt to copulate. Any limitation on introgression after this point can be categorised as post-copulatory barriers, including cryptic female choice, or factors such as hybrid inviability or hybrid sterility where the two species can interbreed, but any young produced are incapable of backcrossing.

These factors each represent a potential barrier to introgression and can therefore be investigated to identify the what most limiting factor preventing introgression, giving insight into the factors that limits or promotes introgression, and therefore influences evolution and speciation processes. We summarise these factors in Table 3.1, with the state of knowledge prior to this study for each species/sex/location combination for *D. aquaticus* and *D. minor*. Given that *D. aquaticus* females and *D. minor* males are capable of interbreeding in the wild, it can be assumed that none of the potential barriers listed in Table 3.1 prevent their interbreeding. We can also assume that *D. minor* females and *D. aquaticus* males within the Introgression Zone are capable of meeting each other, due to the overlap of their successfully introgressing opposite sex conspecifics. However, for both combinations of species and sex outside the Introgression Zone, no assumptions can be made, other than that they are unable to introgress, based on available evidence (Lattimore et al. 2011; Vink and Dupérré 2010).

Table 3.1. Summary of previous knowledge on *D. aquaticus* and *D. minor* introgression. Introgression is known to be possible between *D. aquaticus* females and *D. minor* males inside the Introgression Zone. For *D. minor* females and *D. aquaticus* males inside the Introgression Zone they can be assumed to be able to meet each other. No assumptions can be made about spiders outside the Introgression Zone. A = *D. aquaticus*. M = *D. minor*. Black square = Inside Introgression Zone, Grey Square = Outside Introgression Zone. A green tick indicates that the barrier is capable of being broken, at least in some cases, while a red cross indicates that the barrier prevents introgression. An orange question mark indicates that the status of this barrier is uncertain and requires investigation. Barriers are arranged in order from left to right based on when they appear in the spiders' life history. The final column represents whether the two species are capable of introgressing based on current genetic evidence (Lattimore et al., 2011; Vink & Dupérré, 2010).

Cross by Location	Habitat Overlap	Timing of Reproductive Maturity	♂ Courtship	♀ Receptiveness	Attempted Copulation	Post Copulatory Factors	Capable of Introgression
 [A] ♀ x [M] ♂	✓	✓	✓	✓	✓	✓	✓
 [M] ♀ x [A] ♂	✓	✓	?	?	?	?	✗
 [A] ♀ x [M] ♂	?	?	?	?	?	?	✗
 [M] ♀ x [A] ♂	?	?	?	?	?	?	✗

Here, we aim to address currently unknown factors that may limit or promote introgression for the remaining species/sex/location combinations, up to and including attempted copulation. To do this, we conducted a series of field observations to investigate habitat overlap and the timing of reproductive maturity, and laboratory crossing experiments to assess factors associated with courtship and copulation. From these findings, we will infer what is driving geographic limitations and species limitations of the introgression. This will identify areas of future research for other introgressing species and provide insight into what drives species isolation and speciation in non-introgressing lineages.

## 3.3 Methods

### 3.3.1 In Situ Monitoring

To address whether habitat overlap and/or timing of reproductive maturity may limit introgression outside of the Introgression Zone, we monitored a population of *D. minor* and *D. aquaticus* at Waipara River in Canterbury using fortnightly observations from October 2020 to March 2021. Two 10 m x 50 m transects were used: One transect was placed on the river side (*D. aquaticus* feeding ground) and the other on the border between rocky riverside habitat (*D. aquaticus* habitat) and shrubby/grassy habitat (*D. minor* habitat) (Supplementary Fig. 1). Monitoring consisted of walking each transect at night and identifying all individuals found to species, sex, and life stage, as well as their position within the transect. From these data, we mapped the habitat usage and distribution of both species in time and space. Due to COVID-19 travel restrictions and budget constraints, we were unable to perform a similar series of observations inside the Introgression Zone, which would have been ideal for quantitatively comparing the extent of habitat overlap and the timing of reproductive maturity between the two regions. However, our primary aim of these observations was to determine if the two species are likely to physically interact outside the Introgression Zone, which we can assume is occurring (at least at some times) within the Introgression Zone given this is where we have evidence of introgression occurring.

### 3.3.2 Spider Collection & Rearing

*Dolomedes aquaticus* and *D. minor* were collected from the Waikato, Nelson, Canterbury, and Southland regions of New Zealand from 2020 to 2021, to be used in laboratory experiments (Fig. 3.2). Spiders were hand-collected at night, using eyeshine as outlined by Benson & Suter (2013). All spiders were collected as juveniles or sub-adults to ensure that they were unmated. Collection was permitted by local councils (Waikato Regional Council, Marlborough District Council, Southland District Council) and/or private landowners. Collection locations are listed in Supplementary Table 1.

The best feature to determine species is their colouration, with *D. minor* possessing a distinctive stripe along the centre of the dorsal side of their cephalothorax and abdomen (Vink and Dupérré 2010). However, we cannot exclude the possibility of collecting hybrid

specimens within the Introgression Zone. The introgression is described only through mitochondrial DNA sequences of a small number of *D. minor*, and hybrids have never been found (Lattimore et al. 2011; Vink and Dupérré 2010). Hence, there are no known diagnostic features for hybrids. Without genotyping each specimen, we were unable to definitively rule out the inclusion of hybrids in our experiments, however, we believe this risk is very minimal.

Spiders collected in the field were returned to the laboratory and reared to adulthood in individual containers (150 mm x 95 mm x 60 mm) under controlled environmental conditions (temperature: 20°C; light regime: 12:12 h; lights on at 0800 hours). Each container had mesh holes to allow air flow, a corked hole for depositing food, continuous access to water via a dental cotton bud in the bottom of the container that led to a vial of tap water, and a small egg-carton shelter. Spiders were fed at least twice weekly with a mix of live field crickets (iNZect Direct New Zealand), and woodlice, earwigs and earthworms that were caught locally. Food detritus, dead spiders, and spider moults were removed from the containers daily and containers fully cleaned as required. A subset of spiders from Waikato and Canterbury were weighed to the nearest 0.00001g upon reaching maturity.

### 3.3.3 Laboratory Experiments

We performed crossing experiments between spiders from within the Introgression Zone (sourced from Southland) and crossing experiments between spiders from outside the Introgression Zone (sourced from Canterbury). In both groups, male *D. minor* were given the opportunity to mate with female *D. aquaticus* and male *D. aquaticus* were given the opportunity to mate with female *D. minor*. Single species trials were done to provide a baseline for comparison, where *D. minor* sourced from Waikato and *D. aquaticus* sourced from Nelson and Southland were mated within-species. Experiments took place after spiders reached adulthood in the lab, between November 2020 and January 2022, and individuals from different regions of the country were not intermixed during experiments. Trials occurred under white light, with the temperature at 20°C, between 1200 and 1800. A total of 70 trials were conducted (Supplementary Table 3). Due to uneven population sizes and difficulties in rearing, a small number of spiders were used more than once to increase replicates. However, only spiders that did not mate were reused, every spider was given a

new partner for each experiment, and they were given a minimum of 48 hours between trials to recover.

Females were fed the night before an experiment to ensure they were satiated, and then introduced to the mating arena (265 mm x 235 mm x 120 mm) prior to the male. Initially, we introduced the female at least ten minutes before the male, but in later experiments females were left overnight to increase the amount of chemical cues and webbing in the arena when it was observed that *D. minor* males seemed reluctant to court females in the single-species trials. We address possible issues with this approach below (see 3.4 Discussion). The arenas featured climbable mesh walls and a climbable ceiling. Males were added to the arena far from the female, to reduce the risk of premature male death due to female aggression. Trials were recorded using Sony FDRAX53 4K digital cameras and typically lasted for four hours, although experiments were often truncated by female aggression or cannibalisation of the male. Males would occasionally be removed from the arena if the female would not accept attempts at courtship and there was clear risk to the life of the male. Trials were occasionally extended if a long courtship or mount was underway.

Behaviours were coded using BORIS software (Friard and Gamba 2016). For each trial we recorded: whether a male courted; latency of the male to court (the time from the start of the trial to the male beginning courtship behaviour for the first time); mean courtship duration (the mean length of courtship instances for a trial, this was chosen as a more accurate representation of male behaviour because the total duration of courtship would be strongly affected by female aggression or male success leading to early copulation); whether the female attacked; the number of female attacks per hour (standardised due to variable trial length); whether the female recoiled from a male upon his approach; and whether the pair copulated or attempted copulation.

### 3.3.4 Statistical Analyses

To visually assess overlap of *D. minor* and *D. aquaticus* at the Waipara site, the locations of mature and penultimate spiders of each species were plotted based on their observed location in the transects. To assess population dynamics throughout the mating season, these data were also displayed on a time series plot. An apparent peak in male maturity at the beginning of the season was assessed using binomial exact tests to test for

synchronisation in male maturity. These data were used to address habitat overlap and timing of reproductive maturity.

We compared each of the behaviours described above between all four types of crossing experiments, including the single species trials (*D. minor* only and *D. aquaticus* only), to address the barriers of male courtship and female receptiveness. For each behaviour, we first compared the presence or absence of a behaviour, and then compared the frequency or duration of that behaviour for those trials in which it occurred. This approach modelled the importance of performing or not performing a behaviour and overcame the issue of violating model assumptions when zeros (trials where behaviours were not performed) were included.

Binary variables (probability of courting, female attack, and female recoil) were modeled with binomial or quasibinomial generalised linear models (GLMs). Numeric variables (latency to court, mean courtship duration, attacks per hour, and recoils per hour) were tested using GLMs with a Gaussian distribution, with log transformations when appropriate to meet model assumptions. Where significant differences were found, Tukey's exact tests were undertaken using the multcomp R package (Hothorn et al. 2023). All statistical analyses were performed in R 4.2.2 (R Core Team 2022).

### 3.4 Results

#### 3.4.1 Observations outside Introgression Zone

We found overlap between the two species' habitats outside the Introgression Zone, where several individuals were observed moving from their typical habitat and entering the areas more commonly occupied by the other species (Fig. 3.3).

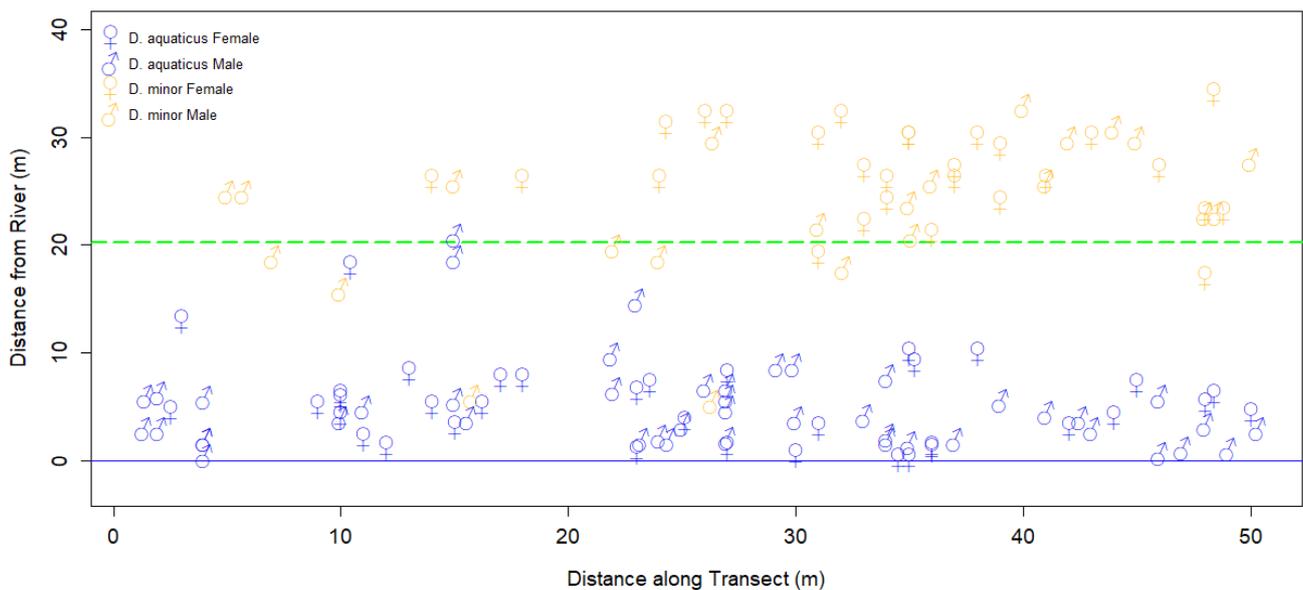


Figure 3.3. Location of spiders in Waipara monitoring transects, showing several wandering spiders are found in the habitat of the other species. Male and female symbols represent the distribution of penultimate and mature spiders at the Waipara Riverside outside the Introgression Zone, over the course of monitoring. The blue line represents the river's edge. Dotted green line represents the approximate location of the habitat boundary between the species.

Both species had a peak in male abundance in October 2020 (Fig. 3.4). Binomial exact tests show that the sex ratio for *D. aquaticus* was significantly male biased in October ( $p = 0.031$ ). There was a similar pattern in *D. minor*, although it was not significant ( $p = 0.054$ ). However, when analysed separately, there was a clear significant male-bias in *D. minor* sex ratio during the 22<sup>nd</sup> of October 2020 sampling period ( $p = 0.015$ ). Throughout the rest of the sampling months there was no significant difference in sex ratio in either species (Supplementary

Table 4).

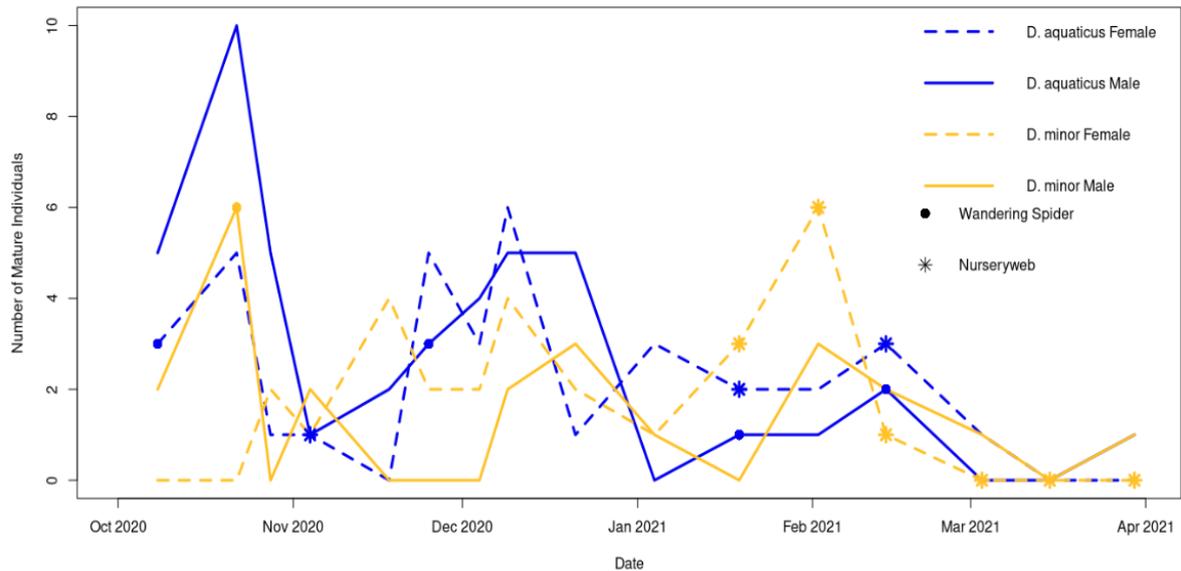


Figure 3.4. Number of mature and penultimate spiders over the 2020-2021 breeding season at the Waipara Riverside, outside of the Introgression Zone. Male abundance in both species is high in October 2020. Wandering spiders denotes spiders outside of their expected habitat. Nurseryweb indicates a spider on a nurseryweb, or a cluster of juveniles emerging from a web.

### 3.4.2 Size Dimorphism

Both species were sexually dimorphic in size (*D. aquaticus*  $t = -6.98$ ,  $df = 32.1$ ,  $p < 0.001$ ; *D. minor*  $t = -22.1$ ,  $df = 105.23$ ,  $P < 0.001$ ), with the females being significantly larger than the males (approximately 2 times in *D. aquaticus* and three and a half times in *D. minor*), and *D. aquaticus* being the larger of the two species (Fig. 3.5).

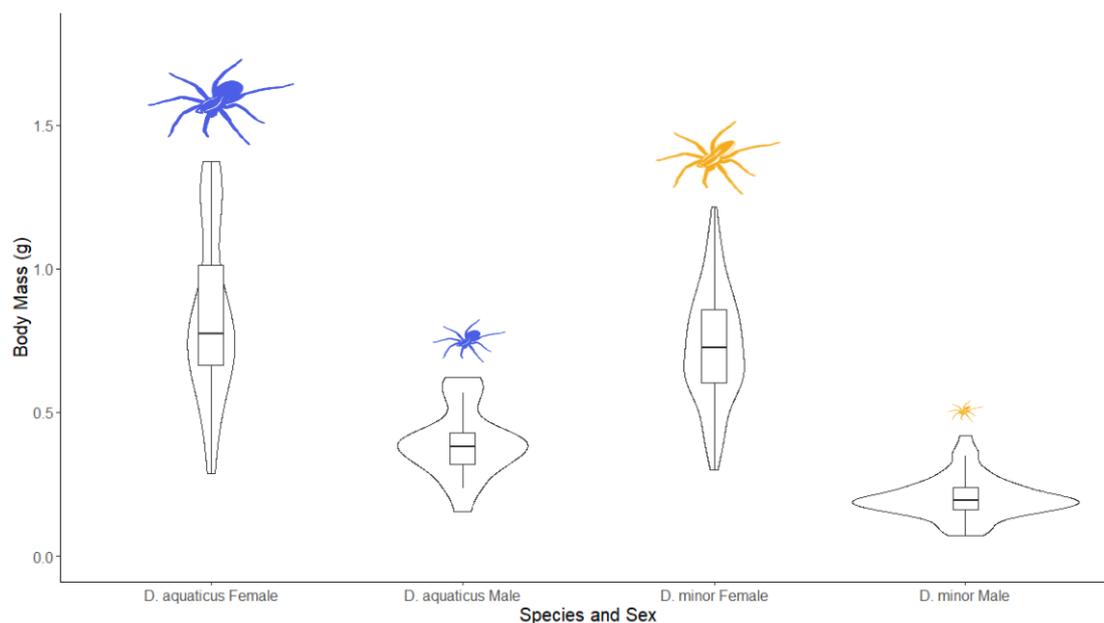


Figure 3.5. *Dolomedes aquaticus* is significantly larger than *D. minor*, with both species showing sexual size dimorphism. Boxes show interquartile range, with the central bar indicating the median. The curved lines represent the full spread of the data. The spider silhouettes above the bars are scaled relative to median body mass.

### 3.4.3 Male Initiation

Males in all types of experiments were willing to initiate courtship. The GLM indicated significant differences between species-sex combinations ( $df = 5$ , Residual Deviance = 14.17,  $p = 0.015$ ), however the Tukey's analysis found no pairwise significant differences (Supplementary Table 5).

We found significant differences in male courtship latencies among groups ( $F_{1,5} = 6.88$ ,  $p < 0.001$ ), although the only significant differences for latency to court was between groups with different species of males, with *D. minor* males overall having higher latency (for example, the *D. aquaticus* only experiments and the *D. minor* only experiments) (Supplementary Table 6, Fig. 3.6). We found no significant differences between males of the same species exposed to different females, or within/outside the introgression zone (i.e. a *D. minor* male has similar latency whether he is courting a *D. aquaticus* female or a *D. minor* female, or whether he is from inside or outside the introgression zone).

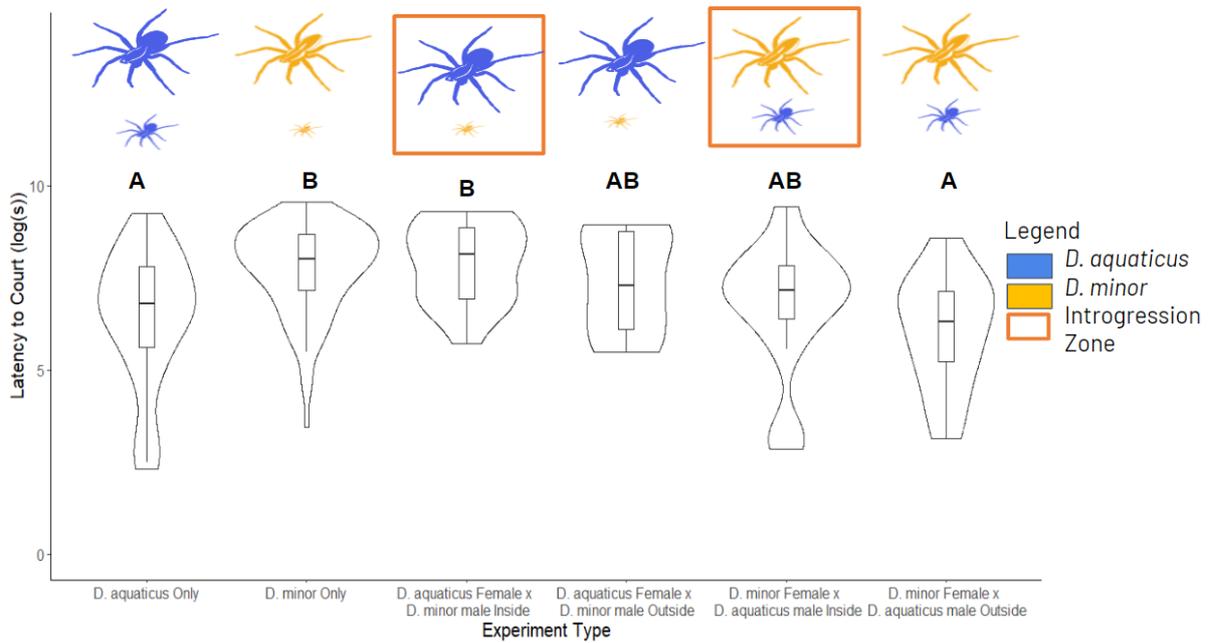


Figure 3.6. Male latency to begin courtship by experiment type showing males of the two species have significantly different courtship latencies, regardless of which female they are placed with. Boxes show interquartile range, with the central bar indicating the median. The curved lines represent the full spread of the data. Significant differences from the Tukey's Exact Test are shown by letter categories.

There was a significant difference in mean courtship duration among groups ( $df = 5$ ,  $F = 6.36$ ,  $p < 0.001$ ), with the groups from outside the Introgression Zone showing lower mean duration than the other groups (Fig. 3.7, Supplementary Table 7).

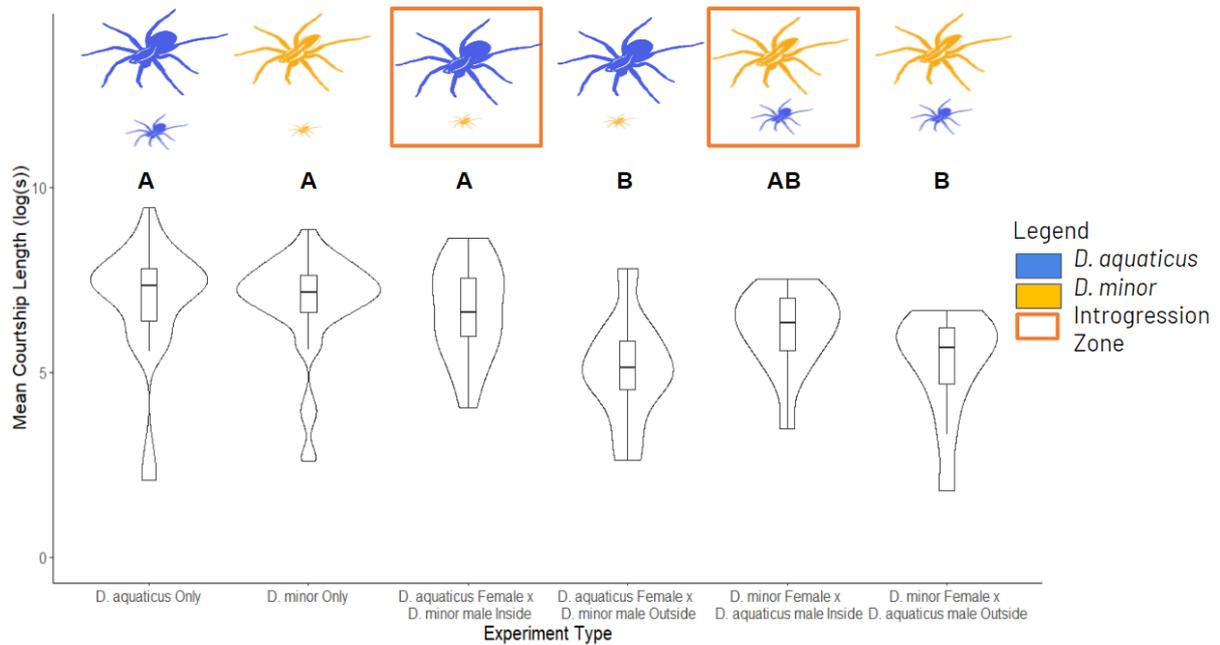


Figure 3.7. The relationship between mean courtship length and experiment type shows that groups from outside the Introgression Zone have significantly shorter courtships than other groups. Mean Courtship Length is the mean length of courtship event in a given experiment in experiments where courtship was initiated, measured in seconds on natural log scale, to meet assumptions of normality. Boxes show interquartile range, with the central bar indicating the median. The curved lines represent the full spread of the data. Significant differences from the Tukey's Exact Test are shown by letter categories.

### 3.3.4 Female Receptiveness

We found significant differences in the likelihood of female attack between different experimental groups ( $df = 5$ , Residual Deviance = 13.62,  $p = 0.018$ ), although there were no significant differences observed in the Tukey's analysis (Supplementary Table 8). However, for those females who did attack males, there were significant differences in the per hour rate ( $df = 5$ ,  $F = 8.00$ ,  $p < 0.001$ ) (Supplementary Table 9, Fig. 3.8). Specifically, *D. minor* females crossed with *D. aquaticus* males outside the Introgression Zone, showed significantly more aggression than any other group.

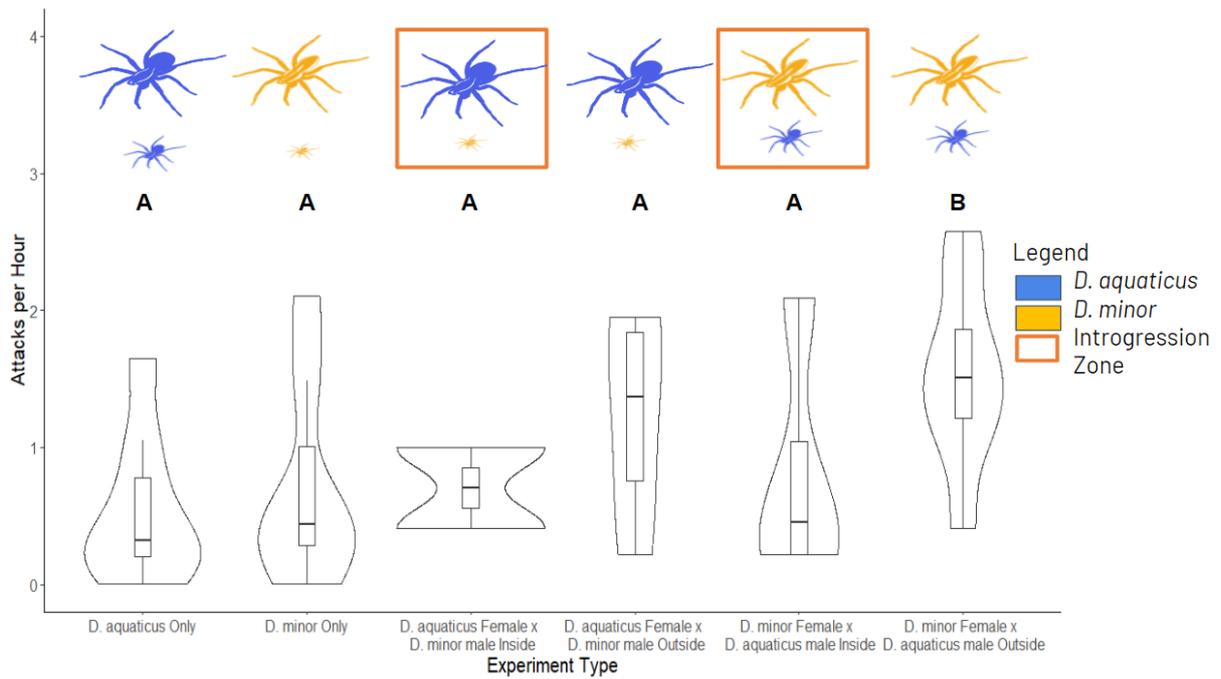


Figure 3.8. The relationship between female attack rates and experiment type, showing that *D. minor* females from outside the Introgression Zone crossed with *D. aquaticus* males show high levels of aggression. Attacks per hour are the number of attacks by females on males standardised by trial duration in experiments where females performed attacks. Boxes show interquartile range, with the central bar indicating the median. The curved lines represent the full spread of the data. Significant differences from the Tukey's Exact Test are shown by letter categories.

There was a significant difference in the probability of female recoil from male copulation attempt ( $df = 5$ , deviance = 29.70,  $p < 0.001$ ), although these were generally uncommon across all trials, with it being entirely absent in one experiment group (*D. aquaticus* females and *D. minor* males outside the Introgression Zone) (Fig. 3.9). Pairwise comparisons found a significant difference between *D. minor* females, who were significantly more likely to recoil when crossed with *D. aquaticus* males (regardless of in vs. outside the Introgression Zone) rather than with males of their own species (Supplementary Table 10).

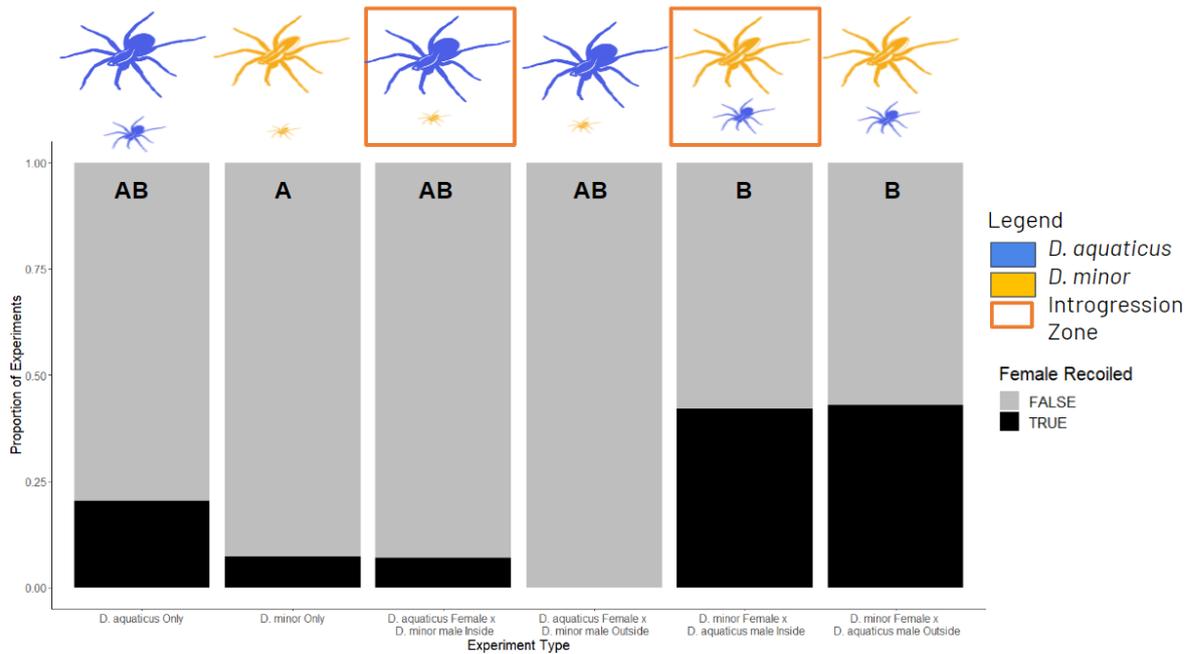


Figure 3.9. Proportion of experiments where females recoiled from male approach during courtship, showing that *D. minor* females had a high probability of recoiling away from *D. aquaticus* males. True represents experiments where any recoil behaviour was observed from females. False represents experiments where no recoil behaviour was shown. Significant differences from the Tukey's Exact Test are shown by letter categories.

### 3.4.5 Copulation Attempts

We found a significant difference in the probability of copulation attempts between experimental groups (df = 5, Deviance = 64.29,  $p < 0.001$ ). However, most experimental groups showed very low rates of copulation attempts, except for the *D. aquaticus* only trials (Fig. 3.10). Copulation rates were significantly higher in this group than in every other group (except *D. aquaticus* females paired with *D. minor* males outside the introgression zone where a lack of copulation attempts prevented statistical comparison) (Supplementary Table 11).

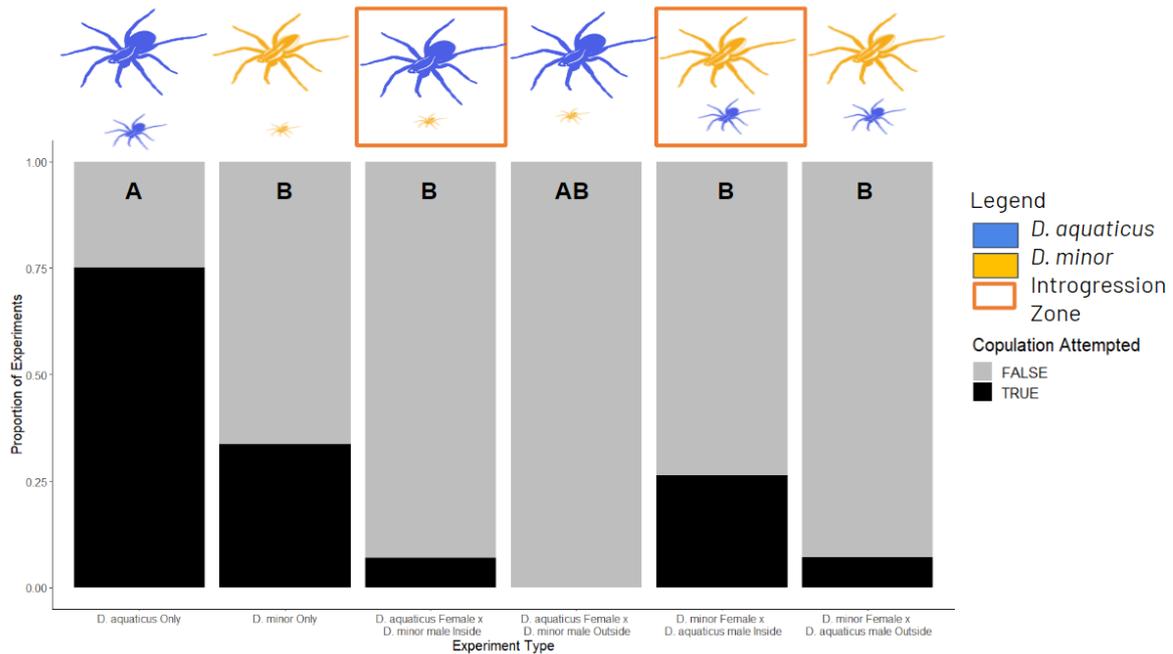


Figure 3.10. Proportion of experiments where copulation was attempted shows *D. aquaticus* only trials had high probability of copulation compared with every other group. “True” represents experiments where any copulation behaviour was observed. False represents experiments where no copulation behaviour was observed. Significant differences from the Tukey’s Exact Test are shown by letter categories.

### 3.5 Discussion

Here, we investigated life history and mating behaviour in promoting and limiting their influence on an introgression that is both one-way and geographically restricted. Our experiments revealed that two different behavioural mechanisms: male and female choice, act to limit the two different forms of introgression (Table 3.2). Male-choice potentially limits the geographic aspect of introgression for these species, with males from outside the Introgression Zone being unwilling to continue courting a non-conspecific female. In contrast, female rather than male behaviour may limit introgression to one way, preventing mating between *D. minor* females and *D. aquaticus* males. High levels of female attacks and recoiling in the *D. minor* female vs *D. aquaticus* male combination suggests that female-choice is key to maintaining the one-way aspect of the introgression. Our study shows mating behaviour can strongly influence introgressive gene transfer.

We found little evidence that either habitat overlap or timing of reproductive maturity are limiting factors to introgression outside of the Introgression Zone. Field observations at the Waipara site showed that both species move into the habitat of the other, albeit rarely. This is consistent with studies on multiple spider species that were found to readily introgress both by sharing habitats and wandering between habitats (De Busschere, Van Belleghem, and Hendrickx 2015; Hedin, Foldi, and Rajah-Boyer 2020; Oxford and Croucher 2014). Our observations also showed that both species' males mature at the same time at this site, indicating that these males would be seeking mates simultaneously. Therefore, encountering reproductively mature individuals of the opposite species is not likely to prevent, introgression. Whilst our methods are limited by lack of comparison between outside and inside the Introgression Zone, they do demonstrate the distinct possibility of the two species meeting regularly outside the zone.

Table 3.2. Summary of evidence for barriers to introgression. *D. aquaticus* males encounter a strong barrier of female receptiveness from *D. minor* females. *D. minor* males encounter a barrier in their own willingness to court *D. aquaticus* females outside of the Introgression Zone. In both these cases, it cannot be assumed that these are the only barriers to introgression (indeed, *D. aquaticus* males have shown attempted copulation behaviour with *D. minor* females), but they are the first substantial barriers to introgression in these combinations. This is why subsequent potential barriers have been marked with question marks rather than crosses, as it is uncertain whether these factors represent further barriers or not. A = *D. aquaticus*. M = *D. minor*. Black square = Inside Introgression Zone, Grey Square = Outside Introgression Zone. Green Tick = That the barrier is capable of being broken, at least in some cases. Red cross = The barrier prevents introgression. Orange Question Mark = The status of this barrier is uncertain and requires further investigation. Barriers are arranged in order from left to right based on when they appear in the spiders' life history. The final column represents whether the two species are capable of introgressing based on current genetic evidence (Lattimore et al., 2011; Vink & Dupérré, 2010).

Cross by Location	Habitat Overlap	Timing of Reproductive Maturity	♂ Courtship	♀ Receptiveness	Attempted Copulation	Post Copulatory Factors	Capable of Introgression
[A♀] x [M♂]	✓	✓	✓	✓	✓	✓	✓
[M♀] x [A♂]	✓	✓	✓	✗	?	?	✗
[A♀] x [M♂]	✓	✓	✗	?	?	?	✗
[M♀] x [A♂]	✓	✓	✓	✗	?	?	✗

However, there are environmental factors that this study cannot address. These include habitat structure inside and outside the Introgression Zone and whether sites where the two species can interact are more common in the south, leading to more likelihood of interbreeding. It could also be the case that historically, sites in Introgression Zone were more favourable to introgression, but such conditions no longer exist even within the zone. It has also been demonstrated that animals can be less choosy for mates when breeding seasons are short (Tinghitella et al. 2013), meaning that spiders in the far south within the Introgression Zone could be less choosy about their mates under natural conditions. Additionally, Wirtz (1999) theorized that females should mate with males of other species, when males of their

own are rarer. This leads to the hypothesis that *D. aquaticus* males are rare in the Introgression Zone compared to *D. minor* males. Whilst both species are common both inside and outside the Introgression Zone, we require further fine-scale investigation of population dynamics at each location to test these hypotheses.

Variation in male courtship behaviour may partially explain the geographic limitation of introgression in this genus. This is consistent with evidence from other taxa, where male ability to secure matings is an important factor in asymmetric hybridization (While et al. 2015). As was expected, we observed no issues with male courtship or female receptiveness for *D. minor* males and *D. aquaticus* females from inside the Introgression Zone, as this is the expected cross that is observed in nature (Lattimore et al. 2011; Vink and Dupérré 2010). While no combination of spiders showed any difference in initiating courtship (except for differences in latency discussed below), spiders from outside the Introgression Zone showed lower mean courtship lengths than other equivalent groups. For *D. aquaticus* males this may be explained by female aggression (discussed below), but this is not the case for *D. minor* males.

*Dolomedes minor* males from outside the Introgression Zone spent less time courting per courtship event compared to other groups, suggesting they were unwilling to pursue courtship effort over time. Shorter courtship does not seem to have been caused by female avoidance or aggression. This shorter courtship duration may indicate that males are only able to identify the female after close inspection. A male beginning to court a female may not have fully identified her as a conspecific or assessed her status, as pheromone cues can be identical between introgressing spider species (Oxford and Croucher 2014). Only upon approaching a female would a male be able to fully assess a female's potential suitability as a mate. Additionally, given that a large role of courtship is to reduce female aggression and avoid pre-copulatory cannibalization (Johnson & Sih, 2005; Zhang et al., 2022a), it would be adaptive to perform courtship in the presence of a female, even if the male has no intention to copulate with her. This lack of courtship effort could also be due to changes in our experimental setup, with females from outside the Introgression Zone being left to acclimatise to the arena for less time, and therefore leave fewer pheromones in the arena. However, given this did not have an effect on male likelihood to court or male latency to court, we find this to be unlikely to have had a significant impact.

It is less clear why a male *D. minor* outside of the introgression zone does not see a *D. aquaticus* female as a suitable mate. Copulation between this combination of spiders may be mechanically impossible. This would imply that the genitalia of one or both *Dolomedes* species are different inside vs. outside the Introgression Zone. North-south clines in the genitalia of New Zealand invertebrates have been described before (Shen and Leschen 2018), and *D. minor* male genitalia are known to have a significant amount of variation (Vink and Dupérré 2010). A morphometric analysis of the relevant genital structures inside and outside of the Introgression Zone is required to test this hypothesis.

Courtship latency was different between the males of the two species. *Dolomedes minor* males had longer latency than *D. aquaticus* males, regardless of which female they were courting. Latency period in spider courtship has been associated with courtship effort in some experiments (Eberhard, Möller, and Uhl 2021; Fisher and Price 2019) so males with longer courtship latency could be choosier about their mates than those with shorter courtship latency. This is supported by the substantially different mating rates between the two species, with *D. minor* having longer latency and fewer matings than *D. aquaticus*. Within this context, *D. aquaticus* court without proper information on the female they are courting, making them maladapted for approaching female *D. minor*. Assuming there is genital incompatibility between *D. minor* males and *D. aquaticus* females outside the Introgression Zone, it follows that a male would not waste time attempting to court and copulate with an unsuitable female. Given *D. minor*'s high courtship latency and apparent choosiness, they would have ample time to gather this information compared with *D. aquaticus* males, which may limit introgression outside of the Introgression Zone. This is a counter-intuitive situation where bolder and less choosy males are unable to take advantage of interbreeding, where cautious and more reserved males are.

Female receptiveness is a significant contributor to the one-directional nature of the introgression between *D. minor* and *D. aquaticus*. Regardless of whether they were located inside or outside of the Introgression Zone, female *D. minor* often moved away at high speed upon *D. aquaticus* male approach. Furthermore, in experiments with spiders from outside the Introgression zone, females showed elevated levels of aggression. Female recoiling behaviour was almost entirely absent from other experimental groups, suggesting there is a large difference in the way that *D. minor* females perceive *D. aquaticus* males, compared to

how they perceive conspecific males and how *D. aquaticus* females perceive males of both species. Female choice plays an important role in other introgressive systems (Morgado-Santos et al. 2015). The relative size of participants also plays an important role in many spider mating systems (Schneider and Fromhage 2010), and may explain the patterns observed here. In *Dolomedes*, larger males (relative to the size of their mate) have been shown to have a significant advantage in achieving copulations (Schoenberg, Hebets, and Sullivan-Beckers 2022). In the *D. aquaticus* male and *D. minor* female cross, the male is almost the same size as the female, however, this is counter to the size difference between *D. minor* and their conspecific males (who have the larger sexual size discrepancy of the two species). This could be responsible for the female's adverse reaction to the male, perceiving this larger male as undesirable. Indeed, in *D. triton* and *D. fimbriatus* larger males are more likely to survive female attack, meaning the female is less in control of the encounter (Kuntner and Coddington 2020). This could indicate a female preference for a small male who can be more easily cannibalised, a criterion the large *D. aquaticus* males do not fulfill. While we cannot test for the effect of body size on mating outcome across all these groups, because size is strongly co-linear with species, it may partially explain the limitation to introgression in this direction.

We note that the rates of courtship and attempted copulations in our experiments may be higher than would be seen in the wild due to the unnatural setting of experimental arena. In the wild, females would be able to flee further from males than the space in the arenas allowed for, and the male would potentially have difficulty reacquiring the female, especially if there are males of the female's own species present. *Dolomedes aquaticus* are known to hide in water when threatened (Forster and Forster 1973). Therefore, a female recoiling from male approach would likely be an even bigger impediment to mating in the field than it was in the lab, supporting the conclusion that female-choice limits the one-way nature of the introgression, despite the witnessed attempted copulations.

Attempted copulations between *D. minor* females and *D. aquaticus* males could, however, happen in the wild, if some other factor prevents introgression. This could be a post-copulatory factor (such as hybrid infertility, or post-mating female choice), or it could be a limitation of the mechanics of the interlocking genitals during copulation (a classic lock-and-key barrier (Eberhard 1992)), with sperm unable to be successfully transferred. Mechanical

incompatibility during copulation has been speculated to be a limiting factor for introgression between other spider species that are even more morphologically similar than *D. minor* and *D. aquaticus* (Oxford and Croucher 2014), and has been demonstrated to be isolating in other taxa including fish (Jennings et al. 2023) and carabid beetles (Sota and Kubota 1998). Such a question would be best answered by morphometric analysis of the relevant genital structures in both species to explore the mechanics involved in attempted copulations between different combinations of spiders. This could help explain the presence of attempted copulations in combinations of spiders known to be unable to interbreed, as well as potentially explain the male choice limitation of the introgression.

The findings of female-choice preventing one aspect of introgression (i.e., the species direction) and male-choice preventing another (i.e., the geographic limitation) presents a fascinating dichotomy for future research. On the one hand, female-choice is known to be an important aspect of spider mating systems in general (Neumann and Schneider 2011; Schneider, Uhl, and Herberstein 2015), and size difference as a predictor of mating success has been demonstrated in *Dolomedes* specifically (Schoenberg, Hebets, and Sullivan-Beckers 2022). This would imply that for some introgressive systems, knowledge about the general mating dynamics of the group can be applied to explain aspects of the introgression. An example where such an application could be applied is the introgression between *Latrodectus* spider species in New Zealand (Vink et al. 2008), given how well *Latrodectus* mating systems have been studied (Golobinek, Gregorič, and Kralj-Fišer 2021; Modanu, Michalik, and Andrade 2013; Neumann and Schneider 2020; Scott 2020; Sivalingham and Mason 2021; Vink et al. 2011). On the other hand, male-choice also represents a barrier when the relative size difference seems favourable to introgression in *Dolomedes*. These are counter-intuitive aspects of this introgression, based on the prevailing knowledge of *Dolomedes* mating systems. Hence, caution clearly needs to be used when generalising about possible causes and limiting factors of introgression, based on general mating system knowledge.

Future work on this topic could seek to address the impact of environmental factors discussed above, by comparing between sites inside and outside the Introgression Zone. Relevant factors would include the rate of encounters along habitat borders, and the timing of the life cycles of the two spider species inside and outside the zone. Also of relevance

would be further work describing the introgression itself. Currently, this introgression is described from a small number of *D. minor* with *D. aquaticus* located within the Southland region (Lattimore et al. 2011; Vink and Dupérré 2010). This is sufficient to indicate the location of introgression, the direction of the species cross and show evidence of recent and/or ongoing introgression with multiple introgression events. However, more intensive sampling and genetic could find the true size and borders of the Introgression Zone, and techniques such as the use of microsatellite loci (Ji et al. 2004) or ddRADseq (Bougie, Brelsford, and Hedin 2024) could provide more information on the rate of hybridisation and the evolutionary timeline of introgression.

Our approach highlights the importance of incorporating behavioural approaches to investigate the causes and maintenance of introgression. Introgression studies typically focus on identifying biogeographic patterns introgression using genetic techniques (Chang, Song, and Zhou 2007; De Busschere, Van Belleghem, and Hendrickx 2015; Hedin and Lowder 2009; Johannesen and Veith 2001), and rarely interrogate behavioural mechanisms. By stepping through each potential barrier in turn using a series of crossing experiments that interrogate the biology of the species from habitat usage through to male and female mating behaviour, we were able to determine the specific barriers for each type of introgression limitation, finding behaviour to be key to determining the extent of introgression in this instance.

**Chapter 4: No evidence for genital shape morphology affecting introgression in New Zealand *Dolomedes* fishing spiders**



*D. minor* nurseryweb with spiderlings – Helen Macky

## 4.1 Abstract

Genital diversity has been a topic of debate among evolutionary biologists for decades. The classical lock-and-key hypothesis states that genital diversity serves to prevent different species from interbreeding. Whilst this hypothesis has been controversial for some time, recent evidence has pointed to a role of genitalia in isolating species in some taxonomic groups. Spiders are a compelling group to study the potential isolation mechanisms of genitalia. Hook structures on the male genitalia attach to the female and are therefore focal points for studies of the role of genital shape. Specifically, the retrolateral tibial apophysis (RTA), found in the RTA clade of spiders, is a structure highly diverse in its structure and key to successful sperm transfer. *Dolomedes aquaticus* and *D. minor* are New Zealand fishing spiders, undergoing introgression (movement of genes between species). The introgression is one-way, with genes flowing from *D. aquaticus* to *D. minor*, and geographically limited to the extreme south of the species' shared range. Here, we used micro-CT and 3D geometric morphometrics to analyse shape variation in the male RTAs. If shape variation isolates *D. minor* from outside the Introgression Zone from the other spiders, then this could explain this pattern of gene transfer. We found highly variable genital shape in both species, and there was no evidence of genital shape or size limiting the interbreeding of these species. Further work should include analysis of the female genitalia to examine its potential role in this introgression.

## 4.2 Introduction

Evolutionary biologists are obsessed with the topic of genitals. Animal genitalia are strikingly diverse, both in males (Eberhard 2010; David J. Hosken and Stockley 2004; Langerhans, Anderson, and Heinen-Kay 2016) and females (Anderson and Langerhans 2015; Orbach et al. 2021; Sloan and Simmons 2019), and for this reason are often used by taxonomists in delineating species (Masly 2011). Explaining this diversity has been a topic of research for decades across many taxa (Eberhard and Huber 2010; Langerhans, Anderson, and Heinen-Kay 2016; Masly 2011; Orbach et al. 2018). Additionally, given their crucial role in reproduction, genitalia are key to studies of speciation and the mating barriers between

species (Alichi 2022; Eberhard and Huber 2010; W. G. Eberhard and Ramirez 2004; Huber 1993). The lock-and-key hypothesis is related to both of these phenomena.

For decades, Dufour's (1844) lock-and-key hypothesis has been controversial. The hypothesis states that the complexity of genitalia is to prevent maladaptive interbreeding between species, thereby drawing a direct link between the diversity of genitalia and their function in mating. The hypothesis is compelling in its intuitive nature but has received heavy criticism over the years. Critics have pointed to examples where extreme differences among species in genital morphology do not prevent crosses (Eberhard and Ramirez 2004; Masly 2011); that female genitalia lack variety "locks" to the male "keys" (Shapiro and Porter 1989) species-specific genitalia in species that do not overlap in range, and therefore should not need mechanical isolation (Eberhard and Huber 2010); and the importance of genitalia to stimulate the female during mating, thus placing genital shape under inter-sexual selection via female choice (Eberhard 2010; Masly 2011).

However, several of these criticisms of lock-and-key have issues of their own. For example, while Masly (2011) notes several cases where lock-and-key does not function to prevent crosses, he also notes several invertebrate taxa where an effective lock-and-key mechanism is well demonstrated, including in Lepidoptera, Araneae, Coleoptera (with a more recent example studied by Alichi (2022)), and Diptera. Recent evidence also shows that female genital diversity is more extensive than previously thought and coevolves with male genitalia, therefore providing the "locks" that were thought to be missing (Anderson and Langerhans 2015; Kuntner et al. 2016; Lupše, Cheng, and Kuntner 2016; Sloan and Simmons 2019). Finally, whilst females of many taxa have genitalia sensitive to male stimulation (Masly 2011; McPeck, Shen, and Farid 2009; Robertson and Paterson 1982) this cannot be the case in taxa where female genitalia have no innervation whatsoever, as is the case in many spider taxa (Eberhard and Huber 2010; Huber 1993). In such cases, it has been argued that instead of being directly selected for by female stimulation, genital structures in these species are selected on their ability to successfully transfer sperm to females of their own species, rather than their ability to prevent sperm transfer to other species (Eberhard and Huber 1998). Whilst lock-and-key is only one selective force driving the evolution of genitalia, many researchers argue that it should not be ignored, and that genital form is still

an important factor in the interactions between species ((Alichi 2022; Anderson and Langerhans 2015; Langerhans, Anderson, and Heinen-Kay 2016; Sloan and Simmons 2019).

Spiders are a compelling group for the study of genital diversity and its impact on speciation. Spiders are well known for extreme courtship practices and mating systems resulting from sexual selection, such as sexual cannibalism and monogyny (Schneider and Fromhage 2010). Female spiders are also well suited to impose strong selection on males, with a significant size advantage to females, male-biased sex ratios, polyandry and sperm storage organs present in many species (Fromhage and Schneider 2012). Most spider taxa also lack nerves in the female copulatory organs (Berendonck and Greven 2005; Huber 1993) (exception found in (Aisenberg, Barrantes, and Eberhard 2015)). This precludes any hypotheses of female stimulation as a selective agent driving genital diversity and provides comparison with other taxa where this is the case (Eberhard & Huber, 2010). Additionally, whilst males have an extremely complex genital system involving intromittent genitalia inflated via hydrostatic pressure, spider copulation relies on male genitalia hooking onto the female genitalia (Eberhard & Huber, 2010). Therefore, these hooks represent a useful focal point for studying spider copulation mechanics (Aisenberg, Barrantes, and Eberhard 2015; Eberhard and Huber 2010; Hernández, Molina, and Aisenberg 2019; Poy et al. 2020). In fact, the most common of these hooks, the retrolateral tibial apophysis (RTA), present in the RTA clade of spiders, has been shown to be highly species-specific and diverse due to sexual selection for its role in copulation (Eberhard and Huber 2010; Huber 1995).

Micro-computed tomography (herein 'Micro-CT') is often used in studies of invertebrate genitalia. It has several key advantages over traditional techniques, including being able to: render a three-dimensional (3D) image of the target structure (as 2D images are often insufficient for understanding complex organs (Orbach et al. 2018; 2021), analyse surface detail and internal structures, and non-destructively sample specimens (Michalik et al. 2013). Micro-CT has been applied to a diverse range of evolutionary problems including describing behaviour during seed beetle copulation (Dougherty and Simmons 2017), investigating coevolution of male and female genitalia (Sloan and Simmons 2019), and quantifying arthropod brain size (Sombke et al. 2015). A key strength of micro-CT is that it can be combined with geometric morphometric analysis, which quantifies and compares variation in trait shape across individuals or populations (Adams, Rohlf, and Slice 2004;

Mitteroecker and Schaefer 2022). Geometric morphometrics has even previously revealed clines in the shape of spider genitalia (Wilson et al. 2021).

Genitalia are typically studied in fully reproductively isolated species (Alichi 2022; Masly 2011), which leads to a lack of knowledge on the role of genital shape in speciation (Langerhans, Anderson, and Heinen-Kay 2016). A model group where species are only partially isolated and the RTA is variable in shape across a geographic range, could be informative on the role of the RTA (and male genitalia generally) in speciation. This is the case for two species of New Zealand fishing spiders: *Dolomedes aquaticus* and *D. minor* (Araneae: Pisauridae). The two species are undergoing one-way introgression, with mitochondrial DNA being transferred from *D. aquaticus* to *D. minor* (Vink and Dupérré 2010). This indicates that *D. minor* males are able to mate with female *D. aquaticus*, but that male *D. aquaticus* are unable to mate with female *D. minor*. The introgression is also geographically limited, only taking place in the extreme south of New Zealand's South Island (the Introgression Zone), despite the species co-occurring throughout the range of *D. aquaticus* (Chapter Three) (Lattimore et al. 2011; Vink and Dupérré 2010). This is consistent with other New Zealand species that show a north-south cline in their genital morphology (Shen and Leschen 2018).

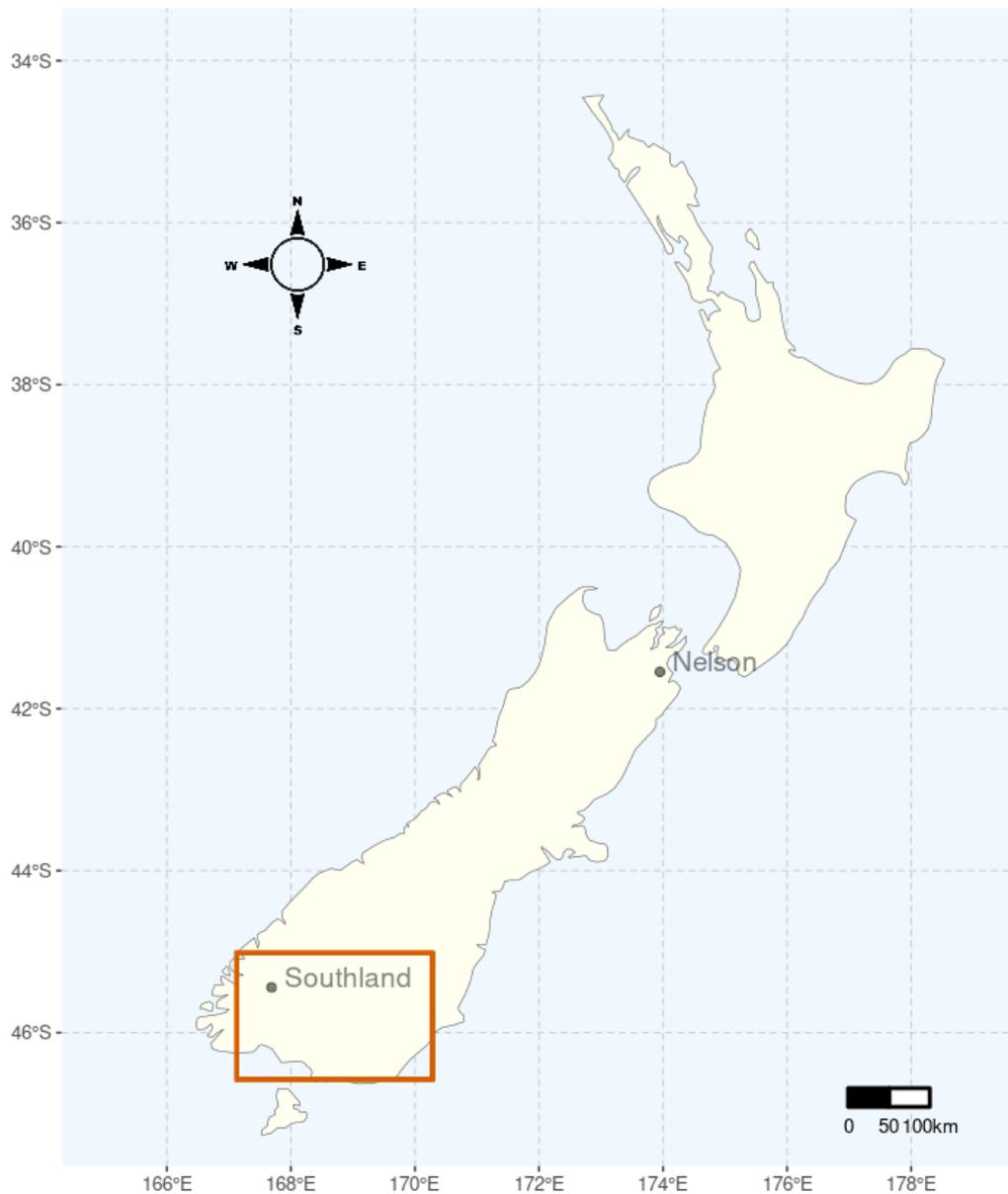
In this study, we used micro-CT imaging and 3D geometric morphometrics to analyse variation in the shape of RTAs of New Zealand *Dolomedes*, both inside and outside their Introgression Zone, in order to elucidate the role of the RTA in the maintenance of species barriers. The limitations on the *Dolomedes* introgression have been partially explained through behavioural work. *Dolomedes minor* females reject male *D. aquaticus* and male *D. minor* from outside the Introgression Zone are less likely to court *D. aquaticus* females. However, we previously witnessed attempted copulations between several combinations of spiders, and we therefore hypothesised that genital morphology shapes the behaviour of these spiders and the nature of their introgression (Chapter Three). Evidence to date suggests that the external genitalia of the females of both species shows little variation, as do the RTAs of *D. aquaticus* (Vink and Dupérré 2010). However, the RTA of *D. minor* appears to be highly variable, with some forms visually resembling the RTA of *D. aquaticus* (Vink and Dupérré 2010). However, these observations were not made with the goal of quantifying shape variation based on geographic location. Here, our aim is to investigate whether the

variation in the RTA of *D. minor* could explain both the ability of these two species to introgress in a small part of their overlapping range, and what the limitations of the introgression are. If the RTA of *D. minor* in the Introgression Zone are more similar to *D. aquaticus*, then this correlation could explain why male *D. minor* are able to mate with female *D. aquaticus* in this part of their geographic range. We predict that (1) the RTAs of the two species will be significantly different; (2) the RTAs of *D. minor* outside the Introgression Zone will be significantly different from *D. aquaticus*; (3) that the RTAs of *D. minor* inside the Introgression Zone will be similar to those of *D. aquaticus*, potentially even (4) the RTAs of *D. minor* inside the Introgression Zone will be an intermediate form between *D. aquaticus* and *D. minor* outside the Introgression Zone, explaining why these spiders can mate with females of both species.

## 4.3 Methods

### 4.3.1 Specimen Collection

*Dolomedes aquaticus* and *D. minor* were collected from the Nelson and Southland regions of Aotearoa New Zealand from 2021 to 2022 with permission from the relevant local councils (Marlborough District Council, Southland District Council) to be used in laboratory experiments (See Supplementary Table 1 for a full list of collection locations) (Fig. 4.1). Five specimens of each species were collected from the two localities, giving five spiders from each species inside and outside of the Introgression Zone and a total sample size of 20. Spiders were hand-collected at night using a headtorch to detect eyeshine Benson & Suter (2013). All spiders were collected as adults, except for one *D. aquaticus* specimen from Southland who was collected in his penultimate moult and raised under laboratory conditions. Specimens were euthanised in a -18 °C freezer and stored in ethanol until preparation.



*Figure 4.1. Map of New Zealand showing collection locations of spiders for CT scanning. Spiders were collected from Nelson and Southland. Orange box indicates the location of the Introgression Zone.*

#### 4.3.2 Specimen Preparation, Scanning and Reconstruction

Specimens were dried using a series of ethanol soakings and hexamethyldisilazane (HMDS) drying (California Department of Food and Agriculture, n.d.; Heel et al. 2022). This series of soakings in increasing ethanol concentrations and HMDS remove all water from the specimens, whilst retaining the original specimen shape (i.e. the specimens do not shrivel when dried in this fashion).

Specimens were scanned at the University of Auckland's Micro-CT facility, using Bruker SkyScan 1272 & 1172 desktop Micro-CT units. Scans were focused on the front portion of the spiders' cephalothoraxes, with both pedipalps in frame. To account for the size differences of the specimens, voxels ranged from 1.3 $\mu$ m to 1.7 $\mu$ m (source voltage: 55kV, source power: 170 $\mu$ A, exposure length: 650 – 700ms, rotation: 0.2° through 360°, total scan time ~42 minutes). Reconstruction was performed in NRecon 2.1.0.1.

### 4.3.3 Segmentation

Using the scans, the left RTA of each spider was manually segmented using Amira 5.3.3. Masking was used to define the outer edges of the RTA, and segments were filled so that the rendered models were not hollow. A challenge was presented in defining where the lower edge of the RTA is, as the RTA is a complex structure and continuous with the tibial cuticle, with no notable difference in tissue density. To maximise consistency, the base of the RTA was defined relative to the angle of the surface leading to the tip of the RTA. That is, starting at the RTA tip and working down, when the tibial cuticle turned away from this surface at a significant angle, this was defined as no longer part of the RTA (Fig. 4.2). This avoided segmenting unnecessary material at the base of the RTA and the rest of the pedipalp, and instead focused on the biologically relevant points and faces of the RTA.

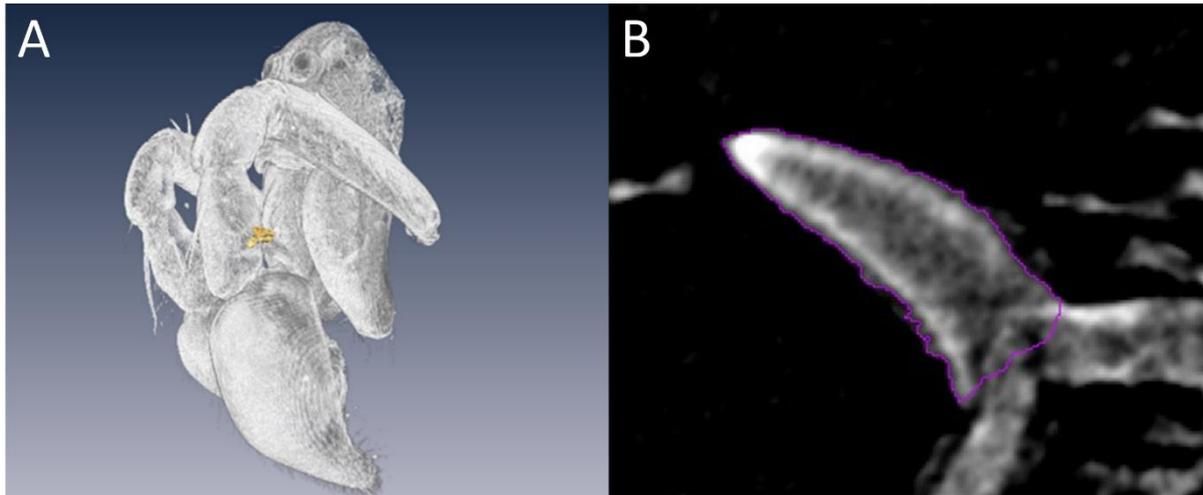


Figure 4.2. A: 3D reconstruction of a *D. aquaticus*. The position of the RTA highlighted in yellow, on the pedipalp. B: Cross section through an RTA of *D. minor*, with the area segmented outlined in purple. The RTA was segmented by starting at the RTA tip and working down towards the tibial cuticle. When the tibial cuticle turned away from this surface at a significant angle, this was defined as the bottom of the RTA.

These segments were rendered into 3D models in .ply format and further processed in Blender 3.6, by smoothing the base of the RTA to remove any segmentation artefacts. Processing was continued in Instant Meshes to standardise the size and distribution of polygons. Any errors (flipped normals and holes) were corrected in MeshMixer, using the Inspector and Auto-Repair functions. Given the issues in standardising the base of the RTA, the bases were sliced with spheres (scaled to have a diameter double the length of the longest axis in the RTA model) using the Boolean Difference function of MeshMixer. This created models with curved bases, allowing redundant material to be removed and preserving as much relevant material as possible in a way that was consistent between specimens. A *D. aquaticus* specimen from the Introgression Zone had a malformed RTA and was removed from further analysis, leaving a total sample size of 19.

#### 4.3.4 Analyses

Generalised Procrustes Surface Analysis (GPSA) was performed on these models using software developed by Pomidor et al (2016). GPSA was used over Generalised Procrustes Analysis (GPA), as GPSA is a landmark free technique. In GPA, researchers place landmarks on points that are homologous between samples. However, this is not practical in cases

where points need to be placed on smooth continuous surfaces, or in cases where homologous points do not exist (Goswami and Clavel 2024; Mitteroecker and Schaefer 2022). RTAs have “teeth”, which protrude from the structure. These teeth would usually be excellent points for landmark placement, but they vary in number between specimens, making it impossible to assign homologous points between samples. Given this, we used GPSA, where points are spread evenly over the surface of the entire model. The resulting dataset are coordinates of homologous points across all the specimens. Pomidor et al (2016) recommend dimension reduction of the resulting dataset of homologised points (which in the case of this analysis had 21,264 dimensions) using Principal Components Analysis (PCA). These principal components were used to visualise the data and detect shape patterns.

The first 6 principal components were extracted and visualised, with each of the subsequent components being compared against the first principal component. Four categories summarising location and species of specimens (*D. aquaticus* inside Introgression Zone, *D. aquaticus* outside Introgression Zone, *D. minor* inside Introgression Zone and *D. minor* outside Introgression Zone) were visually compared using convex hulls, to look for overlap of these categories in the principal components.

We also used PERMANOVA (as data did not meet assumptions for MANOVA) based on the first 6 principal components (Spanke et al. 2023; Wasiljew et al. 2021) to test for significant differences in the shape of the RTA between the two species. PERMANOVA was also used to test for significant differences in the shape of the RTA between four categories: *D. aquaticus* inside Introgression Zone, *D. aquaticus* outside Introgression Zone, *D. minor* inside Introgression Zone and *D. minor* outside Introgression Zone. For the latter PERMANOVA, a pairwise PERMANOVA post-hoc test was performed, with Bonferroni significance correction.

Traditional size measurements of the RTA were also taken from the rendered models. These were performed in Amira, with measurements being scaled based on voxel size to find the true size of the structures. Each RTA was measured for the height and width of the main face, and the length of the outcropping arm-like structure. These measurements were compared using t-tests and ANOVAs to test for significant differences between species and category. RTA height was log transformed to meet the model assumptions. Post-hoc analyses used Tukey HSD.

PERMANOVA was performed using Vegan Community Ecology Package (Oksanen et al. 2022) and Tukey's exact tests were undertaken using the multcomp R package (Hothorn et al. 2023). All analyses following the initial GPSA were done in R 4.2.2 (R Core Team 2022).

## 4.4 Results

The RTAs of *D. minor* and *D. aquaticus* were highly variable. Sites of variation include the top edge of the teeth, the bottom edge, the attachment point for the outcropping arm, and several points on the arm itself (Fig. 4.3). The principal components produced by the analysis explained a relatively low percentage of the variance in the data. The first six principal components were used, based on diminishing returns of explained variance after this point. These components account for ~60% of the variation in the full dataset (Supplementary Figure 2). This indicates there are multiple sources of variation within the data, and no single dominant trait that is consistently different between specimens. Rather, there is a relatively large set of more subtle differences.

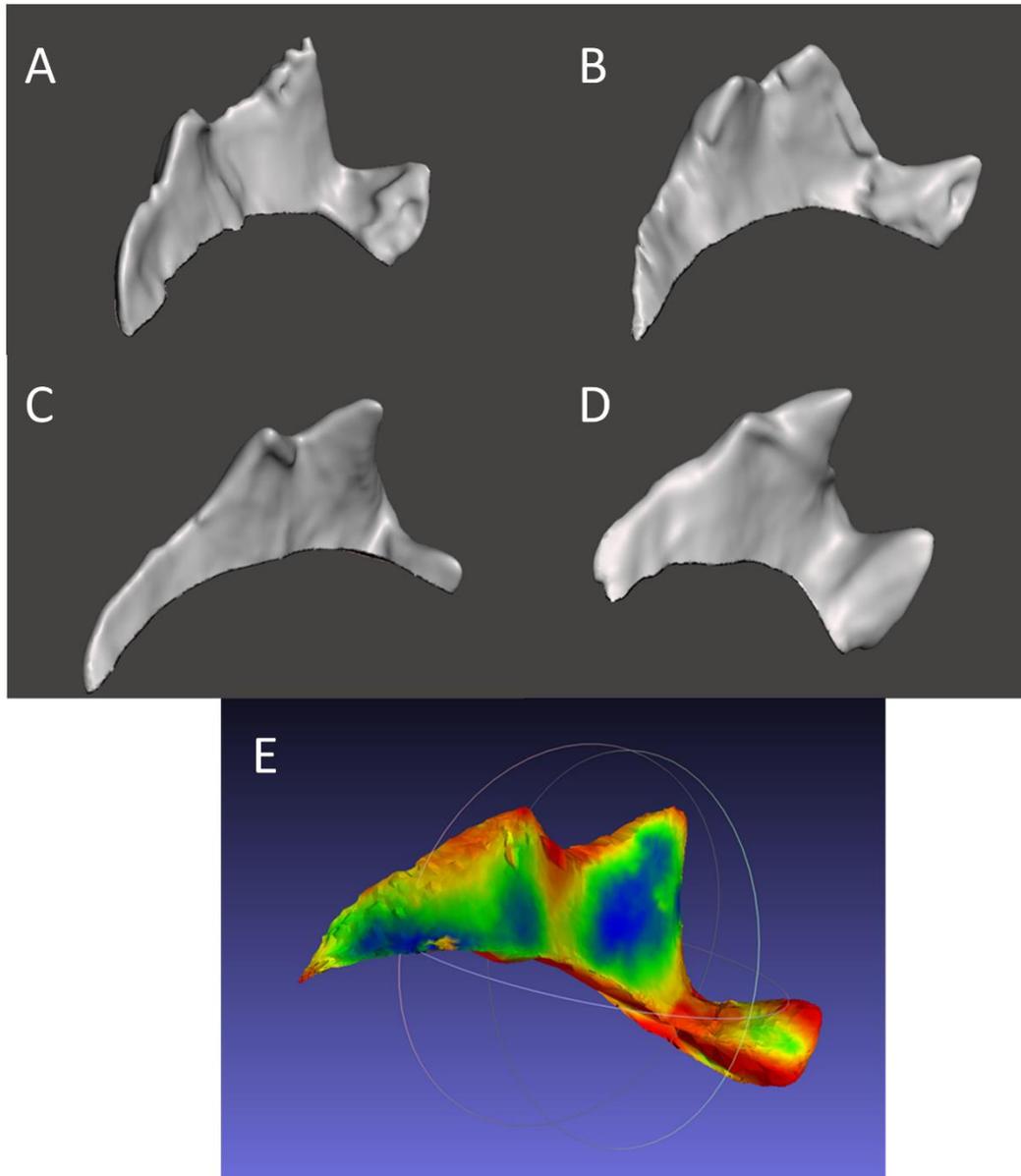


Figure 4.3: 3D models of segmented and processed RTAs. A: *D. aquaticus* specimen from outside the Introgression Zone; B: *D. aquaticus* specimen from inside the Introgression Zone; C: *D. minor* specimen from outside the Introgression Zone; D: *D. minor* specimen from inside the Introgression Zone; E: Heatmap of variation on average specimen generated through GPSA, ranging from blue to red indicating areas of low and high variation respectively. RTA's are highly variable, with variation across the peaks of the main face, and the shape, size, and angle of the outcropping arm, shown on the right side of the models. There is also variation driven by the underside of the models.

Visualisation of the first six principal components shows a large amount of variation in the distributions of both species and regions (Fig. 4.4) There is a relatively consistent division of species, particularly in principal component 1. There is no such pattern for region, with the

only consistent pattern being a strong grouping of *D. aquaticus* inside the Introgression Zone. This indicates that species may be distinguishable from these data, but whether a specimen is from inside or outside the Introgression Zone would be more difficult to determine. The overlaid convex hulls show that there is significant overlap between the four categories of spiders denoting different species and their placement inside or outside the Introgression Zone, across the first six principal components (Fig. 4.4).

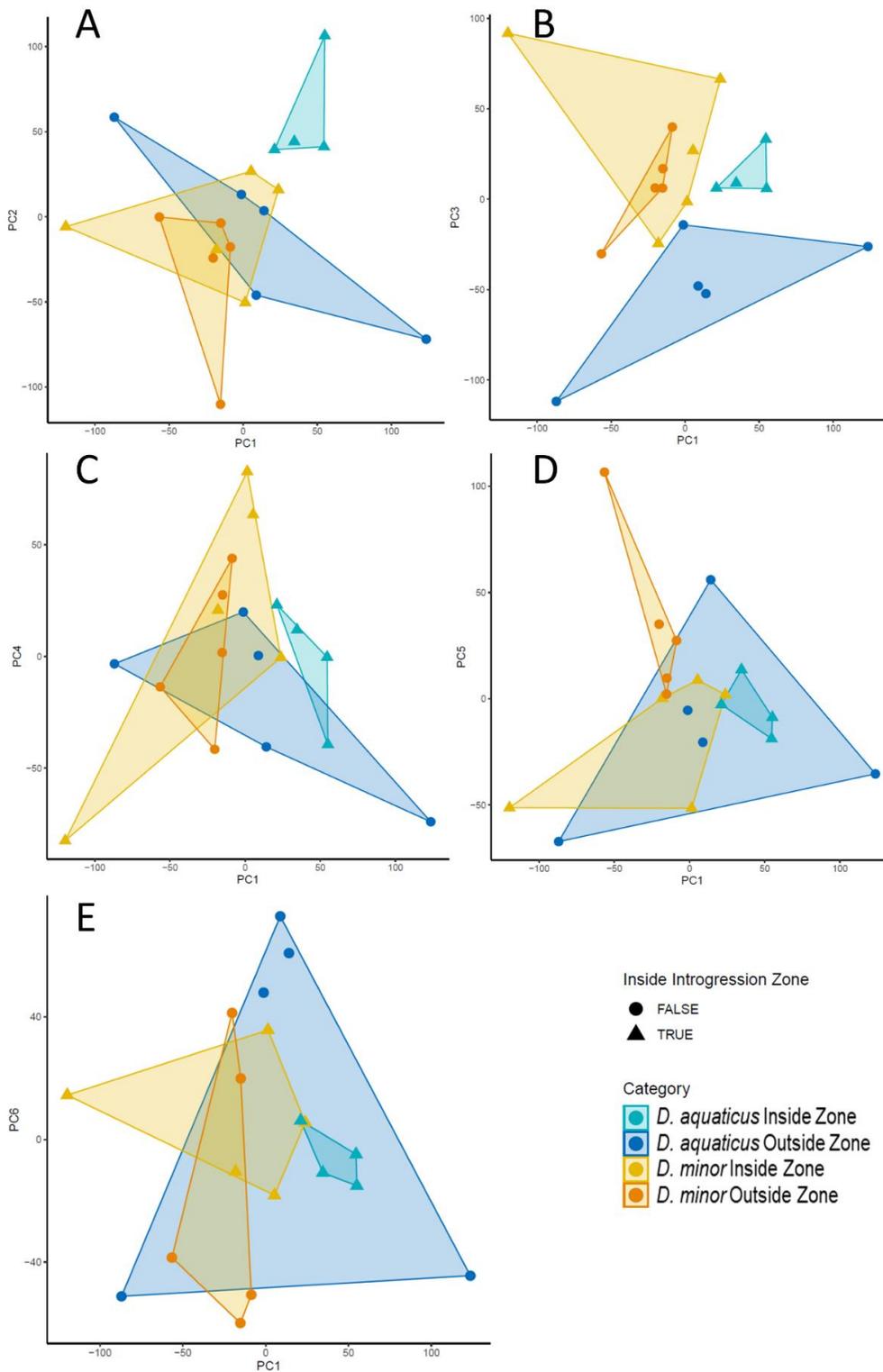


Figure 4.4: Convex hulls overlaid onto Principal Component Analysis plots show significant overlap in shape between all spider categories in the (A) first and second principal component, (B) first and third principle component, (C) first and fourth principal component, (D) first and fifth principal component, (E) first and fifth principal component. Each point represents an RTA from a single specimen, with light blue points representing *D. aquaticus* from inside the Introgression Zone, dark blue points

representing *D. aquaticus* from outside the Introgression Zone, gold points representing *D. minor* from inside the Introgression Zone and orange points representing *D. minor* from outside the Introgression Zone. Triangular points represent specimens from inside the Introgression Zone, whereas square points represent specimens from outside the Introgression Zone. All principal components are plotted against PC1.

PERMANOVA analysis comparing the two species revealed a significant difference in RTA shape (df = 1, F = 2.78, p-value = 0.001). PERMANOVA analysis of the RTA shape categorised by species and location (inside or outside the Introgression Zone) also showed a significant difference in shape between categories (df = 1, F = 2.146, p-value = 0.001). Post-hoc pairwise comparisons showed the largest proportions of the variation are accounted for by *D. aquaticus* inside the Introgression Zone, compared to the other categories. The largest of these is the difference between *D. minor* outside the Introgression Zone and *D. aquaticus* inside the Introgression Zone (Table 4.1), and this effect is statistically significant following False Discovery Rate Correction.

Table 4.1: Pairwise comparisons of species and location categories show a significant difference between the RTAs of *D. minor* outside the Introgression Zone and *D. aquaticus* inside the Introgression Zone. However, there was no significant difference between *D. minor* outside the zone and *D. aquaticus* outside the zone. Summary of pairwise comparisons following PERMANOVA. Significance levels are adjusted based on False Discovery Rate correction.

Category Comparison	Degrees of Freedom	F-value	R <sup>2</sup>	p-value	Adjusted p-value
<i>D. aquaticus</i> outside vs <i>D. aquaticus</i> inside	1	2.166	0.236	0.036	0.064
<i>D. aquaticus</i> outside vs <i>D. minor</i> inside	1	1.628	0.1691	0.053	0.064
<i>D. aquaticus</i> outside vs <i>D. minor</i> outside	1	1.910	0.1927	0.051	0.064
<i>D. aquaticus</i> inside vs <i>D. minor</i> inside	1	2.545	0.2667	0.027	0.064
<i>D. aquaticus</i> inside vs <i>D. minor</i> outside	1	5.489	0.4394	0.007	0.042*
<i>D. minor</i> inside vs <i>D. minor</i> outside	1	1.221	0.1324	0.294	0.294

There was no significant difference RTA width (df = 17, t = 0.833, p = 0.417), height (df = 11, t = -0.08, p = 0.937), or outcrop length (df = 14, t = 0.786, p = 0.445) between species. There was also no significant difference in RTA height (df = 3, F = 0.910, p = 0.460) or outcrop length (df = 3, F = 0.282, p = 0.838) between species and location categories. However, there was a significant difference in width (df = 3, F = 5.272, p = 0.011) (Fig. 4.5). Post-hoc analysis

showed that this difference is between *D. minor* inside and outside the Introgression Zone and *D. aquaticus* outside the Introgression Zone and *D. minor* inside the Introgression Zone (Table 4.2).

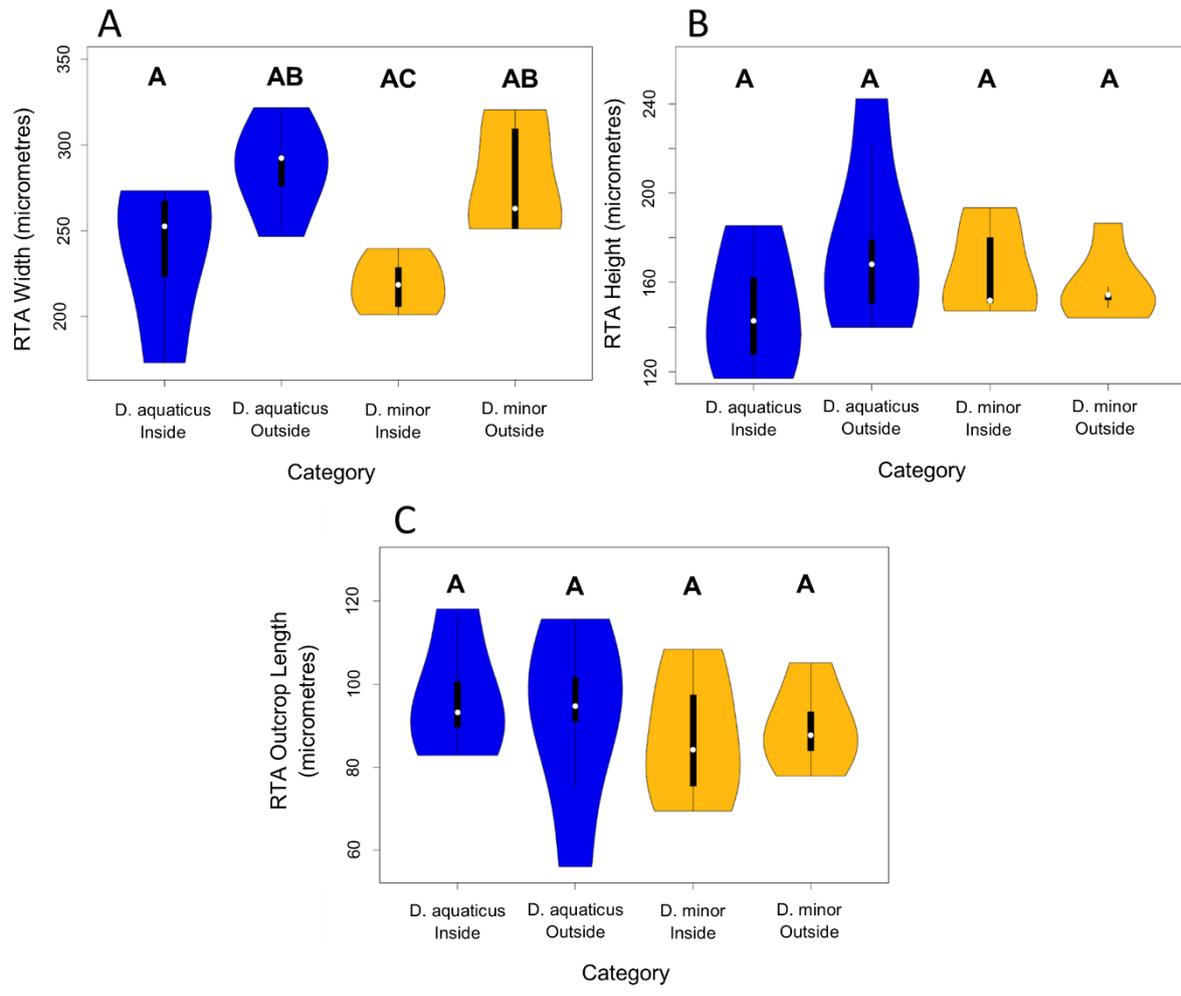


Figure 4.5: RTA measurements for species and location categories. A; Spiders from outside the Introgression Zone have wider RTAs than *D. minor* from inside the Introgression Zone. B & C: There are no significant differences in RTA height or Outcrop length. Boxes show interquartile range, with the central point indicating the median. The curved lines represent the full spread of the data.

Table 4.2: Tukey's HSD comparisons of RTA width for species and location categories.

Category Comparison	Estimate	t-value	p-value
<i>D. aquaticus</i> outside vs <i>D. aquaticus</i> inside	48.37	2.301	0.142
<i>D. aquaticus</i> outside vs <i>D. minor</i> inside	-19.22	-0.915	0.797
<i>D. aquaticus</i> outside vs <i>D. minor</i> outside	41.23	1.962	0.245
<i>D. aquaticus</i> inside vs <i>D. minor</i> inside	-67.60	-3.411	0.018*
<i>D. aquaticus</i> inside vs <i>D. minor</i> outside	-7.14	-0.360	0.983
<i>D. minor</i> inside vs <i>D. minor</i> outside	60.46	3.051	0.036*

## 4.5 Discussion

This study found no evidence that genital shape is responsible for the one-way or geographic limitations on the introgression between *D. aquaticus* and *D. minor*. Across all specimens there was a high level of shape variation in the RTA, with multiple different traits being responsible for the variation in shape. The degree of shape variability was similar between the two species, contrary to the prior evidence that *D. aquaticus* would all have similar RTAs, although *D. aquaticus* from inside the Introgression Zone showed very little variation. This category was shown to be different to other RTA shapes, however, this does not explain the pattern shown in the introgression. The overall variety in shape led to similar shapes in most categories of species and location, rather than any category having a distinct shape of its own that might explain limits on the introgression. There was a significant difference in shape between the two species' RTAs. However, our evidence did not support the hypothesis that *D. minor* outside the Introgression Zone have RTAs that are significantly different from *D. aquaticus* and did not explain why *D. minor* outside the Introgression Zone do not mate with local *D. aquaticus*. Size of the RTA, based on measurements of height,

width and the length of the main outcropping, showed no significant differences between species, and no significant differences between height and outcrop length between categories of species and location. We did find significant differences in RTA width between these categories, but not in a pattern that explains the nature of the introgression. Overall, the variation of RTAs, even within species, is too great for RTA shape to be a significant factor in isolating these species, based on the evidence presented here. This leads to two questions: why are the RTAs of the two species different if the RTA plays no role in introgression; and what maintains the introgression between these species?

A quantifiable difference in genital shape between different spider species is consistent with previous morphometric analyses (Costa-Schmidt and de Araújo 2010; Crews 2009; Wilson et al. 2021). The difference between species is possibly due to the RTAs being adapted for function within their species, without necessarily precluding interbreeding. One factor in this could be that the RTA in each species is adapted to their specific method of copulation. *Dolomedes minor* have extremely rapid copulations, often taking less than a second (Chapter Two), and specialisation would seem to be necessary for such quick copulations to be successful. This would support Eberhard & Huber's (1998) classic argument that genitalia are adapted to promote sperm transfer within a species, rather than prevent transfer with other species, also known as cryptic female choice (Eberhard & Huber, 2010; Sloan & Simmons, 2019). However, such speculation would need to be supported by a more applied study on the functional morphology.

Other factors could also be driving genital diversity in these two species. Another compelling explanation for male spider genital complexity is sexual conflict, with male features being adapted to prevent female remating (Kuntner et al. 2016). There is also morphometric evidence from other spiders that the shape of certain genital structures can affect the likelihood of the males being sexually cannibalised (Hernández, Molina, and Aisenberg 2019), which would be particularly relevant to *D. minor* given the male's specialised behaviour to escape female cannibalism (Chapter Two). However, evidence of these phenomena come from non-RTA spiders, making it difficult to apply this framework to RTA complexity (Kuntner et al. 2016).

Another possible explanation is that the diversity in RTA shape is non-functional, and indeed described arbitrarily. Morphometrics as a field involves the description and quantification of shape, on the assumption that such variation is biologically meaningful (Mitteroecker and Schaefer 2022). This is true of traditional landmarking techniques, where the landmarks are placed in positions assumed to be meaningful by the researcher. However, this criticism has also been levelled at semilandmarking and landmark-free techniques (such as the one used by our study) (Goswami and Clavel 2024; Mitteroecker and Schaefer 2022). Such techniques can capture almost all the variation in a structure or surface, but not all such variation is meaningful. In the specific case of these RTAs, great care was taken to clean the scans so that small surface variations would not have an effect. The scans were also segmented manually so that only the outermost structures of the RTA were included, as these were the parts most likely to come into contact with the female genitalia and therefore be relevant in this context. However, this does still involve some level of subjective decision-making and assumptions. One such decision was to focus segmentation on the outermost structures of the RTA on the assumption that these would be more likely to contact the female and be more biologically relevant than the base that connects the RTA to the pedipalp. It is conceivable that this technique led to differences in specimens being caused by the technique used to delineate the bottom edge, and differences are seen in the bottom edge of the GPSA heatmap (Fig. 4.3). However, given that these techniques were applied systematically, it is more likely that such differences represent changes in the overall structure of the RTA that are most prevalent at the base, such as the thickness of the RTA altering the shape of the base, from the perspective of the GPSA analysis. There is also variation in the data acquired by different landmark free techniques, even when applied to the same surfaces, due to different methods for placing homologous points (Shui, Profico, and O'Higgins 2023). The specific method described by here has also been criticised for being reliant on careful selection of a prototype specimen (Spanke et al. 2023). However, we were careful to select an RTA that was a close visual match to as many other specimens as possible. Despite all of this, it is still generally accepted that the RTA must be under sexual selection (Eberhard & Huber, 2010; Hosken & Stockley, 2004; Huber, 1995; Langerhans et al., 2016).

As for the introgression, this could be maintained by cryptic genital diversity in females. This paper follows an unfortunate trend of ignoring female genitalia in studies of genital function (criticised by Anderson & Langerhans, 2015; Langerhans et al., 2016; Orbach et al., 2021; Sloan & Simmons, 2019). Despite an assumption of a lack of diversity in female genitalia in this group based on the available evidence (Vink and Dupérré 2010), evidence is building across multiple taxa that female genitalia are more diverse than often assumed from initial investigation (Anderson & Langerhans, 2015; Kuntner et al., 2016; Lupše et al., 2016; Sloan & Simmons, 2019), and match diversity with male genitalia (Lupše, Cheng, and Kuntner 2016; Sloan, Kennington, and Simmons 2023; Sloan and Simmons 2019) (though see Ramos et al, (2005) for contrary evidence). The use of genitals as descriptors of species has been criticised for concealing the true diversity of genital characteristics within species (Huber and González 2001). Furthermore, given that *D. aquaticus* male RTAs were also hypothesised to lack diversity, and this analysis has shown them to vary just as much as *D. minor* RTAs, it is also possible that there is more hidden diversity in the female genitalia of these species. If this is the case, the male genitalia would need to be tested for their ability to couple with the variety of female genitalia across both species. Presumably, both species would have no trouble interlocking with females of their own species, but hypothetically *D. minor* males from inside the Introgression Zone could be adapted to the variety of female genitalia in both species, in ways that are obscure through examining male genitalia alone. For future studies, micro-CT imaging and GPSA are well suited for examination of female genitalia (Sloan, Kennington, and Simmons 2023).

Freeze fixing of these spiders would provide more insight into the processes involved in copulation. This involves the pouring of liquid nitrogen onto the spiders whilst they are in copula. This provides a snapshot of the mating process and the interaction of structures involved (Dougherty and Simmons 2017). It would allow for a highly informative investigation into which structures (both male and female) and which parts of those structures, are most important to genital fit (Poy et al. 2020; 2023). For example, the out-cropping “arm” (shown on the right of all models in Fig. 4.3) was shown to be highly variable in shape, size, and angle of attachment to the main RTA. Seeing how this structure interacts with female genitalia would allow appropriate emphasis (or de-emphasis if it is shown to be unimportant) to be given to its shape in future analyses. Freeze fixing would likely be

practical for *D. aquaticus*. However, given the unpredictable courtships, low mating rates and extremely quick copulations of *D. minor*, it would be much more challenging in this species.

In addition to addressing RTA shape, we found no evidence that mismatch in the size of the RTA plays a role in promoting or limiting introgression. Whilst spiders outside the Introgression Zone had significantly wider RTAs than spiders inside the Introgression Zone (for both species), it is difficult to link this observation to the observed patterns of introgression. If the width of an RTA is an important factor in achieving genital lock with a female, then the RTAs of spiders outside the Introgression Zone should be differentiated between the two species, thereby explaining why the two species cannot interbreed in that location. Spider genitalia coevolve to avoid size differences, and this is especially important in species where the females are many times larger than the male (Lupše, Cheng, and Kuntner 2016). However, size may not be as important in this cross, as these species are both relatively modest in the sexual size dimorphism, and relatively similar in size to each other (Chapter Three). The measurements taken were also hampered by a lack of consistency in RTA shape. For example, an extension of the main face of the RTA can increase RTA width without adding much surface area, compared to other RTAs (For example, the bottom left of Fig. 4.3 compared to the bottom right, would have a greatly increased width if the thin section on the left is included). Ultimately, this variety in shape is why we opted for a landmark-free approach to the shape analysis, and points of measurement are analogous to landmarks in that they require consistent placement. However, the results of the measurement analysis are broadly consistent with the GPSA, in that they both show a large amount of variation in both species. Measurements of RTA size would also benefit from freeze-fixing studies described above, as it would provide a basis for which structures are most relevant to measure. The difference in RTA width could also be explained by a difference in overall body size across latitude.

In conclusion, this study found evidence of differences in RTA shape between *D. minor* and *D. aquaticus*, but no conclusive evidence that these differences contribute to the unusual introgression between these species. Further work would involve examination of the female genitalia in these species and freeze fixation of mating pairs for additional information.



## Chapter 5: General Discussion



*A seven-legged D. minor female continues to stand guard over her nurseryweb – Helen Macky*

## 5.1: Summary of Findings

Mating is key to the evolution of almost all animal species. Mating rates control the influence and direction of sexual selection, by determining which traits and strategies will be important and set the direction for the evolution of a species. Mating between species allows the possibility of introgressive gene transfer, allowing traits from one species to drive evolution of another. This thesis studied the mating rates of two endemic New Zealand fishing spiders, *Dolomedes minor* and *Dolomedes aquaticus*, and investigated an unusual introgression between the two species. In Chapter Two I found that *D. aquaticus* has a polygynous and monoandrous mating system, typified by a syndrome of female aggression post mating, and lack of genital damage. Meanwhile, *D. minor* have a mating system with low mating rates, typified by genital damage and low probability of mating. Chapters Three and Four focused on the introgression between the two species, in particular aiming to find explanations for the compelling one-way and localized introgression pattern. Chapter Four found that, despite mixed evidence, genital shape is unlikely to play a role in the nature of the introgression between the two species. Chapter Three showed strong evidence of the role of behaviour in maintaining the one-way and geographical aspects of the introgression, with *D. minor* females being unreceptive to *D. aquaticus* males and *D. minor* males from outside the Introgression Zone being less likely to put effort into courting *D. aquaticus* females.

## 5.2 Notable Contributions

This thesis makes several notable contributions to the literature of spider mating systems and introgression. These include expanding knowledge of genital damage outside of web-building spiders investigating the mating dynamics of endemic New Zealand spiders and building on our knowledge of the correlation between life history, morphological and behavioural traits and how these relate to an animal's mating system. Notable contributions to the introgression literature include highlighting the importance of mating behaviour in maintaining and limiting introgression between species and providing more evidence in the debate about the importance of genital shape.

Much of the work done on spider mating rates and genital damage focuses on web building species (Schneider and Fromhage 2010; Uhl, Nessler, and Schneider 2010). In these species, sedentary females can be monitored over consecutive days, and operational sex ratios can be estimated by counting males on webs of mature females, providing a methodological advantage (Schneider and Fromhage 2010). The use of model taxa reduces issues with experimental or observational methodology or raising animals in laboratory conditions, as generally these issues only need to be solved once (Zuk et al. 2014). There is value in compounding knowledge and building hypotheses in model systems, however, these hypotheses must eventually be tested on new species and systems (Uhl, Nessler, and Schneider 2010). This is particularly true for topics related to sexual selection, where even small differences between taxa can have large impacts on the evolution of desirable traits (Zuk et al. 2014). For example, it is assumed that monogyny in spiders requires a male biased sex ratio due to the risk that the males undergo during their search for females (Fromhage, Elgar, and Schneider 2005). Part of this risk is that the male is away from his web and unable to hunt while searching for a female (Schneider and Andrade 2011). This assumption likely does not apply to *Dolomedes*, or indeed any species that does not build webs for prey capture. It is notable then that this thesis found no evidence of monogyny in either species studied, and no evidence of protandry in *D. minor* (protandry is associated with monogyny as males rush to be the first to mate with a female (Schneider and Fromhage 2010). Indeed, monogyny has only been observed in one *Dolomedes* species to date (Schwartz, Wagner, and Hebets 2014). As further research is undertaken in this group, it may become apparent that there is an evolutionary association between monogyny and web building. Ecology and environment have long been recognised as influences in mating system evolution (Kokko, Klug, and Jennions 2014), and web building vs non-web building spiders is an excellent point for comparison, worthy of further study.

Non-web building spiders have been used as model taxa before. Notably, Salticidae are used for the study of contest behaviour and cognition (Nelson 2023), and Lycosidae are used to study sexual selection and signalling (Starrett et al. 2022). However, there is currently a gap for a group of non-web building spiders to study the effects and evolution of mating systems. The work in this thesis has furthered the case for *Dolomedes* as a taxa to fill the knowledge gap in the mating systems of non-web building spiders. The genus is diverse and distributed

globally (NMBE - World Spider Catalog 2024), and some regions contain a high diversity of species (Yu and Kuntner 2024), leading to excellent potential for comparative work. As discussed in previous chapters, they exhibit a wide range of mating behaviour (Schoenberg, Hebets, and Sullivan-Beckers 2022; Schwartz, Wagner, and Hebets 2014). In the case of New Zealand *Dolomedes*, they can be found in almost all regions of the country (Vink and Dupérré 2010) and form dense populations in the appropriate habitat. They are also easy to capture, requiring no specialised equipment and minimal training, and presenting no threat to human life. They are also relatively easy to rear and experiment on in the lab, with the largest challenge/expense being the sourcing of adequate live food. I found *D. aquaticus* particularly active in the lab, both maturing quickly and being very responsive during experiments.

Another noteworthy contribution of this thesis is the insight into the unknown mating dynamics of two endemic New Zealand spiders. As mentioned in Chapter One, the mating behaviour of *Dolomedes* in New Zealand are highly understudied, with only a brief set of observations by Forster & Forster (1973) on the mating behaviour of *D. aquaticus*. The Forsters would no doubt be delighted that their speculation on the nature of *D. aquaticus* mating (that females mate once shortly after maturing and subsequently become aggressive) has been confirmed by this thesis, through systematic laboratory experimentation with mated and unmated spiders. The research in this thesis presents another step for further research into New Zealand *Dolomedes* mating systems (discussed further below). I would argue this knowledge is valuable for its own sake to enhance our understanding of our native species and provide opportunities for the public to become enamoured with endemic spiders that are often overlooked, despite being abundant, widespread, fascinating, and in my opinion, beautiful.

### 5.3 Limitations and future directions

A significant limitation of this work is that I was unable to confirm successful copulations, either through dissections to confirm sperm transfer or through rearing females through to egg-laying and offspring hatching. I originally planned to dissect females after their experiments to test for sperm deposition in their spermathecae. However, extensive attempts to extract and visualise the sperm were unsuccessful, even when attempted on

male genitalia (seemingly a guaranteed location for sperm). This indicates either an issue with the implementation of methods (which were adapted from Gabel and Uhl (2013) and Modanu et al (2013)) or with issues in the preservation of specimens. The result is that any analysis of sperm competition and deposition is the purview of future work.

*Dolomedes aquaticus* provide an excellent baseline for comparison for any study of sperm competition between the two species, given that sperm competition seems to be rare, with females typically only copulating with one male each. However, given the possibility of genital plugging, lack of a female syndrome of aggression and female remating, sperm competition in *D. minor* seems more likely. This presents the question of whether the male strategy in *D. minor* is pure sperm competition avoidance (through genital plugging), or a combination of avoidance and engagement. Linked to this is the question of the effectiveness of the male genital in acting as a plug. As mentioned previously, broken male emboli need precise positioning to impede subsequent mating (Herberstein et al. 2012), which may explain the *D. minor* females' tendency for cannibalism and the resulting short copulation durations. It is also possible that broken male genitalia could reduce female remating, even if they do not effectively block the genital opening, as mating plugs in other spiders can act as signals that females have been mated with (Kuntner et al. 2012). Hence, a *D. minor* male seeking to avoid sperm competition would be best to avoid a female with male emboli fragments on or in her genitalia. The possibilities for investigating sperm transfer and competition in these two species are extensive.

The duration of *D. minor*'s copulation is also noteworthy. Spider copulation speed is highly variable, from mere seconds to several hours (Elgar 1995). The copulation durations described in this thesis for *D. minor* are in many cases shorter than *Argiope aurantia*, which is described as having the shortest copulations of any spider species at approximately 2-3 seconds (Assis and Foellmer 2019). *Argiope aurantia* has other similarities to *D. minor*, in that the short copulations facilitate escape behaviour from female cannibalism, and the male breaks his palp upon exit, leaving the palp behind as a mating plug (Assis and Foellmer 2019). Such rapid escape behaviours have been described in other species (Zhang, Liu, et al. 2022), and even in other *Dolomedes* (Arnqvist 1992). This is consistent with evidence that shows that copulation speed increases in species where risk of sexual cannibalism is high and undesirable for the male (Zhang, Yu, et al. 2022). However, formal description of a mean

or median speed would require further work to assess sperm transfer. It should also be noted that Assis & Foellmer (2019) speculate that copulations lasting less than a second are not possible in spiders. Whilst this thesis does not definitively disprove this assumption (as copulations have not been confirmed to transfer sperm), this raises questions as to how *D. minor* would transfer sperm if this were the case. As mentioned previously, the copulation behaviour seen in the lab was consistent with that seen in the wild, and given the rapid nature of female attacks, it would be unlikely for males to survive copulations of even a few seconds.

Also worthy of future research is the *D. aquaticus* males who had broken genitals after their mating trials/crossing experiments. In Chapter Two I observed a male *D. aquaticus* who broke an embolus copulating with a female of his own species. However, there were two further males who broke their genitalia during experiments with *D. minor* females during the crossing experiments of Chapter Three. Whilst this is a small set of males, it is interesting that all of these males were from the Introgression Zone, where interbreeding with *D. minor* may still be occurring. I have argued in this thesis that mating behaviour affects evolution through sexual selection within a species, and facilitating or limiting introgression of genes to other species. These broken genitalia represent the intriguing possibility of introgression influencing mating behaviour, with *D. aquaticus* from inside the Introgression Zone showing what was otherwise a hallmark of *D. minor* mating. This deserves further investigation with a larger sample size of *D. aquaticus* from the Introgression Zone.

My field observations of *Dolomedes* also provide several avenues for future study. These include investigating males mating with moulting females, which I observed once in the field. This has been observed in other species as a male method to avoid cannibalism (and dubbed soft-mating), as the female is essentially immobile as she emerges from the moult (Schneider and Andrade 2011). A detailed study of this behaviour in *Argiope bruennichi* found it to be extremely effective in reducing cannibalism, which males took advantage of to leave to find a second mate (Uhl et al. 2015). However, the behaviour was less common in nature, likely due to males needing to find sub-adult females, who do not produce pheromone cues until maturity (Uhl et al. 2015). Given that during my field observations, I also saw one male attempt a “traditional” copulation (but abort it using their escape behaviour), and several instances of males courting mature females, it is likely this is a

limiting factor in *D. minor* as well, and traditional courtship and copulation is more common. Uhl et al (2015) also found evidence of males guarding sub-adult females awaiting their maturity. I would speculate that this is another strategy influenced by the ecology of a web-building spider. Whilst *Dolomedes* are described as sit-and-wait opportunistic predators (Nyffeler and Pusey 2014; Williams 1979), they are likely still more mobile than orb-web spiders, which would make it difficult for a male to guard a female (both in terms of following the female and avoiding becoming a meal for the female). However, the dynamics of soft-mating in *D. minor* deserve further investigation. Chapter Two showed that mating in *D. minor* is risky for the male, requiring rapid escape behaviour to avoid cannibalism, and that most males will only expend a single palp with a female. Investigation of soft-mating would create a scenario where female aggression and female choice are removed from the mating dynamics. This would allow for comparison for the effect on mating rates, mating probability, copulation duration and sperm transfer.

Furthermore, with relation to soft-mating, Lattimore et al (2011) also hypothesise that female moulting is responsible for the breakdown of species barriers in the Introgression Zone, with soft-mating making the female genitalia more malleable to differently shaped male genitalia. Essentially, this would remove the “lock” from the lock-and-key system. However, I now find this to be an unlikely explanation given the findings of Chapter Four, that genital shape is unlikely to influence introgression in the first place.

Another phenomenon worthy of future investigation is aggressive interactions between males in the field. This was observed once between courting males, with the larger male very quickly driving away a smaller male. Aggressive interactions are known in many different spider taxa (Kuntner et al. 2015; Kwek et al. 2021; Walker and Holwell 2018; Zhang, Ai, and Li 2019, with Salticidae in particular being used as a model system for communication in these interactions (Yu et al. 2022; Nelson 2023). In these taxa, direct confrontation provides a significant evolutionary advantage to the males to justify the risks involved. These motivations include mate guarding to assure paternity (Zhang, Ai, and Li 2019) and securing an ideal mate when males have limited sperm supply (Pollo, Muniz, and Santos 2019). Male-male aggression would provide an evolutionary mechanism to maintain relatively large male size relative to females, with male size being an important determinant of fight outcome in other taxa (Walker and Holwell 2018). As it stands, this single aggressive

interaction may be a rare occurrence, but future experiments could be augmented by placing two or more males in an arena with a female. This would test whether males will tend to interact aggressively, or ignore each other and pursue their own courtships (as has also been shown in other spiders (De Young and Wilgers 2016)). This would provide information on how male-male aggression influences the evolution of this species, and whether it is an important factor in determining the outcome of mating.

## 5.4 Concluding Remarks

During the course of this thesis, I have shown the influence of mating rates and sexual selection in two New Zealand *Dolomedes*. The results of this thesis demonstrate that mating systems and sexual selection can have drastic impacts on behaviour, even in such closely related species. I also investigated introgression between the two species, focusing on genital shape and behaviour, rather than genomics. I showed that behaviour is key to introgression between these two species, highlighting the importance of such studies to our broader understanding of introgression. Genital shape, however, was not shown to play a significant role in isolating the two species, providing further evidence in the ongoing debate about the importance of genitalia in isolating species. Important future work would include studies on sperm transfer and the effectiveness of genital plugging in these spiders, as well as potential effects of interbreeding on mating behaviour in the Introgression Zone.

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## Supplementary Tables and Figures

*Supplementary Table 1: Collection Locations and their approximate coordinates*

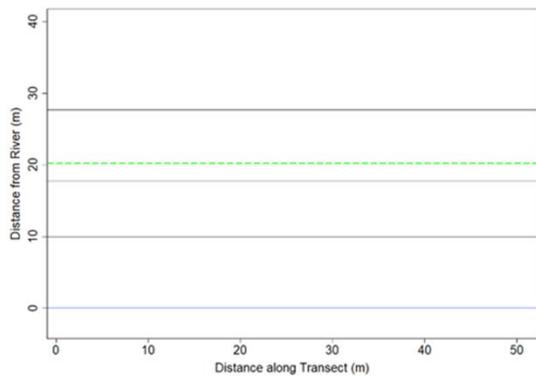
Location Description	Region	Latitude	Longitude
Ashley Gorge, Riverbed by Campground	Canterbury	-43.2289	172.2288
Ashley Gorge Road, near turn of Lees Valley Road	Canterbury	-43.2471	172.2102
Kowai River, near limeworks	Canterbury	-43.3319	171.8661
Garry River, off Birch Hill Road	Canterbury	-43.2257	172.3468
Port Hills, Near Flying Nun Track/ Port Hills, Rhode Spring/ Port Hills, near sign of the Bell Bird	Canterbury	-43.6104	172.6399
University of Waikato, Hamilton Campus	Waikato	-37.786230	175.3154
Hammond Park, Malcom Street end	Waikato	-37.8073	175.3167
Braithwaite Park	Waikato	-37.7382	175.2465
Onamalutu River	Blenheim	-41.4615	173.6915
Leatham River/Branch River	Blenheim	-41.7177	173.1715
Motueka River	Blenheim	-41.2783	172.8076
Wairau Tributry	Blenheim	-41.7162	173.0799
State Highway 6, near Wairau Bridge	Blenheim	-41.4691	173.8038
Hammond Park, Riverlea Road end (Monitoring Site)	Waikato	-37.8093	175.3252
Oreti River	Southland	-45.6605	168.2545
Bald Hill, Knutsford Road	Southland	-46.1761	167.913
Aparima River	Southland	-46.1337	168.0031
Whitestone River	Southland	-45.5177	167.782
Waiau + Tributries	Southland	-45.9329	167.7091
Mararoa River	Southland	-45.6154	167.6918
Eyre River, Woodside Road Bridge	Canterbury	-43.2816	172.0988
Waipara River (Monitoring Site)	Canterbury	-43.0598	172.5916

Supplementary Table 2: Results of Binomial Exact tests on *D. minor* population at Hammond Park

Date	Male Proportion of Mature Population	P-value
27/10/2020	0.667	0.343
11/11/2020	0.400	0.813
23/11/2020	0.375	0.855
10/12/2020	0.333	0.910
21/12/2020	0.333	0.927
5/01/2021	0.286	0.971
20/01/2021	0.333	0.927
3/02/2021	0.071	0.999
17/02/2021	0.25	0.964
2/03/2021	0.643	0.211
16/03/2021	0.435	0.798
2/04/2021	0.345	0.969
14/04/2021	0.381	0.905
27/04/2021	0.414	0.868
13/05/2021	0.211	0.998
25/05/2021	0.333	0.927
10/06/2021	0.263	0.990
22/06/2021	0.214	0.994
14/09/2021	0.091	0.999
2/10/2021	0.181	0.994
20/10/2021	0.278	0.984
2/11/2021	0.5	0.598
15/11/2021	0.533	0.5
29/11/2021	0.231	0.989
15/12/2021	0.4	0.868
20/12/2021	0.357	0.910
3/01/2022	0.091	0.999
17/01/2022	0.2	0.989
1/02/2022	0.4	0.828
15/02/2022	0.1	0.999
1/03/2022	0.571	0.5
14/03/2022	0.6	0.377
28/03/2022	0.6	0.304
1/04/2022	0.308	0.954
11/04/2022	0.308	0.954
26/04/2022	0.5	0.593
10/05/2022	0.157	0.999
24/05/2022	0.2	0.989
8/06/2022	0.091	1.000
21/06/2022	0.25	0.938
4/07/2022	0.125	0.996
18/07/2022	0	1

2/08/2022	0	1
16/08/2022	0	1
29/08/2022	0.2	0.969
27/09/2022	0.571	0.5
10/10/2022	0	1
24/10/2022	0.5	0.623
8/11/2022	1	0.031*
23/11/2022	0.467	0.696
5/12/2022	0.333	0.875
21/12/2022	0.5	0.598
4/01/2023	0.222	0.980
17/01/2023	0.25	0.965
31/01/2023	0.333	0.910
15/02/2023	0.2	0.989
21/02/2023	0.214	0.994
4/03/2023	0.363	0.887
15/03/2023	0.333	0.952
29/03/2023	0.5	0.588
10/04/2023	0.567	0.292
25/04/2023	0.304	0.983
8/05/2023	0.296	0.990

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*Supplementary Figure 1. Left: Diagrammatic representation of the Waipara Site. Blue line represents the river's edge. The dotted green line represents the approximate location of the habitat boundary (variable due to not being precisely parallel to the river and the river varying height over time). The black lines represent the boundaries of transects, with the lower transect also being bound by the river. Right: A photograph of the site showing the habitat border between the shrubby and rocky habitats. Rivers edge also shown in the background of the photo.*

Supplementary Table 3. Number of experimental trials for each crossing type

	Sample size of <i>D. aquaticus</i> female x <i>D. minor</i> male crosses	Sample size of <i>D. minor</i> female x <i>D. aquaticus</i> male crosses	Total number of trials
Outside Introgression Zone	18	13	31
Inside Introgression Zone	29	19	48
Total number of experimental trials			70

Supplementary Table 4. Results of Binomial Exact tests on *D. aquaticus* and *D. minor* populations at Waipara River. *Dolomedes aquaticus* is significantly male biased in October 2020. Results of one-sided binomial exact tests of ratio of males – females of penultimate and mature spiders at the Waipara observation site. \* indicates a significant p value (<0.05)

Month (2020 – 2021)	P <i>Dolomedes aquaticus</i>	P <i>Dolomedes minor</i>
October	0.031*	0.054
November	0.612	0.980
December	0.271	0.867
January	0.984	0.969
February	0.856	0.806
March	0.750	0.250

Supplementary Table 5. Tukey's analysis of probability to court. Males of the two species have no significant differences in their probability of courtship initiation. Results of probability to court. \*\*\* indicates  $p < 0.001$ , \*\* indicates  $0.001 \leq p \leq 0.01$ , \* indicates  $0.01 < p \leq 0.05$ .

Comparison	Estimate	Standard Error	Z Value	P Value
<i>D. aquaticus</i> Female x <i>D. minor</i> Male Inside –				
<i>D. aquaticus</i> Only	-1.50	0.579	-2.59	0.092
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside –				
<i>D. aquaticus</i> Only	-1.62	0.646	-2.51	0.111
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Only	-1.32	0.477	-2.74	0.062
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Only	-0.816	0.681	-1.198	0.824
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside –				
<i>D. aquaticus</i> Only	-0.0540	0.881	-0.061	1.00
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside –				
<i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-0.125	0.606	-0.207	1.00
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	0.192	0.420	0.458	0.997

*D. minor* Female x *D. aquaticus* male Inside –  
*D. aquaticus* Female x *D. minor* male Inside      0.681      0.643      1.060      0.888

*D. minor* Female x *D. aquaticus* male Outside –  
–  
*D. aquaticus* Female x *D. minor* male Inside      1.44      0.852      1.70      0.512

*D. minor* Only –  
*D. aquaticus* Female x *D. minor* male Outside      0.318      0.509      0.624      0.988

*D. minor* Female x *D. aquaticus* male Inside –  
*D. aquaticus* Female x *D. minor* male Outside      0.806      0.704      1.15      0.850

*D. minor* Female x *D. aquaticus* male Outside –  
–  
*D. aquaticus* Female x *D. minor* male Outside      1.57      0.899      1.75      0.479

*D. minor* Female x *D. aquaticus* male Inside –  
*D. minor* Only      0.0.489      0.553      0.884      0.945

*D. minor* Female x *D. aquaticus* male Outside –  
–  
*D. minor* Only      1.25      0.786      1.592      0.581

*D. minor* Female x *D.*  
*aquaticus* male Outside

—

*D. minor* Female x *D.*

*aquaticus* male Inside      0.762              0.925              0.824              0.959

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Supplementary Table 6. Tukey's analysis of courtship latency. Males of the two species have significantly different courtship latencies, regardless of which female they are placed with. Results of latency to court linear model with log transformation. \*\*\* indicates  $p < 0.001$ , \*\* indicates  $0.001 \leq p \leq 0.01$ , \* indicates  $0.01 < p \leq 0.05$

Comparison	Estimate	Standard Error	Z Value	P Value
<i>D. aquaticus</i> Female x <i>D. minor</i> Male Inside – <i>D. aquaticus</i> Only	1.43	0.425	3.36	0.011*
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Only	0.928	0.518	1.79	0.456
<i>D. minor</i> Only – <i>D. aquaticus</i> Only	1.31	0.288	4.54	<0.001***
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Inside – <i>D. aquaticus</i> Only	0.274	0.456	0.601	0.990
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Outside – <i>D. aquaticus</i> Only	-0.397	0.482	-0.825	0.960
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-0.502	0.581	-0.865	0.950
<i>D. minor</i> Only – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-0.120	0.390	-0.308	1.00

<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	-1.16	0.526	-2.20	0.229
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	-1.83	0.549	-3.33	0.0124*
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	0.382	0.490	0.782	0.968
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	-0.654	0.602	-1.09	0.878
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	-1.33	0.623	-2.13	0.263
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. minor</i> Only	-1.04	0.422	-2.45	0.134
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. minor</i> Only	-1.71	0.451	-3.78	0.00264***

*D. minor* Female x *D.*  
*aquaticus* male Outside

–

*D. minor* Female x *D.*

*aquaticus* male Inside

-0.671

0.572

-1.17

0.840

---

Supplementary Table 7. Tukey's analysis of mean courtship length. Groups from outside the Introgression Zone have significantly shorter courtships than other groups. Results of mean courtship length linear model with log transformation. \*\*\* indicates  $p < 0.001$ , \*\* indicates  $0.001 \leq p \leq 0.01$ , \* indicates  $0.01 < p \leq 0.05$

Comparison	Estimate	Standard Error	Z Value	P Value
<i>D. aquaticus</i> Female x <i>D. minor</i> Male Inside – <i>D. aquaticus</i> Only	-0.252	0.403	-0.626	0.988
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Only	-1.84	0.491	-3.75	0.00303***
<i>D. minor</i> Only – <i>D.</i> <i>aquaticus</i> Only	-0.0144	0.275	-0.053	1.00
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Inside – <i>D. aquaticus</i> Only	-0.767	0.432	-1.78	0.466
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Outside – <i>D. aquaticus</i> Only	-1.71	0.457	-3.75	0.00306**
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-1.59	0.551	-2.89	0.0454*
<i>D. minor</i> Only – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	0.238	0.370	0.644	0.986

<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	-0.515	0.499	-1.03	0.899
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	-1.46	0.524	-2.81	0.0570
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	1.83	0.464	3.94	0.00148**
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	1.08	0.572	1.88	0.399
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	0.129	0.592	0.218	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. minor</i> Only	-0.753	0.401	-1.88	0.401
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. minor</i> Only	-1.70	0.428	-3.76	0.00139**

*D. minor* Female x *D.*  
*aquaticus* male Outside

–

*D. minor* Female x *D.*

<i>aquaticus</i> male Inside	-0.947	0.544	-1.743	0.487
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Supplementary Table 8. Tukey's analysis of likelihood to attack. Females show no significant difference in their probability to attack in the Tukey's comparison. Results of linear model of female probability to attack male. \*\*\* indicates  $p < 0.001$ , \*\* indicates  $0.001 \leq p \leq 0.01$ , \* indicates  $0.01 < p \leq 0.05$

Comparison	Estimate	Standard Error	Z Value	P Value
<i>D. aquaticus</i> Female x <i>D. minor</i> Male Inside –				
<i>D. aquaticus</i> Only	-0.343	0.480	-0.715	0.978
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside –				
<i>D. aquaticus</i> Only	0.681	0.608	1.12	0.863
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Only	-0.747	0.356	-2.10	0.270
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Inside –				
<i>D. aquaticus</i> Only	0.265	0.565	0.469	0.997
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Outside –				
<i>D. aquaticus</i> Only	0.313	0.635	0.493	0.997
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside –				
<i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	1.02	0.644	1.59	0.585
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-0.404	0.415	-0.973	0.920

*D. minor* Female x *D. aquaticus* male Inside –  
*D. aquaticus* Female x  
*D. minor* male Inside      0.608              0.604              1.01              0.908

*D. minor* Female x *D. aquaticus* male Outside  
–  
*D. aquaticus* Female x  
*D. minor* male Inside      0.657              0.670              0.980              0.918

*D. minor* Only –  
*D. aquaticus* Female x  
*D. minor* male Outside    -1.43              0.557              -2.56              0.099

*D. minor* Female x *D. aquaticus* male Inside –  
*D. aquaticus* Female x  
*D. minor* male Outside    -0.417              0.709              -0.587              0.991

*D. minor* Female x *D. aquaticus* male Outside  
–  
*D. aquaticus* Female x  
*D. minor* male Outside    -0.367              0.767              -0.480              0.997

*D. minor* Female x *D. aquaticus* male Inside –  
*D. minor* Only              1.01              0.510              1.98              0.333

*D. minor* Female x *D. aquaticus* male Outside  
–  
*D. minor* Only              1.06              0.587              1.81              0.441

*D. minor* Female x *D.*  
*aquaticus* male Outside

—

*D. minor* Female x *D.*

<i>aquaticus</i> male Inside	0.05	0.733	0.067	1.00
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Supplementary Table 9. Tukey's analysis of attack rate. *D. minor* females from outside the Introgression Zone crossed with *D. aquaticus* males show high levels of aggression. Results of linear model female attacks per hour with log transformation. \*\*\* indicates  $p < 0.001$ , \*\* indicates  $0.001 \leq p \leq 0.01$ , \* indicates  $0.01 < p \leq 0.05$

Comparison	Estimate	Standard Error	Z Value	P Value
<i>D. aquaticus</i> Female x <i>D. minor</i> Male Inside – <i>D. aquaticus</i> Only	0.144	0.295	0.490	0.996
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Only	0.294	0.302	0.971	0.922
<i>D. minor</i> Only – <i>D. aquaticus</i> Only	-0.167	0.218	-0.767	0.971
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Inside – <i>D. aquaticus</i> Only	0.182	0.310	0.588	0.991
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Outside – <i>D. aquaticus</i> Only	1.84	0.343	5.34	<0.001 ***
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	0.150	0.340	0.438	0.998
<i>D. minor</i> Only – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-0.311	0.269	-1.16	0.848

<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	0.0381	0.348	0.109	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	1.69	0.378	4.48	<0.001***
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	-0.460	0.276	-1.67	0.544
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	-0.111	0.354	-0.314	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	1.54	0.383	4.025	0.00143**
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. minor</i> Only	0.350	0.285	1.23	0.816
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. minor</i> Only	2.00	0.321	6.24	<0.001***

*D. minor* Female x *D.*  
*aquaticus* male Outside

–

*D. minor* Female x *D.*

*aquaticus* male Inside

1.65

0.390

4.24

<0.001\*\*\*

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Supplementary Table 10. Tukey's analysis of likelihood of recoiling. *D. minor* females showed high probability of recoiling away from *D. aquaticus* males. Results of binomial general linear model of female recoils. \*\*\* indicates  $p < 0.001$ , \*\* indicates  $0.001 \leq p \leq 0.01$ , \* indicates  $0.01 < p \leq 0.05$

Comparison	Estimate	Standard Error	Z Value	P Value
<i>D. aquaticus</i> Female x <i>D. minor</i> Male Inside – <i>D. aquaticus</i> Only	-1.24	0.823	-1.51	0.604
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Only	-16.2	932	-0.017	1.00
<i>D. minor</i> Only – <i>D. aquaticus</i> Only	-1.20	0.509	-2.35	0.140
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Inside – <i>D. aquaticus</i> Only	1.04	0.596	1.74	0.446
<i>D. minor</i> Female x <i>D.</i> <i>aquaticus</i> male Outside – <i>D. aquaticus</i> Only	1.07	0.657	1.63	0.523
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-15.0	932	-0.016	1.00
<i>D. minor</i> Only – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	0.0463	0.810	0.057	1.00

<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	2.28	0.868	2.63	0.0697
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Inside	2.32	0.910	2.54	0.0879
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	15.0	932	0.016	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	17.2	932	0.018	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. aquaticus</i> Female x				
<i>D. minor</i> male Outside	17.3	932	0.019	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. minor</i> Only	2.24	0.579	3.86	0.001**
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside				
–				
<i>D. minor</i> Only	2.27	0.641	3.54	0.00398 **

*D. minor* Female x *D.*  
*aquaticus* male Outside

–

*D. minor* Female x *D.*

<i>aquaticus</i> male Inside	0.0308	0.712	0.0043	1.00
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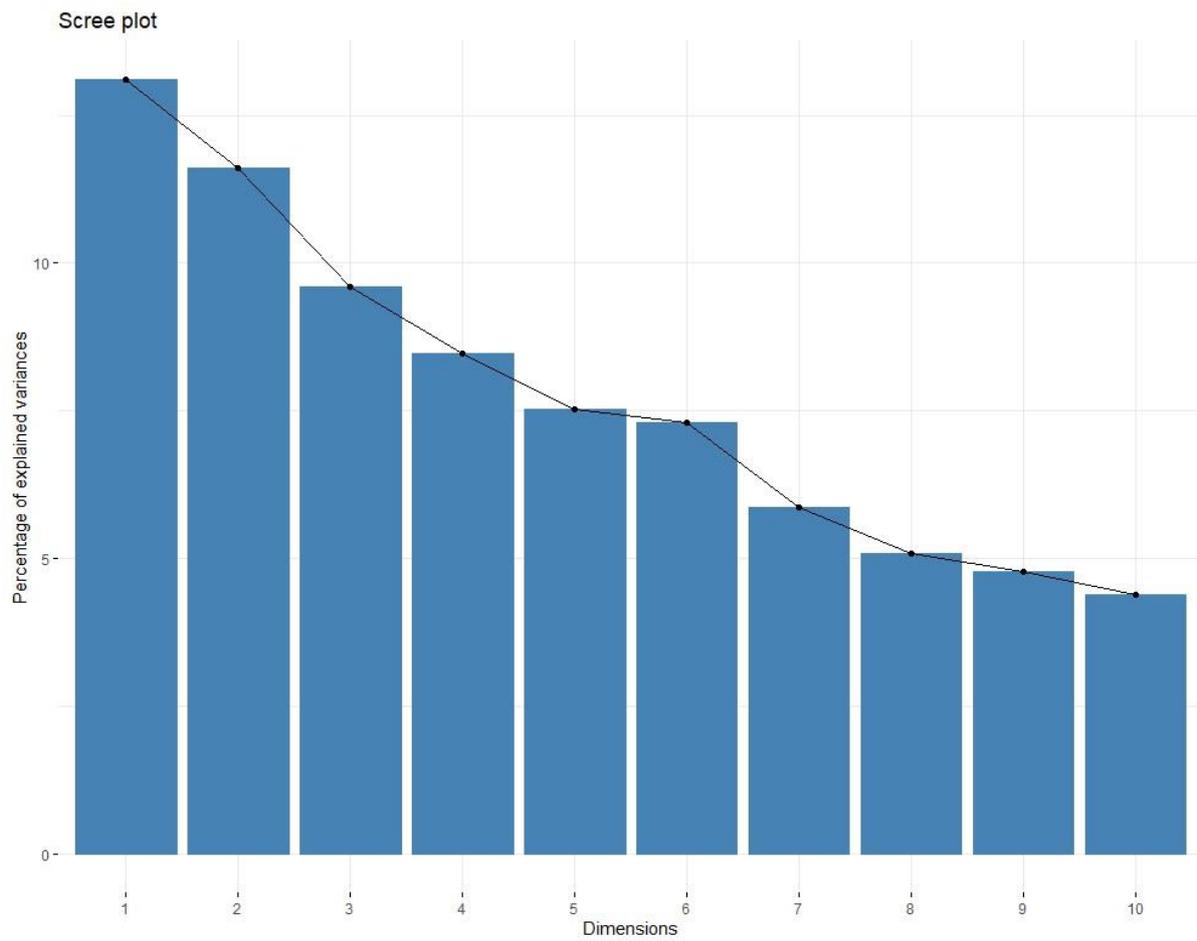
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Supplementary Table 11. Tukey's analysis of probability of attempted copulation. *Dolomedes aquaticus* only trials had high probability of attempted copulation compared with every other group. Results of binomial general linear model of attempted copulations. \*\*\* indicates  $p < 0.001$ , \*\* indicates  $0.001 \leq p \leq 0.01$ , \* indicates  $0.01 < p \leq 0.05$

Comparison	Estimate	Standard Error	Z Value	P Value
<i>D. aquaticus</i> Female x <i>D. minor</i> Male Inside –				
<i>D. aquaticus</i> Only	-3.70	0.811	-4.56	<0.001***
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside –				
<i>D. aquaticus</i> Only	-18.7	932	-0.020	1.00
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Only	-1.74	0.396	-4.40	<0.001***
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Only	-2.13	0.627	-3.40	0.006**
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside –				
<i>D. aquaticus</i> Only	-3.66	1.09	-3.35	0.00703**
<i>D. aquaticus</i> Female x <i>D. minor</i> male Outside –				
<i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	-15.0	932	-0.016	1.00
<i>D. minor</i> Only –				
<i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	1.96	0.757	2.59	0.0722
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside –				
<i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	1.57	0.899	1.75	0.423

<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside – <i>D. aquaticus</i> Female x <i>D. minor</i> male Inside	0.0377	1.27	0.030	1.00
<i>D. minor</i> Only – <i>D. aquaticus</i> Female x <i>D. minor</i> male Outside	16.9	932	0.018	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside – <i>D. aquaticus</i> Female x <i>D. minor</i> male Outside	16.5	932	0.018	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside – <i>D. aquaticus</i> Female x <i>D. minor</i> male Outside	15.0	932	0.016	1.00
<i>D. minor</i> Female x <i>D. aquaticus</i> male Inside – <i>D. minor</i> Only	-0.384	0.554	-0.693	0.976
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside – <i>D. minor</i> Only	-1.92	1.05	-1.82	0.378
<i>D. minor</i> Female x <i>D. aquaticus</i> male Outside – <i>D. minor</i> Female x <i>D. aquaticus</i> male Inside	-1.54	1.16	-1.32	0.714

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*Supplementary Figure 2: Scree plot of Principal Component explained variance from 1 to 10 dimensions.*