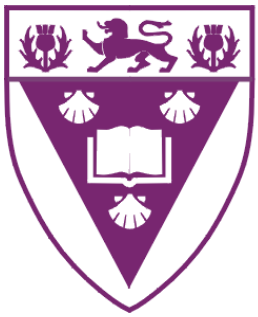
Reproductive isolation mechanisms of two cryptic species of *Eccritotarsus* (Hemiptera: Miridae), biological control agents of water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae).



RHODES UNIVERSITY

Where leaders learn

Thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

of

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by

Sandiso Mnguni

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Abstract

Water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae), is one of the world's worst alien invasive plants. It is indigenous to the Amazon basin in South America but has become a problematic alien invasive in other parts of the world. As such, several host-specific biological control agents have been sourced from the native distributions in South America and have been released to control this plant where it has become problematic. Two of these agents include the geographically and reproductively isolated cryptic species of *Eccritotarsus* (Hemiptera: Miridae). One of these species was collected in the upper reaches of the Amazon River in Peru, while the other was collected over 3500km away from that site, in Florianopolis, southern Brazil. These cryptic species were thought to be a single species until recently, when DNA barcoding indicated that they were likely to be two species, and the species status has now been confirmed by interbreeding experiments and detailed morphological studies. The Brazilian population remains *Eccritotarsus catarinensis* (Carvalho), while the Peruvian population is now known as *Eccritotarsus eichhorniae* (Henry). The aim of this project was to investigate the mating behaviour and other behavioural traits of the two species that have resulted in reproductive isolation, and which could have led to speciation. In addition, investigations involving analysis of chemical compound compositions of the two species aimed to determine the extent to which the compounds played a role in the development and maintenance of reproductive isolation. To achieve the aims, behavioural-observation experiments were conducted in the form of no-choice, bi-choice and multi-choice tests in 1:1, 2:1 and 3:1 sex ratio assessments, both within and between species. Chemical compound compositions of *E. catarinensis* and *E. eichhorniae* were also assessed using Nuclear Magnetic Resonance (NMR), Solid-phase micro-extraction (SPME) and Gas-Chromatography Mass-Spectrometry (GC-MS) techniques. In no-choice experiments, the highest number of single and multiple copula incidences, and average total copula duration was found within species while copulation between species was much rarer. In bi-choice experiments, *E. eichhorniae* females and *E. catarinensis* males only chose to mate with their respective conspecifics, and within species copulations continued to have higher average total copula duration. In multi-choice experiments, the highest number of single and multiple copula incidences and average total copula duration was also found within species. GC-MS analysis suggested that *E. catarinensis* females and *E. eichhorniae* males have unique chemical compounds missing in their conspecifics and same sex of the other species. Further analysis

suggested that *E. catarinensis* females and *E. eichhorniae* males have similar chemical compound compositions, whereas as *E. eichhorniae* females and *E. catarinensis* males have similar chemical compound compositions. These results suggest that there are behavioural differences that led to the development and maintenance of prezygotic reproductive isolation mechanisms, and that this is probably driven by pheromones in chemical compound compositions. These two species were geographically isolated in the native range and the populations have diverged to the point that they are now reproductively incompatible and therefore, distinct species. The main driver of the speciation is most likely mate recognition and attraction, as only reproductively important traits such as pheromones, genitalia, the scent glands and antennae have changed, while other traits, including host range and morphology, have remained remarkably stable. This provides evidence that differences in sexual selection in isolated populations may be important drivers of speciation and reproductive isolation in cryptic species.

PLAGIARISM DECLARATION

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The following thesis has not been submitted to a university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.

Signature: _____ Date: _____

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List of Abbreviations

et al – Et alia (and others)

RIM – Reproductive isolation mechanisms

& – And

MOBAT – Morphological-based-alpha-taxonomy

DNA – deoxyribose nucleic acid

mtDNA – mitochondrial DNA

rDNA – ribosomal DNA

RCD – Reproductive character displacement

COI – cytochrome oxidase subunit 1 gene

COII – cytochrome oxidase subunit 2 gene

ArgK – Arginine kinase

ITS1 – Internal transcribed spacer 1

ITS2 – Internal transcribed spacer 2

28S-DS – Large ribosomal subunit

RAPD – Random amplified polymorphic DNA

PCR – Polymerase chain reaction

AD – Anno Domini (Year of our Lord)

SADWA South African Department of Water Affairs

DATS – Department of Agricultural Technical Services

PPRI – Plant Protection Research Institute

2n – diploid

US$ – United States dollar

2.4-D – 2.4-dichlorophenoxyacetic acid

P – Phosphorus

N – Nitrogen

ha – Hectares

cm – centimetres

m – metres

°C – Degrees Celsius

e.g. – Exempli gratia (for example)

pH – potential hydrogen

% – percentage

mg/h – milligrams per litre

USA – United States of America

N and S – North and South

etc. – Et cetera (and other things)

mm – millimetre

km – kilometre

ml – millilitre

g – grams

ISSRs – Inter Simple Sequence Repeats

B – Brazilian

P – Peruvian

F – female

M - male

MDS – multidimensional scaling plot

F2 – 2nd generation

i.e. – id est (that is)

min – minutes

L/h – litres per hour

MSG – Metathoracic scent gland

SPME – solid phase micro-extraction

NMR – nuclear magnetic resonance

GCMS – Gas Chromatography Mass Spectrometry

PCO – Principal Components Ordination

Hg – Mercury

CHAPTER 1: General Introduction

Biological control is considered an important management tool for the control of invasive alien plants worldwide (McFadyen 1999). To develop safe and effective biological control agents, research scientists need to have an intricate understanding of the relationship between phytophagous insects and their host plants. This ensures that the safety record of biological control is maintained (Suckling & Sforza 2014), and that effective agents are continued to be released for the protection of both agricultural and natural ecosystems (Van Driesche et al. 2010). The need to understand biological control agents and their host plants has resulted in an impressive number of publications covering a diverse range of disciplines including pest management, conservation biology, taxonomy, phylogeography, biochemistry, ecology, evolutionary biology, and many more. These studies have important applied implications for biological control; but have also made significant contributions to the broader scientific disciplines. This thesis has investigated the mechanisms responsible for the reproductive isolation of two recently discovered cryptic species of biological control agents, *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae) and *Eccritotarsus eichhorniae* (Henry) (Hemiptera: Miridae), for the problematic invasive alien plant, water hyacinth *Eichhornia crassipes* (C. Martius) Solms-Laubach (Pontederiaceae). The results of the study have implications for the biological control of water hyacinth in South Africa and worldwide, but also contributes towards our understanding of the evolution of species through reproductive isolation and more specifically, the mechanisms driving speciation of cryptic species.

1.1 Cryptic species

Cryptic species are two or more distinct species classified as one because of their morphological resemblance (Darlington 1940). Cryptic species are usually recently diverged (although not always), reproductively isolated, occur in sympatry and are usually separable by molecular data only (Pfenninger and Schwenk 2007). The recognition of cryptic and sibling species has formed an integral part in the success of many fields of study, and a proper identification of cryptic species is likely to enhance the success rates of many biological control programmes. A proper identification of cryptic species will ensure that the correct biological control agent is used. It will also lead to further host specificity tests that often target co-evolved herbivores that primarily feed on one host. However, concerns related to the establishment of cryptic species are likely to arise because an inappropriate identification could mean that the new species may feed on non-target native plant species, which is not desirable and could lead to the rejection of its release. Focus should be placed on releasing the correct species.

Cryptic species are known to hide divergence in habitat, physiology, life cycle traits and have ecological implications since two species are named as one based on morphological traits (Amato et al. 2007). This is because most of the time, cryptic species end up being delineated as separate species, following reassessments. The greatest challenge they impose is distinguishing them from other species based solely on morphological traits (Amato et al. 2007). Amato et al. (2007) compared morphological and genetic species definitions in sympatric populations of the planktonic diatom genus *Pseudo-nitzschia*, an important constituent of coastal and oceanic phytoplankton blooms. Each of the two entities consisted of multiple genetically distinct and reproductively isolated taxa, all occurring in sympatry, suggesting that they are reproductively isolated and, therefore, biologically distinct units. Little work has been done to investigate the gradual evolutionary processes and diversification mechanisms in cryptic species due to not understanding phylogenetic patterns, biogeography, degree of sympatry and diversification timeframes that result from disconcerting effects such as mutation, hybridization and phenotypic plasticity (Amato et al. 2007).

Identification of cryptic species is often difficult due to the morphological similarity between the lineages in question, so techniques have been developed to determine subtle differences at the genetic, molecular and behavioural level. DNA sequences such as the cytochrome oxidase I (COI) from the mitochondrial gene, and many other molecular markers have been used to discover new species, including cryptic species (Jung et al. 2011). This is largely because these DNA fragments have proved useful as DNA barcodes to identify and distinguish between animal species. For example, a 648 bp, the COI fragment size, has been shown to be able to identify cryptic species (Jung et al. 2011).

DNA barcoding is a tool used by taxonomists for identification or classification, by using a short genetic sequence (barcode) from a standardized region of a genome present in an organism's DNA (Hebert and Gregory 2005). It can distinguish individuals of a population belonging to a species by using the fact that genetic variation between species is greater than within species (Hebert et al. 2003). It is commonly used to identify unknown species and to assess whether they should be combined or separated. DNA barcoding is believed to speed up the discovery of species among many other things, suggesting that it can be used as a useful tool in teasing apart cryptic and sister species (Hebert and Gregory 2005). It is deemed to be a reliable, cost-effective and relatively easy-to-use molecular identification tool that can be used across metazoan taxa (Virgilio et al. 2010). It is worth mentioning that DNA barcoding has yielded contradictory results as it has been shown to be a useful tool for molecular identification in some insect families of Lepidoptera, Hymenoptera, Coleoptera and Diptera (e.g. Burns et al. 2008; Fisher and Smith 2008; Greenstone et al. 2005; Hebert et al. 2004; Smith et al. 2006, 2007, 2008) whereas its usefulness has also been questioned in some insect families of Lepidoptera and Orthoptera (e.g. Elias et al. 2007; Trewick 2007).

Mitochondrial DNA has proven to be the more reliable tool than other parts of the genome for DNA barcoding because it evolves so much quicker than other regions, and because it is passed directly from the mother to the offspring via the egg cytoplasm (Amato et al. 2007). Mitochondrial DNA evolves much quicker than other sequences because it lacks repair mechanisms and proofreading capabilities, causing it to be prone to base substitutions, resulting in high mutation rates (Neiman and Taylor 2009). Because mtDNA is passed directly from the mother to the offspring, it provides researchers with a reliable technique of investigating evolutionary relationships among individuals and species at large, by assessing their short variable sections located in the 'non-coding' region (Neiman and Taylor 2009).

Apart from partial DNA sequences (nuclear and ribosomal internal transcribed spacers; as well as the mitochondrial, nuclear and ribosomal DNA sequences), acoustic mating signals are commonly used to distinguish between closely-related species in insects and other animals (Bickford et al. 2007). Acoustic signals can be detected from far away and are known to facilitate mate choice, induce defence mechanisms and promote species recognition in many taxa; and as such, many researchers believe that divergence in acoustic signals could help to explain speciation (Wilkins et al. 2013). Acoustic signals are known to denote the emitter's identity, location and condition in one package, providing a good tool for the receiver to be able to discriminate within and between species, subsequently minimising the costs associated with direct contact (Wilkins et al. 2013). Although the processes that lead to divergence in acoustic signals and the negative attributes that result from those processes are not well understood; ecological selection, sexual selection and genetic drift have been proposed as some of the processes (Wilkins et al. 2013). Reproductive character displacement (RCD), the phenomenon where differences among similar species whose distributions overlap geographically are accentuated in regions where the species co-occur but are minimized or lost where the species' distributions do not overlap, is also known to have a role to play in acoustic signals, as a powerful tool for diversification in geographically and reproductively isolated populations. Therefore, reproductive character displacement needs to be assessed in many living organisms.

In addition to acoustic signals, the subtle differences in mating pheromones can be used to distinguish between cryptic species, although the physical appearance of the glands used to emit those olfactory signals may be identical (Bickford et al. 2007). Also, pheromones of cryptic species are distinct and species-specific, differing in subtle and minute, yet significant ways, such as the composition of the chemical compounds, as well as the ratios or proportions at which the chemical compounds are produced (Bickford et al. 2007; Groot et al. 1999; McBrien et al. 2002; Moraes et al. 2005; Yang et al. 2015; Zhang et al. 2015). Mayr (1963 as cited in Bickford et al. 2007) claimed that sibling or cryptic species are more common in species whose chemical sense is more developed than

the sense of vision, while it is rarest in species whose sense of vision is highly developed than the chemical sense. It has become widely evident that nonvisual communication, by means of sound, vibrations, pheromones, electric signals and magnetic fields, is also a key distinguishing feature in discovering cryptic species (Bickford et al. 2007).

In many ecological systems, biodiversity estimates continue to place heavy reliance upon morphological traits, yet molecular taxonomic assessments have revealed an underestimation of biodiversity, because new lineages and genetically distinct entities within morphologically delineated species are emerging at a rapid rate (Bickford et al. 2007). The underestimation of biodiversity estimates could result in hidden, unknown and unexplored ecological interactions and inappropriate conservation efforts, thus more work is needed in different taxonomic groups, phyla and biomes at large to ensure that every species is discovered and described adequately, especially taxonomic groups that have high variations (Pfenninger and Schwenk 2007). Schlick-Steiner et al. (2007) highlight that plesiomorphic, cladogenesis and convergently evolved characters are problematic in this regard, and because morphologically identical species may differ in their mitochondrial DNA, it then becomes a necessity to combine morphological traits with other methods available such as molecular assessments and semiochemistry. One of the problems posed by cryptic species is that the newly discovered species might have limited distribution and population losses could go largely unrecorded, and as such, many researchers believe that there has already been a substantial cryptic loss of population diversity (Ceballos and Ehrlich 2009).

Authors have suggested that the discovery of cryptic species might bring issues for conservation, because newly discovered cryptic species are likely to provide previously unrecognised ecosystem services (Ceballos and Ehrlich 2009). Some authors have further argued that conserving biodiversity is important for maintaining ecological function and critical ecosystem services (Hughes et al. 1997; Ceballos and Ehrlich 2002, 2009). The maintenance of ecological functions and critical ecosystem service could be important for biological control programmes. The establishment of cryptic species in biological control programmes could enhance its success rates, as more suitable biological control agents could be found, that would contribute in controlling a problematic weed in many regions.

1.2 Cryptic species in biological control

Cryptic species are likely to utilise different ecological niches, have varied host range preferences (and this is probably the most important consideration for biological control), have varied temperature tolerance regimes and have distinct behavioural patterns (Rosen 1986). Therefore, the correct identification of biocontrol agents may determine the success or failure of introductions when released against a targeted invasive weed in any region (Rosen 1979). Also, misidentifications of biocontrol agents could lead to surveys for importation in incorrect

geographical regions. Cryptic species may differ significantly in development times (Taylor et al. 2011), further justifying the need to accurately identify the biocontrol agents. Cryptic species are also likely to differ in other biological traits (diet, water loss, behavioural patterns, progeny etc.) that contribute towards their efficiency as biocontrol agents (Rosen 1979). Lastly, Rosen (1979) stipulates that the correct species identification is also key in mass-production and recovery phases of biological control programmes due to unforeseeable events such as genetic bottlenecks and population crashes.

Studying speciation is at the forefront of evolutionary biology and great strides have already been made in investigating its aspects, from sympatric speciation to reinforcement, and to postzygotic isolation mechanisms. Some authors argue that theories of speciation mainly describe how mechanisms cause reproductive isolation without using mathematical analyses to test the possibility of such predictions (Turelli et al. 2001). Other researchers argue that theories of speciation are mostly verbal because mechanisms responsible for speciation are many and they may be intertwined, ranging from ecology, behaviour and interaction between multi-locus genotypes (Turelli et al. 2001). Mechanisms responsible for reproductive isolation mechanisms include vicariance, parallel speciation, reinforcement, genetic drift and these will be thoroughly discussed in the next chapter.

1.3 Biological control

Biological control involves using a living organism to reduce the population density of another living organism known as a pest or an invader, by placing complete reliance on the exploitation of a natural control approach that uses phytophagous insects, mites and pathogenic fungi (Bale et al. 2008). Biological control is an ancient concept, with one of the earlier uses of biological control being in AD 300, involving predatory ants *Oecophylla smaragdina* Fabricius (Hymenoptera: Formicidae) used against citrus orchard pests such as *Tessaratoma papillossa* Stål (Hemiptera: Tessaratomidae) in China (Van Lenteren 2005). Controlling the cottony-cushion scale, *Icerya purchase* Maskell (Hemiptera: Monophlebidae), on citrus crops in California in the 1880s by transporting a ladybird beetle, *Radolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae), and the dipteran parasitoid, *Gryptochaetum iceryae* (Williston) (Diptera: Cryptochaetidae), have paved the way for biological control in the modern era (Van Lenteren 2005). These early successes in controlling insect pests were followed by attempts to use biological control to control alien invasive plants. The first biological control agent for an invasive alien plant was a cochineal insect, *Dactylopius ceylonicus* Signoret (Hemiptera: Dactylopiidae) which successfully reduced infestations of the invasive cactus, *Opuntia monacantha* Haw. (Cactaceae) in Australia and South Africa in the early 1900's.

1.4 Biological control of invasive alien plants

An invasive alien plant is a plant that is not native in an ecosystem and that is likely to cause adverse economic and environmental effects, and potentially affect human health (Vilà et al. 2011). Defining invasive alien plants often results in conflict of interest, because a plant may be considered as a serious invasive of natural ecosystems in one area and still be considered a valuable plant in another area (McFadyen 1998). Invasive alien plants negatively impact native biodiversity by either eliminating or reducing it through competition, altering trophic interactions and transmission of pathogens (Vilà et al. 2011). Invasive alien plants are responsible for over 40% of animal extinctions and their negative effects always result in socio-economic, health, conservation and ecological costs (Nel et al. 2004). They have also been shown to intensify poverty and impede development through their impact on agriculture, forestry, fisheries and natural systems (Vilà et al. 2011). Their effects are known to be enhanced by climate change, pollution, habitat loss and human-induced disturbances (Nel et al. 2004). Alien invasive plants tend to flourish in introduced areas due to favourable environmental conditions, climatic compatibility and lack of natural enemies and competitors (Hill and Olckers 2001). Therefore, re-uniting the invasive plants with their natural enemies through biological control is one of the key management strategies for controlling invasive alien plant populations (Bale et al. 2008). Biological control of weeds is rich in history, has a notable success rate and traditionally, the biological control of invasive alien plants puts greater emphasis on host specificity than in insect biocontrol (McFadyen 1998). This is to ensure that the biological control agents that have been released have no non-target effects. Biological control agents that have undergone host-specificity tests assist in combating the negative effects brought by weeds.

The philosophy behind biological control is utilising co-evolved natural enemies of the weed for control purposes. The philosophy revolves around the idea that weakening the invasive alien plant results in its reduced competitiveness and as a result, indigenous species will have a greater chance of competing and surviving. The biocontrol of weeds programmes are governed by procedures that involve several steps: (1) exploration (involving the correct identification of the weed and its country of origin), (2) agent selection (crucial step that aims to choose the best agent based on the combination of post-hoc analyses and pre-release studies), (3) host-specificity testing (tests of feeding preferences involving the problematic weed and its closely related plant species to determine the potential host range of the agent and the potential plants at risk), (4) rearing and release (the stage involving mass production of the agent) and (5) evaluation or monitoring (assessment that justifies continued expenditure, done after the agent has been released and has somewhat established itself and also aims to determine reasons for success or failure of an agent to control the weed) (McFadyen 1998). Success and failures of any biological control programme of weeds is usually dependent on several factors such as climatic compatibility, favourable habitat

conditions, sufficient number of agents released, plant phenology and the level of predation and parasitism upon agents and (Coetzee and Hill 2008).

Biological control is an ideal method for controlling high densities of floating invasive aquatic weeds as it uses co-evolved biological organisms. Biological control of aquatic weed species has been particularly successful in South Africa (Coetzee et al. 2011). For example, red water fern (*Azolla filiculoides*), water lettuce (*Pistia stratioles*) and salvinia (*Salvinia molesta*) have been completely controlled to the extent that they now have minimal effects on the aquatic ecosystems (Coetzee and Hill 2008). This demonstrates the efficiency and the substantial effectiveness of biological control for aquatic invasive alien species in the country. Substantial control of water hyacinth, *E. crassipes* has been reached in South Africa, but is limited under eutrophic conditions, and in the cooler, high lying areas of the country that receive winter frost (Coetzee et al. 2007a). This has necessitated the release of more control agent species than anywhere else in the world in an attempt to improve control.

1.5 Water hyacinth

1.5.1 Water hyacinth description

First described in 1824, *E. crassipes* belongs to the family Pontederiaceae which has always been known to be a troublesome family that contains many weeds. The genus *Eichhornia* only possesses eight species and in those, only *E. crassipes* is classed as a pantropical aquatic weed, whereas others are classed as neotropical and palaeotropical weeds (Coetzee et al. 2009). *Eichhornia crassipes* is an erect free-floating herbaceous aquatic macrophyte that is a prominent figure on the list of the worst weeds in the world (Coetzee et al. 2005) (Fig 1.1). It is native to the Amazon Basin in South America (Coetzee et al. 2009). *Eichhornia crassipes* is known for its attractive shiny round leaves and striking purple flowers which contributed to its spread throughout the world by gardeners (Fig. 1.2). *Eichhornia crassipes* has a unique polymorphism in its flower structure (tristyly), because it has three different style lengths that lead to three possible configurations (floral trimorphism): short, intermediate and long (Julien et al. 1999). The short style (about 25 cm long) is common in tropical regions whereas the intermediate and long lengths (about ±60 cm long) are common in native and invaded areas found in temperate regions (Julien et al. 1999). The short style has medium and long stamens, the medium style has short and long stamens while the long style has short and medium stamens (Julien et al. 1999). The initial leaves of seedling *Eichhornia crassipes* are elongated and strap-like but later grow into a spathulate form measuring only a few centimetres, whereas mature plants usually measure 1 m in height (Julien and Griffiths 1998). The plant has singular shoots/crowns that each has about 10 expanded leaves arranged spirally and separated by short internodes (Coetzee et al. 2011). The

axillary buds of *Eichhornia crassipes* develop as stolons by growing horizontally for 20-60 cm before becoming daughter plants, whereas the inflorescence develops from the apical meristem (Julien et al. 1999). As with many plants, roots develop at the base of each leaf, forming a dense mass that ranges from 20-300 cm in length, with the ratio of root to shoot depending entirely on the nutrient conditions (Coetzee et al. 2011). *Eichhornia crassipes* has submerged roots and rhizome, aerial leaves and has air filled petioles which enable the plant to float on the water surface. The inflated petioles are a distinguishing feature of the weed from other members of the family Pontederiaceae (Julien et al. 1999; Ajuonu et al. 2009). *Eichhornia crassipes* is a perennial that can undergo both sexual reproduction through the production of seeds, and asexual reproduction through the development of clonal daughter plants which breakoff and form new plants (Coetzee et al. 2009; Coetzee et al. 2011). *Eichhornia crassipes* has high phenotypic plasticity, high growth rate and development, and produces highly resistant viable seeds (Ajuonu 2007).

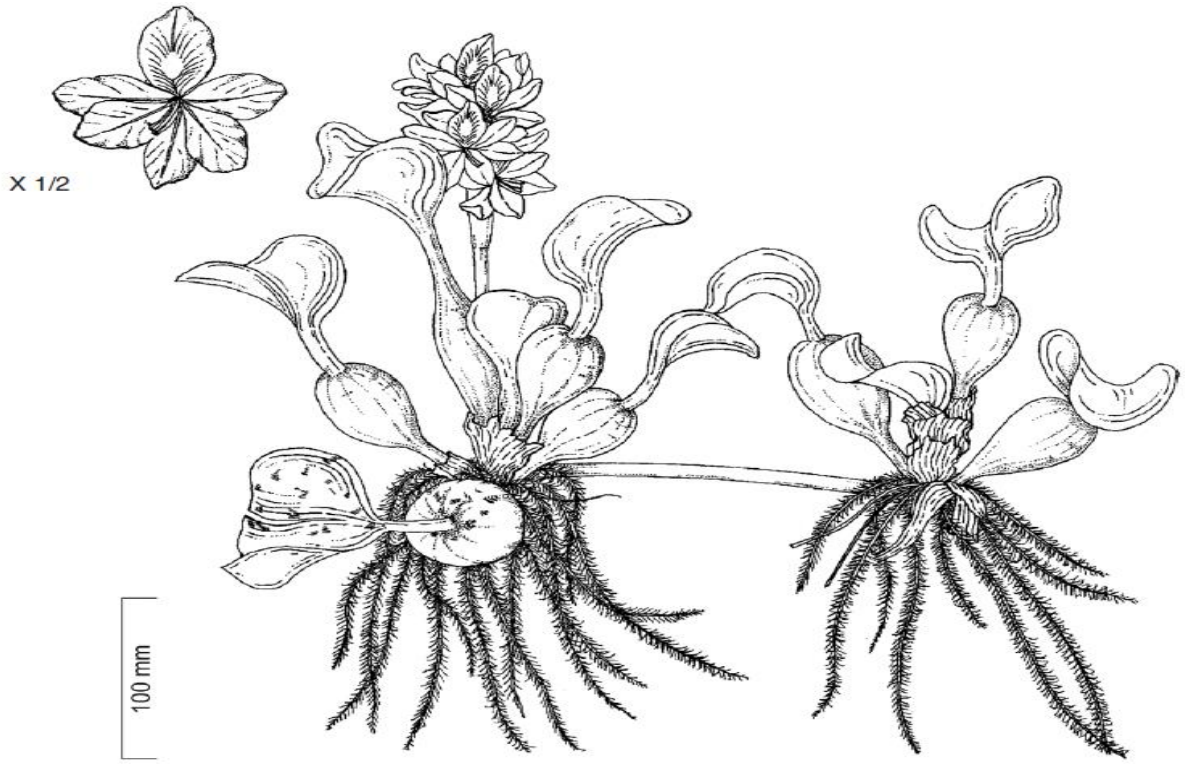


Figure 1.1 A schematic representation of an invasive aquatic macrophyte, water hyacinth *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae) image. Adopted from (Coetzee et al. 2011) [Drawn by W. Roux and first published in Henderson and Cilliers 2002].

Figure 1.2 A flowering *Eichhornia crassipes* plant, illustrating the beautiful lavender-blue flowers, largely responsible for its anthropogenic spread around the world. (From Coetzee et al. 2017).

1.5.2 Uses of water hyacinth

Water hyacinth has often served as an ornamental plant, animal feed, mulch, manure, fertiliser, and for making compost, fuel bricks, making paper, generating methane biogas, removing nutrients and toxic chemicals present in the water, waste water treatments, improving soil quality, assessing bioelectricity production, hydrothermal liquefaction and for anaerobic co-digestion with poultry litter (Gopal 1987). Furthermore, Ndimele and Ndimele (2013) established that water hyacinth can absorb petroleum hydrocarbon and can thus be utilised in phytoremediation of crude oil-polluted aquatic ecosystems. Some researchers believe that water hyacinth could be useful in sewage and waste water treatment because of its fast growth and absorption rates (Hill et al. 1999). The lengthy list of water hyacinth uses serves to somewhat demonstrate why water hyacinth has proved to be a problematic invasive alien plant throughout the world. It is possible that because the plant has invaded in so many areas throughout the world, people have been forced to find uses for it and this paved the way for the requirement of control measures, because water hyacinth has the ability to proliferate under suitable climatic conditions.

1.5.3 Water hyacinth distribution

Water hyacinth is considered indigenous in the Amazon basin in South America (Center et al. 1989; Julien et al. 1999). The first time it was recorded outside of its native distribution was in Louisiana, U.S.A., in 1884, followed by Florida, U.S.A., in 1890. Contestants at the New Orleans Cotton Exposition competition in U.S.A in 1884 were each given a live water hyacinth plant as a gift and many of the first introductions of this plant to other countries can be linked back to this meeting (Julien et al. 2001). It was later reported in Java (1894) in Vietnam (1902) and China (1902). It was first reported in South Africa in 1910 when it was deliberately introduced into botanic gardens as an ornamental plant because of its attractive flowers (Hill et al. 1999). It was later noted in Hong Kong, where someone admired its beauty and took the plant to Sri Lanka; while on the other hand a Chinese resident imported the plant from Hong Kong to his garden to feed livestock (Burkil 1935). Its distribution now extends through tropical, subtropical and warm temperate regions of the world (limited to latitudes of 40° N and S) (Fig. 1.3), and has the status of being the most important aquatic weed in the world, including South Africa (Hill et al. 1999). During the early stages of its establishment, its spread in most parts of the world has been facilitated by either downstream flow or by seeds in mud that are attached to animals as vectors, or by anthropogenic spread via gardeners, aquarium owners and boating enthusiasts (Hill et al. 1999). In South Africa, water hyacinth offers the greatest challenge in eight of the nine provinces, and is found to be least problematic in the Northern Cape.

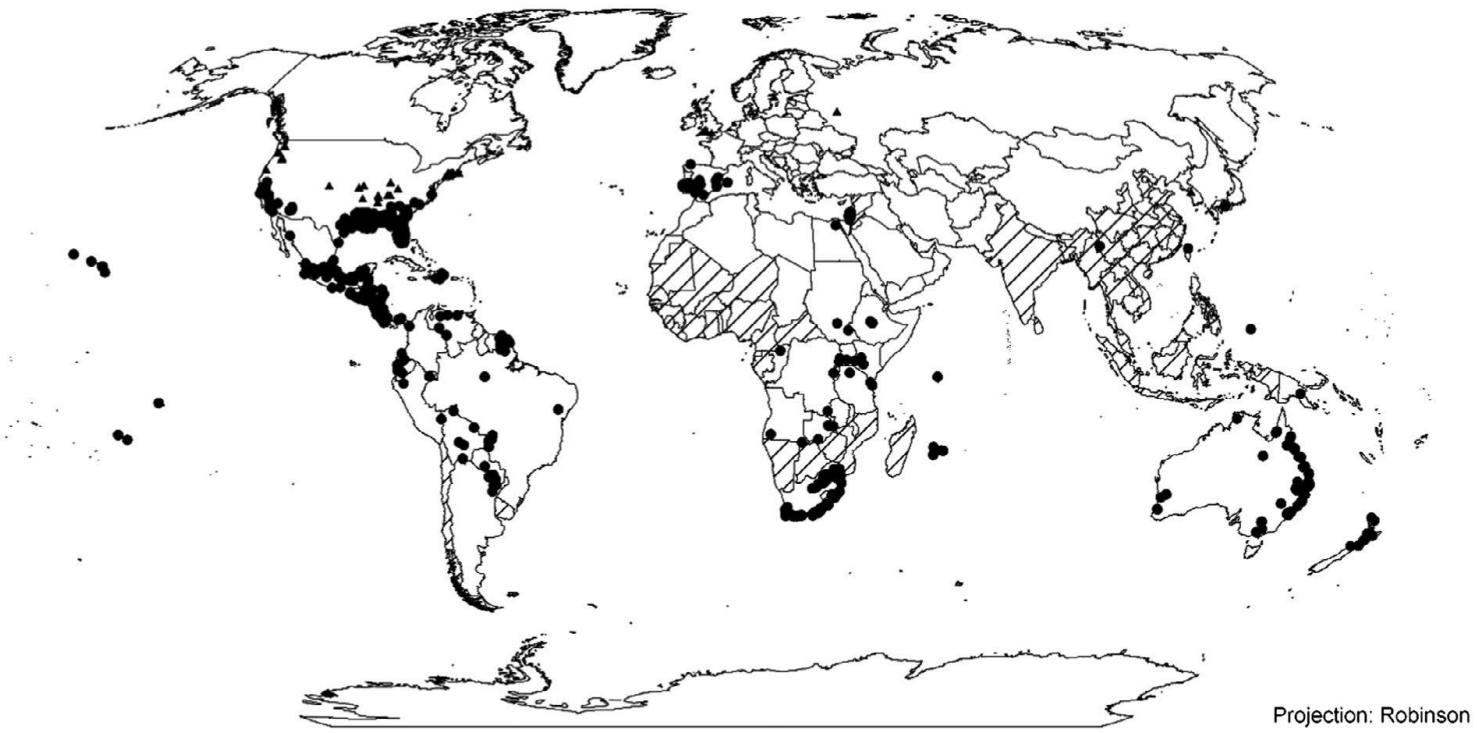


Figure 1.3 Global distribution of *Eichhornia crassipes*, including established and casual populations. Where information has been provided by country, these administrative areas have been shaded. Where more precise distribution data is available this is indicated as dots, with established population indicated as circles, and ephemeral populations as triangles. (Source: Kriticos and Brunel 2016. From Coetzee et al. 2017).

1.5.4 Water hyacinth impacts

Water hyacinth has a major impact on biodiversity in general. It is now the most problematic aquatic weed in the world, forming thick extensive mats that impede water flow in rivers and dams (Coetzee et al. 2009) (Fig. 1.4). It is known as the most troublesome invasive aquatic weed because of the ecological and socioeconomic effects that it causes (Coetzee et al. 2009). It has been declared as a noxious weed in many countries since the beginning of the 20th century (Gopal 1987). For example, water hyacinth is a noxious weed in USDA, a class 1 noxious weed in Australia, a prohibited weed in New Zealand, a declared invader in Botswana and a Category 1b weed in South Africa (Coetzee et al. 2017). In many of these countries, the transportation and sale of water hyacinth is prohibited, and land users and water authorities are required to control infestations in an appropriate manner (Holm et al. 1977; Coetzee et al. 2017). Coetzee et al. (2017) further highlight the fact that although no recent legislation is in place, this plant is still considered a threat, and governments consider it an unwanted non-native species. This is primarily because water hyacinth infestations cause restricted utilization of still or moving water, resulting in thick extensive mats which then affect the ecosystem's health (Cilliers 1991). Water hyacinth impacts include changes in the physio-chemistry found underneath water hyacinth mats which lead to greater dissolved carbon dioxide concentrations, lesser dissolved oxygen, lesser phosphorus concentrations, and inevitably those changes in physio-chemistry reduce phytoplankton populations (Ajuonu 2007). Water hyacinth also causes increased evapotranspiration and increased siltation (Cilliers 1991). In conservation and ecological terms, the invasive weed reduces biota diversity in the bottom of aquatic biomes as well as shallow waters closer to the shore. Ultimately, water hyacinth invades and destroys native flora by competing for things such as space, sunlight and it uses most of the nutrients available in the water, proving to be a problematic pest to native flora (Coetzee and Hill 2008). This invasive aquatic weed also offers refuge and breeding place and therefore promotes vectors of human and animal diseases such as malaria, cholera, bilharzia, to name just a few (Coetzee and Hill 2008). The thick and extensive mats formed by the presence of the invasive weed prohibits water usage, thus having an indirect major impact in fisheries and related commercial and recreational activities, hydroelectric programmes, efficient navigation and transport, functioning of irrigation canals, tourism and communities with high dependence of freshwater waterways for food, transport and clean water (Coetzee and Hill 2008).

Figure 1.4 Hippopotamus appear out above an infestation of *Eichhornia crassipes* on the Mkhadzi River, in the Kruger National Park, South Africa. Hippopotamus are known to disperse plants within and between waterbodies. (From Coetzee et al. 2017).

1.5.5 Control measures used against water hyacinth

Many attempts have been made to control the weed using a variety of methods. Complete eradication of water hyacinth has not been attained in most areas due to the ability of the weed to regrow from remaining viable seeds and daughter plants (Ajuonu 2007). Manual clearing and mechanical control, which are labour intensive and often expensive were the initial control options used against the weed, but can only offer temporary control because the weed can quickly increase in biomass from the remaining plants in a very short space of time under favourable conditions (Coetzee and Hill 2008). Mechanical control involves utilising floating booms or fixed barriers to prevent movement in undesirable areas, followed by physical removal and crushing of the weed. Chemical control is expensive, only temporarily effective, requires frequent application, affects non-target organisms and is effective for small infestations (Cilliers 1991). This largely involves the use of 2,4-dichlorophenoxyacetic acid (2,4-D) and

glyphosate which is most successful where there is rapid growth of water hyacinth, high temperature and high humidity measures (Findlay et al. 1996). Other herbicides commonly used are paraquat, diquat, aminotriazol (amitrole), ametryn and terbutryn and penoxsulam (Findlay et al. 1996). Herbicides in the imidazolinone, sulfonylurea and other groups have also been shown to be effective against the weed (Findlay et al. 1996). However, chemical control of water hyacinth in South Africa relies on the use of glyphosate.

Biological control is the most important control approach that has been used in the past and is considered as the most promising long-term solution against the water hyacinth problem because it is environmentally-friendly, economical, poses no health risks and is self-sustainable (Ajuonu 2007). In low nutrient warm areas, biological control alone results in complete control and no other control measures are required, whereas in cold and high nutrient systems, integrated control is required – this means that in many systems it is essential to include chemical control in an integrated strategy to achieve control (Coetzee and Hill 2008).

1.6 Biological control of water hyacinth

1.6.1 Challenges to the biological control programme

The control of water hyacinth is variable and is impacted by factors such as nutrients in water bodies, climate suitability, herbicide usage and the number of biocontrol agents released (Julien et al. 1997). The expected time for a successful biological control programme of water hyacinth to start yielding results relies upon many factors, but in suitable conditions such as regions that resemble those found in tropical regions, it takes about 3-5 years (Coetzee and Hill 2008). The range of temperatures that the invasive alien plant and its natural enemies face have been shown to affect growth and development rates, nutrient acquisition, reproduction rate, thereby extending the duration that it takes for biocontrol agents to reduce weed infestations (Coetzee et al. 2007b). The nutrients in the water, quality of the plant, and the size and density of individual plants are also shown to influence the success of biocontrol agents (Coetzee et al. 2007a). The number of individuals for each biocontrol agents released against an invasive alien plant have also been shown to have a role to play.

In tropical regions, where nutrient availability is not limited, water hyacinth multiplies in about 6-18 days at optimum temperature; the growth rate either ceases or is prolonged at 10°C (and stored carbohydrates from the stem coming into play as energy reserves). The plant dies when the temperature is below 5°C or above 40°C, with 22-35°C favouring its growth and development (Wright and Purcell 1995; Coetzee et al. 2009; Julien et al. 1997). Water hyacinth thrives in highly eutrophic, nutrient-rich environments that have low light intensities (Coetzee et al. 2009). There is a positive correlation between growth rate and the percentage concentration of nitrogen in its

leaves, and there is also a positive correlation between its growth rate and nutrient concentrations in the water (Center and Wright 1990). Water hyacinth can tolerate many pH conditions, thus only waters having salinity greater than 0.06% can kill this invasive aquatic weed but pH below 4.5 or above 10 can also be damaging (Ajuonu 2007). Calcium concentrations are also key as growth ceases if below 5 mg/L (Ajuonu 2007).

In South Africa, water hyacinth infestations are more challenging in high altitude cold winter regions, with eutrophic waters (Hill et al. 1999). In such regions, frost is seen on frequent occasions, thus the active growing season for both the water hyacinth and its natural enemies is slightly shifted by being restricted to 6 months (Hill et al. 1999) and there are mismatches between the plants and insects (Hill and Olckers 2001). Water hyacinth then regrows during spring months while its natural enemies only become effective during mid-summer months (Hill et al. 1999), in the process allowing the weed to grow in the absence of the agents (Hill et al. 1999). Because several invasive weeds, as well as their natural enemies are native in warm tropical regions, it is of utmost importance to select cultures that are from an area of similar climate when considering an introduction of any biocontrol agent (Williamson 1996). This will probably maximise the effectiveness of the introduced biocontrol agents since it is likely to quickly adapt into the new area. The variability seen in temperate regions can be attributed to many factors such as eutrophication of water bodies, herbicide operations interference, hydrology of small bodies, droughts and floods in non-impounded water systems and insufficient effort and quantity of released biocontrol agents (Hill et al. 1999). South Africa is known to have lots of nitrates (N) and phosphates (P) which cause a seemingly, though deceivingly insufficient feeding damage of biocontrol agents against the weed, as a result of an exponential increase in biomass of the weed, since water hyacinth outbreaks are correlated with nutrient levels in water bodies (Hill et al. 1999). This led to Hill and Olckers (2001) to envisage new ways to improve the control of water hyacinth, such as research into other potential agents, a deep understanding of the hindering factors and finding ways to integrate biological control with other management procedures to enhance the currently estimated 10% success rate (Coetzee et al. 2011).

1.6.2 Biocontrol agents released against water hyacinth

Globally, ten arthropods and four pathogens have been released as biocontrol agents to combat water hyacinth (Coetzee et al. 2009). The earliest biocontrol agents that have been used included the two weevils stem boring weevils, *N. eichhorniae* and *N. bruchi*, and these are known to cause a considerable damage and are the most widely established in the world (Hill and Cilliers 1999; Coetzee et al. 2011). The two co-existing *Neochetina* weevils have been successful in controlling water hyacinth in the USA, Argentina, Australia and Sudan, reducing infestations by 80-99 % (Julien 2001). These weevils have also had a significant impact on water hyacinth infestations in Uganda, Papua New Guinea, Mexico, Benin, South Africa, Zimbabwe and Malawi (Julien 2001). The

mite, *Orthogalumna terebrantis* Wallwork (Acarina: Sarcoptiformes: Galumnidae), which forms window-like mines on the leaves of water hyacinth was also one of the earliest biocontrol agents to have been released and it is known to cause considerable damage and is established in many areas of the world (Hill and Cilliers 1999; Coetzee et al. 2011). Its feeding, as often observed in South Africa, acts as an intermediate and causes pathogens to enhance the effectiveness of the damage caused by insects (Julien 2001). The moth or petiole borer, *N. albiguttalis* also forms part of the biocontrol agents released against water hyacinth. It is known to cause a considerable damage and is established in many areas of the world (Hill and Cilliers 1999). For example, it is reported that *N. albiguttalis* has been successful in the USA and Sudan, whereas it failed to establish in Benin and Ghana (Oke et al. 2012). The mirid, *Eccritotarsus catarinensis* (Hemiptera: Miridae) is known to have considerable damage and is also established in Ghana, South Africa and Malawi (Hill & Cilliers, 1999; Coetzee *et al.*, 2005; Coetzee *et al.*, 2007b; Coetzee *et al.*, 2011). The cryptic species, *Eccritotarsus eichhorniae* (Hemiptera: Miridae) has only been released in South Africa and its impact and establishment have not been recorded yet. On many occasions, biocontrol agents that can interact with each other are often used in combination, such as *Neochetina* spp. and *Eccritotarsus* spp.

Fungi such as *Cercospora piaropi* Tharp. and *Cercospora rodmanii* Conway (Mycosphaerellales: Mycosphaerellaceae), *Acremonium zonatum* Link (Hypocreales: Hypocreaceae) as well as *Alternaria eichhorniae* NagRaj and Ponnappa (Pleosporales: Pleosporaceae) have been observed on water hyacinth, and have been redistributed onto other areas because they are all classed as suitable bioherbicides and mycoherbicides (Cilliers 1991). *Cercospora rodmanii* is known to have a considerable damage and is established in several sites (Hill & Cilliers, 1999; Morris *et al.*, 1999). *Cercospora piaropi*, *A. zonatum* and *A. eichhorniae* occur locally in South Africa, and as such, they have not been released as leaf pathogens that target water hyacinth in some sites (Morris et al. 1999). Pathogens form part of an integrated approach of controlling water hyacinth and are known to enhance the efficacy of biocontrol agents.

There have been promising agents in amongst the most recently-released biocontrol agents in South Africa including the grasshopper, *Cornops aquaticum* (Brüner) (Orthoptera: Acrididae: Leptysminae), released in 2013, and despite showing promise, has not established in South Africa (Coetzee, J.A. pers. comm.). The leaf hopper, *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) was released in South Africa in 2013 and is known to have established, but its impact is yet to be assessed (Hill and Coetzee 2017). The moth or petiole borer, *Xubida infusellus* (Walker) (Lepidoptera: Pyralidae) and the fungus *Uredo eichhorniae* Gonz. Frag. & Cif. (anamorphic fungus: Pucciniales) have been shelved in South Africa (Morris *et al.*, 1999; Coetzee *et al.*, 2009), while several *Thrypticus* species (Diptera: Dolichopodidae) and *Taosa* species Remes Lenicov (Hemiptera: Dictyopharidae) are

under investigation in Argentina (Coetzee et al. 2011). The moth, *Bellura densa* (Walker) (Lepidoptera: Noctuidae) was rejected in South Africa following host-specificity testing (Center and Hill 2002).

1.6.3 History of biological control of water hyacinth in South Africa

The biological control campaign that targets the invasive water hyacinth started in 1962, when entomologists from the United States of America attempted to find biocontrol agents of the weed in its indigenous distribution in South America (Cilliers 1991). The South African Department of Water Affairs (SADWA) and the Department of Agricultural Technical Services (DATS) also contemplated introducing a snail, *Marisa cornuarietis* Linnaeus (Gastropoda: Ampullariidae), a potential herbivore that had been seen feeding on water hyacinth but it was later rejected because of its lack of host-specificity and voracity since it is a generalist herbivore (Cilliers 1991). After this, no work had been done until 1973, when the Plant Protection Research Institute (PPRI-ARC) assigned an entomologist expert to work on the weed on a part-time basis, but the agreement only continued until 1977 (Cilliers 1991). The campaign was then resumed in 1985, along with several other biological control programmes against other aquatic weeds (Cilliers 1991). It is now 55 years since the first attempt to search for natural enemies in native range of water hyacinth and the success and efficacy of biocontrol agents varies from complete control to minimal control (McFadyen 1998). To date, water hyacinth biocontrol agents have been introduced in about 39 countries world-wide where the two weevils, *Neochetina eichhorniae* Warner and *Neochetina bruchi* Hustache (Coleoptera: Curculionidae), as well as the moth, *Niphograpta albiguttalis* (Warren) (Lepidoptera: Crambidae) have been the most effective and successful at establishing themselves all over the world (Julien and Griffiths 1998; Julien et al. 1999; Julien et al. 2001; Winston et al. 2014). Furthermore, recently in South Africa, additional biocontrol agents such as *Eccritotarsus* spp. (Hemiptera: Miridae) and *Megamelus scutellaris* (Hemiptera: Delphacidae) have been released, resulting in moderate control (Winston et al. 2014). Some biocontrol agents such as the *Xubida infusella* (Walker) Lepidoptera: Crambidae) have been shown to have a slight impact on water hyacinth (Winston et al. 2014). Other biocontrol agents such as *Cornops aquaticum* (Brüner) (Orthoptera: Acrididae) have been release and post-release assessments are yet to be done (Winston et al. 2014).

1.7 The mirid, *E. catarinensis*: one of many biocontrol agents released against water hyacinth

1.7.1 The mirid, *E. catarinensis* morphology and life cycle (biology)

The mirid *E. catarinensis* is a plant bug that measures 2-3 mm in length, is primarily black with darkly marked but mostly transparent wings (Stanley and Julien 1999). This bug is a leaf sap-sucking, biocontrol agent of water

hyacinth (Hill et al. 1999). Both the nymphs and adults are mobile and feed gregariously underneath water hyacinth leaves, causing chlorosis (and therefore yellowing and browning), and eventually leaf death because of excessive acquisition of chlorophyll from the palisade parenchyma (Hill et al. 1999). Feeding stunts the growth rate of the weed by reducing photosynthesis, that eventually leads to a reduction in overall biomass (Coetzee et al. 2007b). Development takes about 23 days, with 15 of those days used for undergoing four to five nymphal instars, after which the adult lifespan is approximately 50 days (Hill et al. 1999). The insect can live for several weeks in suitable laboratory conditions and can persist even when the leaves are subjected to chlorosis (personal observation under laboratory conditions).

1.7.2 The introduction of the mirid *E. catarinensis* into South Africa

Eccritotarsus catarinensis was originally collected from Florianopolis (Santa Catarina) in Brazil in 1992 (Hill et al. 1999). During host specificity testing in quarantine in South Africa, the population suffered a massive bottleneck due to an air-conditioning malfunction (Hill, M.P. pers. comm.). Only one gravid female survived this bottleneck and all host specificity testing and subsequent release of this agent were the progeny of this single female (Taylor et al. 2011). Host specificity testing showed that it was suitably host specific for release in South Africa, and as a result, it was released in 1996 and successfully established populations at more than 30 sites in the country (Taylor et al. 2011). Although the agent established widely, successful control was variable and limited. This limited success was thought to be due to climatic incompatibility and the lack of genetic diversity within the agent population. A new population of the mirid was then collected from the upper Amazonian basin (near Iquitos in Yarapa River), in Peru in 1999 (Taylor et al. 2011), a site more than 3500km from the Brazilian population source (Paterson et al. 2016). The intention of this second collection of the agent was to increase genetic diversity and variability in the hope that this would increase the efficacy of the biological control agent (Paterson et al. 2016). It was also thought that interbreeding of these two populations would increase the chances of establishment in colder regions. The underlying assumption was that the resulting population from these two interbreeding populations would yield a more resistant, resilient, efficient and effective biological control agent that would easily overcome unforeseen catastrophic events in the future (Paterson et al. 2016). As such, they have been released in the country.

1.7.3 The discovery of cryptic species in *E. catarinensis* (Carvalho)

Taylor et al. (2011) found a clear distinction between the Brazilian and Peruvian populations since the comparison of the mitochondrial cytochrome oxidase subunit 1 gene (mtDNA) revealed a 5.2% haplotype sequence divergence between the two imported populations in South Africa, and this led to them being considered as cryptic species. Genetic analyses have been conducted in about 16 species in Miridae thus far and the 5.2% haplotype sequence

divergence is a higher mean divergence value than what has usually been recorded in interspecific comparisons of the CO1 region for this taxonomic group (Park et al. 2011; Taylor et al. 2011; Paterson et al. 2016). The Inter Simple Sequence Repeats (ISSR) loci results involving the same populations were in unison with mtDNA results reported above, as 29 fixed differences were observed. Collectively, the mtDNA and ISSRs findings suggested that the two populations now represent separate species altogether (Taylor et al. 2011; Paterson et al. 2016).

1.7.4 Interbreeding experiments involving Brazil and Peru populations

Taylor et al. (2011) findings led to further investigations, involving a 'no choice interbreeding experiment' (1st) and a 'choice interbreeding experiment' (2nd). In the first experiment, where pairs of virgin adults were placed on a small water hyacinth plant within and between species, it was established that there were very few or no offspring produced (Paterson et al. 2016). In the second experiment, where eight populations were set up in pools covered with mesh, sequenced at COI and fingerprinted using Inter Simple Sequence Repeats (ISSRs), two clear groups were identified, and when the populations were mixed, the Brazilian population was more dominant, and had thus out-competed the Peruvian population (Paterson et al. 2016). These findings revealed little or no hybridization and supported the findings in the no-choice breeding experiment. More detailed morphological studies were subsequently conducted on these two populations, and it was established that they have unique and subtle morphological differences in the scent glands and the antennae (Henry 2017). As such, they have now been described as different species based on the detailed morphology differences and prior findings reported above, with the Peruvian population described as *Eccritotarsus eichhorniae* Henry. So, in essence, the findings of Taylor et al. (2011), Paterson et al. (2016) and Henry (2017) have provided the rationale for this study, which aimed to supplement their findings by assessing interbreeding behaviour and chemical compound compositions of *E. catarinensis* and *E. eichhorniae*, in an attempt to further investigate the mechanisms or barriers responsible for reproductive isolation between the two cryptic species. This will improve our understanding of the mechanisms that resulted in speciation in these cryptic species of *Eccritotarsus* and the evolution of cryptic species more generally. The mating behaviour and chemical compound compositions of these insects have not been assessed.

The overarching aim of the project was to assess the reproductive isolation mechanisms of two cryptic species of *Eccritotarsus* (Hemiptera: Miridae), biological control agents of water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae). To achieve that, mating behaviour and chemical compounds were assessed.

CHAPTER 2: Mating behaviour of two cryptic species of *Eccritotarsus* (Hemiptera: Miridae).

Introduction

This chapter assessed the mating behaviour of two geographically and reproductively isolated cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae) that are now known to be separate species. The Brazilian population remains *Eccritotarsus catarinensis* (Carvalho) while the Peruvian population has recently been described as *Eccritotarsus eichhorniae* (Henry) (Henry 2017). The two species are morphologically similar and were thought to be close relatives but interbreeding between the species is either extremely rare or non-existent (Paterson et al. 2016; Henry 2017). The mechanisms responsible for reproductive isolation have not been thoroughly explored, and investigating these mechanisms could increase our understanding of the process of speciation in these cryptic species of *Eccritotarsus* and for other herbivorous arthropods.

Speciation - the formation of a new species, remains a complex issue in the biological sciences even though many centuries of investigations and debates have been invested in it (Hewitt 2001). Gene flow barriers are known to result in either sympatric or allopatric speciation, from which reproductive and ecological selection mechanisms are known to maintain the genome of the population and possibly lead to formation of a new species. One of the fields of research that has increased our understanding of how new species evolve is phylogeography, which is the study of the geographical distribution of genealogical lineages (Avise 1998). Phylogeography largely uses, but is not limited to mitochondrial DNA (mtDNA), to investigate phylogenetic relationships in populations, subspecies and species of all animals and then plots their geographical distribution. Using DNA sequences allows one to deduce the phylogenetic relationship history from the point of its divergence (Taberlet et al. 1998). It combines several types of data and has proved to be a useful tool in answering many evolutionary questions for taxonomists (Bernatchez and Wilson 1998). A large geographic distance separates the two chosen species of this study and it is clear from the mitochondrial DNA that this distance has acted as a barrier to genetic transfer resulting in the formation of two distinct species (Paterson et al. 2017).

Other gene flow barriers or mechanisms responsible for reproductive isolation include genetic drift and chance events (e.g. mutation, chromosome rearrangements etc.), and these have been shown to lead to an initial divergence (Curie 2012). Initial divergence proves to be a great challenge for organisms when populations are faced with similar selection regimes, and it is widely accepted that the magnitude of gene flow influences genetic drift, but quantifying the amount of gene flow has proved to be difficult; so our understanding of the potential impacts of genetic drift and speciation is limited (Curie 2012). This has been further complicated by the inability to separate natural selection from sexual selection, relatively known important drivers of speciation in all living

organisms, with natural selection contributing more frequently towards speciation than sexual selection (Curie 2012). But Kirkpatrick and (Ravigne 2002) report that sexual selection contributes more than natural selection through one-allele mechanism driven by survival or fecundity selected characters.

Mayr (1963) postulated that allopatric speciation associated with geographical vicariance leading to reproductive isolation in organisms provides the only mechanism of speciation. But it is worth mentioning that dispersal barriers appear and disappear in random fashion throughout evolutionary history and most importantly, different species are likely to respond differently to that incident (Ronquist 1997). Nevertheless, barriers to gene flow play an important role in speciation. Speciation, which divides a single population into sub-populations, usually leads to reproductive isolation between divergent populations (Lee et al. 2008).

Bush and Butlin (2004) list ways in which speciation can occur in specialist insects and these are: by co-speciation, in allopatry on the same resource, in allopatry leading to a resource shift, in sympatry leading to a resource shift and by interspecific hybridization. Co-speciation, also known as co-cladogenesis is when an ancestral association between two or more species splits into descendant associations concurrently. It occurs through a co-ordinated allopatric speciation process, and as a result, cospeciated clades are usually tightly associated and are of the same age. An example of co-speciation involves lice and their hosts, because a phylogenetic study using mitochondrial cytochrome oxidase I found clear evidence of co-speciation between most of the 15 allopatric pocket gophers (Geomyidae) and their associated lice (see Hafner et al. 1994). It has been proposed that whether or not co-speciation occurs in lice may depends on the degree of allopatry between host species (Bush and Butlin 2004).

Allopatry on the same resource occurs when specialists have undergone allopatric speciation without a host shift. An example of allopatry on the same resource involves *Heliconius* spp. (Lepidoptera: Nymphalidae) butterflies because they specialise on *Passiflora* spp. (Passifloraceae) in the subgenus *Plectostemma* but evolution of an alternative mimetic colour pattern, now associated with strong assortative mating has driven speciation (see (McMillan et al. 1997)). Few studies document this form of speciation, but many of these speciation events involve shifts in habitats and use of host-plant resource (Bush and Butlin 2004).

Allopatry leading to a resource shift involves three broad possibilities: sympatry, allopatry without a restriction in population size or vicariance and allopatry with a period of reduced population size. This type of speciation occurs when a population of widely distributed species becomes geographically isolated on only one of its normal hosts. An example of allopatry leading to a resource shift involves *Larinus* spp. weevils (Coleoptera: Curculionidae) on *Ornopodium* (Asteraceae) and *Cynara* (Asteraceae) thistles in the Mediterranean region, and is attributed to an association between phylogeography, based on allozymes, and host-plant use (see Briese et al. 1996).

Sympatry leading to a resource shift occurs when there is a development of a biologically distinct host race from inhabiting a new host. An example for this involves a tephritid fruit fly *Rhagoletis pomonella*, whereby it is widely accepted that sympatric speciation is a progressive process and whether a complete reproductive isolation happens quickly or slowly depends on the intensity of the divergence selection, the degree of host preference and assertive mating. It would appear that finding behaviour genes responsible for speciation has proved to be key in phylogeography and evolutionary biology studies in general (see Roderick and Gillespie 1998). It is important to understand how speciation occurred in *E. catarinensis* and *E. eichhorniae*, to understand reproductive isolation.

Parallel speciation is yet another possible mechanism that may lead to reproductive isolation. Parallel speciation is a special form of parallel evolution where traits that are responsible for reproductive isolation evolve in independent, closely related populations as a by-product of adapting to different environments. Geographic isolation results in parallel evolution and it plays a key role in the evolution of reproductive isolation and divergent morphology (Worsham et al. 2017). Parallel speciation by natural selection can occur through: non-adaptive evolution of premating isolating mechanisms via pleiotropy with morphological/physiological traits, mate preferences subjected to direct selection through phenotypic evolution causing a sequence of selections, or new environments causing changes to sensory mode for improved mate choice or detection of mates and post-mating isolation evolving as a by-product of environmental selection pressures (Schluter and Nagel 1995).

Mechanisms that are responsible for reproductive isolation can be group into two and these are termed as prezygotic isolation mechanism (resulting from mating discrimination or unsuccessful gamete recognition) and postzygotic isolation mechanism (resulting from hybrid invariability or sterility, chromosomal rearrangement, DNA divergence and genetic incompatibility); and these respective mechanisms are difficult to tease apart because they may work concurrently (Lee et al. 2008). Several studies have led many to believe that prezygotic isolation and pre-copulatory mechanisms have a greater role to play as a gene flow barrier than postzygotic isolation and post-copulatory mechanisms, and are the driving force behind speciation (Curie 2012). This idea originates from research involving speciation that showed substantial prezygotic isolation, and the fact that it can contribute more towards total barrier to gene flow than postzygotic isolation, but some authors have argued that the findings have largely ignored the role played by extrinsic postzygotic isolation due to differential adaptations, which might be the first step in speciation (Sobel et al. 2010). Their arguments goes as far as postulating that genotype-by-environment interactions, such as the effects of larval diet on adult behaviour might influence both pre- and postzygotic isolation measures, so it becomes difficult to assess which mechanism has played a greater role (Curie 2012). Findings have revealed that if closely related sister species are reproductively isolated by a pre- or a post-mating barrier, they can co-exist without hybridizing or completely excluding each other, even when they have the

same resource and mate recognition systems (Butlin 1990). Additionally, findings reveal that if these closely related sister species were to hybridize, they would either form a stable hybrid zone (Butlin 1990) or a new parthenogenetic species (Bullini 1994), and this has been the main driving force behind many species concepts.

On another hand, recently-diverged specialist insects are likely to inhabit different hosts, reducing the chances of coming across each other when feeding or looking for mates. For these insects, mating may be limited to their respective hosts or habitats for each species, and host preferences may then play a pivotal role in their mate-recognition systems (Bush and Smith 1997), but that does not seem to be the case in *Eccritotarsus* spp..

Nosil et al. (2005) distinguishes pre-mating, pre-zygotic barriers from post-mating, prezygotic barriers and post-mating, postzygotic barriers. Pre-mating, prezygotic barriers include: temporal-allochronic-isolation, habitat-ecological-isolation, immigrant-invariability-premating and sexual-behavioural-ethological-isolation. Temporal-allochronic-isolation is caused by reduced encounters of potential mates due to differing mating times while habitat-ecological-isolation is caused by reduced encounters of potential mates due to differing mating sites. These will not affect *Eccritotarsus* spp. as they do not have differences in mating times and mating site as they the same host. Immigrant-invariability-premating is caused by reduced encounters of potential mates due to the extinction of poorly suited non-natives while sexual-behavioural-ethological-isolation is caused by reduced mating due to divergent courtship signals and/or mating preferences. The sexual-behavioural-ethological-isolation could be playing a significant role in maintaining the reproductive isolation in *E. catarinensis* and *E. eichhorniae*. Post-mating, prezygotic barriers include: mechanical-isolation and gametic-incompatibility. Mechanical-isolation is caused by reduced transfer of sperm during a mating encounter due to poor genitalia compatibility while gametic-incompatibility is caused by reduced fertilisation of eggs due to an ill-suited sperm. It is also possible that one or both post-mating, prezygotic barriers could also be playing a role in these *Eccritotarsus* spp. because the crossing the interpopulation pairs yielded very few or no offspring (Paterson et al. 2016). It is worth assessing which barrier is more prominent in these two *Eccritotarsus* species. Cross-breeding experiments is one way of investigating premating, prezygotic barriers and this study has attempted to do exactly that (Nosil and Crespi 2006).

Nosil et al. (2005) further highlights that post-mating, post-zygotic barriers include: immigrant-invariability-postmating, zygotic-mortality, hybrid-invariability-genetic, hybrid-invariability-ecological, sexual-selection-against-hybrids, hybrid-sterility and F2-generation-breakdown. Immigrant-invariability-postmating is caused by reduced production of offspring due to the mortality of mated, maladapted immigrants while zygotic-mortality is caused by reduced survival of zygotes soon after fertilisation. Hybrid-invariability-genetic is caused by reduced survival of hybrid offspring independent of the environment whereas hybrid-invariability-ecological is caused by reduced survival of hybrid offspring where variability is environment dependent. Sexual-selection-against-hybrids

is caused by reduced mating success of hybrid offspring while hybrid-sterility is caused by reduced fertility of hybrid offspring. Lastly, F2-generation-breakdown is caused by reduced survival or fertility of subsequent hybrid generations. Both pre- and post- mating and zygotic barriers form an integral part of phylogenetics and evolutionary biology. Therefore, it will be important to investigate which postmating, post zygotic barrier has played a role in *E. catarinensis* and *E. eichhorniae* that yield very little or no offspring when crossed together. It is possible that the immigrant-invariability-postmating is responsible for Paterson et al. (2016) findings.

There are many other possible mechanisms responsible for reproductive isolation that could lead to speciation and one of them is *Wolbachia*. Even though the mechanisms of *Wolbachia* are not well understood, it is estimated to be present in about 5 million insect species (Werren 1997a). Cytoplasmic incompatibility is the most common effect of *Wolbachia*. The *Wolbachia* are a recently studied group of bacteria found in reproductive tissues (ovaries and testes) of insects and other arthropods that can either be vertically transported through the cytoplasm of eggs or horizontally between species as rickettsiae (Werren 1997b). The rickettsiae are parasitic bacteria that have an intracellular association with their host and belong to the Alpha subdivision of Proteobacteria (Weisburg et al. 1992). This group of bacteria tampers with the reproduction performances of their hosts by causing reproductive isolation that leads to speciation through reproductive and/or cytoplasmic incompatibility (zygotic death), parthenogenesis and feminization (Werren 1997b). Bordenstein et al. (2003) reports that the incompatibility causes reduced fitness in uninfected females when they mate with infected males. It does this by either causing populations to diverge genetically, by promoting the development of parthenogenetic species or by causing quick evolutionary changes involving sex-determining mechanisms. *Wolbachia* is deemed to be widespread and brings implications to evolutionary processes in many living organisms since they are known to alter early development and mitotic processes (Werren 1997a). For example, Bordenstein et al. (2003) found cytoplasmic-incompatibility-type variations in parasitic wasps expressed primarily as conversion in one species but expressed as embryonic mortality in sibling species. These group of microorganisms have been used in biological control as genetically-engineered microbial agents to elevate the effectiveness of natural enemies because they are known to be tolerant of the highly variable cellular environments of their many hosts (Werren 1997b). Paterson et al. (2016) screened for *Wolbachia* in *Eccritotarsus* spp. and he found that both species are not infected with *Wolbachia*.

The evolution of male genitalia has proven to be one of the most reliable tool for species identification even though the responsible evolutionary processes are not well understood (Arnqvist 1998). Among many taxonomists, there has long been a 'lock and key hypothesis' that links with mechanical isolation mechanisms that have been discussed above. The hypothesis suggests that the male genitalia evolve structurally to be species-specific, non-changing and unique to best fit the female genitalia in what is termed a pre-insemination hybridization avoidance (Arnqvist

1998). When it comes to this hypothesis, morphologically, the male genitalia are expected to correspond to female genitalia, and if the fit is “poor”, the insemination is expected to be “more difficult” (Arnqvist 1997). It also assumes that the male genitalia adapts to female genitalia and not the other way around, and as such genitalia morphology should not be correlated with other morphological traits (Arnqvist 1997). For example, Henry (2017), found slight differences in the male genitalia of *E. catarinensis* and *E. eichhorniae* in the form of left paramere, endosoma and right paramere, even though the insects look morphologically very similar. The hypothesis was proposed during the pre-Darwinian era and it claims that pre-insemination reproductive isolation is expected to be more pronounced in monandrous than polyandrous species (Arnqvist 1997). The *Eccritotarsus* spp. (Hemiptera) are possibly polyandrous species because copula incidences between species have been recorded in the interbreeding experiments of this study, even though they were expectedly rarer than within species. The light differences in their male genitalia may be responsible for the few copula incidences involving between species.

The ‘sexual selection hypothesis’ on the other hand suggests that the male genitalia evolve based on sexual selection through variation in post-insemination paternity success that arises from differences in mating success in males (Arnqvist 1998). Sexual selection potentially results from: sperm competition (i.e. sperms from several males competing for fertilisation in female reproductive tract), cryptic female choice (i.e. post-mating ability to discriminate between male sperms, and presence or absence of storage of sperms influenced by the stimulation of male claspers) and sexual conflicts (i.e. control of reproduction leading to antagonistic co-evolution or arms race) (Hosken and Stockley 2004). A study has supplemented the sexual selection hypothesis by suggesting that sexual conflicts brought by differing reproductive interests between the sexes can promote the evolution of both female and male genitalia (House and Lewis 2007). It is also possible that cryptic female choice exists in these *Eccritotarsus* spp., explaining why Paterson et al. (2016) found very few and no offspring involving between species. It is possible that the females have the post-mating ability to discriminate between male sperms, and presence or absence of storage of sperms was influenced by the stimulation of male claspers. The same cryptic female choices may be explaining why the between species recorded very few copula incidences between them.

Researchers strongly believe that combining behavioural, biochemical and genetic experiments could prove to be fruitful in broadening our understanding about possible speciation mechanisms (Andersson and Simmons 2006). For example, it was widely accepted that mating behaviour could also have a part to play towards the formation of a new species. As a result, there are multiple behavioural experiments performed that have denoted that females accept more sperm by mating longer with attractive males to ensure the success of their progeny, and this is demonstrated in a flood of recent exciting research (e.g. Williams 1966; Trivers 1972; Parker 1979; Holland and Rice 1998; Gavrilets et al. 2001; Arnqvist and Rowe 2005). To elaborate on this, Bateman (1948) suggested that

mating more than once benefits females as they will have offspring with high fertilisation success with good traits inherited such as genitalia morphology, quantity and quality of sperm, as well as seminal fluid composition, amongst other traits of evolutionary importance.

Reinforcement, the process by which natural selection increases reproductive isolation is another possible mechanism contributing to speciation and reproductive isolation but it is not yet known when reinforcement prevails (Wood et al. 1999). The ecological divergence has always been shown to be a stepping stone towards the evolution of reproductive isolation through reinforcements, assortative mating and/or sexual preference (van Doorn et al. 2009). Researchers have long demonstrated that the incompatibility between genes from different species is one mechanism responsible for reproductive isolation because the so-called "speciation genes" are not able to mix with alleles from another species and that may lead to reinforcement mechanisms (Lee et al. 2008). The presence of speciation genes has always been attributed to adaptive evolution (Orr et al. 2004). Speciation genes are specific genes known to cause reproduction isolation barriers.

The evolution of gene expression divergence could also have a big role to play in reproductive isolation but underlying factors responsible for it are yet to be thoroughly investigated (Curie 2012). Gene expression divergence is a phenotypic trait that reflects evolution of gene regulation and identifying dissimilarity between cells and tissues within the same species (Glazko and Mushegian 2010). It has been proposed that genomic neighbourhood could play a role in the evolution of gene expression divergence. Other proposed possible mechanism attributed to gene expression divergence are changes in *cis*-regulatory factors and coding factors, but some findings suggest that it is both (Shapiro et al. 2006). Curie (2012) acknowledges that it is rather difficult to pinpoint whether regulatory genomic differences outweigh coding sequence differences or not when it comes to reproductive isolation. Large efforts have been made to try and address this issue. For example, in *Drosophila*, transcriptional profiling has been utilised to track down candidate genes that show miss-expression in hybrids. The candidate genes showed that both genomic differences and coding sequence somewhat play equal important roles in reproductive isolation. Therefore, a holistic approach in dealing with speciation is advised.

Anthropogenic changes enhance or suppress speciation, and an example here could be an introduction of a biocontrol agent that diversifies and speciates (Curie 2012). Evolution can occur after the introduction of the biocontrol agent. It is reported that habitat disturbance suppresses speciation whereas fragmentation of natural populations promotes speciation by disturbing the gene flow (Curie 2012). Anthropogenic changes are also likely to elevate the occurrence of secondary contact, leading to an opportunity for reinforcements, however, different taxa are likely to respond differently to that stressor (Hendry et al. 2001). Most importantly, researchers should consider that speciation rate (i.e. the number of branching events per lineage per unit time) and the speciation

duration (i.e. the time taken for one species to branch into two reproductively isolated groups) differ between taxonomic groups (Funk et al. 2006; Curie 2012). Often, reproductive isolation could result in cryptic species.

This chapter aimed to assess the mating behaviour of the two geographically and reproductively isolated cryptic species, *Eccritotarsus* spp., to investigate whether there are any possible behavioural traits that have resulted in reproductive isolation, due to the 'lock and key' hypothesis or cryptic female choices. I specifically assessed the number of copula incidences, presence or absence of multiple copula incidences, duration of copula incidences as well as the time taken to start mating. The behavioural cues assessed for both intra- (within species) and inter-population (between species) were in the form of pairs, triads and tetrads.

Materials and Methods

Insect culture

Eccritotarsus spp. populations were kept in the Department of Zoology and Entomology, at Rhodes University in Grahamstown. *Eccritotarsus catarinensis* was maintained at the Biological Control Quarantine Facility, while *E. eichhorniae* was housed at the Waainek Mass Rearing Facility, approximately 1km away from the Quarantine Facility. Both species were kept at 25±1°C and 65±5% relative humidity for many generations over 10 years, under 12:12 light vs. dark conditions. The cultures were kept separate from each other to avoid interbreeding between the two species. Both separated species were exposed to the same conditions: enclosed in several cages in buckets filled with water hyacinth plants growing in water under ideal nutrient regimes. These plants were constantly changed to provide optimum nutrition for the mirids.

Insect collection and maintenance

Mirids were collected as 3rd to 5th instar nymphs from the respective *E. catarinensis* and *E. eichhorniae* cultures using an aspirator to perform behavioural observations in the form of no-choice, bi-choice and multi-choice interbreeding experiments. Forty Petri-dishes (90mm, Munktell, Lasec, Ahlstrom) lined with filter paper, with one nymph in each were prepared, for both species. Water hyacinth leaves were placed into the Petri-dishes. Humidity in the Petri-dishes was maintained by adding distilled water using a 3ml Pasteur pipette. Petri-dishes were then inserted into plastic wrappers to increase humidity and placed under a 24-hour fluorescent growing light. The experiment was conducted in the Rhodes University Behavioural and Chemical Ecology Laboratory, Life Sciences Building, Barratt Square Complex, Rhodes University in Grahamstown, under 23±5°C and 45±20 relative humidity.

When the nymphs had developed into adults, the insects were individually taken out of the Petri-dish using a small paint brush and were viewed either ventrally or laterally, to observe their genitalia. The genitalia identification followed Hill et al. (1999) (Fig. 2.1). Nymphs were placed individually to ensure that all adults used for the respective interbreeding experiments had not mated. Each Petri-dish was labelled with the species and sex of the individual inside. When sufficient numbers of adult insects were recorded, 'no-choice', 'bi-choice' and 'multi-choice' mating experiments within and between the two species were conducted. These experiments are described below.

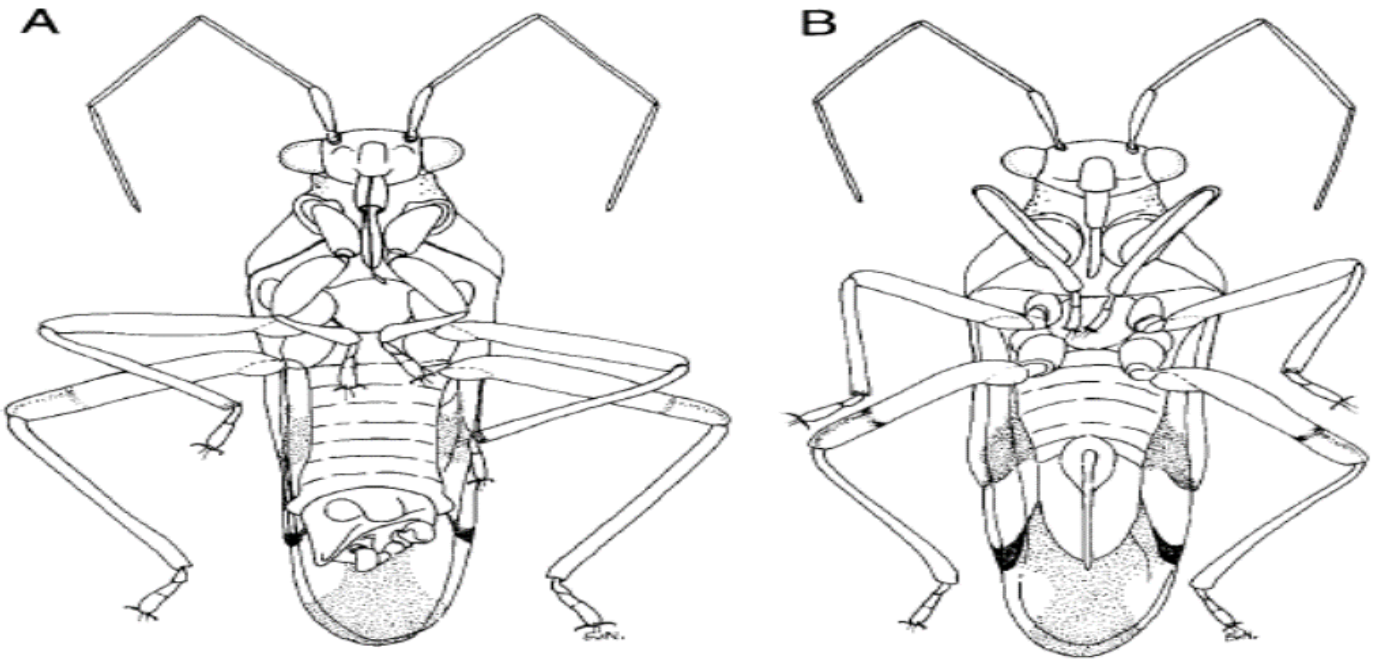


Figure 2.1 The morphology of female and male genitalia representations of an adult *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae): (A) male and (B) female. Sexes are indistinguishable when using the dorsal view while they are distinguishable when using the lateral and ventral views. Nymphs can also be sexed similarly to adults, following the same protocol shown here. *Eccritotarsus eichhorniae* sexes can be distinguished in the same way. (From Hill et al. 1999).

Pre-copulatory behavioural-observation gestures

Behavioural gestures performed before insects started copulation were considered, to assess whether there were any differences between them. These were also recorded to assess whether they play a role in copula incidences.

Behavioural-observations: interbreeding experiment

No-choice interbreeding experiments

Each Petri-dish had half a water hyacinth leaf. The leaves were lightly sprinkled with distilled water. The process of putting a leaf in the Petri-dish and sprinkling water was very similar for all three breeding experiments. The four Petri-dishes were then inoculated with adults from both species. The combination for each trial was: *E.catarinesis*$_{female}$×*E.catarinensis*$_{male}$, *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$, *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ and *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$. Each replicate of four Petri-dishes was observed simultaneously for a period of 3 hours to assess behavioural patterns in the four interbreeding pairs (conducted between 06h00-18h00). The experiment was replicated 30 times. Treatments were used as independent variables, while the number of copula incidences, number of multiple copula incidences, average total copula duration and copula latency were used as dependent variables. Mating in this study was defined as the point where a male was seen climbing onto the female, in which they would be joined together in copula forming a V-shape (Fig. 2.2). The number of single and multiple copula incidences were counted. A multiple mating incident was defined as an incident where the male separated from the female for a period of time and then copulation between the same individuals occurred again. The copula duration was recorded as the difference between the start and end of a mating encounter and this was counted in minutes. The time taken to start mating was also recorded in minutes and was the difference from the time the experiment started until the time insects started mating.

Bi-choice and Multi-choice interbreeding experiments

The 'bi-choice experiment' combinations were: *E.catarinensis*$_{female}$×*E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$, *E.catarinensis*$_{female}$×*E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$, *E.catarinensis*$_{male}$×*E.eichhornaie*$_{male}$×*E.catarinensis*$_{female}$, *E.catarinensis*$_{male}$×*E.eichhorniae*$_{male}$×*E.eichhornae*$_{female}$ while the 'multi-choice experiment' combination for each trial was: *E.catarinensis*$_{female}$×*E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$×*E.eichhorniae*$_{male}$. These were also watched for a period of 3 hours to assess behavioural patterns in the four interbreeding triads and one interbreeding tetrad (conducted between 06h00-18h00). Both these experiments were replicated 30 times. Treatments were the independent variables, while the number of copula incidences, number of multiple copula incidences, average total copula duration and copula latency were the dependent variables. These parameters were measured in the same way as in 'no-choice experiments' as described above. In all interbreeding experiments, insects were observed and consistent behavioural gestures involving these species were also recorded. The underlying purpose for doing the choice tests was to assess whether competition is going to have an effect on copula incidences and copula duration.

Dye preparation

Four different food colourings (Robertsons, Libstar Manufacturing Solutions (PTY) LTD, Gauteng) in blue, pink, yellow and green were used to distinguish females and males of both species. 125g of corn flower was mixed with 200ml of distilled water in a small evaporating dish and then few drops of each food colouring were added. These evaporating dishes were then left to dry for 3 days. The dried corn flower was then crushed to a fine powder. Six grams of each dye was weighed and used as a marker by dipping insects in them, in both 'bi-choice' and 'multi-choice' experiments. The *E.catarinensis*$_{female}$ were represented by pink, *E.eichhorniae*$_{female}$ were represented by green, *E.catarinensis*$_{male}$ were represented by blue while *E.eichhorniae*$_{male}$ were represented by yellow throughout the experiments.

Figure 2.2 An example of a mating incident observed in choice experiments described above, involving *Eccritotarsus* spp. (Hemiptera: Miridae). Four respective colours from a dye preparation were used to be able to distinguish between the females and males of each species. This image represents a *E.catarinensis*$_{female}$ dyed pink in copulation with *E.eichhorniae*$_{male}$ dyed yellow as well as *E.catarinensis*$_{male}$, dyed blue.

Statistical analysis

Statistica Software version 13 (Statistica 2015) was used for all statistical analyses for this study. Data for copula latency and average total copula duration were checked for normality for all interbreeding experiments. Both copula latency and average total copula duration did not meet the normality assumptions, thus Generalized Linear Modelling using a Log Linear Link ($P < 0.05$) and Kruskal Wallis Test were used to analyse the data, depending on

the assumptions. Tukey's HSD Post-Hoc and the Multiple Comparisons of Mean Ranks For All Groups were then used to test for significant differences between the treatments. The data for the copula latency and average total copula duration only involved pairs, triads and tetrads that recorded copula incidences in all the interbreeding experiments. Chi Square Tests (50:50 distribution) were used to determine significant differences between single and multiple copula incidences. The tests have been analysed similarly to make interpretation relatively easy.

Results

Pre-copulatory behavioural-observation gestures

Pre-copulatory behavioural-observation gestures were performed by both females and males of the two species. No notable or distinct differences in these gestures were observed between *E. catarinensis* and *E. eichhorniae*. Prior to copulation, the insects would rub their prothoracic legs together and straighten and preen their antennae using their prothoracic legs. The mirids could also be seen straightening their flattened wings with their metathoracic legs. As with prothoracic legs, the metathoracic legs were rubbed against each other, before and after rubbing their wings. Lastly, another behavioural-observation gesture involved mesothoracic legs rubbing against either prothoracic or metathoracic legs. Some individuals performed all the pre-mating behavioural-observation gestures while others only performed two or three gestures. These gestures were seen in every replicate of each interbreeding experiment. These behavioural-observation gestures were only performed when the insects were close to each other (within ±3 cm), suggesting that they may be using vibrations for locating mates when near to each other, and only using pheromones when far from each other. Alternatively, they may be doing these behaviours due to high levels of pheromones near to the mates.

Males from both species always approached the females and there were no cases where the female moved towards a male. On several occasions, females would move away from advancing males in what was interpreted as a rejection of the male for copulation. These observations suggest that for both *Eccritotarsus* species, males initiate and are the most active in the copulation process, and females are responsible for selecting their copulatory partners, and they can move away from males and reject them. This might also suggest that for both *Eccritotarsus* species, females emit pheromones and males respond to them, but the male still needs to be attractive enough.

No-choice interbreeding experiments

Number of copula incidences

Although not significantly higher at the 95% confidence limit (but significant at the 93.3% limit), the highest number of copula incidences was found within species (*E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ and *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$) than between species (*E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ and *E.eichhorniae*$_{femle}$×*E.catarinensis*$_{male}$) (χ^2 = 6.84, df = 3, *P* = 0.077). Although not significantly higher, the highest number of copula incidences was found in *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair which recorded 11 copula incidences. The second highest number of copula incidences was found in *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair which recoded 7 copula incidences. The lowest number of copula incidences was found in *E.catarinesis*$_{female}$×*E.eichhorniae*$_{male}$ pair which recorded 2 copula incidences. The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair recorded 5 copula incidences and therefore had higher copula incidences than their *E.catarinesis*$_{female}$×*E.eichhorniae*$_{male}$ counterpart (Fig. 2.3).

Number of multiple copula incidences

Although not significantly higher, the highest number of multiple copula incidences was found in *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair which recorded 3 multiple copula incidences, which also had the highest number of single copula incidences (χ^2 = 3.33, df = 3, *P* = 0.343). The second highest number of multiple copula incidences was found in *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair which recorded 2 multiple copula incidences. The *E.catarinesis*$_{female}$×*E.catarinesis*$_{male}$ pair which had the second highest number of copula incidences had only one multiple copula incidence. The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair which had the lowest number of copula incidences had no multiple copula incidences recorded. The *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair which had higher copula incidences had higher multiple copula incidences than their *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ counterpart. The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair which had higher copula incidences had higher multiple copula incidences than their *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ counterpart (Fig. 2.3).

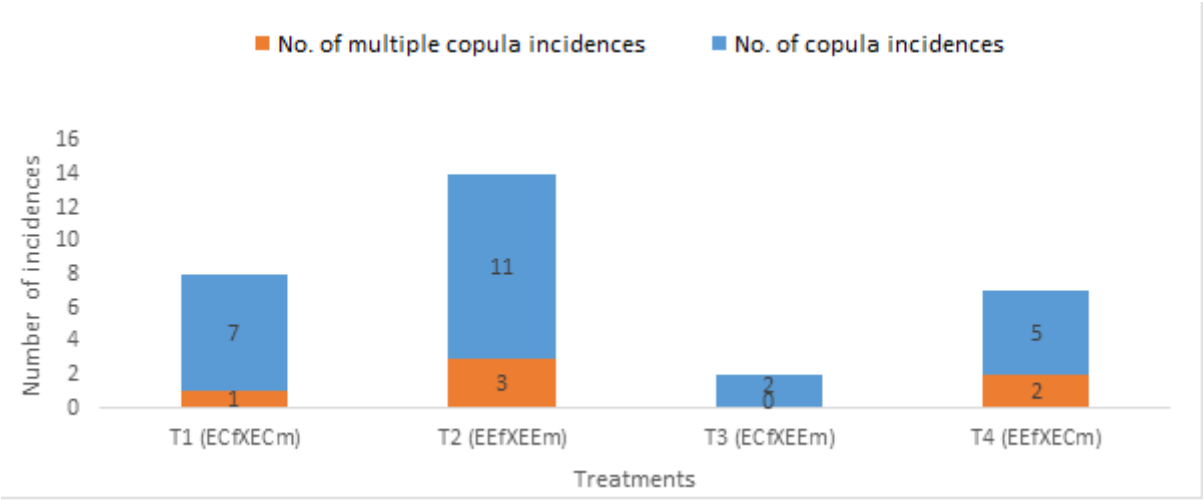


Figure 2.3 The number of single (χ^2 = 6.84, df = 3, *P* = 0.077) and multiple (χ^2 = 3.33, df = 3, *P* = 0.343) copula incidences of four breeding pairs of each interbreeding treatment under no-choice experiments. EC is *E. catarinensis*, EE is *E. eichhorniae*, f is female and m is male. Thirty replicates were done and used to calculate percentages, and each replicate had four different combinations as shown above (n= 30).

Copula latency

The highest copula latency was found in the *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair (187.6±23.35 min) and this was significantly higher than other treatments (Wald $\chi^2(2) = 74.645$, $P < 0.001$). The second highest copula latency was found in the *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair which started to mate 34.4min sooner than *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ on average. The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair, which had the lowest average total copula duration had the lowest copula latency. The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair had a higher copula latency than their *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ counterpart by a margin of 102.6 min (Fig. 2.4). Pairs that did not mate were excluded from the analysis.

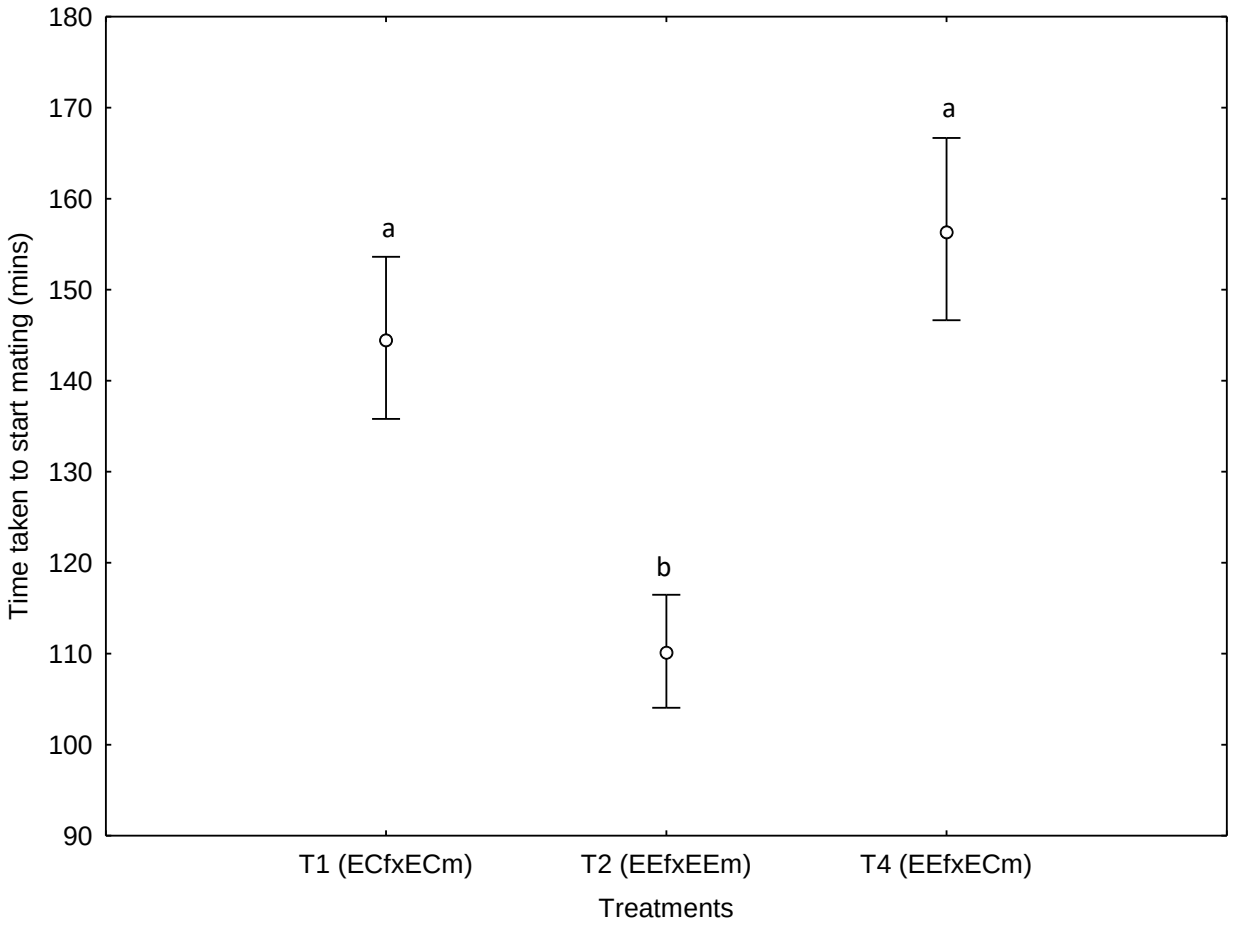


Figure 2.4 The average copula latency (mins) in no-choice experiment using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 74.645$, $P < 0.001$) to test for significant differences. T3 (ECf×EEm) pair was excluded in the analysis due to having 2 copula incidences. EC is *E. catarinensis*, EE is *E. eichhorniae*, f is female and m is male. (Error bars = standard error, means followed by the same letter are not significantly different (P > 0.05)).

Average total copula duration

The highest average total copula duration was found in *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair and this was significantly higher than other treatments (Wald $\chi^2(2) = 47.833$, $P < 0.001$). The second highest average total copula duration was found in the *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair. The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair had the lowest average total copula duration and also had the lowest single and multiple copula incidences, as well as the lowest copula latency. The *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair had a higher average total copula duration than their *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ counterpart by a margin of 3.4 min. The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair had higher average total copula duration than their *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ counterpart by a margin of 6.2 min (Fig. 2.5). Pairs that did not mate were excluded from the analysis.

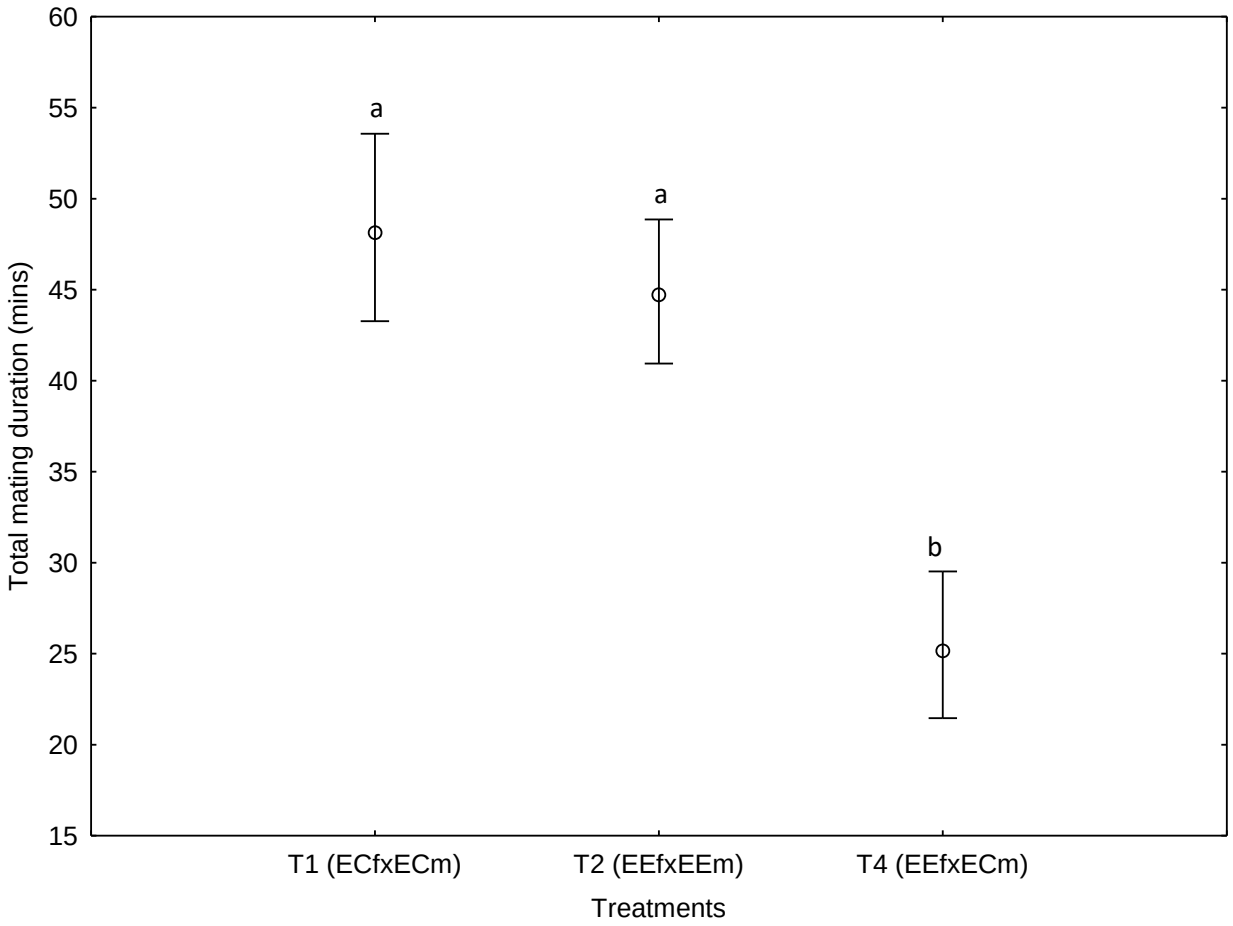


Figure 2.5 The average total copula duration (mins) in no-choice experiment using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 47.833$, $P < 0.001$) for significant differences. T3 (ECf×EEm) pair was excluded in the analysis due to having 2 copula incidences. EC is *E. catarinensis*, EE is *E. eichhorniae*, f is female and m is male. (Error bars = standard error, means followed by the same letter are not significantly different (P > 0.05)).

Bi-choice interbreeding experiments

The bi-choice interbreeding experiments results are presented here in the form of average total copula duration and copula latency. Each triad is treated separately and will be presented separately.

***E.catarinensis*$_{female}$×*E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$:** This triad only recorded 2 copula incidences with no multiple copula incidences recorded. The *E.catarinensis*$_{male}$ in this triad only mated with its conspecific, the *E.catarinensis*$_{female}$ on both occasions. As a result, no copula incidences or multiple copula incidences were recorded involving the *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair in this triad. The high average total copula duration found in this triad in comparison to other triads, involving the *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair (29±2.12 min) could be because of very few copula incidences recorded overall, and as a result may not be a true reflection of the average total copula duration for this pair. This figure proved to be the highest for all mating pairs found in all four treatments used in this experiment (Table 2.1).

***E.catarinensis*$_{female}$×*E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$:** This triad recorded 15 copula incidences with 3 multiple copula incidences in total. The *E.eichhorniae*$_{male}$ in this triad mated with both *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{female}$ but 13 out of 15 times, it mated with its conspecific, the *E.eichhorniae*$_{female}$. Three of those 13 copula incidences recorded involving the *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair were multiple copula incidences and this *E.eichhorniae*$_{male}$ had no multiple copula incidences with the *E.catarinensis*$_{female}$. The *E.eichhorniae*$_{male}$ in this triad had a higher average total copula duration when it mated with its conspecific, the *E.eichhorniae*$_{female}$ (27.15±1.51 min) compared to when it mated with the *E.catarinensis*$_{female}$ (25±10.61 min). It was not possible to do statistics involving the triads due to low number of cross-copula incidences. Interestingly, this *E.eichhorniae*$_{male}$ had a lower copula latency when it mated with its conspecific, the *E.eichhorniae*$_{female}$ (120±46 min) compared to when it mated with the *E.catarinensis*$_{female}$ (137.5±15.59 min).

***E.catarinensis*$_{male}$×*E.eichhorniae*$_{male}$×*E.catarinesis*$_{female}$:** This triad recorded 17 copula incidences with 5 multiple copula incidences in total. The *E.catarinensis*$_{female}$ in this triad mated with both *E.catarinensis*$_{male}$ and *E.eichhorniae*$_{male}$, but 8 out of 17 times it mated with its conspecific, the *E.catarinensis*$_{male}$ while surprisingly 9 out of 17 times it mated with *E.eichhorniae*$_{male}$. Only in 1 of those 8 copula incidences recorded involving the *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair was a multiple copula incidence while 3 of those 9 copula incidences recorded involving the *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair were multiple copula incidences. In this triad, one particular *E.catarinensis*$_{female}$ was also seen mating with both *E.catarinensis*$_{male}$ and *E.eichhorniae*$_{male}$ in this treatment in one replicate only. The *E.catarinensis*$_{female}$ in this triad had a higher average total copula duration when it mated with its conspecific, the *E.catarinensis*$_{male}$ (28.75±2.06 min) compared to when it mated with the *E.eichhorniae*$_{male}$ (20.11±1.28 min). Interestingly, in this triad, *E.catarinensis*$_{female}$ had a lower copula latency when they mated with their conspecifics, the *E.catarinensis*$_{male}$ (87.13±7.37 min) compared to when they mated with the *E.eichhorniae*$_{male}$ (156±6.20 min).

***E.catarinensis*$_{\text{male}}$×*E.eichhorniae*$_{\text{male}}$×*E.eichhorniae*$_{\text{female}}$:** This triad recorded 13 copula incidences with 5 multiple copula incidences recorded in total. The *E.eichhorniae*$_{\text{female}}$ in this triad only opted to mate with its conspecifics, the *E.eichhorniae*$_{\text{male}}$ in all 13 occasions. As a result, no copula incidences or multiple copula incidences were recorded involving the *E.eichhorniae*$_{\text{female}}$×*E.catarinensis*$_{\text{male}}$ pair in this triad. The relatively low average total copula duration found in this triad (in comparison to other intra-population pairs) involving the *E.eichhorniae*$_{\text{female}}$×*E.eichhorniae*$_{\text{male}}$ pair (23.92±1.06 min) is likely to be a better reflection due to a higher number of copula incidences recorded in this triad. This figure proved to be the lowest for all 8 possible mating pairs found in all four treatments or triads used in this bi-choice interbreeding experiment (Table 2.1).

In summary, the highest average total copula incidence and duration was recorded within species rather than between species. The *E.eichhorniae*$_{\text{males}}$ showcased a greater probability of recording copula incidences than *E.catarinensis*$_{\text{males}}$. The *E.eichhorniae*$_{\text{males}}$ mated more with conspecifics and also mated more relative to *E.catarinensis*$_{\text{males}}$ when only exposed to the other species. The *E.eichhorniae*$_{\text{females}}$ and *E.catarinensis*$_{\text{males}}$ opted to mate only with their respective conspecifics.

Table 2.1 The average total copula duration including unmated triads (±SE) and the copula latency including unmated triads (±SE), involving 30 replicates of mated pairs of the mirid *Eccritotarsus* spp. (Hemiptera: Miridae) observed for a period of three hours. ECf = *E.catarinensis*$_{female}$, EEf = *E.eichhorniae*$_{female}$, ECm = *E.catarinensis*$_{male}$ and EEm = *E.eichhorniae*$_{male}$. The ***** next to **ECm×EEm×ECf** triad indicates the presence of a single replicate whereby a female mated with both males.

Trial	Mating pair	Single copula incidence	Multiple copula incidence	Average copula duration (mins)	Average copula latency (mins)
ECf×EEf×ECm	ECm×ECf	2	N/A	29±2.12	99±20.51
	ECm×EEf	0	0	0	0
ECf×EEf×EEm	EEm×EEf	13	3	27.15±1.51	120.46±3.68
	EEm×ECf	2	0	25±10.61	137.5±15.59
ECm×EEm×ECf *	ECf×ECm	8	1	28.75±2.06	87.13±7.37
	ECf×EEm	9	3	20.11±1.28	156±6.20
ECm×EEm×EEf	EEf×EEm	13	5	23.92±1.06	108.46±6.21
	EEf×ECm	0	0	0	0

Multi-choice interbreeding experiments

Number of copula incidences

The significantly highest number of copula incidences was observed within species (*E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ and *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$) rather than between species (*E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ and *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$) (χ^2 = 13.25, df = 3, *P* = 0.004). The highest number of copula incidences was found in *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ which recorded 13 copula incidences and this was significantly higher than other treatments. The second highest number of copula incidences was found in *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ which recoded 12 copula incidences. The lowest number of copula incidences was found in *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ which recorded 0 copula incidences while the other between cross in *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ recorded 7 copula incidences.

Number of multiple copula incidences

Although not significant, the highest number of copula incidences was observed within species (*E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ & *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$) rather than between species (*E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ & *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$) (χ^2 = 2.20, df = 3, *P* = 0.532). The highest number of multiple copula incidences was shared by *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ and *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ which recorded 2 multiple copula incidences each (Fig. 2.6). The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair which had no copula incidences also had no multiple copula incidences recorded.

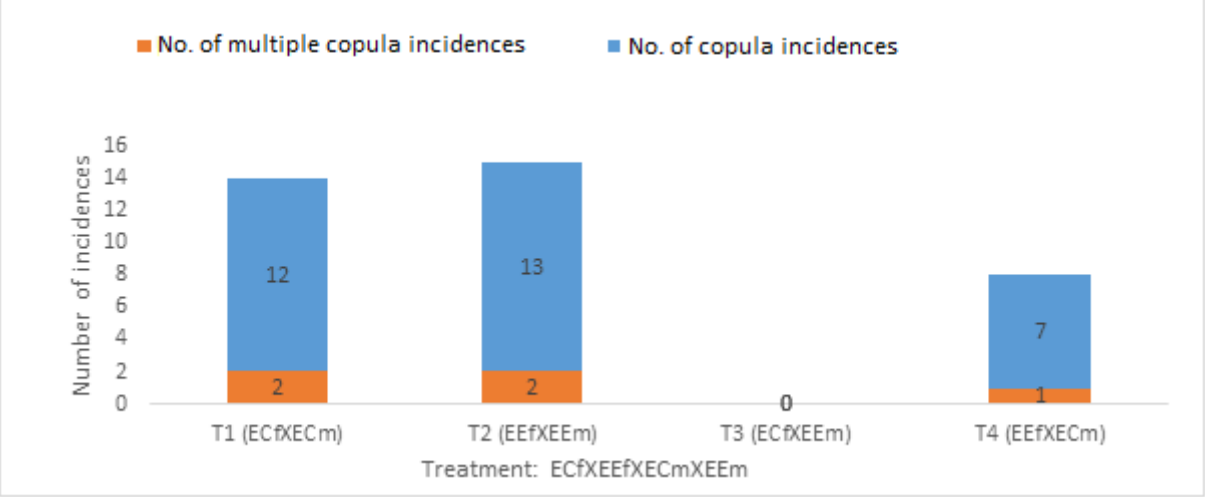


Figure 2.6 The number of single (χ^2 = 13.25, df = 3, *P* = 0.004) and multiple (χ^2= 2.20, df = 3, *P* = 0.532) copula incidences of four possible breeding pairs involving both sexes of both populations as a single interbreeding treatment under multi-choice experiments. EC is *E. catarinensis*, EE is *E. eichhorniae*, f is female and m is male. Thirty replicates were done and used to calculate percentages, and each replicate had only one combination as shown above.

Copula latency

The significantly highest copula latency was observed in the *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair (160.80±14.40 min) (Wald $\chi^2(2)$ = 187.850, *P* < 0.001). The second highest copula latency was observed in the *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair (127.70±5.82 min). The *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair had higher copula latency than their *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ counterpart by a margin of 63.05 min. The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair had a higher copula latency than their

E.catarinensis$_{female}$×*E.eichhorniae*$_{male}$ counterpart by a relatively big margin of 127.7 min. The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair had no copula incidences (Fig. 2.7). The pair that had no copula incidences was excluded in the analysis.

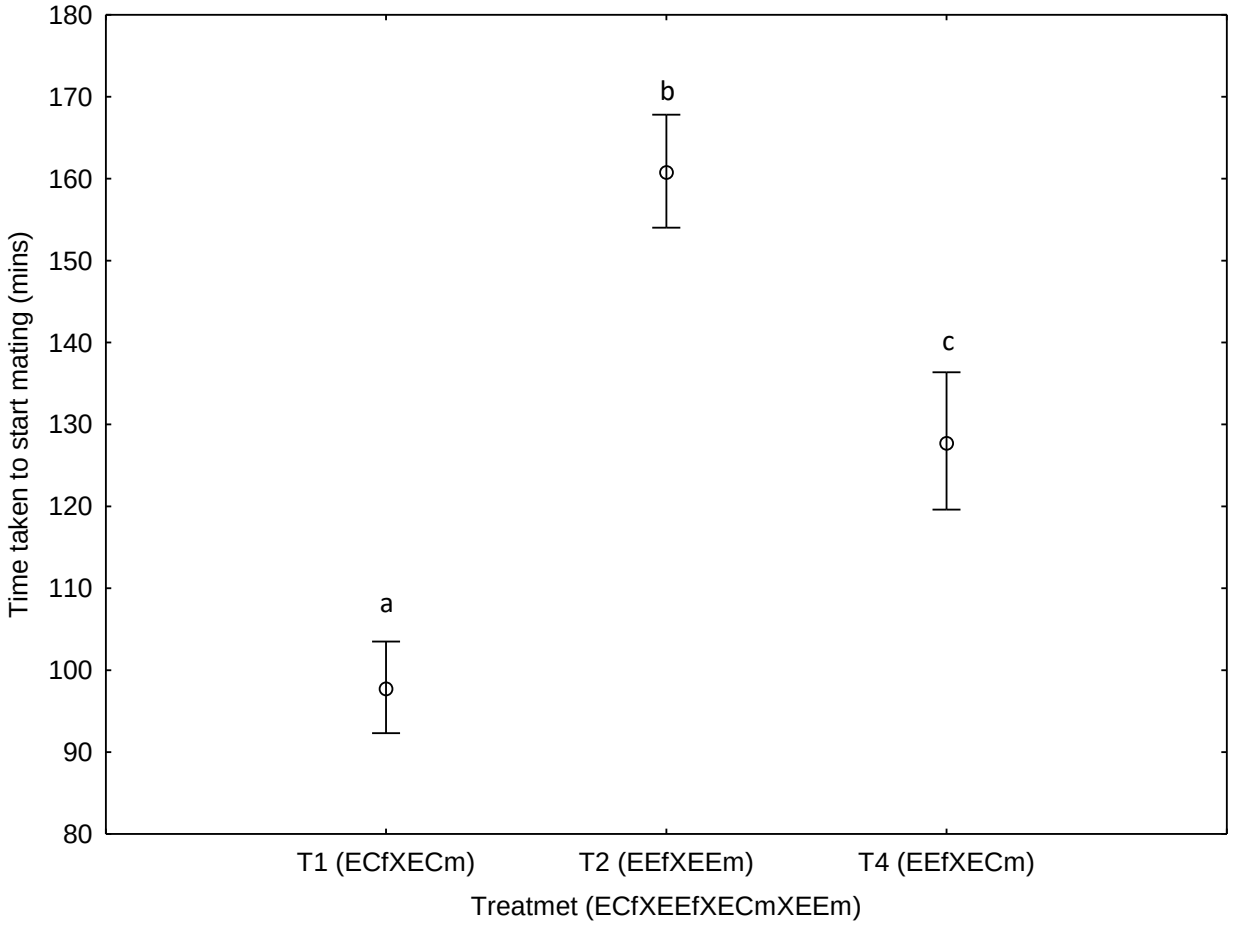


Figure 2.7 The copula latency (mins) in multi-choice experiment, analysed using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 187.850$, $P < 0.001$) for significant differences. T3 (ECf×EEm) pair was excluded in the analysis due to having no copula incidences. EC is *E. catarinensis*, EE is *E. eichhorniae*, f is female and m is male. (Error bars = standard error, means followed by the same letter are not significantly different (P > 0.05)).

Average total copula duration

The significantly highest average total copula duration was observed within species (*E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ and *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$) than between species (*E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ and *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$) (Wald $\chi^2(2) = 779.220$, $P < 0.001$). The highest average total copula duration was observed in the *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair

(107.20±16.10 min). The second highest average total copula duration was observed in the *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ pair (30.31±0.94 min). The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair had no average total copula duration as it had no single and multiple copula incidences. The *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair had a higher average total copula duration than their *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ counterpart by a margin of 76.89 min. The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair had higher average total copula duration than their *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ counterpart by a margin of 17.14 min. The pair that had no copula incidences was excluded in the analysis (Fig. 2.8).

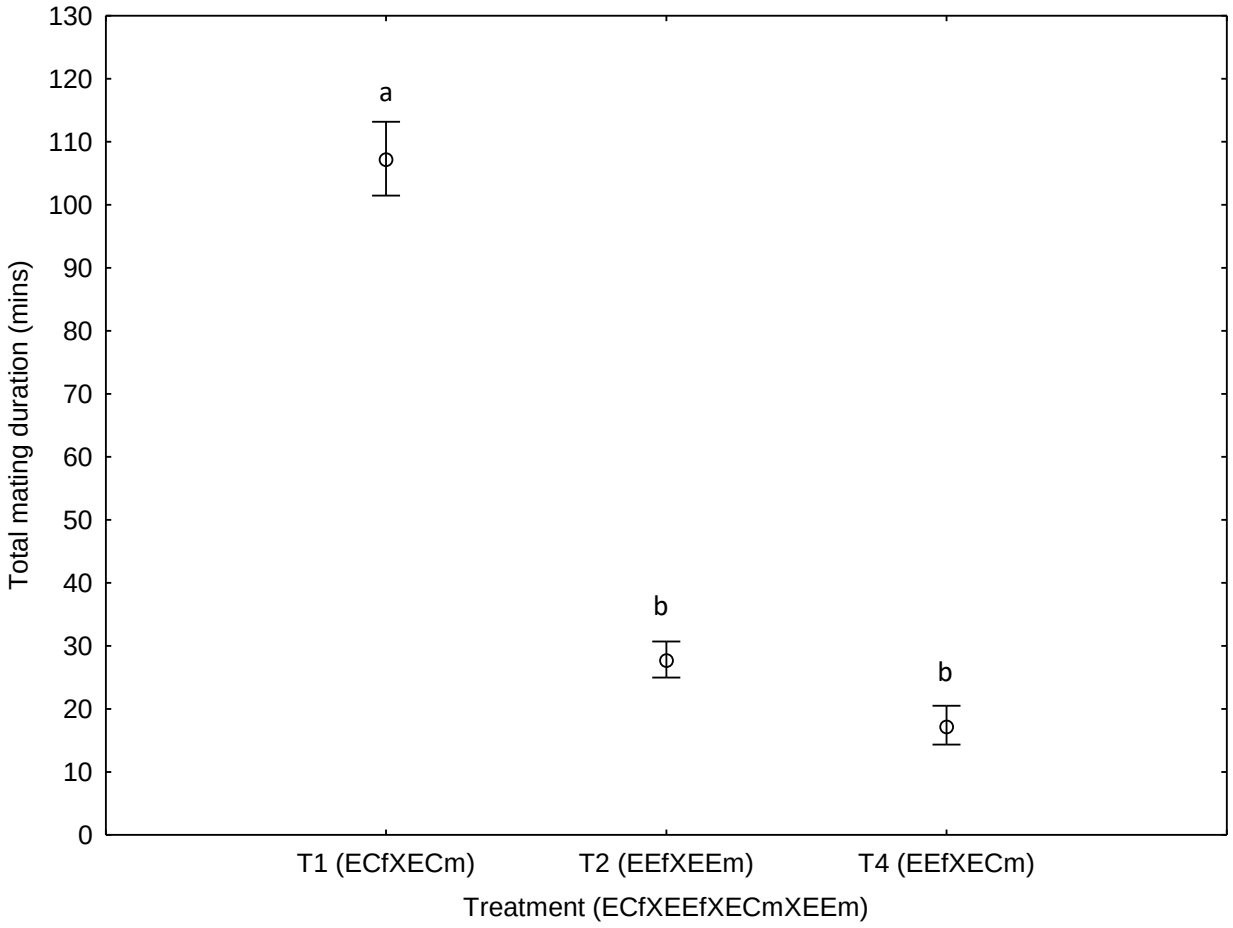


Figure 2.8 The average total copula duration (mins) in multi-choice experiment using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 779.220$, $P < 0.001$) for significant differences. T3 (ECf×EEm) pair was excluded in the analysis due to having no copula incidences. EC is *E. catarinensis*, EE is *E. eichhorniae*, f is female and m is male. (Error bars = standard error, means followed by the same letter are not significantly different (P > 0.05)).

Comparison of results between experiments

The parameters that were measured between the three experiments were compared to determine which breeding experiment the pairs performed better in, and which sex performed better in all breeding experiments.

A – copula latency

***E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$**: This pair took a significantly shorter period of time to start mating in bi-choice and multi-choice experiments than in the no-choice experiment (Wald $\chi^2(2) = 128.640$, $P < 0.001$) (Fig. 2.9). No-choice experiment was significantly different from bi-choice and multi-choice experiments.

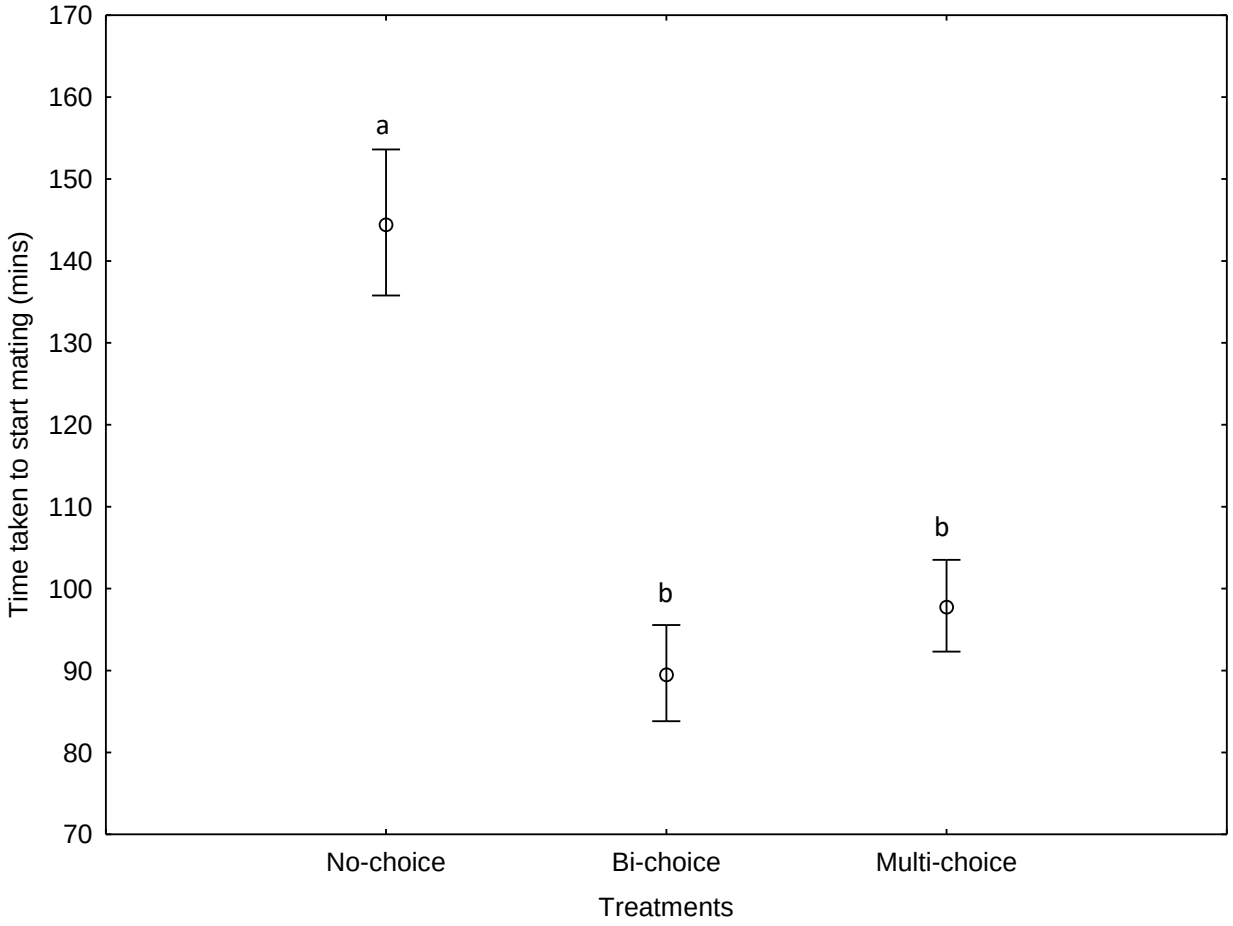


Figure 2.9 The *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ copula latency (mins) in no-choice, bi-choice and multi-choice experiments, analysed using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 128.640$, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different ($P > 0.05$)).

***E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$**: This pair showed an opposite trend to the *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ pair, as it took a significantly shorter period of time to start mating in the no-

choice and bi-choice experiments, but a significantly longer period of time to start mating in multi-choice experiment (Wald $\chi^2(2) = 173.050$, $P < 0.001$) (Fig. 2.10). The multi-choice experiment was significantly different from no-choice and bi-choice experiments.

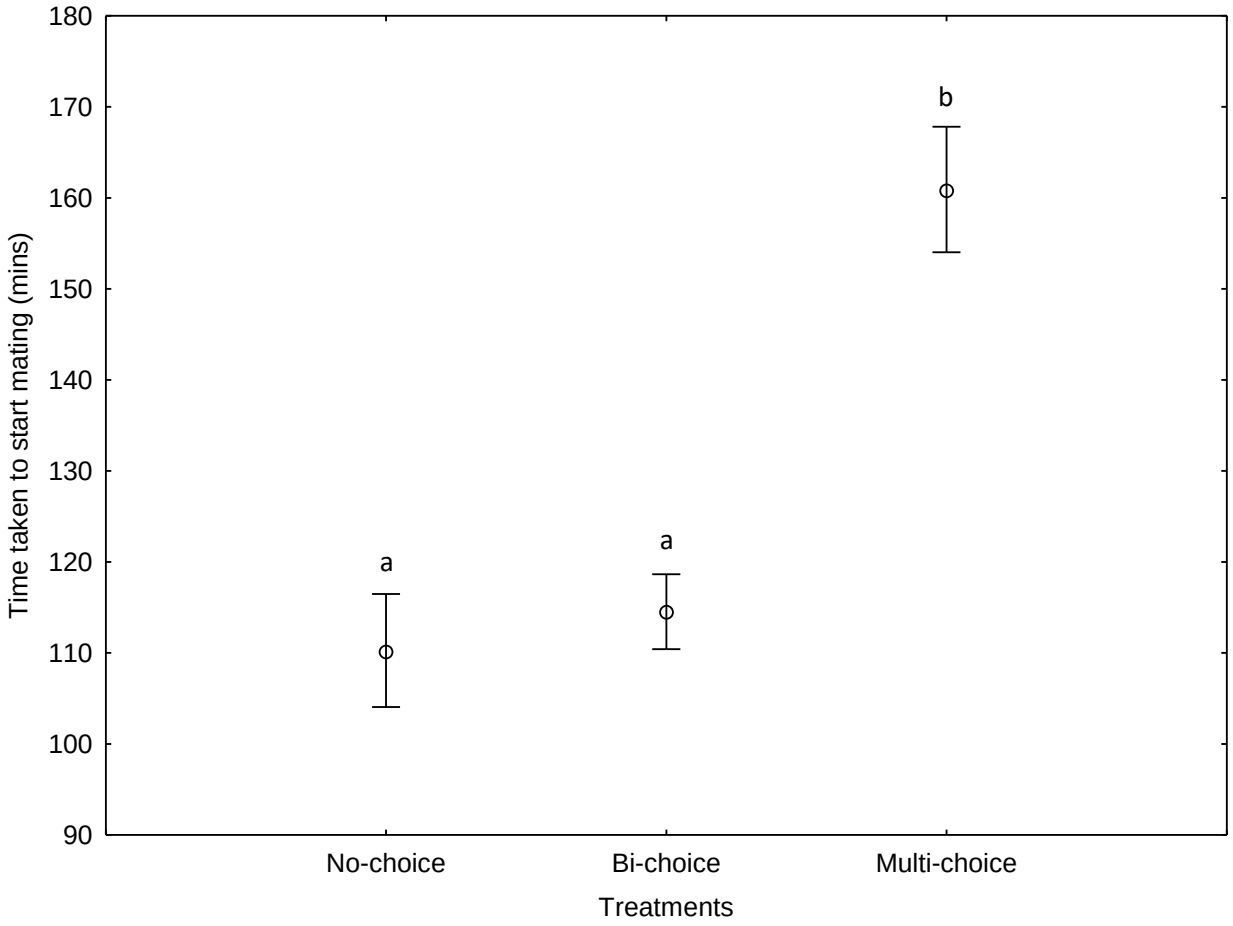


Figure 2.10 The *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ copula latency (mins) in no-choice, bi-choice and multi-choice experiments, analysed using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 173.050$, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different ($P > 0.05$)).

***E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$**: This pair took a significantly longer period of time to start mating in no-choice and bi-choice experiments, but took a significantly shorter period of time to start mating in multi-choice experiment (Wald $\chi^2(2) = 12E11$, $P = 0.001$) (Fig. 2.11). All treatments were significantly different from each other.

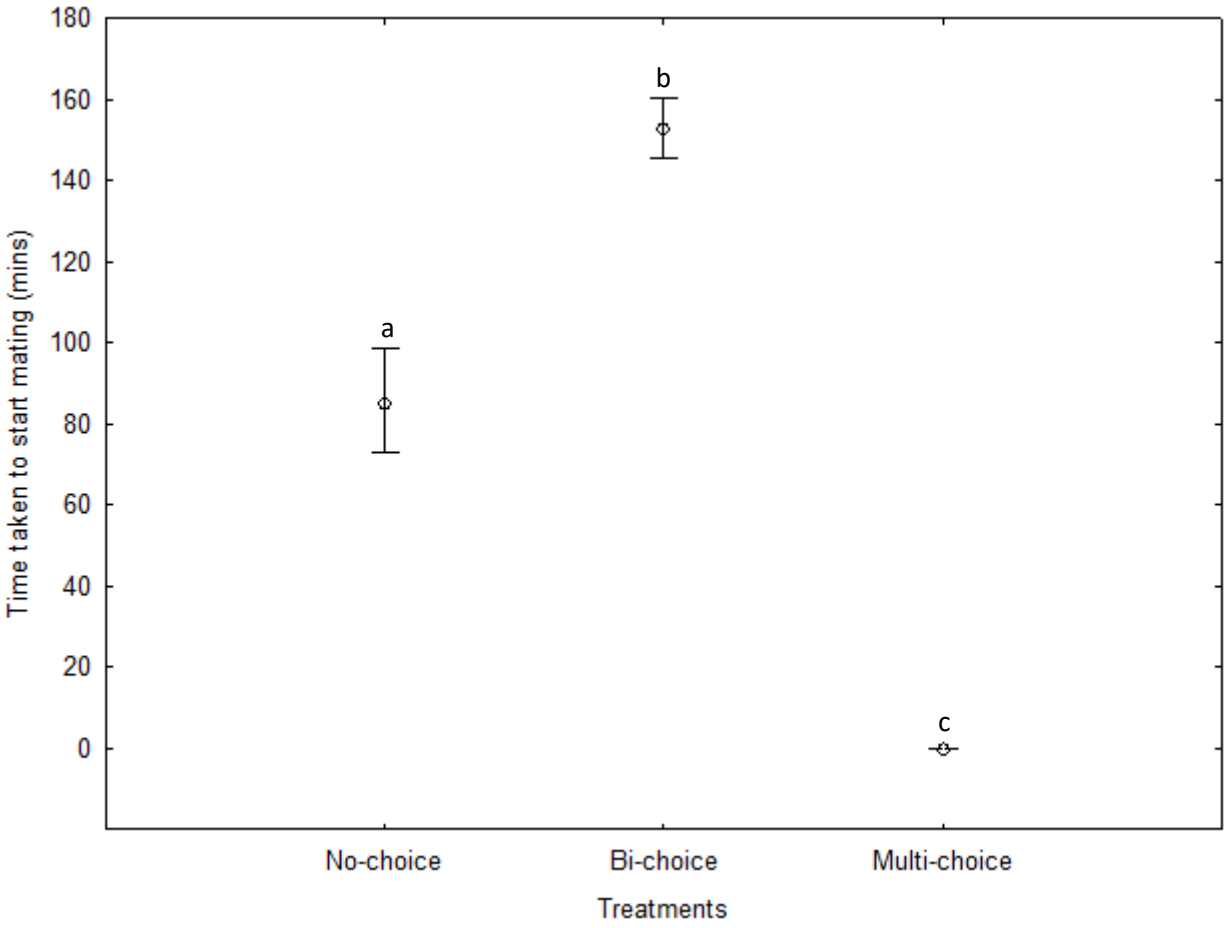


Figure 2.11 The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ copula latency (mins) in no-choice, bi-choice and multi-choice experiments using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2)$ = 12E11, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different (P > 0.05)).

***E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$**: This pair took a significantly longer period of time to start mating in no-choice and multi-choice experiments but took a significantly shorter period of time to start mating in bi-choice experiment (Wald $\chi^2(2)$ = 34E11, $P < 0.001$) (Fig. 2.12).

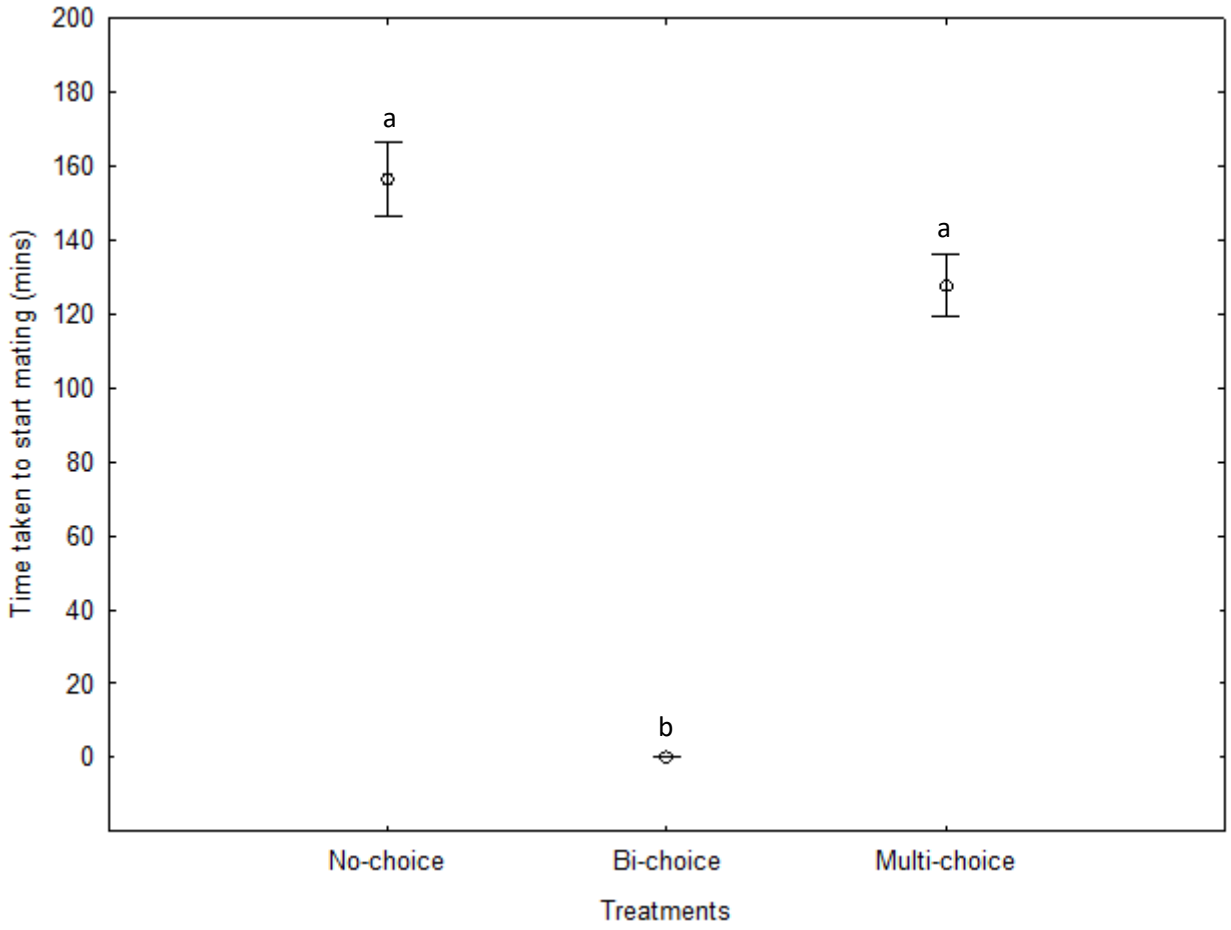


Figure 2.12 The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ copula latency (mins) in no-choice, bi-choice and multi-choice experiments using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2)$ = 34E11, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different (P > 0.05)).

B – average total copula duration

***E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$**: This pair had significantly shorter copula durations in no-choice and bi-choice experiments but had significantly longer copula duration in multi-choice experiment (Wald $\chi^2(2) = 493.260$, $P < 0.001$) (Fig. 2.13).

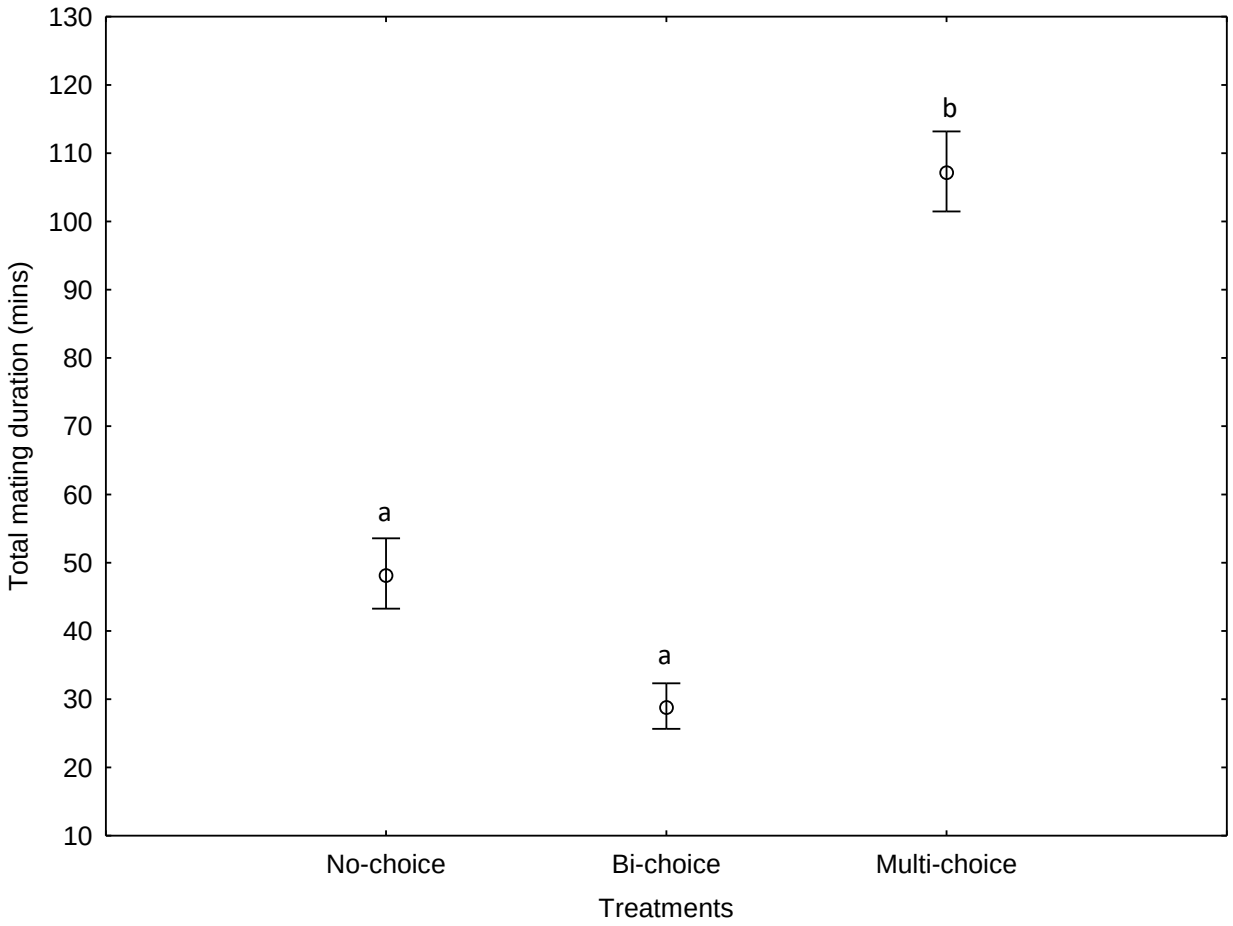


Figure 2.13 The *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$ average total copula duration (mins) in no-choice, bi-choice and multi-choice experiments, analysed using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 493.260$, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different ($P > 0.05$)).

***E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$**: This pair had significantly shorter copula durations in bi-choice and multi-choice experiments but had significantly longer copula duration in no-choice experiment (Wald $\chi^2(2) = 95.563$, $P < 0.001$) (Fig. 2.14).

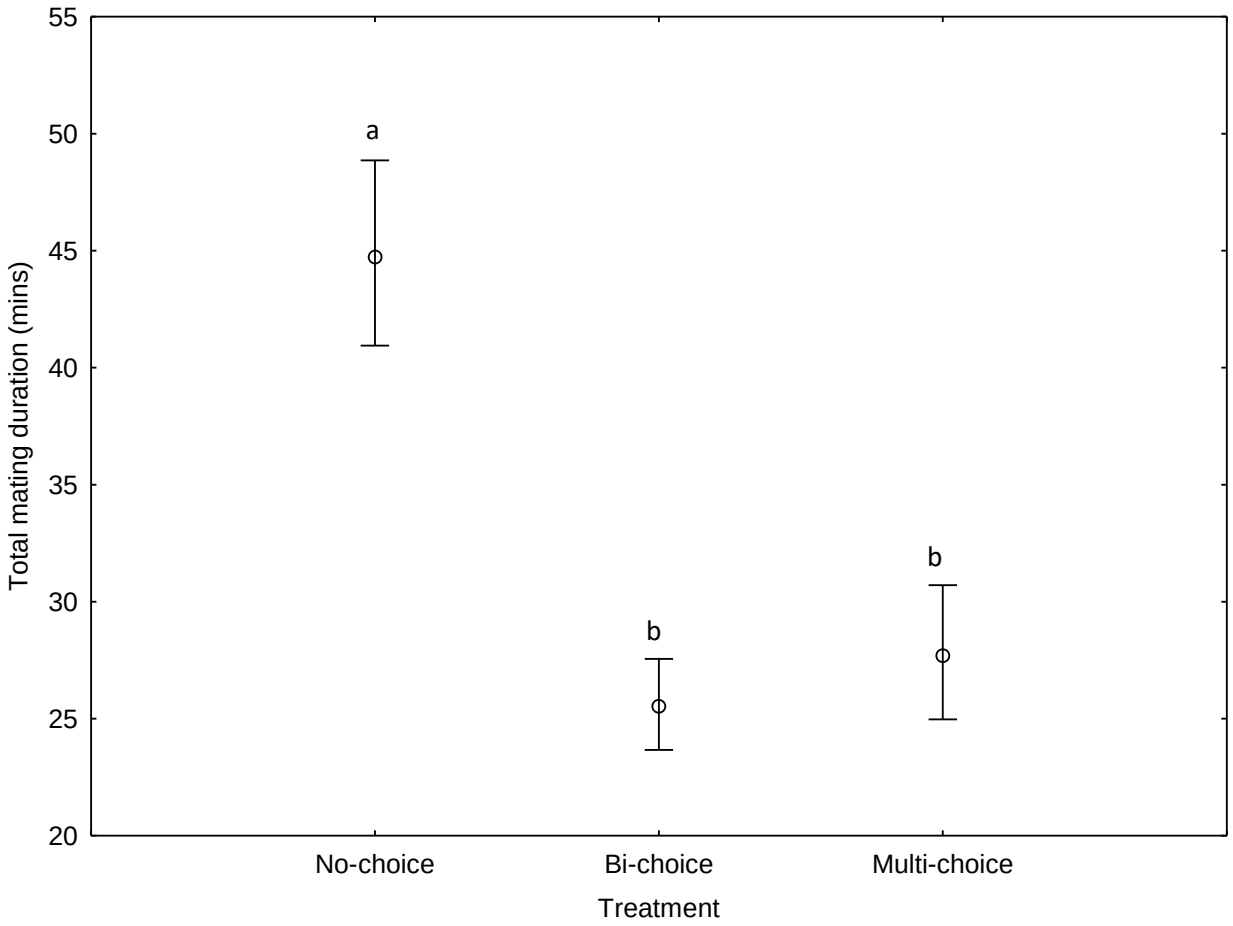


Figure 2.14 The *E.eichhorniae*$_{female}$×*E.eichhorniae*$_{male}$ average total copula duration (mins) in no-choice, bi-choice and multi-choice experiments, analysed using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 95.563$, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different ($P > 0.05$)).

***E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$**: This pair had significantly longer copula durations in no-choice and bi-choice experiments but had significantly shorter copula duration in multi-choice experiment (Wald $\chi^2(2) = 906E8$, $P < 0.001$) (Fig. 2.15).

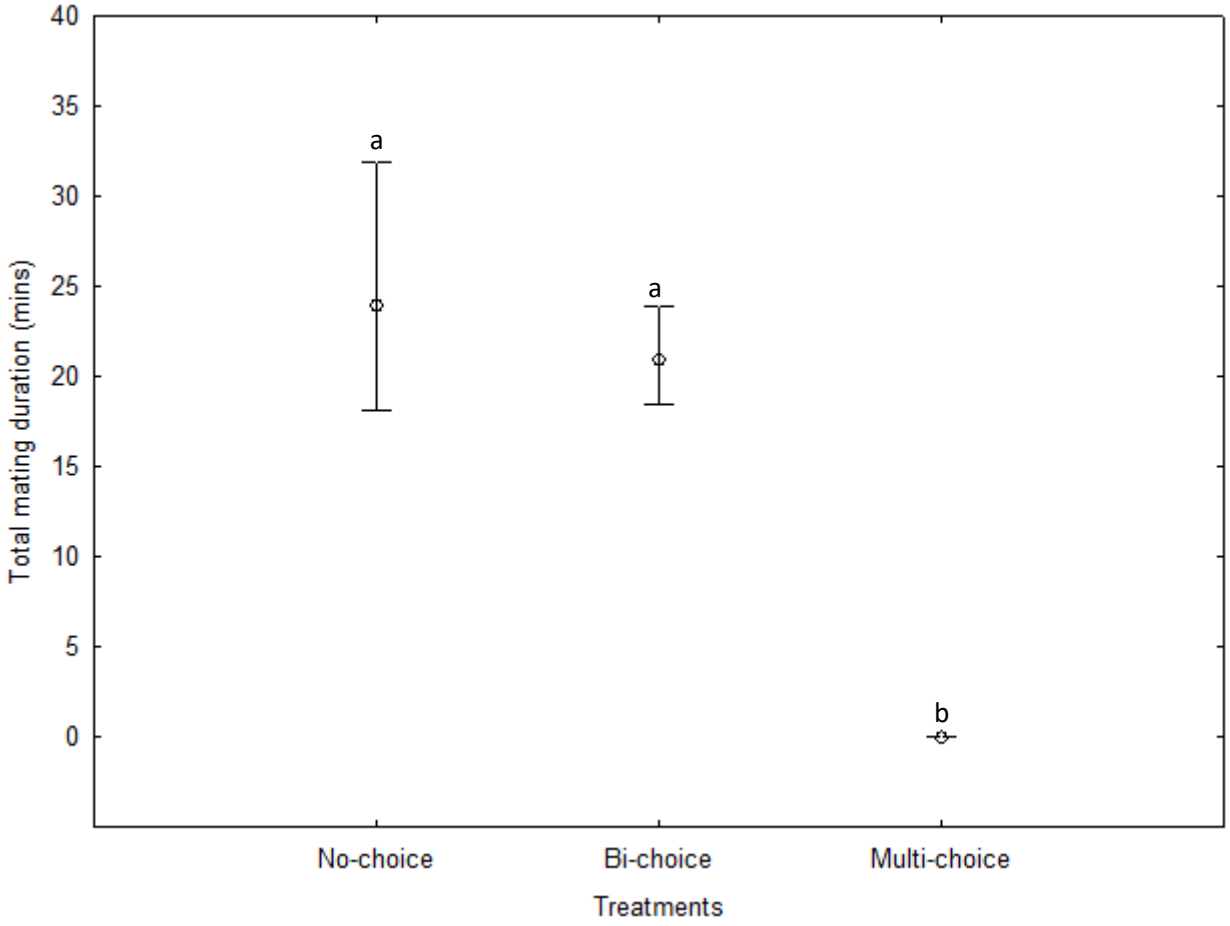


Figure 2.15 The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ average total copula duration (mins) in no-choice, bi-choice and multi-choice experiments, analysed using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 906E8$, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different ($P > 0.05$)).

***E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$**: This pair had long copula durations in no-choice and multi-choice experiments but had a short copula duration in bi-choice experiment (Wald $\chi^2(2) = 39E10$, $P < 0.001$) (Fig. 2.16). The bi-choice experiment was significantly different from the no-choice and multi-choice experiments.

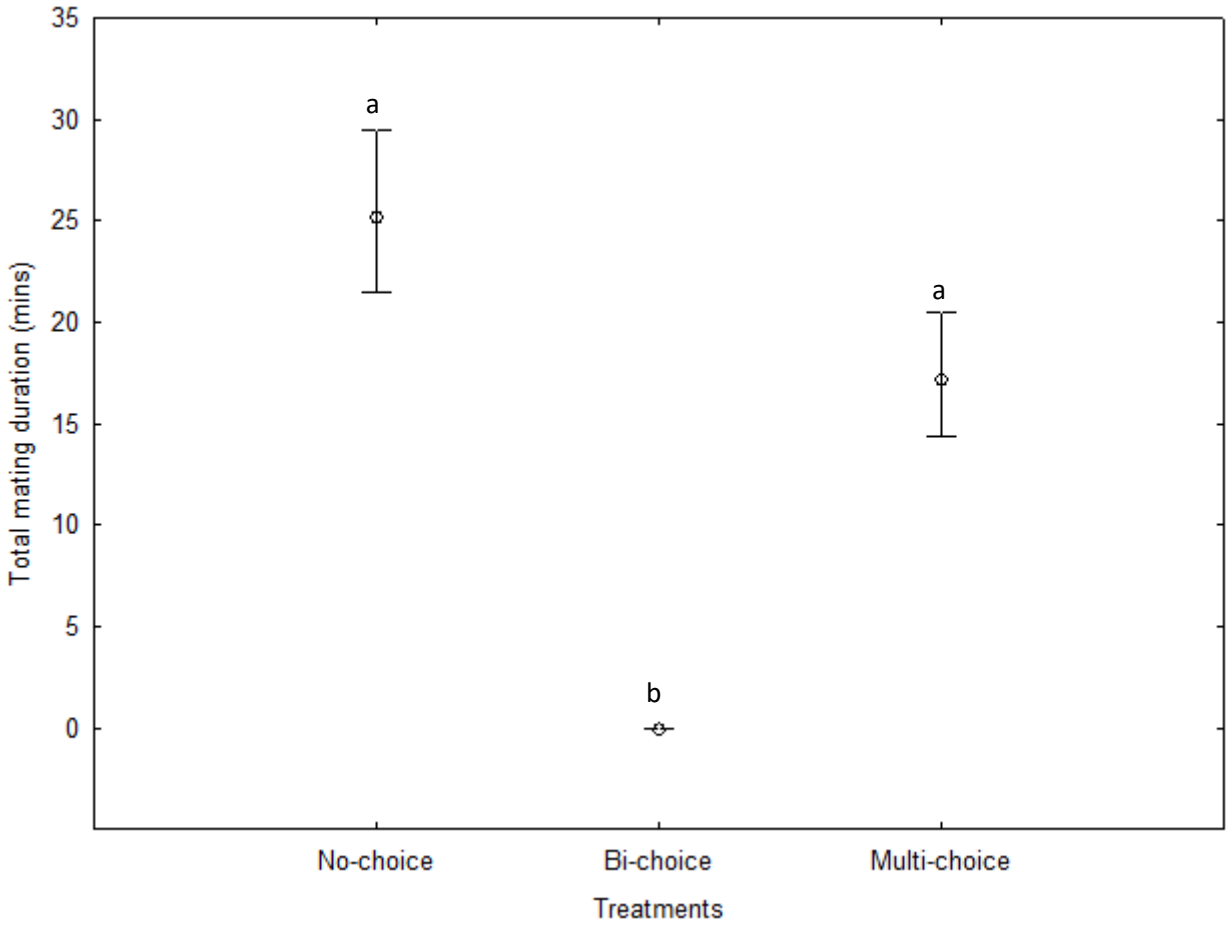


Figure 2.16 The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ average total copula duration (mins) in no-choice, bi-choice and multi-choice experiments using Generalized Linear Model (Log-Linear Link) (Wald $\chi^2(2) = 39E10$, $P < 0.001$). (Error bars = standard error, means followed by the same letter are not significantly different ($P > 0.05$)).

C – copula incidences

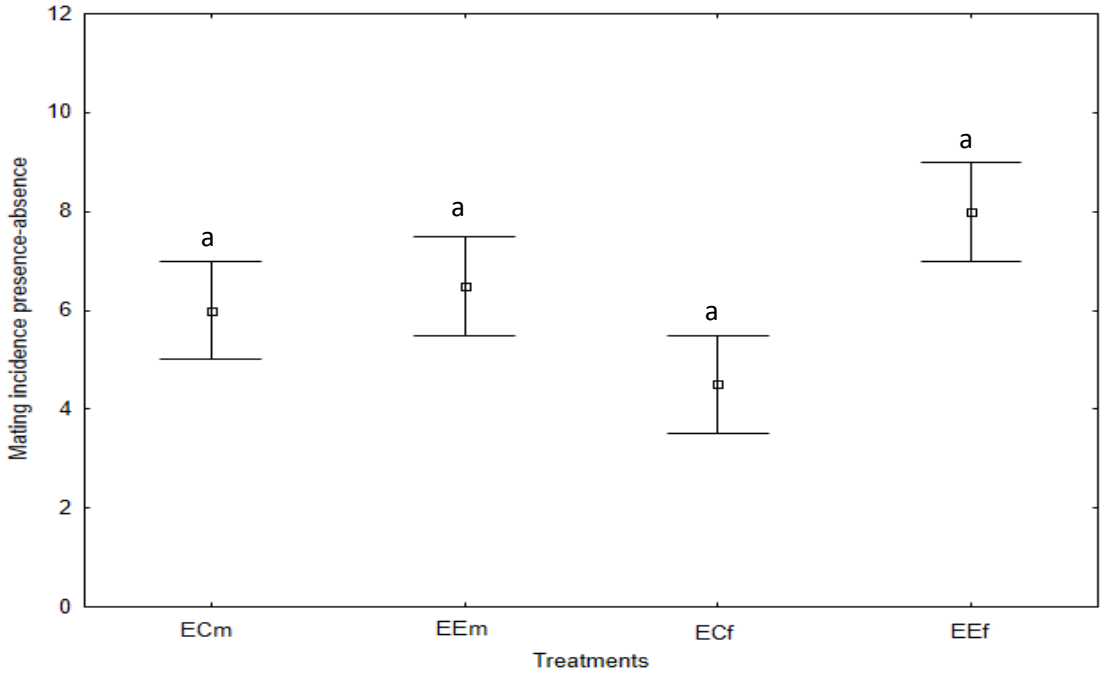
No-choice experiment: Although not significant, *E.eichhorniae*$_{females}$ had higher copula incidences than *E.catarinensis*$_{females}$. Likewise, *E.eichhorniae*$_{males}$ had higher copula incidences than *E.catarinensis*$_{males}$. There were no significant differences between the sexes of both species ($H_{3,8} = 0.70$, df = 3, p = 0.87) (Fig. 2.17).

Bi-choice experiment: Similarly to no-choice experiments, *E.eichhorniae*$_{females}$ had higher copula incidences than *E.catarinensis*$_{females}$. Likewise, *E.eichhorniae*$_{males}$ had higher copula incidences than *E.catarinensis*$_{males}$. There were no significant differences between both sexes ($H_{3,12} = 3.19$, df = 3, p = 0.36) (Fig. 2.17).

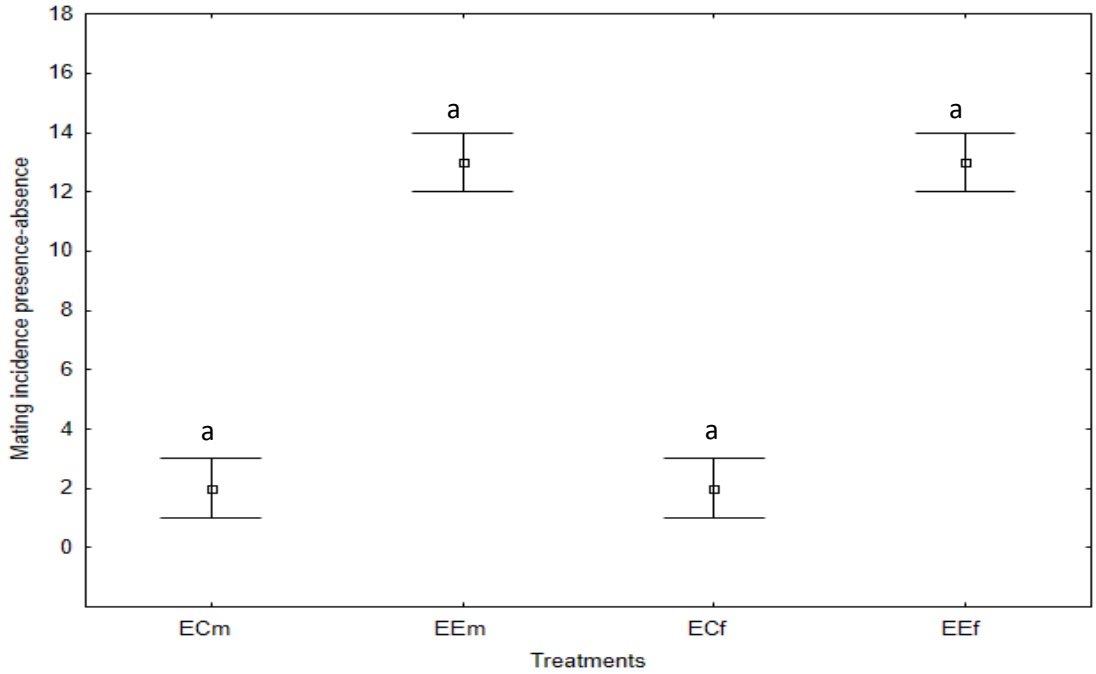
Multi-choice experiment: *E.eichhorniae*$_{females}$ had more copula incidences than *E.catarinensis*$_{females}$, while *E.catarinensis*$_{males}$ had more copula incidences than *E.eichhorniae*$_{males}$. Coincidentally, the cross-breeding involving the *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair yielded no offspring in Paterson et al. (2016) and these respective

sexes only mate with their conspecifics in bi-choice experiment of this study (Table 2.1). There were no significant differences between both sexes ($H_{3,8}$ = 0.70, df = 3, p = 0.87) (Fig. 2.17). Their respective conspecifics being the cross-breeding involving *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair produced very few offspring in Paterson et al. (2016) and had the lowest measures for all parameters in both no-choice and multi-choice experiments.

A



B



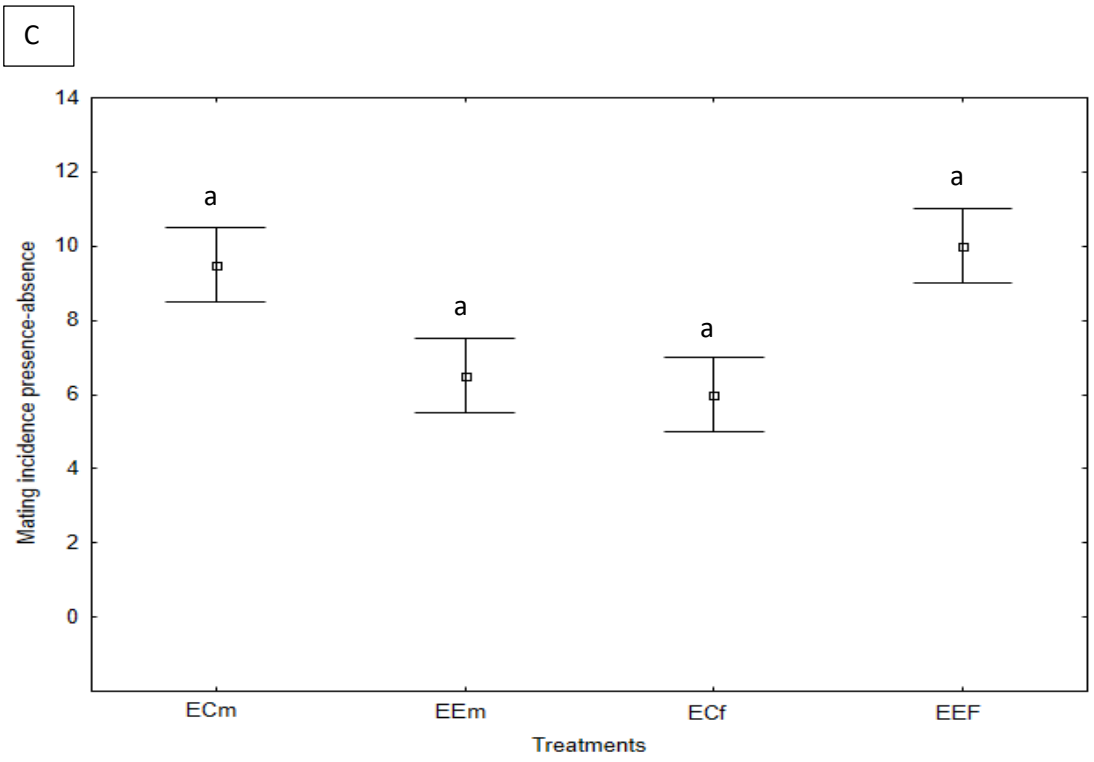


Figure 2.17 Female (ECf & EEf) and male (ECm & EEm) copula incidence presence-absence comparisons in: (A) no-choice ($H_{3,8}$ = 0.70, df = 3, p = 0.87), (B) bi-choice ($H_{3,12}$ = 3.19, df = 3, p = 0.36) and (C) multi-choice ($H_{3,8}$ = 0.70, df = 3, p = 0.87) interbreeding experiments compared using a Kruskal Wallis Test. The final values were achieved by cross referencing copula incidences within and between species. The EC is *E. catarinensis*, EE is *E. eichhorniae*, f is female and m is male. Thirty replicates were done for each interbreeding experiment. (Error bars = standard error, means followed by the same letter are not significantly different (P > 0.05)).

Discussion

The outcome of a copula incidence is the transfer of sperm from males to females, and that has been shown to have profound effects on female reproductive behaviour (Franco et al. 2011). Copula duration affects egg production rate and fertility through the act of mating, presence of sperm and transfer of accessory substances (Gröning and Hochkirch 2008). A copula incidence is known to carry many ecological costs to females and these include general time and energy cost, increased predation rates, physical injury risk and pathogen infections (Watson et al. 1998). A copula incidence also offers positive outcomes such as stimulating female egg production and increase fertility. Females with male ejaculates are known to have effects on female reproductive performance (Gröning and Hochkirch 2008), and it would be important to validate this claim in these *Eccritotarsus* species. Research has shown that males of several groups of insects provide females with a voluminous ejaculate that is

either absorbed or ingested by females, and this nuptial feeding is known sometimes to increase female life span (Arnqvist and Rowe 2005). The most notable result from this study is that there were more copula incidences within species than between species, although these differences were not always significant due to small sample sizes. This was expected and may explain how reproductive isolation is maintained in *Eccritotarsus* spp. The other notable result was that the *E. eichhorniae* pair had more single and multiple copula incidences, higher average total copula duration and higher copula latency. The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair had lower single and multiple copula incidences, lower average total copula duration and lower copula latency. It is highly possible that such findings can be linked to Paterson et al. (2016) who report that the *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair produced no offspring whatsoever.

The *E.eichhorniae*$_{male}$×*E.catarinensis*$_{female}$×*E.echhorniae*$_{female}$ triad had higher mating assessment recordings than their *E.catarinensis*$_{male}$×*E.catarinensis*$_{female}$×*E.eichhorniae*$_{female}$ counterpart in bi-choice experiments, and this result could suggest that *E.eichhorniae*$_{males}$ are more proactive or less selective than their counterpart, the *E.catarinensis*$_{males}$. Alternatively, the *E.catarinensis*$_{males}$ are more passive or more selective than their counterpart, and thus they rarely mate with females from the other species. The *E.catarinensis*$_{males}$ were also less active in terms of copulating with their own species, suggesting that *E.catarinensis*$_{males}$ are less stimulated to mate in general. This could explain the lower fitness recorded for *E. catarinensis* (Paterson et al. 2016). On the other hand, when it comes to females, the *E.catarinensis*$_{female}$×*E.catarinensis*$_{male}$×*E.eichhorniae*$_{male}$ triad had higher mating assessment recordings than their *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$×*E.eichhorniae*$_{male}$ counterpart in bi-choice experiments, suggesting that *E.catarinensis*$_{females}$ are more proactive or less selective than their counterpart, the *E.eichhorniae*$_{females}$. In contrast to the results of the no-choice experiment, the bi-choice experiments suggest that *E.eichhorniae*$_{females}$ are more passive or more selective than their counterpart. The contradictory trends between no-choice and bi-choice experiments may be caused by the fact that the behaviour of the insects change in the presence of competition.

This trend is further supported by multi-choice experiments of this study. *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ did not mate with each other, yet their conspecifics only mated with them in bi-choice experiments, as more proactive or less selective as they may have proven to be. The *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ (deemed to be more passive or more selective) failed to produce any offspring while the *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ (deemed to be more proactive or less selective) produced very little offspring in Paterson et al. (2016). The varying genetic diversity and variability that exists between these two populations could also have come into play (Taylor et al. 2011). For example, it is possible that the low performance of the *E.catarinensis*$_{males}$ could be explained by the genetic bottleneck effect which may have resulted in an inbreeding depression (Leberg and Firmin 2008).

Further interrogation of the bi-choice experiments indicates that when the *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ were given their respective choices, they only mated with their conspecifics. This indicates that both females and males can distinguish their conspecific opposite sex, and both *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ were only stimulated and prepared to mate with their conspecifics. However, interspecific copula incidences were recorded in bi-choice experiments. It is possible that there are pre-zygotic mechanisms allowing the *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ to only mate with their conspecifics. This is the same pair that could not produce any offspring in Paterson et al. (2016). However, it is worth noting that both *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ did record copula incidences in no-choice experiments. This then suggests that when *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ are not given a choice, they will mate with the opposite species but have fewer copula incidences and reduced copula durations. But when given a choice, they will only mate with their conspecifics and that could be key in explaining the reproductive isolation and effectively speciation found in these cryptic species of *Eccritotarsus*. In addition to that, multi-choice experiments, where both sexes of both populations were assessed, has shown that the counterparts of the sexes that opted to only mate with their conspecifics (being *E.catarinensis*$_{females}$ and *E.eichhorniae*$_{males}$) failed to record a single interbreeding incident, thereby further exposing the interbreeding incompatibility that exists between *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$. The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ interpopulation pair findings could also explain why Paterson et al. (2016) reported very few offspring produced by this pair. The fact that there were fewer copula incidences for *E.catarinensis*$_{females}$ than for *E.eichhorniae*$_{females}$ in crosses could tell us that there is some other sort of incompatibility between *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ (they do copulate but do not produce offspring). While it is possible for offspring to be produced in *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$, the low copula incidences limit this possibility.

The differences that exist between the *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ could be playing a key role in these cryptic species being reproductively incompatible and this is suggested by the lack of any copula incidences between these two in bi-choice experiments and the lack of any copula incidence involving their counterparts in multi-choice experiments. These results could explain Paterson et al. (2016) findings, who found that the cross-breeding of these populations yielded very few offspring in *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ and no offspring at all in *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ (which happened to choose to only mate with their conspecifics in this study). Therefore, assessing the chemical compound compositions of these two geographically and reproductively isolated cryptic species of *Eccritotarsus* spp. becomes a necessity. Assessing the presence/absence of a mechanical precopulatory reproductive isolation barrier is also needed which Henry (2017) addressed through

examination of the male parameres in great detail. This could be the reason why there were some copula incidences between *E.eichhorniae*$_{females}$ and *E.catarinensis*$_{males}$ but no offspring were produced.

When it comes to copula incidence differences, particularly in both bi-choice and multi-choice experiments, it might be safe to predict that the *E.eichhorniae*$_{male}$ (more proactive or less discerning) could out-compete *E.catarinensis*$_{male}$ (more passive or discerning) if females were to choose between them, as evidenced by the third triad tested in this study. Being discerning will limit copula incidences.

The highest measure in mating assessments of *E. eichhorniae* was an expected result because the findings of Taylor et al. (2011) suggested that *E. catarinensis* has less genetic variability and diversity than *E. eichhorniae* (represented by the average within population Jaccard similarity index), and thought that would result in few copula incidences and would not be able to overcome environmental stressors. Our study concurs with Taylor et al. (2011) because *E. catarinensis* had fewer copula incidences than *E. eichhorniae*.

In terms of mate recognition, the higher copula incidences recorded for *E. eichhorniae* was an unexpected result. Higgie et al. (2000) report that high or low genetic diversity often has a role to play towards mate recognition, and this was achieved using experimental sympatry of *Drosophila birchii* (Diptera: Drosophilidae) and field sympatric and allopatric populations of *Drosophila serrata* (Diptera: Drosophilidae) species. They found that reproductive character displacement (RCD) evolved within nine generations, indicating that there was strong selection on mate recognition. They concluded that the substantial number of cases involving RCD in field populations in *Drosophila* spp. and in many other taxonomic groups suggests that natural selection on mate recognition may be a major component of the evolution in many species. RCD is the phenomenon where differences involving similar species whose distributions geographically overlap are pronounced in regions where the species co-occur, but are minimized or lost where the species' distributions do not overlap (Higgie et al. 2000). Furthermore, there is growing evidence that genetic incompatibility has a role to play in female mate choices, with mate preference potentially tweaking the evolutionary dynamics of sexual selection (Tregenza and Wedell 2000), and that might be the case with the cryptic species of *Eccritotarsus*. Tregenza and Wedell (2000) highlight the fact that mate choice may be exercised by either sex, and because they invariably invest more in their gametes, females are generally choosier than males and that females may choose mates on the basis of material benefits such as the quality of the male's territory or the size of a food gift she receives on mating. Behavioural observations have suggested that both *E. catarinensis* and E. *eichhorniae* females demonstrate the ability to choose suitable males because there were incidences where they rejected males by running away. Tregenza and Wedell (2000) further stipulate that males may be chosen because they have genes which will confer greater fitness (including mating success) on the female's offspring. This could somewhat explain why few copula incidences were recorded between species in

these cryptic species of *Eccritotarsus*. If males differ in heritable traits, then even if females do not choose their mate before mating, polyandrous females have been shown to still have an opportunity in post-mating to choose between the sperm of several males (known as cryptic female choice), and may also be able to invest differentially in embryos that result from the sperm deposited by different males (Rabosky 2013).

Females choosing males based on the traits they possess is a well-known phenomenon. It is also known that females tend to choose the best possible traits available to them as a mechanism that ensures their reproductive success (Mays and Hill 2004). Studies also show that genetic diversity can be associated with fitness benefits since females have been shown to use genetic dissimilarity as a distinguishing tool for mate choices, and this is often referred to as 'genetic compatibility hypothesis' (Mays and Hill 2004). The hypothesis revolves around the simple idea that the genetic dissimilarity of potential mates can also result in fitness benefits for offspring in the form of genetic compatibility (Mays and Hill 2004). The philosophy behind the hypothesis is that mixing similar genes with dissimilar genes could have fitness benefits and could help the next generation to be able to quickly adapt and overcomes selection pressures. This could explain why *E. catarinensis* is less fecund than *E. eichhorniae*, it has much lower genetic diversity (Paterson et al. 2016). This hypothesis is further discussed and elaborated in Taylor et al. (2011). The dissimilarity hypothesis differs from the similarity hypothesis discussed in the previous paragraph, and it is possible that they both play a role in mate selection. Nevertheless, it is important to keep in mind that when two sympatric populations interbreed, the resulting hybrids will either be sterile or non-viable due to epistatic interactions present between different genomes (Mays and Hill 2004). It is possible that sterile or non-viable hybrids can be found when two allopatric populations interbreed and that could prove to be the case in these two geographically and reproductively isolated cryptic species that have subtle yet key morphological differences that help to tease them apart (Henry 2017).

The patterns observed in bi-choice experiments could suggest that the *E.eichhorniae*$_{\text{males}}$ attract their females effectively, because *E.eichhorniae*$_{\text{females}}$ only mated with their conspecifics. On the other hand, these results could suggest that the *E.catarinensis*$_{\text{females}}$ are not effective at attracting males, because *E.catarinensis*$_{\text{males}}$ also only mated with their conspecifics too. These finding are further elaborated when comparing copula incidences across all interbreeding experiment because both the *E.catarinensis*$_{\text{females}}$ and *E.eichhorniae*$_{\text{males}}$ had lower copula incidences than their respective counterparts, the *E.eichhorniae*$_{\text{females}}$ and *E.catarinensis*$_{\text{males}}$; the interbreeding pair that did not produce any offspring in Paterson et al. (2016). Genetic differences, together with morphological differences and other possible differences such as behavioural observations and chemical compound composition could have led to the reproductive isolation as a result of sexual selection in these cryptic species (Henry 2017). The reproductive isolation could have then led to speciation and therefore separated the two *Eccritotarsus* species.

Understanding the failure to record any mating assessments between *E.catarinensis*$_{females}$ and *E.eichhorniae*$_{males}$ in the multi-choice experiment, after their conspecific opposite sexes chose to mate only with them should be made a priority and thoroughly investigated.

The average total copula duration was not significantly different between the two species, which is consistent with Wheeler Jr. (2001), who found that the copula duration for mirids lasts from a period of several minute to several hours. *Eccritotarsus catarinensis* had the highest copula duration in multi-choice experiments. An example of a mirid that has a short copula duration is *Lygus pabulinus* (Linnaeus) (Hemiptera: Miridae) that only mates for about 2 minutes (Groot et al. 1998), whereas *Nesidiocoris caesar* Ballard (Hemiptera: Miridae) mates for about 180 minutes (Chatterjee 1984).

The extended mating periods recorded in several mirids has often been linked with sperm competition mechanisms that exist between males, and it is believed to be a way in which males minimise their intra-sexual and inter-sexual competitions (Franco et al. 2011). Another finding associated with a relatively long and extended mating period has had to do with females being less likely to accept another male for copulation. This finding has been backed by another one, which suggests that an extended mating period results in more ejaculatory transfer of sperms by males, thereby causing the females not to see another reason to mate again, due to the sufficient number of sperms received by the female in that one copulating incident (Franco et al. 2011). In *Nezara viridula* (Linnaeus), an extended mating period has been attributed to reduced fertilization success of other males (McLain 1980); but in *Eccritotarsus*, one female was recorded mating with both males from *E. catarinensis* and *E. eichhorniae* in choice experiments (personal observation, denoted by a * in the third triad in bi-choice experiments), although this was rare. In this study, *E.catarinensis*$_{males}$ have been observed mating for a very long time in both bi-choice and multi-choice experiments, perhaps competition influenced this result. It is possible that competition in bi-choice and multi-choice experiments forced them to mate for a very long time to minimise intra- and inter- sexual male competitions. It is therefore advisable to carry out experiments that will measure the ejaculatory substance or sperm count of these two *Eccritotarsus* species. The recorded findings in no-choice, bi-choice and multi-choice are possible mating patterns likely to be occurring out there in the field for these cryptic species of *Eccritotarsus*.

In continuing putting things into perspective, both *E. catarinensis* and *E. eichhorniae* individuals generally showed similar behavioural-observation gestures. More specifically, females and males of these cryptic species showed the same pre-mating behaviour, which has been shown to be consistent in the Miridae. The pre-mating behaviour topic has previously been discussed in *Macrolophus tenuicornis* (Wheeler Jr. and Herring 1979) (Hemiptera: Miridae), *Lygus hesperus* (Knight) (Hemiptera: Miridae) (Strong et al. 1970), as well as *Macrolophus pygmaeus*

(Rambur) (Hemiptera: Miridae) and *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) (Franco et al. 2011). However, in mirids, it is reported that behavioural cues are seldom used for species identification; but subtle and species-specific behavioural cue could prove to be useful for *Eccritotarsus* species, because females are believed to be able to distinguish between males using subtle, species-specific cues (Lundgren 2011). Additionally, in several species, cuticular hydrocarbons have been shown to be a more reliable species identification and mate-recognising tool than behavioural cues (Franco et al. 2011). The findings reported in bi-choice experiment of this study suggest that the *E.eichhorniae*$_{female}$ (speculated to be more passive or discerning) is not attracted to *E.catarinensis*$_{male}$ and vice versa, whereas the *E.catarinensis*$_{female}$ (speculated to be more proactive or discerning) has no preference, possibly because the traits to recognise the different species have been lost during the bottleneck (Taylor et al. 2011; Paterson et al. 2016; Henry 2017). Going forward, it is important to understand why *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{male}$ chose not to mate with each other at all in the multi-choice experiments, when their respective conspecifics were present, and only do so when they do not have a choice. There must be sexual selection mechanisms responsible for explaining these patterns.

It is clear that very limited interbreeding is likely to occur in South Africa. Therefore, assessing the establishment of the *E. eichhorniae* in the country and comparing it to *E. catarinensis* is a necessity. *Eccritotarsus eichhorniae* seems to have a higher mating fitness than *E. catarinensis*, and is likely to out-compete the *E. catarinensis* in the field, but this will depend on temperature. Brazil is a subtropical region with a maximum temperature of 28±8°C whereas Peru is a tropical region with a maximum temperature of 33±11°C (Ismail and Brooks 2016b), thus choosing a species that is climatically suitable will be key in the success of these water hyacinth biological control agents. Differences in temperature as a result of latitude could be responsible for the differences in mating patterns. These interbreeding experiments can be used to supplement Taylor et al. (2011) and Paterson et al. (2016) findings. For example, the lack of differences in pre-mating behavioural-observation gestures suggests that they may not be key in helping one sex to be able to distinguish between the other sex within and between species.

In summary, there were no notable differences involving the pre-mating behavioural gestures. I have learnt that there are pre-copulatory isolation mechanisms, but that there is likely to be further isolation mechanisms, given that there were copulatory incidences between *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$, the same pair that produced no offspring in Paterson et al. (2016). So it is possible that there may be post-copulatory mechanisms involved in these *Eccritotarsus* species. Paterson et al. (2016) have paved the way for this study that is the first to look into the mating behaviour involving the cryptic mirids. The next chapter looks into a possible mechanism responsible for the findings of this chapter by assessing the chemical ecology of the two species.

CHAPTER 3: Chemical compound compositions of cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae).

Introduction

Chemical compounds, visual cues and acoustic signals all form a critical part of insect communication and mate recognition, and these have a long history in evolution (Teal and Tumlinson 1992). Pheromones are used by many living organisms and each phylum has unique and species-specific pheromones emitted, which will inevitably induce different responses and effectively serve different functions in different phyla (Teal and Tumlinson 1992). Most pheromones are classed as lipid pheromones, but there has since been an exponential increase in establishment of protein and peptide pheromones (Singer 1991). Lipid pheromones are said to be conducive to insects because of their varied solubility and vapour pressures that are suitable in many environmental conditions (Teal and Tumlinson 1992). The lipid pheromones range from highly volatile compounds, aggregation pheromones, alarm pheromones, volatile aromatic components to trail pheromones (Teal and Tumlinson 1992). The importance of functional groups often needs to be stressed because some species have been reported to be responsive only when a specific functional group is present, and could be hindered by similar compounds having a different functional group (Schwarz et al. 1990). When other atoms or functional groups are added to the chain of a chemical compound or substituted, the chemical nature of the compound changes. The naming of compounds gives out the length of the carbon chain as well as what and where the functional group exists. The interconversion of the functional groups (e.g. ester, alcohol, carboxylic acid, phenols, aldehyde and others) can explain differences in the pheromone blends used by closely related species. Also, the number, position and geometry of double bonds as well as fluorine, halogen, proton or methyl group substitutions; together with the length of a carbon skeleton or shortening of alkyl chain are important factors in pheromone efficacy (e.g. Bengtsson et al. 1990; Clearwater et al. 1991; Dickens et al. 1991; Jonsson et al. 1991; Millar et al. 1997). Also, the deletion of isoprene units with hydrocarbon or benzyl group usually limits the effectiveness of pheromones in some insects (e.g. Marcus et al. 1991; Xue et al. 1991). While there is still much about pheromones in mirids that is not well understood, it is clear that in most cases the pheromone receptors of mirids are highly specific, and play a key role in mate recognition and attraction. These pheromones and receptors are therefore likely to have played a significant role in speciation in this family and may be important in the development and maintenance of reproductive isolation in *Eccritotarsus*.

The Miridae is the most abundant insect family in the order Heteroptera, having well over 11 000 described species comprising 1400 genera (Zhang and Aldrich 2003b). This Miridae in particular has females that attract males by means of short and long-range chemical compounds known as sex pheromones (Groot et al. 1998; Yang et al. 2015). Paradoxically, Moraes et al. (2005) report that in Miridae, males also attract females. Teal and Tumlinson

(1992) define pheromones as chemical compounds emitted by an individual in an attempt to induce a behavioural and/or physiological response in another individual of the same species. Pheromones that stimulate behavioural responses can be grouped as 'releaser pheromones' whereas those that stimulate physiological responses can be grouped as 'primer pheromones', because of the different observable responses that an individual can be subjected to (Teal and Tumlinson 1992). Several mirids have been reported to have straightforward and easily identifiable sex pheromones (e.g. *Phytocoris* spp.) (Hemiptera: Miridae) while others have been reported to have sex pheromones that are more difficult to identify (e.g. *Lygocoris* spp.) (Hemiptera: Miridae) (Zhang and Aldrich 2008). For this reason, sex pheromones have already been assessed in about 16 species thus far, with findings suggesting that mirids utilise saturated and unsaturated, long and short-chain esters, as well as unsaturated ketoaldehydes to find their mates (Yang et al. 2015). Consistent chemical compounds found present in 10 of the 16 mirids assessed thus far include hexyl butyrate, (E)-2-hexynyl butyrate (E)-2-hexenal and (E)-4-oxo-2-hexenal, indicating that they could be major sex pheromones in other mirids that still need assessing (Yang et al. 2015).

In the Miridae, the metathoracic scent gland (MSG) is known to be a gland that produces defensive chemical compounds that mirids emit when disturbed or provoked (Zhang and Aldrich 2003a). In addition to having defensive chemical compounds, it has also been shown to have sexual, aggregation, alarm and dispersal pheromones that function intra-specifically (e.g. Aldrich et al. 1991; Smith et al. 1991; Aldrich 1994, 1996; Millar et al. 1997; Millar and Rice 1998; McBrien and Millar 1999; Wardle et al. 2003; Zhang and Aldrich 2003b), but can also be used by various other insects inter-specifically as kairomones, a semiochemical that elicits an interspecific communication that benefits a receiver and harms the sender (e.g. Aldrich and Barros 1995; Eisner et al. 1991; Zhang and Aldrich 2003b). However, Millar et al. (1997) found that sex pheromones can also be emitted from the thorax, so investigating the source of chemical compounds emitted by *Eccritotarsus* spp. is needed to explain the behavioural observations (Chapter 2). This will be key in investigating the mechanisms/barriers responsible for reproductive isolation and speciation in *Eccritotarsus*.

Several chemical compounds make up a pheromone bouquet, and findings suggest that the complexity of pheromone blends has a strong correlation to an evolutionary hierarchy (Teal and Tumlinson 1992). In insects, highly derived taxa are reported to use multi-component blends having compounds synthesized by different biochemical pathways (e.g. Hymenoptera, Diptera & Hemiptera) while less derived taxa use single-component blends having compounds synthesized by the same biochemical pathway (e.g. Lepidoptera) (Clearwater et al. 1991; Rocca et al. 1992; Millar et al. 1997). The complexity in pheromone blends lies in the ability of some insects in being able to alter their scents by changing the composition and proportions of their blends, to such an extent that single components of some blends are often ineffective and fail to attract males alone (Teal and Tumlinson 1992).

In many instances, only binary components of pheromone blends are most effective in attracting males in many insects (Groot et al. 1999). So, precise components of pheromone blends are critical in insect communication and usually determine the presence or absence of an observable behavioural response (Aldrich et al. 1991; Dickens et al. 1991; McLaughlin et al. 1991).

Insects are said to be able to pick up components of any pheromone blend by means of many specialised porous sensilla in their antennae (Teal and Tumlinson 1992). The sensilla differ in structure but all have a similar function of inducing a behavioural response, effectively resulting in efficient air sampling for insects (Teal and Tumlinson 1992). Each sensillum reportedly possesses one or more neurons, and each neuron targets a specific component of the pheromone blend, thereby allowing for an accurate detection of any pheromone bouquet (Mankin 1991; Akers and O'connell 1991). Additionally, chemoreceptors present in small numbers also assist in direct detection of trail pheromones using a comparison of pheromone composition and proportions (Peterson and Fitzgerald 1991). Most importantly, pheromones play an important role in mate recognition and attraction (Sorensen and Hoye 2010).

Here I tested the hypothesis that the chemical compound composition of the two geographically and reproductively isolated cryptic species of *Eccritotarsus* spp. are different. This is because Henry (2017) found slight morphological differences in the scent glands of these *Eccritotarsus* spp. that are known to store and emit chemical compounds. *E. eichhorniae* was shown to have a shorter antennal segment II than *E. catarinensis* (Henry 2017). The antennae are used by insects to pick up pheromones, thus they play a significant role in insect communication. The differences in the scent gland and antennae could have significant implications for the chemical compound compositions of the pheromones produced by these two *Eccritotarsus* species. It is possible that these differences in scent glands and antennae may be responsible for maintaining the reproductive isolation in these insects, and could explain the patterns found in Chapter 2. It is possible that the differences in scent glands and antennae may enable or disable one species from picking up a particular chemical compound from a bouquet of pheromones released in an ecological timeframe. The differences in the shape of the scent glands may go as far as changing the functional groups of some of the chemical compounds, causing these two species to have one or two different chemical compounds. These differences may also cause a particular compound that these *Eccritotarsus* spp. share to be emitted at different ratios, proportions or relative abundances. Therefore, the aim of the study was to assess the chemical compound composition present in these two geographically and reproductively isolated cryptic species, to investigate what role chemical compounds have played in speciation or reproductive isolation.

Materials and Methods

Insect culture

Insect culture followed the same protocol as for Chapter 2.

Insect collection & maintenance

Insect collection and maintenance followed the same protocol as for Chapter 2 (Fig. 3.1).

Insect adult assessment

Insect adult sexing followed the same protocol as for Chapter 2 (Fig. 2.1). When a sufficient number of adults was obtained, Solid-Phase Micro-Extraction (SPME), Nuclear Magnetic Resonance (NMR) and Gas-Chromatography Mass-Spectrometry (GCMS) were conducted. SPME is a technique used for many applications including flavours and fragrances, forensics and toxicology, environmental and biological matrices, and product testing to name a few. NMR is an analytical chemistry technique used in quality control and research for determining the content and purity of a sample as well as its molecular structure. GC-MS application includes drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples.

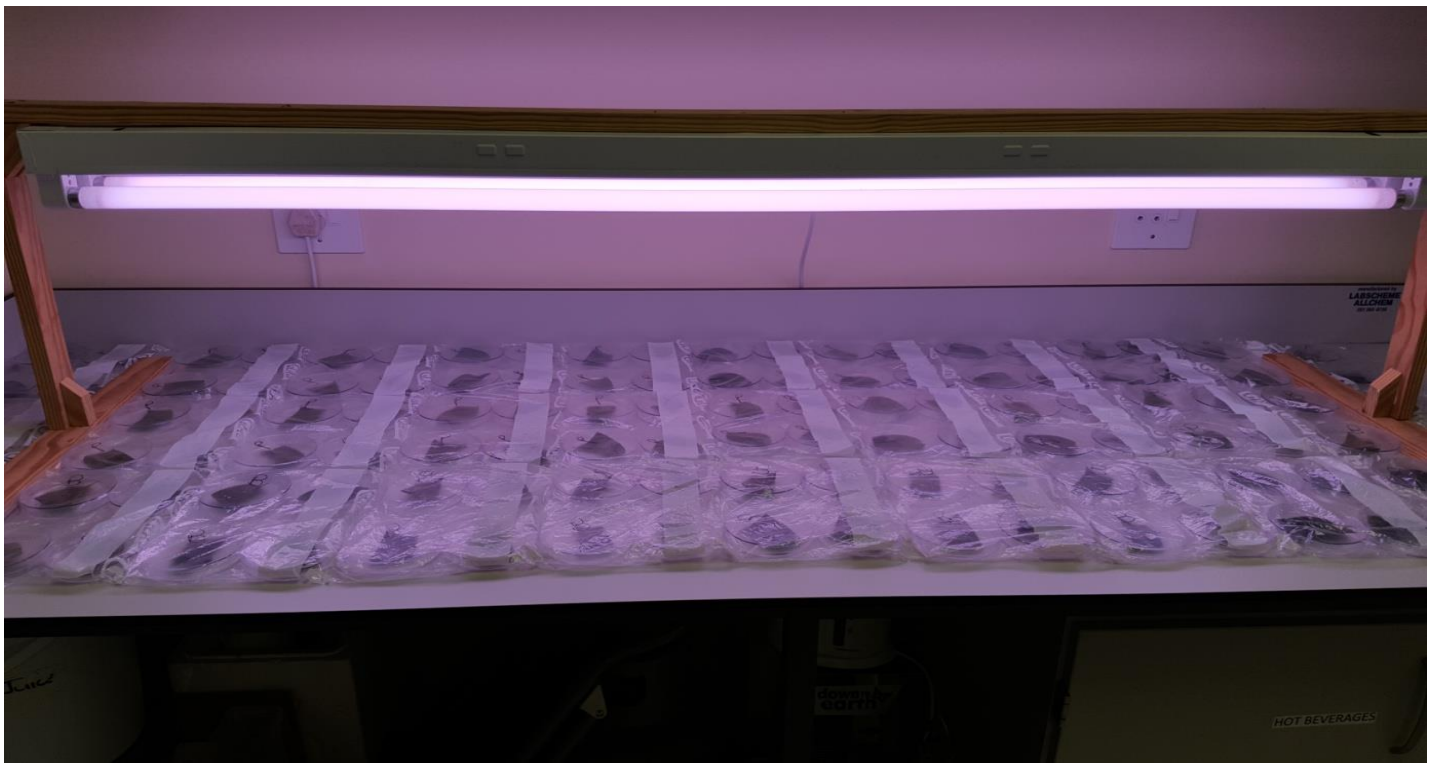


Figure 3.1 Forty small Petri-dishes of both *E. catarinensis* (Carvalho) and *E. eichhorniae* (Henry) species. This was setup at the Rhodes University Behavioural and Chemical Ecology Laboratory as discussed.

Solid-Phase Micro-Extraction

Live specimens (newly emerged, 6-8days old adults and older adults) of *E. catarinensis* and *E. eichhorniae* were packed into plastic containers and transported to the Central Analytical Facility (CAF) at Stellenbosch University for analysis. Three different ages were chosen to account for possible similarities and differences between them because newly emerged adults could have slightly different chemical compounds based on composition, quality and quantity compared to adults that have lived for a few days and those that have lived longer.

Volatile Collection Chamber experiments

Volatiles emitted by the adults were collected in chambers that trapped chemical emissions from test organisms in the Rhodes University Behavioural and Chemical Ecology Laboratory (Fig. 3.2). The test organisms were females and males from both *Eccritotarsus* species. Throughout the experiment, the air flowed through Teflon tubes (ClearAir Engineering Inc., Chicago, USA). A pump (Sonic Silent Power 108, Sonic Aquarium Air Pump, Zhenhua Electric) was used to generate air throughout the system. The air passed through a conical flask that had charcoal in it for trapping impurities, to purify the air. The air then passed through another conical flask that had 500 ml of distilled water in it for adding moisture or humidity in the possibly dry air that had just undergone purification. The air would then pass through one side of an adjustable flow meter, and then through the volatile chamber, which had two openings to it; one side designed to allow air to enter the chamber while the other side of the opening was to allow air to exit the chamber. Air entering was adjusted at 35±5 L/h using one side of the flow meter. Insects were placed inside the volatile chamber. The Tenax tube (SKC Inc; PA, USA) was placed in the opening where air was exiting, to trap all the insect emissions of those that had been placed inside the volatile chamber. Tenax tubes are glass tubes made up of several layers that consist of beads, cotton, sponge, more beads and more cotton (Fig. 3.3). When emissions were trapped from test organisms, the Tenax tube was sealed and stored in a refrigerator at -20°C. Air exiting was also adjusted at 35±5 L/h using the other side of the flow meter, to match the flow of air that entered. A vacuum pump was used to facilitate the air to exit the system, and this was set at 1 Hg.

Five replicates of 10 female and 10 male individuals each were tested concurrently for both *E. catarinensis* and *E. eichhorniae* species. All replicates ran for 12 hours. Only 2-3-day-old adults were used. The volatile collection experiment was conducted in the Rhodes University Behavioural and Chemical Ecology Laboratory, in the Zoology and Entomology building, Life Science Building at Rhodes University (Grahamstown) under 23±5°C and 45±20 relative humidity lab conditions. The volatiles were analysed using Nuclear Magnetic Resonance (NMR) and Gas Chromatography Mass Spectrometry (GCMS). The NMR analysis was done at Rhodes University (Chemistry Department) while SPME and GCMS analysis was done at Stellenbosch University (Central Analytical Facility).

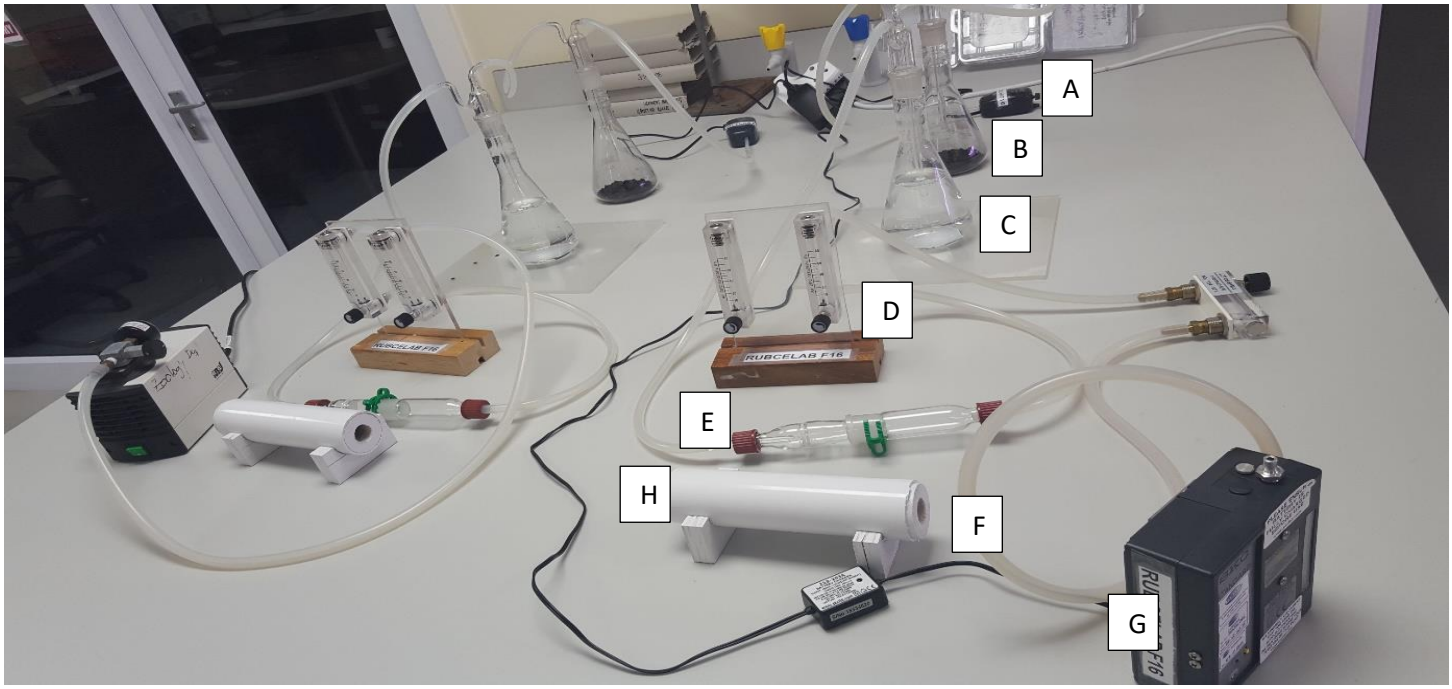


Figure 3.2 The volatile collection experiment: (A) pump, (B) charcoal conical flask, (C) distilled water conical flask, (D) flow meter, (E) volatile chamber, (F) Teflon tube, (G) Vacuum pump and (H) chamber cover. The females were inserted inside the volatile chamber on the left-hand side while the males were inserted inside the volatile chamber on the right-hand side. Collection of 10 females and 10 males' volatiles from each species for each replicate was conducted concurrently (n= 5). All aeration chamber experiments were conducted at the Rhodes University Behavioural and Chemical Ecology Laboratory and analysed using NMR and GCMS as discussed above.

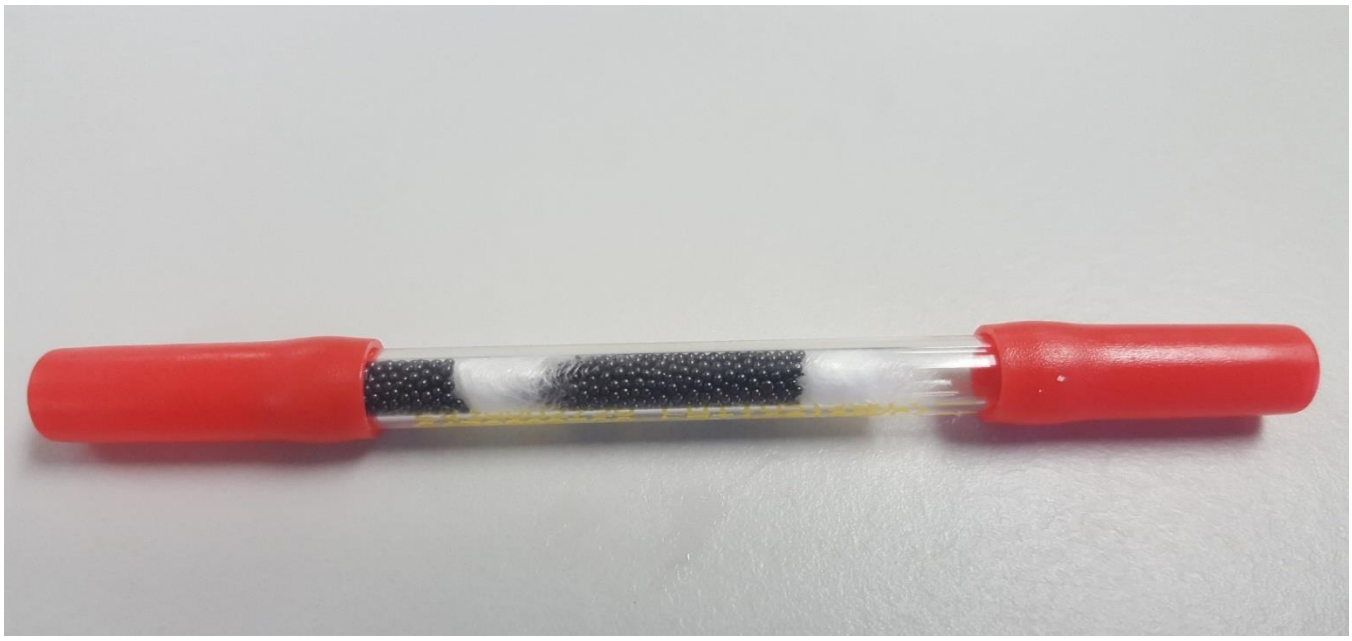


Figure 3.3 Tenax tube (249µm) used to trap the emissions of the two *Eccritotarsus* species (Hemiptera: Miridae).

Nuclear Magnetic Resonance (NMR)

Once the volatiles of male and female from both *Eccritotarsus* species were collected in Tenax tubes, the tubes were transferred into 16 Nuclear Magnetic Resonance (NMR) vials, and 2 ml deuterated chloroform ($CHCl_3$) was added to the tubes and left to settle for 2-3 hours. This allowed for extraction and desorption of the chemical with the possible volatiles present in the Tenax tubes. Chloroform was then extracted using 16 different needles and transferred into long glass vials which were suitable for the NMR machine. These were analysed using 600 MHz Nuclear Magnetic Resonance (NMR) Spectrometer with Protein Capabilities. The 600 MHz NMR spectrometer contained a 14.1 Tesla super-conducting shielded magnet, a 3-channel spectrometer with sample temperature control and two probes (both with z-gradients), *viz*., a triple resonance H, C, N probe (suitable for acquiring protein NMR data) and a broad band indirect observe probe (for routine small molecule NMR analysis).

Gas Chromatography Mass Spectrometry Sample Preparation and Analysis Conditions

Solid-phase micro-extraction (SPME)

This technique was done as an alternative to Gas-Chromatography Mass-Spectrometry (GC-MS). Live specimens (newly emerged adults, 6-8days old adults and older adults) were analysed this way. The Tenax tubes were transferred into 20 SPME vials, and 1 ml 70% methanol and 2 ml 20 sodium chloride were added. Subsequently, a 50/30 m divinylbenzene/-carboxen/-polydimethylsiloxane (DVB/CAR/PDMS) coated fibre was exposed to the sample headspace for 10 min at 70°C. After extraction, desorption of the volatile compounds from the fibre coating was carried out in the injection port of the gas chromatography-mass spectrometry (GC–MS) for 10 min. The fibre coating was inserted in a fibre conditioning station for 15 minutes between samples for cleaning and to prevent cross-contamination between the samples. The samples were analysed on a Thermo Scientific TRACETM 1310 GC which was coupled to a TSQ 8000 Mass Spectrometer detector (MSD).

Chromatographic Separation

Tenax tubes used in the volatile chamber experiment (Fig. 3.3) were transported to CAF (Stellenbosch University). Analysis of volatile compounds was performed on a Thermo Scientific TRACE^{TM} 1300 gas chromatograph coupled to TSQ 8000 Mass Spectrometer detector (MSD). Separation was performed on a non-polar (ZB-MultiResidue-1 (30 m, 0.25 mm ID, 0.25 µm film thickness) part number 7HG-G016-11. The Initial oven temperature was adjusted to 40^{o}C, held for 1 min and finally adjusted to 250^{o}C at 15°C/ min, and held for 5 min. The injector temperature was maintained at 250^{o}C. The injection was splitless and the carrier gas that was used for this experiment was helium gas. The transfer line temperature was held at 300°C. The ionization source temperature was set at 250^{o}C.

The NMR analysis paved the way for solid-phase micro-extraction (SPME) and gas-chromatography mass-spectrometry (GCMS) analysis after having shown the presence of chemical compounds. If NMR did not detect the presence of chemical compound, SPME and GCMS analysis would not have been done. For NMR, four replicates of both females and males for both species were conducted and subsequently analysed. NMR does not have a library of its own so it does not offer the name of chemical compounds. NMR needs to be linked with another machine known as the flame ionization detector for one to have chemical compound names. This led to having to do GCMS which has a library of its own and is able to give out chemical compound names. For GCMS, five replicates of both females and males for both species were conducted and subsequently analysed.

Statistical Analysis

Statistical Analysis was conducted using PRIMER 6.1.8 & PERMANOVA+. Gas-Chromatography Mass-Spectrometry (GCMS) analysis was returned as graphs with peaks of relative abundance of chemical compounds emitted by female and males of *Eccritotarsus* species. Each peak has a name based on its retention time. Names of the chemical compounds were given with the graphs. These names come from the GCMS library (Mokoena, L. pers. comm.) Chemical compound composition data from GCMS of each sex from both *Eccritotarsus* species (mean±se) was log transformed using Log (X+1). The measure of distance for the cluster analysis and MDS was done using the Euclidean distance. The Log (X+1) transformed data was then used to create a cluster analysis, and a multidimensional scatter plot (MDS plot) to assess the similarities and differences in the chemical compound composition of the cryptic species of *Eccritotarsus* based on relative abundances (mean±se).

Results

Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) results revealed the presence of chemical compounds that are similar in both sexes and both species (Peaks: 0; 0.9; 1.3; 1.6; 2.1; 6.9; 7.3 & 7.6) with only one chemical compound (Peak: 3.5) that differs between the sexes and species (Table 3.1). NMR graphs do not have peak identities since the analysis was used for compound presence or absence assessment purposes (Appendix).

Table 3.1 Nuclear Magnetic Resonance (NMR) breakdown in terms of presence and absence of *Eccritotarsus* spp. (Hemiptera: Miridae) chemical compound compositions. The left column represents 4 replicates of each sex for both *Eccritotarsus* species. Each replicate consisted of 10 females and 10 males placed in volatile collecting chambers. The top column represents the observed peaks based on retention times (mins). The presence of the peak is represented by (I) while the absence is represented by (0). EC stands for *E. catarinensis*, EE stands for *E. eichhorniae*, f stands for females and m stands for males (n=4).

Sex/Rep	Peak: 0	0.9	1.3	1.6	2.1	3.5	6.9	7.3	7.6
ECf1	I	I	I	I	I	0	I	I	I
ECf2	I	I	I	I	I	0	I	I	I
ECf3	I	I	I	I	I	0	I	I	I
ECf4	I	I	I	I	I	0	I	I	I
ECm1	I	I	I	I	I	0	I	I	I
ECm2	I	I	I	I	I	0	I	I	I
ECm3	I	I	I	I	I	0	I	I	I
ECm4	I	I	I	I	I	0	I	I	I
EEf1	I	I	I	I	I	0	I	I	I
EEf2	I	I	I	I	I	0	I	I	I
EEf3	I	I	I	I	I	0	I	I	I
EEf4	I	I	I	I	I	0	I	I	I
EEm1	I	I	I	I	I	I	I	I	I
EEm2	I	I	I	I	I	I	I	I	I
EEm3	I	I	I	I	I	I	I	I	I
EEm4	I	I	I	I	I	I	I	I	I

Major chemical compounds (based on high peak means) are peaks 0.0, 1.6, 2.1 and 7.3 (Table 3.2). The *E.eichhorniae*$_{female}$ had the highest mean for peak 0.0, 1.6 and 2.1 while *E.catarinensis*$_{male}$ had the highest mean for peak 7.3 (Table 3.2). The *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ is the same breeding cross that did not produce any offspring (Paterson et al. 2016). Furthermore, this breeding cross only mated with their conspecifics in the bi-choice experiment (Table 2.1 in Chapter 2).

Table 3.2 The relative abundances of chemical compound compositions (mean±se) as expressed by Nuclear Magnetic Resonance (NMR) spectra found in the metathoracic scent glands (MSG) of cryptic species of *Eccritotarsus* (Hemiptera: Miridae).

Peak	***E.catarinensis*$_{female}$**	***E.catarinensis*$_{male}$**	***E.eichhorniae*$_{female}$**	***E.eichhorniae*$_{male}$**
0.0	200±71.66	241,25±102.36	383,75±129.66	187,5±49.55
0.9	5	6,25±0.62	23,75±7.73	5
1.3	16,25±2.77	11,25±1.19	33,75±6.40	16,25±3.28
1.6	1336,25±51.97	1471,25±166.55	1913,75±255.11	1550±64.18
2.1	678,75±38.73	618,75±60.03	825±42.63	560±26.71
3.5	0	0	0	15
6.9	10	10	15±1.44	10
7.3	1605±75.25	1868,75±142.93	1680±288.74	1748,75±74.68
7.6	10	10	15±1.44	10

Solid-Phase Micro-Extraction (SPME)

The chemical compound with the highest area percentage for *E. catarinensis* newly emerged adults was 2-hexen-1-ol acetate whereas hexadecanoic acid was the highest for *E. eichhorniae* newly emerged adults. These chemical compounds were produced at differing area percentages. An area percentage showcases the abundance of the chemical compounds in the form of a percentage. *E. catarinensis* newly emerged adults produced this chemical compound three times more than *E. eichhorniae* newly emerged adults (Table 3.3). Both *E. catarinensis* and *E. eichhorniae* newly emerged adults had hexadecanoic acid as a chemical compound with the second highest area percentage, but this chemical compound was produced at differing area percentages. *E. catarinensis* newly emerged adults produced this chemical compound at a greater area percentage than the *E. eichhorniae* newly emerged adults (Table 3.3). *E. catarinensis* and *E. eichhorniae* 6-8 days old adults as well as older *E. catarinensis* and *E. eichhorniae* adults all had trans-2-hexenyl acetate as a chemical compound with the highest area percentage. This chemical compound was produced at differing area percentages. *E. eichhorniae* 6-8 days old and *E. eichhorniae* older adults produced more of this chemical compound than *E. catarinensis* 6-8 days old and *E. catarinensis* older adults (Table 3.3). *E. catarinensis* and *E. eichhorniae* 6-8 days old adults as well as *E. catarinensis* older adults all had trans-2-hexenal as a chemical compound with the second highest area percentage. This chemical compound was also produced at differing area percentages. *E. catarinensis* 6-8 days old and *E. catarinensis* older adults produced more of this chemical compound than *E. eichhorniae* 6-8 days old adults. *E. eichhorniae* older adults had trans-2-hexenol as a chemical compound with the second highest area percentage, and instead, the trans-2-hexenal was present as a chemical compound with the third highest area percentage.

Table 3.3 Solid-phase micro-extraction (SPME) of geographically and reproductively isolated cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae) showing major chemical compounds based on area productivity involving newly emerged adults, 6-8days old adults and older adults of *E. catarinensis* and *E. eichhorniae*.

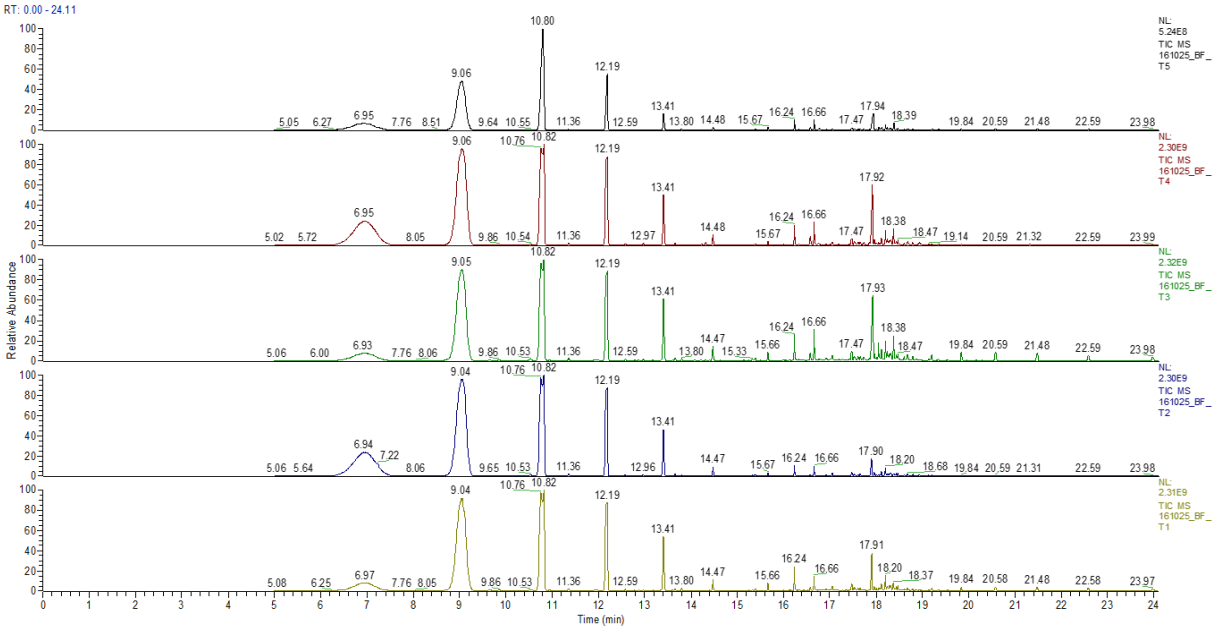
***E. catarinensis* newly-emerged adults**	**Chemical Compound**	**Retention time (in min)**	**Quality**	**Area Productivity**
1	2-Hexen-1-ol acetate	10.72	93	50.07
2	Hexadecanoic acid	27.89	99	15.45
3	Cyclohexasiloxane	11.24	94	6.99
***E. eichhorniae* newly-emerged adults**				
1	Hexadecanoic acid	27.94	94	14.19
2	Hexadecanoic acid	24.52	25	12.29
3	Tetradecanoic acid	27.75	92	6.59
***E. catarinensis* (6-8days) old**				
1	Trans-2-hexenyl acetate	10.85	94	53.74
2	Trans-2-hexenal	8.72	97	21.73
3	Trans-2-hexenol	11.92	91	13.07
***E. eichhorniae* (6-8days) old**				
1	Trans-2-hexenyl acetate	10.84	94	77.93
2	Trans-2-hexenal	8.67	97	6.65
3	Trans-2-hexen-1-ol	11.89	93	6.36
***E. catarinensis* older adults**				
1	Trans-2-hexenyl acetate	10.84	94	36.75
2	Trans-2-hexenal	8.71	98	16.82
3	Trans-2-hexen-1-ol	11.90	90	9.29
***E. eichhorniae* older adults**				
1	Trans-2-hexenyl acetate	10.85	93	70.59
2	Trans-2-hexenol	11.92	91	7.28
3	Trans-2-hexenal	8.67	97	6.63

*Retention time (RT) is a measure of the time taken for a solute to pass through a chromatography column, and it is calculated as the time from injection to detection. ** Quality is the degree of surety of the chemical compound. *** Area percentage finds the percentage of a portion of an object by dividing the area of the portion by the area of the whole original object.

Gas-Chromatography Mass-Spectrometry (GCMS)

The chemical compound composition of the cryptic species of *Eccritotarsus* is identical but slight differences can be found when comparing the sexes based on relative abundances of the peaks (Fig. 3.4, Fig. 3.5 and Fig. 3.6).

a



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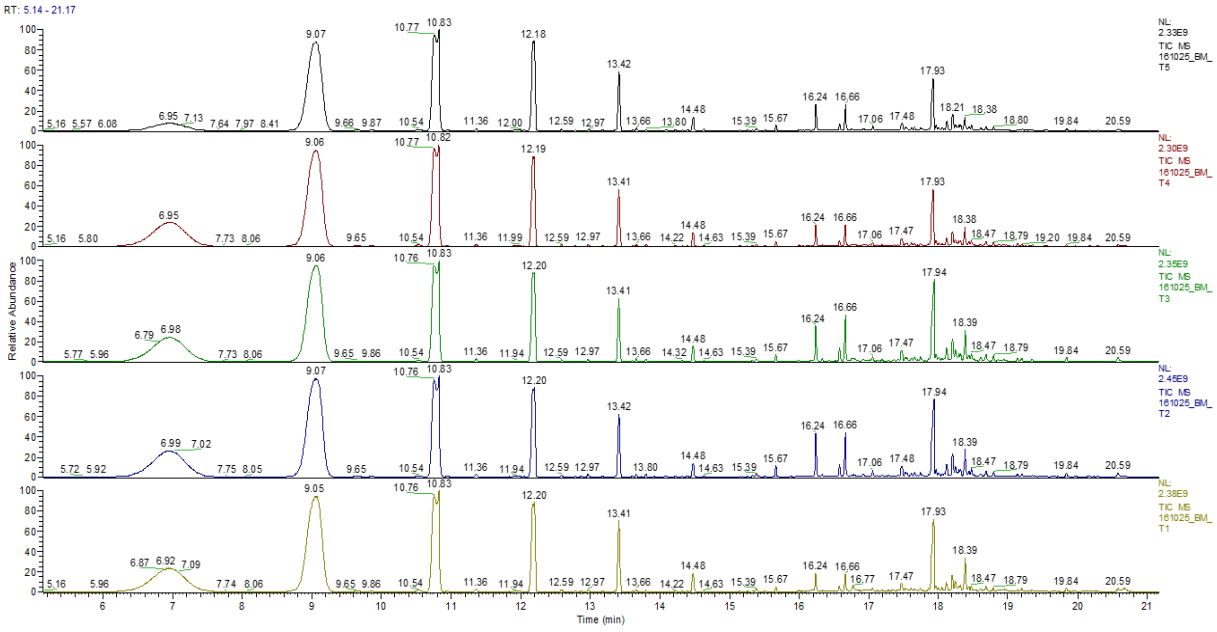
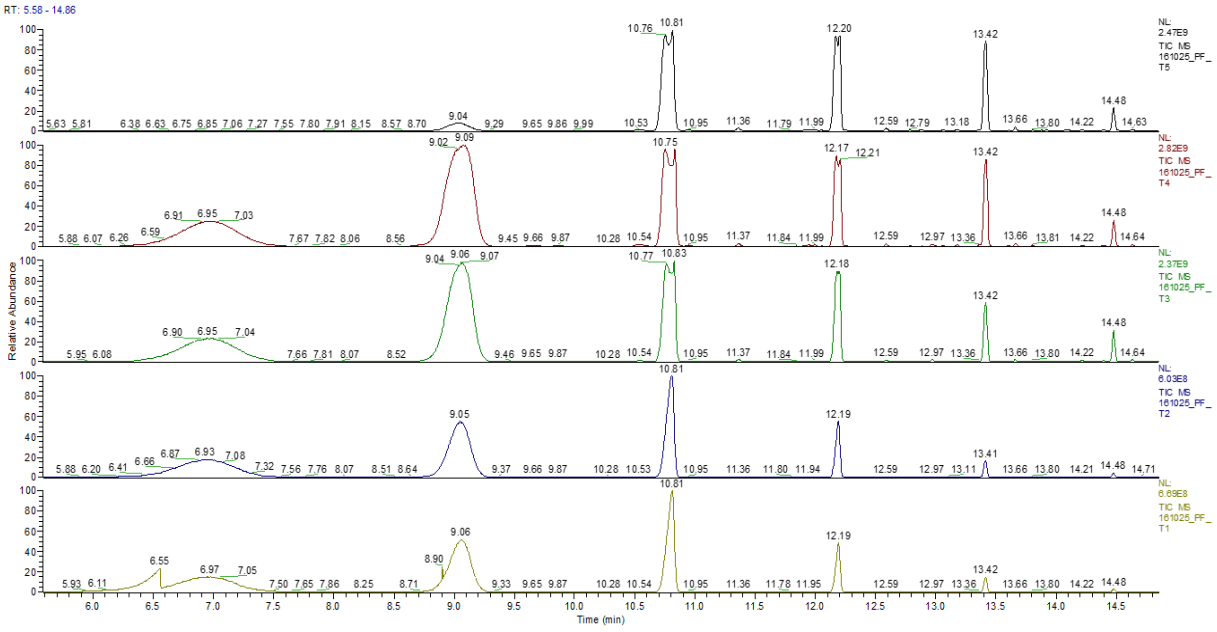


Figure 3.4 Gas chromatographs of the geographically and reproductively isolated cryptic species of *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae): (a) *E.catarinensis*$_{female}$ (0.00-24.11min retention time), (b) *E.catarinensis*$_{male}$ (5.14-21.17min retention time). Five replicates of both sexes are colour-coded as: (T1) gold, (T2) navy, (T3) green, (T4) red and (T5) black respectively.

a



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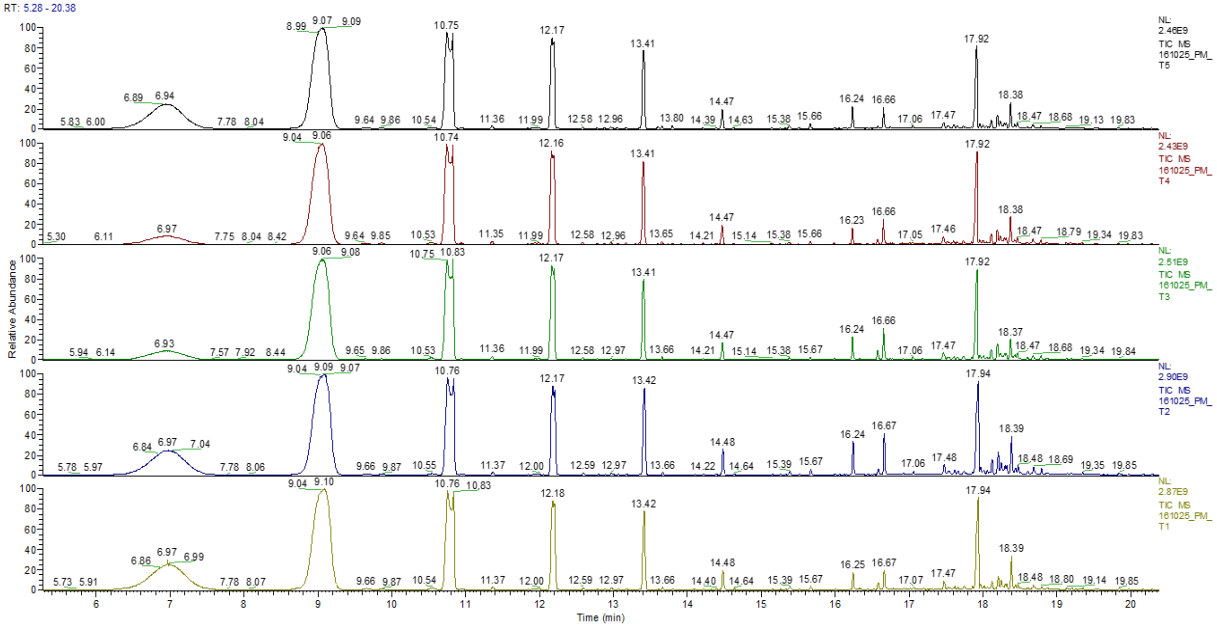
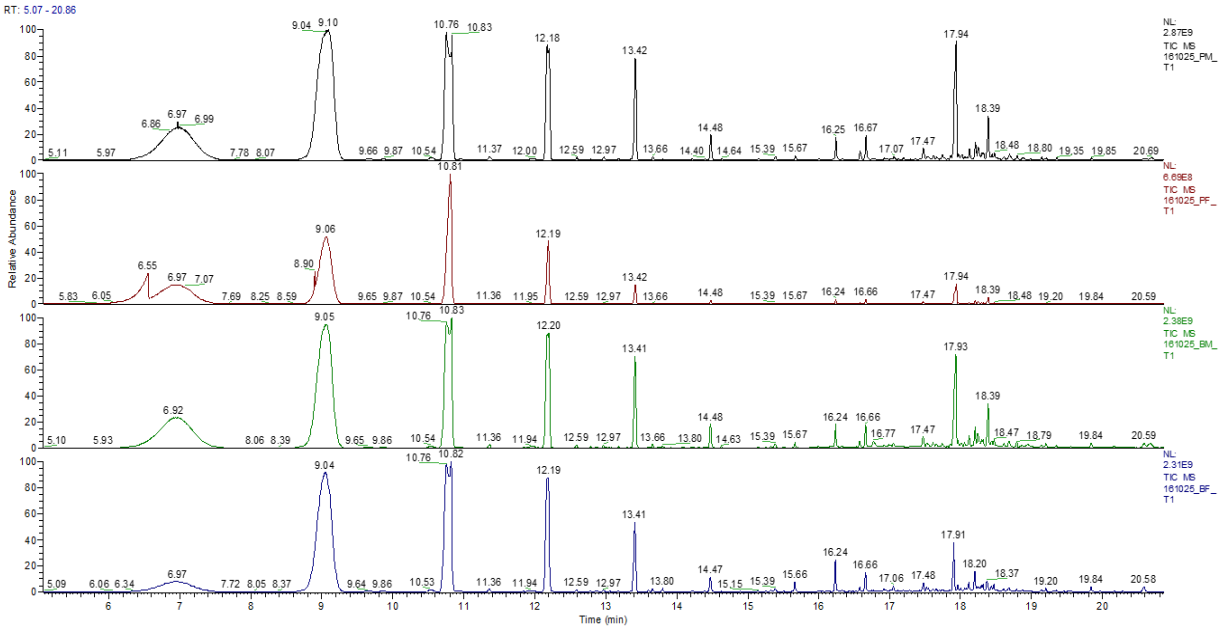
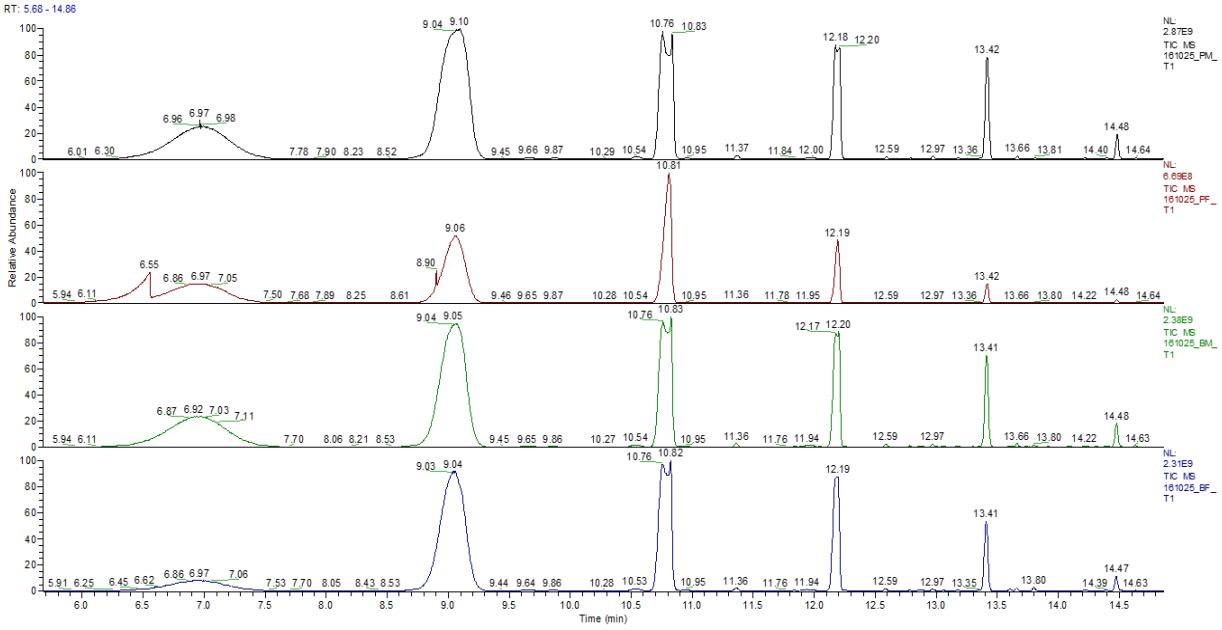


Figure 3.5 Gas chromatographs of the geographically and reproductively isolated cryptic species of *Eccritotarsus eichhorniae* (Henry) (Hemiptera: Miridae): (a) *E.eichhorniae*$_{female}$ (5.58-14.86min retention time), (b) *E.eichhorniae*$_{male}$ (5.28-20.38min retention time). Five replicates of both sexes are colour-coded as: (T1) gold, (T2) navy, (T3) green, (T4) red and (T5) black respectively.

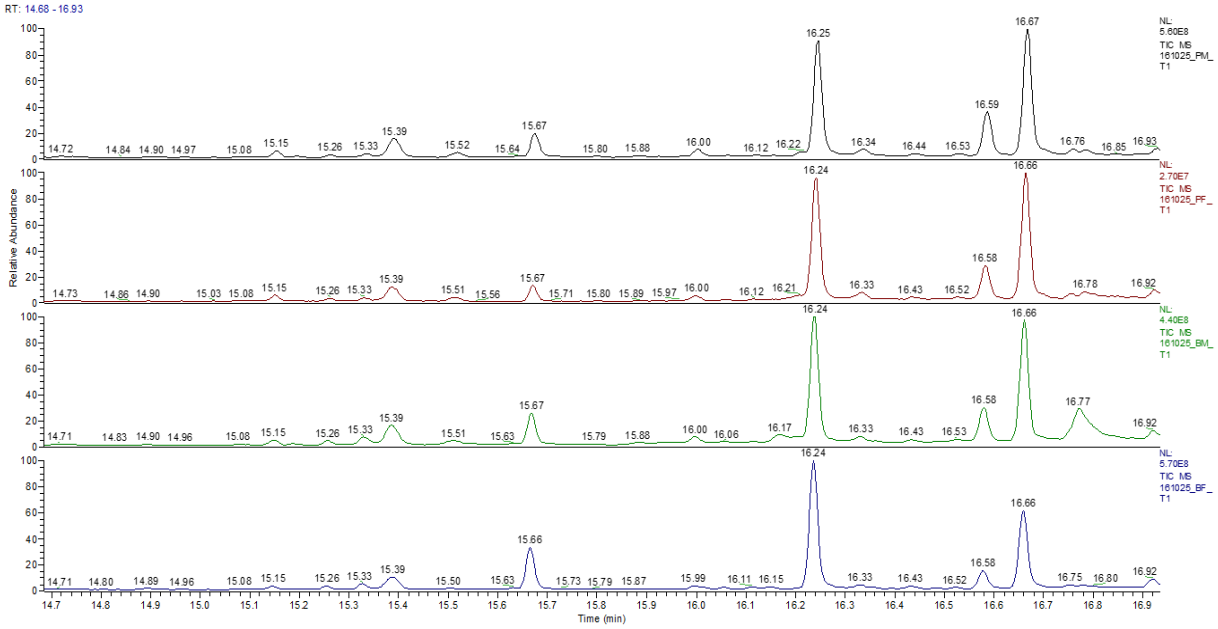
a



b



c


RT: 14.68 - 16.93
Relative Abundance
Time (min)
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NL: 2.70E7 TIC MS 161025_PF_ T1
NL: 4.40E8 TIC MS 161025_BM_ T1
NL: 5.70E8 TIC MS 161025_BF_ T1

d

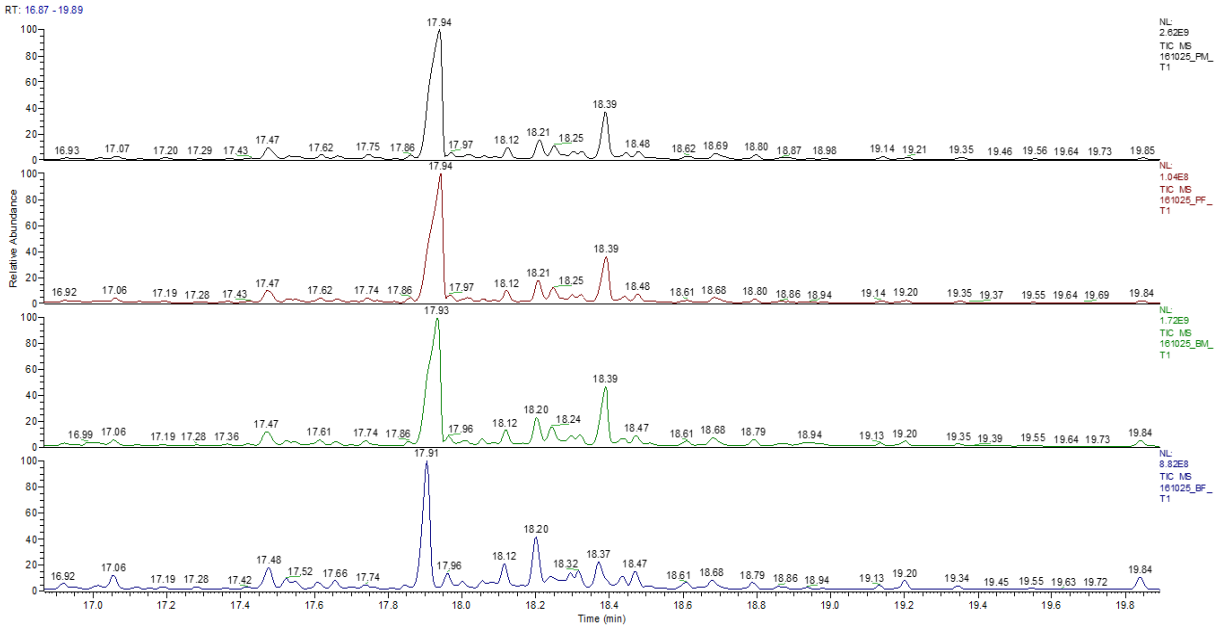

RT: 16.87 - 19.89
Relative Abundance
Time (min)
NL: 2.62E9 TIC MS 161025_PM_ T1
NL: 1.04E8 TIC MS 161025_PF_ T1
NL: 1.72E9 TIC MS 161025_BM_ T1
NL: 8.82E8 TIC MS 161025_BF_ T1

Figure 3.6 Gas chromatographs of the two geographically and reproductively isolated cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae), showing only the first replicate (T1) of both females and males for both *E. catarinensis* (Carvalho) (Brazil pop.) and *E. eichhorniae* (Henry) (Peru pop.) respectively: (a) 5.07-20.86min retention time, (b) 5.68-14.86min retention time, (c) 14.68-16.93min retention time and (d) 16.87-19.89min retention time. The four colour representations are: *E.catarinensis*$_{female}$ (navy), *E.catarinensis*$_{male}$ (green), *E.eichhorniae*$_{female}$ (red) and *E.eichhorniae*$_{male}$ (black).

Table 3.4 Chemical compounds of *Eccritotarsus* spp. (Hemiptera: Miridae) as denoted by the retention time (in minutes) of both females and males, for both *E. catarinensis* (Carvalho) and *E. eichhorniae* (Henry) in the Gas Chromatographs shown above (n= 5).

Retention time (in minutes)	**Chemical compound name**
9.06; 10.80; 12.19; 13.41 & 17.94	Siloxanes (breakthrough material from the Tenax) - ignored
12.89	2-Phenylpropenal
14.89	Benzenebutanoic acid (methyl ester) (ç-methyl-)
15.65-15.66	3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid
16.58	5-t-Butyl-1,2,3-trimethylbenzene
16.66	3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid
17.00	Phenol (2-cyclohexyl)
17.01-17.01	Phenol (4-cyclohexyl)
17.05-17.06	Benzene: 1,1'-(1-methyl-1,3-propanediyl)-bis-
17.12-17.12	Benzene: 1,1'-(1-methyl-1-propene-1,3-diyl)-bis-
17.15-17.16	Naphthalene: 1,2,3,4-Tetrahydro-6-(phenyl methyl)-
17.28-17.28	1H Indene: 2,3-dihydro (1,1,3-trimethyl-3-phenyl)
17.55	Benzene: 1,1'-(2-pentene-1,5-diyl)-bis-
17.89-17.90	Benzenesulfonamide (N-ethyl-2-methyl)
17.96-17.97	Sclareoloxide
18.19-18.20	Naphthalene: 1,2,3,4-Tetrahydro-1-phenyl
18.37	Benzenesulfonamide (N-ethyl-4-methyl)

Both *Eccritotarsus* females and males share chemical compounds 15.66, 16.58, 16.66, 17.06, 17.89, 18.20 & 18.37. There is no chemical compound unique to either *E.catarinensis*$_{female}$ or *E.eichhorniae*$_{female}$. *E.catarinensis*$_{male}$ lacks 14.89 that the other individuals have, while *E.eichhorniae*$_{male}$ has 17.16 that other individuals lack. When comparing within species in *E.catarinensis*$_{female}$ has chemical compounds 14.89, 17.01, 17.12 & 17.28 that *E.catarinensis*$_{male}$ lacks. However, *E.eichhorniae*$_{male}$ has chemical compounds 17.01, 17.12, 17.16 & 17.55 that *E.eichhorniae*$_{female}$ lacks. When comparing between species *E.eichhorniae*$_{male}$ has 17.16 that *E.catarinensis*$_{female}$ lacks while *E.eichhorniae*$_{female}$ has 14.89 that *E.catarinensis*$_{male}$ lacks. When comparing the sexes between species, *E.catarinensis*$_{female}$ has chemical compounds 17.01, 17.12 & 17.55 that *E.eichhorniae*$_{female}$ lacks while *E.eichhorniae*$_{male}$ has chemical compounds 14.89, 17.01, 17.12, 17.16 & 17.28 that *E.catarinensis*$_{male}$ lacks.

Gas-Chromatography Mass-Spectrometry (GCMS) results suggest that *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{male}$ have chemical compounds that their conspecifics and same sex of the other species lack (Table 3.4). *E.catarinensis*$_{male}$ is unique because it lacks peak 14.89 [Benzenebutanoic acid (methyl ester) (ç-methyl-)] that is present in *E.catarinensis*$_{female}$, *E.eichhorniae*$_{female}$ and *E.eichhorniae*$_{male}$ (Table 3.4). *E.eichhorniae*$_{male}$ is unique because it has peak 17.16 [Naphthalene: 1,2,3,4-Tetrahydro-6-(phenyl methyl)-] that is absent in *E.eichhorniae*$_{female}$, *E.catarinensis*$_{female}$ and *E.catarinensis*$_{male}$ (Table 3.4). If the males are doing the calling in *Eccritotarsus*, then the absence of peak 14.89 [Benzenebutanoic acid (methyl ester) (ç-methyl-)] in *E.catarinensis*$_{male}$ and the presence of peak 17.16 [Naphthalene: 1,2,3,4-Tetrahydro-6-(phenyl methyl)-] in E.*eichhorniae*$_{male}$ could prove to be significant when it comes to the above-mentioned female and male differences in pheromones of these geographically and reproductively isolated cryptic species. It is possible that those differences are maintaining reproductive isolation. The peak 14.89 [Benzenebutanoic acid (methyl ester) (ç-methyl-)] is present in *E.eichhorniae*$_{female}$, and investigating its absence in *E.catarinensis*$_{male}$ becomes important.

Table 3.5 Gas-Chromatography Mass-Spectrometry (GCMS) breakdown in terms of presence and absence of *Eccritotarsus* spp. (Hemiptera: Miridae) chemical compound compositions. The left column represents 5 replicates of each sex for both *Eccritotarsus* species. Each replicate consisted of 10 female and males placed in volatile collecting chamber experiment. The top column represents the observed peaks based on retention times (mins). The presence of the peak is represented by (I) while the absence is represented by (0). In terms of symbols, EC stands for *E. catarinensis*, EE stands for *E. eichhorniae*, f stands for females and m stands for males (n=5).

Retention time (in min) of peaks denoting chemical compounds of mirids using GCMS analysis excluding siloxanes															
Sex	**12.89**	**14.89**	**15.66**	**16.58**	**16.66**	**17.00**	**17.01**	**17.06**	**17.12**	**17.16**	**17.28**	**17.55**	**17.89**	**17.97**	**18.20**	**18.37**
ECf1	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECf2	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECf3	0	I	I	I	I	0	0	I	I	0	I	I	I	I	I	I
ECf4	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECf5	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECm1	0	0	I	I	I	0	0	I	0	0	0	0	I	0	I	I
ECm2	0	0	I	I	I	0	0	I	0	0	0	I	I	I	I	I
ECm3	0	0	I	I	I	0	0	I	0	0	0	I	I	0	I	I
ECm4	0	0	I	I	I	0	0	I	0	0	0	0	I	I	I	I
ECm5	0	0	I	I	I	0	0	I	0	0	0	I	I	I	I	I
EEf1	0	I	I	I	I	0	0	I	0	0	0	0	I	I	I	I
EEf2	0	I	I	I	I	0	0	I	0	0	I	0	I	I	I	I
EEf3	0	I	I	I	I	0	0	I	0	0	I	0	I	I	I	I
EEf4	0	I	I	I	I	0	0	I	0	0	0	0	I	I	I	I
EEf5	0	I	I	I	I	0	0	I	0	0	0	0	I	I	I	I
EEm1	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm2	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm3	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm4	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm5	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I

Major chemical compounds (based on high peak means) are peak 15.66 (3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid), 16.58 (5-t-Butyl-1,2,3-trimethylbenzene), 16.66 (3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid), 18.20 (Naphthalene: 1,2,3,4-Tetrahydro-1-phenyl) and 18.37 (Benzenesulfonamide (N-ethyl-4-methyl)) (Table 3.5). The *E.catarinensis*$_{female}$ produced the highest mean for peak 15.66 (3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid) and 18.20 (Naphthalene: 1,2,3,4-Tetrahydro-1-phenyl) while *E.catarinensis*$_{male}$ produced the highest mean for peak 16.58 (5-t-Butyl-1,2,3-trimethylbenzene) and 18.37 (Benzenesulfonamide (N-ethyl-4-methyl)) (Table 3.5). The *E.eichhorniae*$_{male}$ produced the highest mean for peak 16.66 (3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid) (Table 3.5) while *E.eichhorniae*$_{female}$ had no highest mean produced for any peak (Table 3.5). Peak 15.66 (3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid), 16.58 (5-t-Butyl-1,2,3-trimethylbenzene), 16.66 (3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid), 18.20 (Naphthalene: 1,2,3,4-Tetrahydro-1-phenyl) and 18.37 (Benzenesulfonamide (N-ethyl-4-methyl)) all need further investigation because they could be sex pheromones. These peaks can be assessed individually and in combination. Literature has suggested that chemical compounds with the highest peak measure are sex pheromones (Zhang et al. 2015). Individual testing and combination testing of chemical compounds has proved to be a very useful tool in isolating sex pheromones from a bouquet of pheromones (Groot et al. 1999). Comparing such findings in sympatric species has also proven to be useful in assessing pheromones compositions in mirids (Yang et al. 2015).

Table 3.6 Relative abundances (mean±se) as expressed by Gas-Chromatography Mass-Spectrometry (GCMS) spectra found in the metathoracic scent glands of cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae).

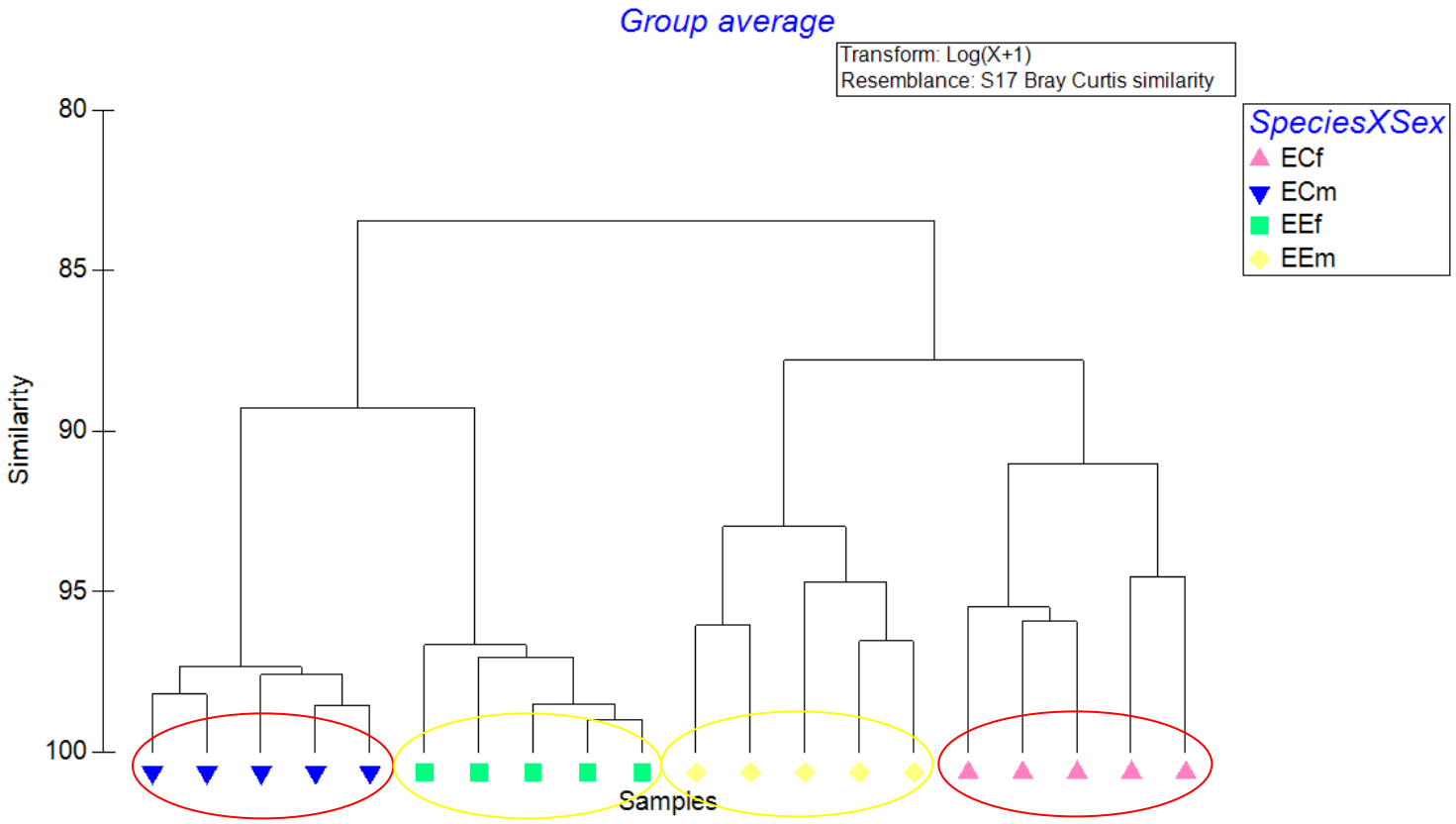
Peaks (Rel. Abund.)	***E.catarinensis*$_{female}$**	***E.catarinensis*$_{male}$**	***E.eichhorniae*$_{female}$**	***E.eichhorniae*$_{male}$**
12.89	0	0	0	0
14.89	2,4±1.8	0	2	2
15.66	30,4±1.24	22,4±0.82	20,4±0.82	17,2±1.43
16.58	12,8±0.66	28,4±0.44	26,8±0.92	20,4±2.23
16.66	50,8±2.40	99,6±0.18	98,8±0.36	100
17.00	0	0	0	0
17.01	3,2±0.22	0	0	6±0.40
17.06	10,4±0.95	7,6±0.33	5,6±0.18	11,2±0.54
17.12	3,2±0.36	0	0	3,6±0.33
17.16	0	0	0	2
17.28	3,6±0.33	0	2	2
17.55	7,6±0.52	6,8±0.22	0	4,4±0.18
17.89	5,6±0.18	4,4±0.18	4,4±0.18	3,6±0.18
17.97	14±1.65	10,4±0.18	8,4±0.33	7,2±0.36
18.20	36±2.61	27,6±0.72	22,4±0.52	10,8±0.96
18.37	29,6±2.25	36,8±1.34	34±1.44	23,6±1.78

Cluster plot results

The *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ have similar chemical compound compositions based on relative abundances (Fig. 3.7). These happen to be the breeding cross that failed to produce offspring (Paterson et al. 2016). These were also the same breeding cross that only mated with their conspecifics in the bi-choice experiment (Table 2.1). The *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{male}$ also have similar chemical compound compositions based on relative abundances. These were the breeding crosses that produced very few offspring in Paterson et al. (2016), and the same breeding cross that had no copula incidences in multi-choice experiment (Fig. 2.9). With regards to sex comparisons between species, both females and males are distant and are in opposite end to each other, suggesting that they have different relative abundances (Fig. 3.7).

Multidimensional Scatterplot (MDS) results

The multi-dimensional scaling plot (MDS-plot) results suggest that *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ have similar chemical compound compositions that have very little variability. This is the breeding cross that failed to produce offspring in Paterson et al. (2016), and the same breeding cross that only mated with their conspecifics in the bi-choice experiment (Table 2.1). It also suggests that *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{male}$ have similar chemical compound compositions and very high variability (Fig. 3.7). This is the breeding cross that produced very few offspring in Paterson et al. (2016), and the same breeding cross that had no copula incidences in the multi-choice experiment (Fig. 2.9). Also, *E.catarinensis*$_{male}$ and *E.eichhorniae*$_{male}$ as well as *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{female}$ are in opposite ends of the graph from each other and therefore have different relative abundances (Fig. 3.7).The female and male differences found in the cluster analysis plot and multidimensional scatterplot (Fig. 3.7) could be explaining the reproductive isolation maintenance that could have led to speciation.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Transform: Log(X+1)
Resemblance: S17 Bray Curtis similarity
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ECf
ECm
EEf
EEm
Similarity
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85
90
95
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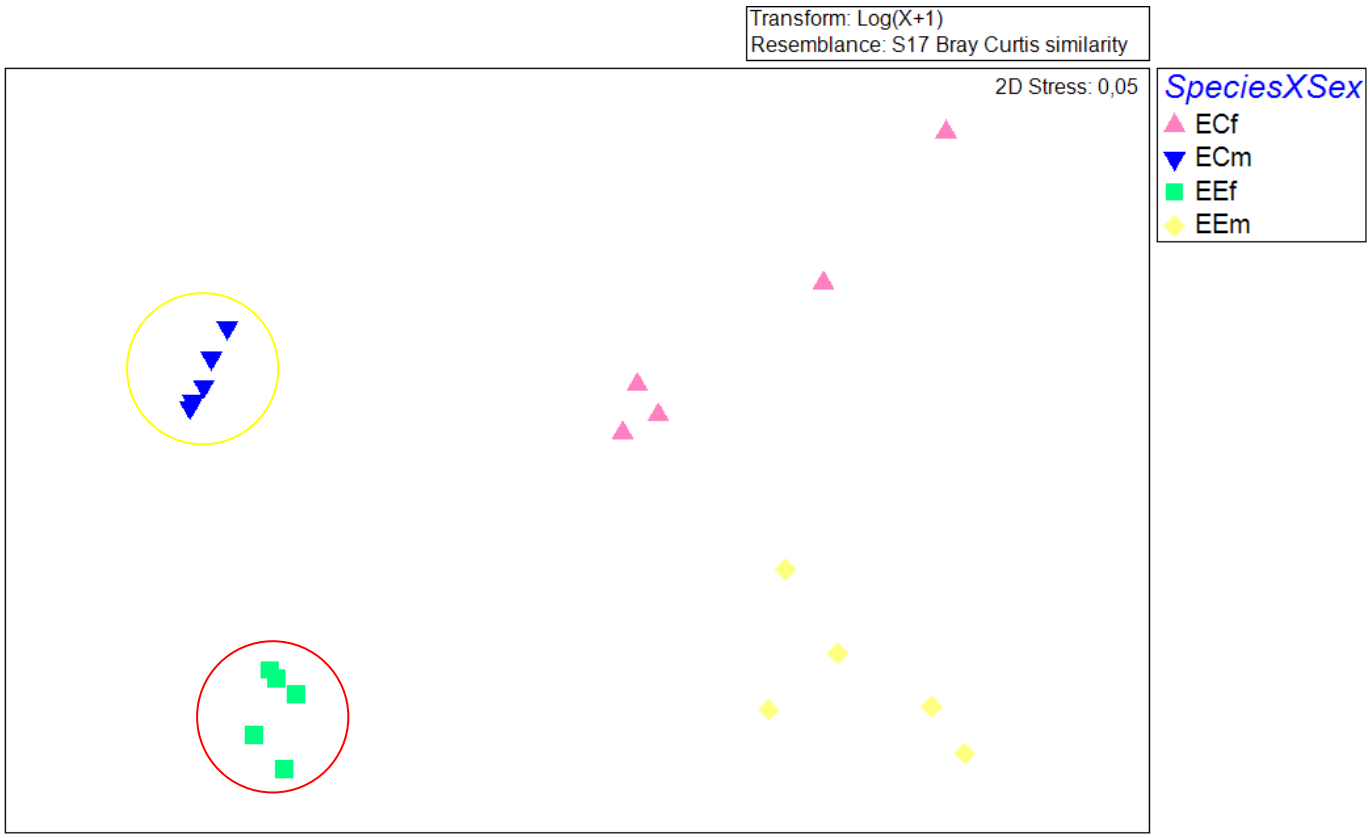
Samples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Resemblance: S17 Bray Curtis similarity
2D Stress: 0,05
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ECf
ECm
EEf
EEm

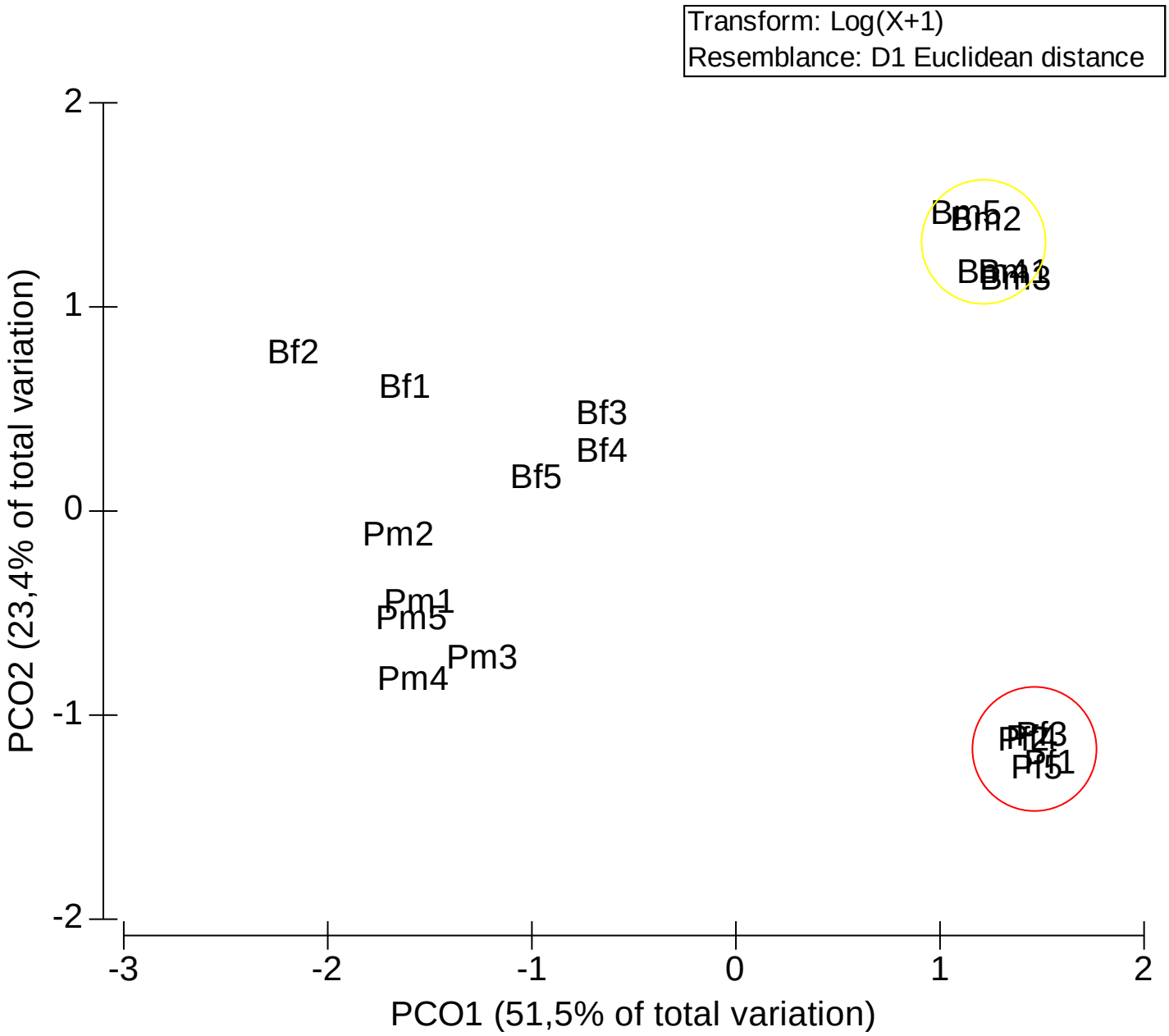

Figure 3.7 Cluster analysis plot, Multi-Dimensional Scaling (MDS) scatterplot (2D Stress: 0.06) and Principal Component Ordination (PCO1 51,5% and PCO2 23,4% of total variation) graphs using chemical compound compositions of two populations of cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae). 'EC' & 'B' indicates *E. catarinensis*, 'EE' & 'P' indicates *E. eichhorniae*, 'f' indicates female and 'm' indicates male. The *E.catarinensis*$_{female}$ were represented by pink, *E.eichhorniae*$_{female}$ were represented by green, *E.catarinensis*$_{male}$ were represented by blue while *E.eichhorniae*$_{male}$ were represented by yellow (n= 5).

Discussion

The differences in chemical compound compositions between the two cryptic *Eccritotarsus* spp. could be key in explaining their interbreeding incompatibility that was reported by Paterson et al. (2016). Missing chemical compounds could be the barrier that exists between these cryptic species and could therefore be responsible for the reproductive isolation and may have led to speciation. Also, these unique attributes could explain the absence of any copula incidences in the multi-choice experiments. Lastly, these unique attributes could explain why crosses had few copula incidences and low copula durations. The results suggest that although the chemical compound compositions of the two geographically and reproductively isolated cryptic species are similar, there are some consistent differences present between species and sexes. Furthermore, these chemical compound compositions differed in terms of quantity, quality, ratio or proportion at which they were produced. The *E.catarinensis*$_{female}$ and *E.catarinensis*$_{male}$ had a greater average chemical compound production than the *E.eichhorniae*$_{female}$ and *E.eichhorniae*$_{male}$. Major chemical compounds are likely to be potentially used as sex pheromones as with other mirids (Zhang et al. 2015).

This study provides evidence that species-specific chemical compounds and their respective quality and quantity, proportions, ratios, concentrations, and relative abundance among many other things significantly contribute to the behavioural pre-mating reproductive isolation barriers reported in behavioural observations (Chapter 2). Chemical compound composition quantity and quality has proven to be a key determinant in helping one to understand the reproductive isolation in the cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae) as suggested in Millar et al. (1997); Millar and Rice (1998); Groot et al. (1999); Zhang and Aldrich (2003a) and most recently in Yang et al. (2015). Quantifying the chemical compounds reported here has given insight into the behavioural-observation mating patterns seen in Chapter 2. Also, the sex differences involving *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ reported in this chapter could be key in tracing the interbreeding incompatibility found in Paterson et al. (2016) and could also support Taylor et al. (2011) findings delineating these two populations as cryptic species. It appears that chemical compound composition played a role in the reproductive isolation and speciation involving these cryptic species of *Eccritotarsus*.

So far, chemical compounds found to be consistent in other several mirid species involve hexyl butyrate, (E)-2-hexenyl butyrate and (E)-4-oxo-2-hexenal which have been shown to act as allomones, anti-sex pheromones and sex pheromones respectively (Yang et al. 2015). Hexyl butyrate is known to be the only chemical compound produced in large amounts by both females and males in all mirids studies (Zhang and Aldrich 2003b). These compounds have been present as female sex pheromones in three Asian mirids: *Stenotus rubrovittatus*

(Matsumura) (Yasuda et al. 2008), *Apolygus spinolae* (Meyer-Dür) (Yang et al. 2014) and *Adelphocoris fasciaticollis* Reuter (Zhang et al. 2015); three North-American mirids: *Lygus hesperus*, *L. lineolaris* and *L. pabulinus* Hahn (Byers et al. 2013) and four European mirids: *Lygus pratensis* Hahn, *L. rugulipennis* Hahn, *Lygocoris pabulinus* (Linnaues) and *Liocoris tripustulatus* (Fabricius) (Fountain et al. 2014). This study is the first to identify the pheromone in a South American mirid but the role of this pheromone in the two *Eccritotarsus* species, and whether the pheromone plays a similar role in both species is not known. Establishing which sex attracts the other sex in *Eccritotarsus* spp. will be important going forward, since the chemical compound composition is mostly shared by these cryptic species with very few differences that could prove to be important towards the chemical communication of these insects. Also, establishing the role played by these chemical compounds reported here, either individually or binary, would drive towards establishing the major chemical compounds of these cryptic species. The quantity might be more of a contributing factor than the quality of compounds present. From then on, it would be ideal to use those major compounds to try to pinpoint specifically the sex pheromones of the cryptic species.

It is highly possible that even compounds present in small amounts could also have a big role to play in the attraction of one sex towards the other. For example, Yang et al. (2015) report that the elimination of (E)-2-octenyl butyrate, a sex attractant present in small amounts in females of *Apolygus lucorum* Meyer-Dür and *Taylorilygus apicalis* (Fieber) from a full four-compound-blend (consisting of hexyl butyrate: (E)-2-hexenyl butyrate: (E)-4-oxo-2-hexenal: (E)-2-octenyl butyrate) failed to attract their respective males and only attracted their conspecifics. The four-compound-blend acts as an attractant, but removing one compound from it often fails to attract the opposite sex. Paradoxically, an addition of large amounts of hexyl buyrate in the full four-compound-blend also failed to attract males of another species, *A. spinolae* (Meyer-Dür). In essence, a small amount taken out of the four-compound-blend attracts one species but adding large amounts did not attract another species. This has then suggested that the quantity and quality of chemical compounds present in either females or males of any mirid species have a critical role to play in the attraction of one sex towards the other. The quantity and quality of chemical compounds could then prove to be a key distinguishing feature in the geographically and reproductively isolated cryptic species of *Eccritotarsus* spp. assessed in this study. The above-mentioned four-compound-blend (consisting of hexyl butyrate: (E)-2-hexenyl butyrate: (E)-4-oxo-2-hexenal: (E)-2-octenyl butyrate) has led many to believe that the four prominent compounds could be major compounds in mirids that still need to be assessed. However, as with many other things, there is a twist to the story since mirid species which have hexyl butyrate have been shown not to have (E)-2-octenyl butyrate for one reason or the other (apart from Millar *et al.*, (1997) who found the two compounds to be present in *Phytocoris relativus* Fallén), adding uncertainty and a new dimension in the evolution of chemical composition of mirid species (Yang et al. 2015). The cryptic species of

Eccritotarsus spp. (Hemiptera: Miridae) did not produce the four-compound blend. For this reason, further assessment of chemical compounds in many other mirids is still needed to enhance and broaden our understanding even further.

In the Miridae, it is common for females and males to have very similar chemical compositions within and between species, but the reason for that is not clear yet. Some authors have postulated that the same compounds that females use to attract males in mirids are instead used by males of the same species as defence pheromones, allomones or by individuals from other species as kairomones (Millar et al. 1997), highlighting how one chemical compound bouquet could be used for many functions both intra- and inter-specifically. However, in most chemical compound bouquets assessed for both mirid females and males to date, only two or three compounds of simple esters (acetates, butyrates or hexanoates) serve as sex pheromones, with one compound being present in both sexes, while other compounds will have other functions (Zhang and Aldrich 2003b). For example, hexyl butyrate is one of the major compounds in many female mirid species yet it can also serve as anti-sex pheromone to inhibit the attraction of sympatric congeners in other male mirid species, or prevent females from releasing pheromones (Millar et al. 1997). An example of this involves *Phytocoris difficilis* Fallén (Zhang and Aldrich 2008), *Lygocoris pabulinus* (Linnaeus) (Groot et al. 2001) and *Lygus lineolaris* Hahn (Zhang et al. 2007). This is possible because even though chemical compounds are similar; the ratios in which they are emitted differ slightly, enabling the receiver to be able to distinguish between the senders through pheromone concentrations. The ability to be able to distinguish between females and males using porous sensilla and chemoreceptors intra-specifically as well as inter-specifically, from a complex of chemical compounds emitted, is important for insect communication evolution considering that mirids are known to produce a complex of chemical compound bouquet from several glands (Millar et al. 1997). The Solid-Phase Micro-Extraction (SPME) results of this study have shown that *E. catarinensis* and *E. eichhorniae* nymphs and adult have the same chemical compounds, but produce them at different area percentages. The differences in area percentages could also play a role in the maintenance of reproductive isolation that exists in these two species. It is possible that a chemical compound produced at a greater proportion may end up acting as a repellent. In that case, a chemical compound that has the second highest area percentage may come into play. Those sequence of events may have a big role to play in the evolution of pheromones.

Although there are differences in the pheromones produced by the two species, the similarities and differences of the functions served by the major compounds is not known. The function of these compounds could help explain further how reproductive isolation developed between these two species and how it is maintained. Other compounds present in small amounts could also be investigated since they also from part of sex pheromones of other mirid species that have been studies in the past (Groot et al. 1999). The female chemical compound

compositions prove to be a distinguishing feature and could be the underlying factor causing reproductive incompatibility. Also, the *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{male}$ are key in the differences observed between the sexes of the cryptic species assessed in this study, and could also play an important role in maintenance of reproductive incompatibility and speciation. This is largely because the *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{male}$ did not record a single mating incident with each other in a multi-choice experiment of this study.

It is evident that the pheromone differences explain Paterson et al. (2016) findings and the behavioural observation mating patterns in Chapter 2 of the study. These pheromones differences could be key in enabling one species to recognise its conspecific, and possibly enabled *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ to mate with their conspecific only in the bi-choice experiments of this study. It is also possible that these differences in pheromones could be prominent in the field and not allow *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{male}$ to mate with each other as evidently seen in the multi-choice experiments of this study. Perhaps there is a chemical compound that restricts and enables *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ to only mate with their conspecifics, while restricting *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ from mating with each other. These chemical compounds will therefore have an indirect effect in maintaining reproductive isolation and speciation in these *Eccritotarsus* species. They will also have an indirect effect in maintaining the genetic makeup of these two *Eccritotarsus* species unchanged. There will be very few cross interbreeding incidences involving *E. catarinensis* and *E. eichhorniae* resulting in little or no genetic sharing. These insects will either have to be introduced singularly or in combination, depending on the climatic needs of the region. Interaction studies will have to be done to assess whether one species will affect the fitness of the other species positively, negatively or neutrally. It is possible to share sex pheromones and have one or two distinct chemical compounds, so it is worth mentioning that sharing some of the chemical compounds may have made it possible for these two *Eccritotarsus* species to be able to mate with each other in no-choice experiments of this study, even though *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ demonstrated that these insects exhibited female choice in bi-choice experiments, and may not necessarily mate with each other as demonstrated by the *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair in multi-choice experiments. These findings have implications for biological control of water hyacinth, because these insects will not interbreed with each other, and as a result, their genes will not mix and their genetic diversity and variability will not be changed. The other implication for biological control is that we are now dealing with another release of a biocontrol agent that we did not anticipate, and *E. eichhorniae* may have very different responses to the environment than *E. catarinensis*.

Future studies could give more insight to a variation caused by genetic drift occurrence due to the geographic isolation seen in these cryptic species of *Eccritotarsus*, since they could indicate a reproductive character displacement (RCD), because of chemical communication interference as suggested by Yang et al. (2015). More

pheromone communication investigations are needed to make a better distinction between these two geographically and reproductively isolated cryptic species assessed in this study, and one way in which to do this could be through antennal stimulation. Zhang and Aldrich (2008) have suggested that assessing the specificity of receptor neurons involving the behaviourally and physiologically-active chemical compounds using a single-cell recording technique could prove to be useful. Another aspect worth considering are the sensilla on the antenna, as well as the neurons and chemoreceptors that pick-up pheromone blends, as they have also been shown to play a key role in chemical communication (Teal and Tumlinson 1992). Lastly, investigating how the MSG regulates the production of pheromones between the cryptic of *Eccritotarsus* spp. could also give an indication as to what might have caused the reproductive isolation and eventual speciation caused by sexual selection mechanisms. This could prove to be key since the compartmentalized MSG produces many forms of pheromones that serve different functions and could be intertwined, making it difficult to be able to tease apart sex pheromones from other pheromones produced and released for other purposes (Zhang and Aldrich 2008).

The chemical compound composition of these cryptic species of *Eccritotarsus* spp. consists of saturated or unsaturated esters, as well as unsaturated ketoaldehyde, and this is known to be consistent throughout the Miridae (Yang et al. 2015). However, it will be interesting to assess the functional group of the chemical compounds reported here, to compare between the *E. catarinensis* and *E. eichhorniae* because a change to the functional group may cause species to react very differently to a pheromone (Schwarz et al. 1990). Schwarz et al. (1990) highlighted the importance of functional groups, to an extent that they may be a key determinant of whether one sex succeeds in attracting the opposite sex or not. Perhaps they could also give an indication of the mechanisms that resulted in speciation between these cryptic species. Testing the effects of the chemical compounds reported here should also be a priority, and this could be done singularly or in combination with other chemical compounds, following the same protocol as in other mirids already studied.

In conclusion, the chemical compositions of cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae) has shown a sex difference involving *E.catarinensis*$_{\text{female}}$ and *E.eichhorniae*$_{\text{male}}$ which could be key mechanisms that led to reproductive isolation and the eventual speciation between the two populations. The two species do share several compounds, but *E.catarinensis*$_{\text{female}}$ and *E.eichhorniae*$_{\text{male}}$ have compounds that are unique to them, but their conspecifics and the same sex of the other species lack those compounds.

CHAPTER 4: General Discussion

The biological control programme of water hyacinth started in 1962, collapsed several years later, and was resumed in 1985 (Cilliers 1991). Since then, many biological control agents have been released against this invasive aquatic weed, particularly in South Africa, yet it continues to pose problems in some sites but has also reduced the problem and resulted in complete control in others (Coetzee et al. 2011). *Eccritotarsus catarinensis* is native to Brazil while *E. eichhorniae* to native in Peru. The Brazilian population was released in South Africa in 1996 whereas the Peruvian population was released in South Africa in 2007, with the intention to increase genetic variability of the *E. catarinensis* biological control population. The two mirids were initially thought to be one species, but genetic studies showed a 5.2 haplotype sequence divergence which led to the revision of the insect (Taylor et al. 2011). Interbreeding experiments also complemented the genetic studies that established about 29 fixed differences between the two haplotypes using Inter-Simple Sequence Repeats (ISSRs), by reporting that they are reproductive incompatibility (Paterson et al. 2016), and as a result, morphological assessments were performed again and it was established that they have subtle yet consistent differences in the morphology of the metathoracic scent glands (MSG) that store pheromones as well as the antennae which would presumably respond to the pheromones produced in the scent glands (Henry 2017). As a result, for the first time ever, the behavioural observations and chemical compound compositions of the two species were assessed. In this study, the role of behavioural and chemical traits in the speciation and maintenance of reproductive isolation was investigated.

4.1 Summary and synthesis of findings

Interbreeding experiments, under choice and no-choice conditions, confirmed the status of these cryptic species and indicated that selective mating within each species plays a role in maintaining the reproductive isolation between the two species. We have established that if these two species were to be found co-existing in the field, it is possible that there will be no copula incidences between *E. catarinensis* females and *E. eichhorniae* males. No-choice experiments highlighted that *E. eichhorniae* have more copula incidences (Chapter2) and that could suggest that there is a greater mutual attraction between the two sexes in *E. eichhorniae*, which stimulates copulation more strongly than in *E. catarinensis*. Perhaps this could also suggest that *E. eichhorniae* has a better mate recognition system or prezygotic reproductive isolation mechanisms than *E. catarinensis*. This could be further linked with genetic diversity and variability between these two species (Taylor et al. 2011). The *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair were quicker to mate than their counterpart in no-choice experiments, suggesting that they have a better mate recognition system but have low mating frequency or performance. Mating for a very long time is possibly a way in which males use to ensure they have deposited enough sperm.

4.2 Implications of findings in biological control in South Africa

The other notable result was that the *E. catarinensis* pair tend to mate for a very long time. Copula durations have been shown to have a positive correlation with sperm competition mechanisms that exists between males, and it is believed to be a way in which males minimise their intra-sexual and inter-sexual competitions (Franco et al. 2011). It is therefore worth assessing the male sperm count of these species to have a better understanding of the relatively long copula duration seen. The *E. catarinensis* is likely to be found mating for a very long time in the field and that could potentially result in little time spent controlling water hyacinth, thereby diminishing their performance in controlling the invasive weed. This might mean that *E. catarinensis* might not be a better control agent, even though it offers a better chance in coping with cold winter temperatures (Ismail and Brooks 2016). The *E. eichhorniae* that has greater fecundity may be a better agent, especially now that we know that have they have higher single and multiple copula incidences than other crosses. Together, Paterson et al. (2016) and the findings involving bi and multi-choice experiments of this study suggest that there is a presence of a prezygotic reproductive isolation mechanism involved that are maintaining the reproductive isolation in these *Eccritotarsus* species.

Franco et al. (2011) discuss the importance of a copula duration, and how males maximise the transfer of sperm. It is possible that males from both *Eccritotarsus* species maximise the transfer of sperm. However, it appears that the males of the two *E. catarinensis* (Carvalho) and *E. eichhorniae* (Henry) species do this in different settings. The *E. catarinensis* pair mated for a long period of time in multi-choice experiments that had 3:1 sex ratio whereas the *E. eichhorniae* pair mated for a long period of time in no-choice experiments that had 1:1 sex ratio. This gives one the impression that *E. catarinensis* pair are more responsive to competition whereas the *E. eichhorniae* pair are not responsive at all. This then suggests that if the two species were to be found co-existing in the field, the *E. catarinensis* pair would easily identify each other and mate for a very long period, to ensure that their female conspecific do not see the need to mate again. It would also shorten the possibility of another male that belongs to the same or another species from interbreeding with the mated female conspecific. It is possible that the male of another species would either lose an interest to mate with that particular female or perhaps that particular female would disregard any other further copula incidences since that particular female may have enough sperms in the spermatheca. It is worth determining whether the *Eccritotarsus* species are monogamous/polygamous and whether these two species might displace each other out there in the field. *E. catarinensis* needs to mate for longer when in the presence of others. Males are holding on to ensure that only they pass on their genetic material. This has massive implications for biological control because the two species will not interbreed, and therefore not mix any genetic information with each other. This then also means that two species will not increasing genetic diversity and variability and instead might place the two species in competition with one another.

Short copula duration between some individuals could suggest the presence of reproductive incompatibility. For example, the *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ pair mated for a short period in multi-choice experiments whereas the *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair mated for a short period in bi-choice experiments. This suggests that in the field, these respective crosses will not do so well with regards to copula duration, although the females are known to end copula incidences prematurely. Perhaps these short copula durations are ideal for *E. eichhorniae* pair and are responsible for them having a higher fecundity than *E. catarinensis*. The lack of interbreeding between the two species could be a negative thing for biological control. Causes of sexual selection theory become important in understanding mating system, mate selection and female choices (Emlen and Oring 1977), so understanding why only sexual selection has played a role in *Eccritotarsus* species would be important.

Multiple copula incidences could also be a way to minimise the desire of mating with another male, whether it is of the same species or a different one. Minimising the desire of a female to mate with another male of the other species, if successful could limit the genetic diversity and variability of these insects (Taylor et al. 2011). Maintaining their genetic diversity and variability ensures that they can easily recognise each other out there in the field, and will reduce the fitness costs since little energy will have to be invested in mate recognition. This could allow the insects to focus on growth and development, as well as reproduction. However, the desire to mate multiple times when there is no competition nearby needs to be assessed thoroughly.

In some species, females have been shown to mate with multiple partners and this has biological implications, both at population and molecular levels. Both *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{female}$ did not mate with multiple partners and this suggests that they will remain separate and not mix (Birkhead and Pizzari 2002). However, in field conditions, it is possible that *E.catarinensis*$_{female}$ and *E.eichhorniae*$_{female}$ might have conspecific multiple copulations since it recorded multiple copula incidences, and so it remains important to understand why it is advantageous for females to have several copulation partners or encounters, especially when that is often associated with rapid molecular evolution, high inter-sexual specialization and population divergence (Gröning and Hochkirch 2008). In males, it is widely reported that having multiple partners or encounters increases their reproductive fitness. Nevertheless, cryptic female choice are known to either result in directional or non-directional sexual selection; with directional sexual selection favouring the spread of the attractive alleles while non-directional sexual selection favours the sperm of the males with compatible genotypes irrespective of their phenotype, but how females identify the genotype of their partners from their sperm is rather unknown (Birkhead and Pizzari 2002).

Eccritotarsus eichhorniae was introduced to increase genetic variability of *E. catarinensis* in South Africa. However, that is not possible when the two species fail to interbreed with each other. *E. catarinensis* sub-populations formed in the country all resulted from a single gravid female that survived a massive bottleneck in Quarantine. Having said that, it is possible that *E. catarinensis* may be found in cooler areas or regions with high altitudes in the country since it is native to Brazil, a subtropical region; whereas *E. eichhorniae* may be found in warmer areas or regions with low altitudes in the country since it is native to Peru, a tropical region (Ismail and Brooks 2016). It is then possible that *E. catarinensis* may cope better with cold winter temperatures than *E. eichhorniae*. It is also possible that *E. eichhorniae* might only flourish in summer months whereas *E. catarinensis* might flourish in both summer and winter months. It is also possible that the two species may not be found co-habiting and therefore it may not be possible for them to interact in the field. This would then also eliminate the need for one species to compete or displace the other. This could mean there will be no synergy or interaction between the two species out in the field. This could have massive implication for the control of water hyacinth in the country, because the two species will still need to be released with other biocontrol agents. But what it does mean is that in South Africa, we have introduced an additional biocontrol agent against water hyacinth, making it now 8 arthropod species against water hyacinth, more than anywhere else in the world. An additional biocontrol agent could lead to better control of water hyacinth, particularly in hotter regions, where the new biocontrol agent could possibly thrive. An additional biocontrol agent could also form a positive or negative interaction with already existing biocontrol agents against water hyacinth in South Africa, working cohesively or against each other in combating the problematic plant in many regions of the country. An additional biocontrol agent will require assessment, in an attempt to understand its biology, distribution and impacts in the country, so a lot of effort will have to go into it. Additional, host-specificity tests will have to be conducted to ensure that the new biocontrol agent does not feed on non-target plants, and that will assist in drawing up effective biological control programme for water hyacinth in South Africa.

4.3 Implications for biological control in the world

It is also possible that only one species might be useful in controlling water hyacinth in a particular region while the other is not able to do so, whereas both species might be suitable in controlling water hyacinth in other regions. Phenotypic plasticity (thermal, physiological, biochemical etc.) might come into play in this regard of enabling the insect to quickly adapt in the introduced region and be able to co-exist or outcompete the other species. Fraham (2010) highlights that some species can adapt genetically to projected future environments and others cannot. It is unlikely that the two *Eccritotarsus* species will work together in combating the effects of the problematic water hyacinth invasive aquatic plant. The interaction of the two *Eccritotarsus* species with other biocontrol agents will also need to be thoroughly assessed. Impact studies involving these two *Eccritotarsus* species are warranted.

Many biological control programmes tend to use several biocontrol agents, with the assumption that they will co-exist and work together in combating the problematic weed. Such programmes assume that several biocontrol agents will yield better results than using only one biocontrol agent. It is then important to assess the interaction between the two *Eccritotarsus* species. Weyl and Hill (2012) discuss how interactions involving two or more different species of herbivorous insects are key population abundance and dynamics regulators, and how they have been demonstrated to be spatial segregation and distribution drivers. They further highlight the importance of fully understanding the ecology of the insects by using insect-insect interaction as an important aspect, and how that can have massive implications in determining the success or failure of biological control programmes. Therefore, it is highly possible to come across a negative relationship involving the two *Eccritotarsus* species in that when one species feeds on water hyacinth, the other species might not find the plant suitable for it, and that may then reduce the fitness and performance of that species. Alternatively, co-habiting in one water hyacinth plant may lead to a positive relationship by enhancing the fitness and performance of both or either species, or having no effect on either species. Hoffmann and Moran (1998) argue that the success of any biological control programme is merely a quantifiable impact or damage caused by an agent or agents and that the number of biological control agents against the problematic weed is often not so important. The post-release assessments of *E. catarinensis* and *E. eichhorniae* species then becomes a necessity, to determine their prevalence and effectiveness against the weed, because it is possible that countries may have accepted or rejected a wrong species. Reassessment *Eccritotarsus* species will ensure that appropriate tests were conducted.

The implications of using the two *Eccritotarsus* species lies in the uncertainty of knowing whether one is releasing *E. catarinensis* or *E. eichhorniae*, wherever there is a need to control water hyacinth. Separating these two species has uncovered a new diversity with the genus *Eccritotarsus*, but they remain morphologically very similar to each other and almost indistinguishable with the naked eye. This study is the first to assess the behavioural mating cues of these two species and is very much a continuation from Taylor et al. (2011) and Paterson et al. (2016) studies. The study is important because it supports Henry (2017) who denotes *Eccritotarsus* as separate species despite being morphologically identical, because there are subtle differences in the metathoracic scent gland and antennae, which may have resulted in the pheromonal differences that have been discussed above. The study also provides the first assessment of what is likely to happen in the field if the two species were to be found co-existing in the same region in the form of multi-choice interbreeding experiments. One of the ways that one can discover cryptic species is through mate calling behaviour (Aldrich et al. 1991; Dickens et al. 1991; Teal and Tumlinson 1992; Groot et al. 1998).

It is possible that speciation governed by reproductive isolation, that was sorely driven by the sexual selection theory in *Eccritotarsus* was recent and rapid, but was only limited to sexual selection as the two species are morphologically very similar (Wiens et al. 2010). This study demonstrates that speciation can occur almost certainly due to genetic drift, and that in speciation there could be no other changes except those that have implications for reproduction. Sexual selection seems to play a strong role in speciation and reproductive isolation, because only changes that are associated with mating changed in the species (Henry 2017). This finding indicates that prezygotic reproductive isolation mechanisms will ensure that the two species do not interbreed and potentially lead to another diversification, especially now that we certainly know that *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ copulation does not yield to any offspring.

4.4 Implications of biological control in general

Collecting populations of what appears as the same species is risky, because we are potentially collecting an unknown species, and there are always risks involved with the introduction of additional control agents. The study has demonstrated that the presence of copula incidences with conspecifics, and subsequently what may be responsible for consequent reproductive isolation barriers, has a strong chemical compound composition link to it as the possible driving force behind it. In essence, it has become apparent that pheromones differences have also played a key role in speciation and reproductive isolation in *Eccritotarsus*. Furthermore, the structural relatedness of the chemical compounds involving *E.eichhorniae*$_{female}$ and *E.catarinensis*$_{male}$ inferred from average peaks (mins.) using multi-dimensional scaling and principal components ordination may further suggest a common biochemical pathway emission for *E. catarinensis* (Carvalho) and *E. eichhorniae* (Henry) pheromones, as it suggests that they have similar chemical compound compositions. With perhaps one or just a few genes likely to be responsible for the chemical compound composition differences seen in Chapter 3 that control speciation and reproductive isolation in *Eccritotarsus*, rapid speciation in allopatry seems plausible for this species (Gandhi and Herms 2010). It has become apparent that the chemical compound composition chapter ties in nicely with the behavioural observation chapter. The question now remains, why does the *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair mate more than their counterpart when there will be no offspring produced at the end of it all? The following question from that would be, does that have any fitness costs? The fitness cost possibility needs to be evaluated.

Chemical compound composition data has suggested that *E. catarinensis* (Carvalho) and *E. eichhorniae* (Henry) have similar signals, suggesting that out there in the field, it will be difficult to avoid the heterospecific sexual interactions. Additionally, it is possible that the occurrence of cross copulation (as few as they may have been) can be explained by what is termed signal jamming, an incident whereby conspecific signals are significantly reduced or interrupted by the presence of heterospecific signals, thereby resulting in decreased mating success as evidently

seen in *E.catarinensis*$_{female}$×*E.eichhorniae*$_{male}$ and *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ crosses in all interbreeding experiments, particularly in the multi-choice interbreeding experiments. Signal jamming, a phenomenon where the transmission, quality or detection of signal is altered by other signal sources, complicating mate recognition by confusing individuals in their search for mates and this may result in fitness costs of their own (Gröning and Hochkirch 2008). In *Eccritotarsus* spp. it resulted in the mistaken interspecies events, and possibly the hybrids. It is also possible that the two species may have gone through what is known as heterospecific rivalry, an incident whereby heterospecifics are mistaken for conspecific individuals of the same sex (usually males) rather than mates (Mallet 2005). The fact that there was a female pheromone from one species in the male of the other supports this claim. If either species is territorial species between *E. catarinensis* and *E. eichhorniae,* it is likely to chase the heterospecifics out of their territory but heterospecific rivalry is closely associated with resource competition, and because *Eccritotarsus* species utilise the same resource, they may compete for space. Such incidents may inevitably lead to fitness costs and might limit the success of biological control programme of water hyacinth in many regions. Gröning and Hochkirch (2008) further claim that weak premating barriers result in low heterospecific copula incidences as evidently seen in *E. catarinensis* and *E. eichhorniae*, and these weak premating barriers are allowing the interspecific mating to take place and have rather resulted in heterospecific copula incidences that could potentially have fitness costs involved. Many authors believe that the fitness costs of heterospecific copula incidences are particularly more pronounced if postmating barriers have fully evolved because that often leads to the identification of the presence of unviable offspring, in what may be termed hybrid infertility. It is rather surprising that hybrid infertility is not given much attention and assessing it in *Eccritotarsus* spp. is warranted.

Sexual selection could be linked with fitness costs and potentially have ecological and evolutionary consequences, but its potential in determining species coexistence has often been neglected. Gröning & Hochkirch (2008) believe that similarly to competition, reproductive isolation due to sexual selection has the potential to lead to displacement of one species by another species through sexual exclusion/displacement, changes in life history parameters, and reproductive character displacement. Therefore, future studies should aim to look into ecological mechanisms that could potentially assist sexually interacting species to co-exist, because sexual selection has dire consequences for the management of many species and might be helpful in providing new insights when it comes to reproductive isolation and speciation for living organisms, particularly in insects (Gröning and Hochkirch 2008). Some authors have argued that predation, mutualism, and competition have been thoroughly investigated while sexual interactions between several species in the form of reproductive interference receives little attention, even though the interaction between species remain key determinants of community compositions (Mallet 2005), and have been addressed in speciation and evolutionary studies.

4.5 Sexual attraction in Miridae

Misdirected or unintended courtship, in other words, an incident where one sex occasionally mates with the opposite sex of another species has been demonstrated by these two *Eccritotarsus* species, and that is also known to result in fitness costs. This is because heterospecifics that resemble high-quality conspecifics based on visual cues may appear as attractive mates. But they are clearly not all that attractive in this system. They do not like mating with each other, hence the copula incidences were few. Fitness cost are worsened when the two species are reproductively incompatible, especially when some males might invest into mating encounters even when females may not be interested. It is possible that *E.eichhorniae*$_{female}$×*E.catarinensis*$_{male}$ pair that does not produce any offspring might be explained by the fact that there is no sperm transfer when this pair attempts to copulate, and this has been shown to be the case in many insects. The males suffer fitness costs in terms of energy invested in copulation while females suffer in terms of attempting to reject the males. The *Eccritotarsus* spp. may be subjected to these fitness costs, and this may have dire consequences for the control of water hyacinth in many regions because it could reduce the fitness of both species at sites where they are both present (Mallet 2005).

Species-specific signals such as chemical compounds play a key role as premating barriers and they may determine the frequency of conspecific encounters (Fisher et al. 2006), this might explain the low copula incidences between species in all interbreeding experiments. This pattern is likely to be exacerbated in the field. This is because findings suggest that in some environments, the localization, detection and identification of conspecific signals may be hindered by abiotic noise, and animals may be forced to change the signal or switch communication channels (Gröning and Hochkirch 2008). Authors argue that calls of several species may be so identical to such an extent that they either mask the perception of conspecific songs or cause individuals to mistake heterospecifics for mates, and that is possibly the case in the two species of *Eccritotarsus* because males of one species also reacted to heterospecific females and successfully mated with them. In addition, it is possible that this may also impair the identification of high-quality mates, influence female or male sex pheromone emissions or production.

Insects use species-specific signals to attract mates to avoid heterospecific sexual interactions because if two or more species use too similar signals, the chances of heterospecific interactions increases (Mallet 2005). Females that are commonly known to be the choosing sex have fitness costs of their own when it comes to choosing heterospecific males (Andersson and Simmons 2006). Heterospecific sexual interactions could potentially increase metabolic rates in dominant species, and researchers have shown that the energy costs of living constitute an important component of life histories, and that the standard metabolic rate (SMR) is linked with the fitness of individuals within a population (Janča and Gvoždík 2017). At the population level, metabolic rates negatively correlate with population density, and thus competition strength, across taxa and possible mechanisms causing

that include a competition-induced shift in feeding as well as activity rates (Janča and Gvoždík 2017). As a result, the density-dependence of metabolic rates may affect the energy budget of individuals and population dynamics (Janča and Gvoždík 2017). According to the "increased intake hypothesis", a higher standard metabolic rate is customary in active individuals with relatively larger internal organs, and that such individuals should be favoured under conditions with good availability of resources (Janča and Gvoždík 2017). However, if resources are limited, more energy will have to be spent on maintenance (i.e. up to 50% of total energy budget), and that reduces the energy that can be invested into somatic growth, reproduction, and survival (Janča and Gvoždík 2017). Assessing the SMR in these two species of *Eccritotarsus* is warranted.

4.6 Future work, predictions and implications of *Eccritotarsus* species

Current studies involve assessing the species distributions in South Africa, and how they interact together in the field, if they do interact at all. It is important to assess the presence or absence of synergy between the two species of *Eccritotarsus*, particularly now that they are known to be separate species, since that could have implications for the biological control of water hyacinth in South Africa. It is also important to assess whether one species displaces or outcompetes the other, or whether they work together in combating the problematic weed. It has been well documented that *E. catarinensis* has successfully established in close to 20 out of a possible 33 sites where it has been released (Coetzee et al. 2007b), but now one needs to confirm whether it is *E. catarinensis* or *E. eichhorniae*, or both. The next step would then be to try and understand why a species is present or absent, singularly and/or in combination.

Assessing the genetic and phenotypic relationship between male traits that are favoured under pre-insemination and post-insemination in sexual selection has been at the forefront of evolutionary biology and therefore also need to be assessed in the genus *Eccritotarsus*, because they are speculated to be associated with cryptic female choices. Because females have been shown to invest more than males in sexual selection, females are expected to gain from reproductive isolation, and therefore, female reproductive strategies might contribute to the promotion of speciation (Birkhead and Pizzari 2002). It is possible that *E.catarinensis*$_{\text{females}}$ and *E.eichhorniae*$_{\text{females}}$ are responsible for the reproductive isolation mechanism that had led to speciation in these two species of *Eccritotarsus*. In the majority of species females are highly selective when it comes to selecting mates and some females are congruent in their mate preference for a particular male, while in other females are incongruent in their preference, with each female preferring a different male (Neff and Pitcher 2005). This theory goes as far as suggesting that females have preference for physical traits such as body length and many other traits measured in Paterson et al. (2016), so it is possible that physical traits had a role to play in the mating assessment in *E. catarinensis* and *E. eichhorniae*. It is well documented that female multiple copula incidences evolved as a

mechanism to increase overall genetic quality of the offspring to ensure that they are able to withstand unsuitable environmental conditions. Neff and Pitcher (2005) point out that across 14 experimental studies that have been done in about 12 different species; researchers have found anywhere from no significant difference in offspring fitness between single mated and multiple mated females to about 189% increase in fitness for offspring from multiple mated females, showcasing the importance of females when it comes to mate selection that could lead to reproductive isolation mechanisms.

In conclusion, literature suggests that choosy females are able increase the genetic quality of their offspring by mating only with males that will contribute good genes or compatible genes to their offspring so that they can cope with harsh conditions. Females are sorely responsible for reproductive isolation and speciation mechanisms, in that they may have driven the change in the males. There are fitness costs associated with mate selection in general, and these are known to affect both sexes. We now know that *E. catarinensis* (Carvalho) and *E. eichhorniae* (Henry) will not interbreed and this is attributed to sexual selection. There are premating reproductive isolation mechanisms that explain the findings of this study. Only sexual selection explains the reproductive isolation that has led to speciation in these two *Eccritotarsus* species, and going forward, it will be important to fully understand why it only has reproductive implications.

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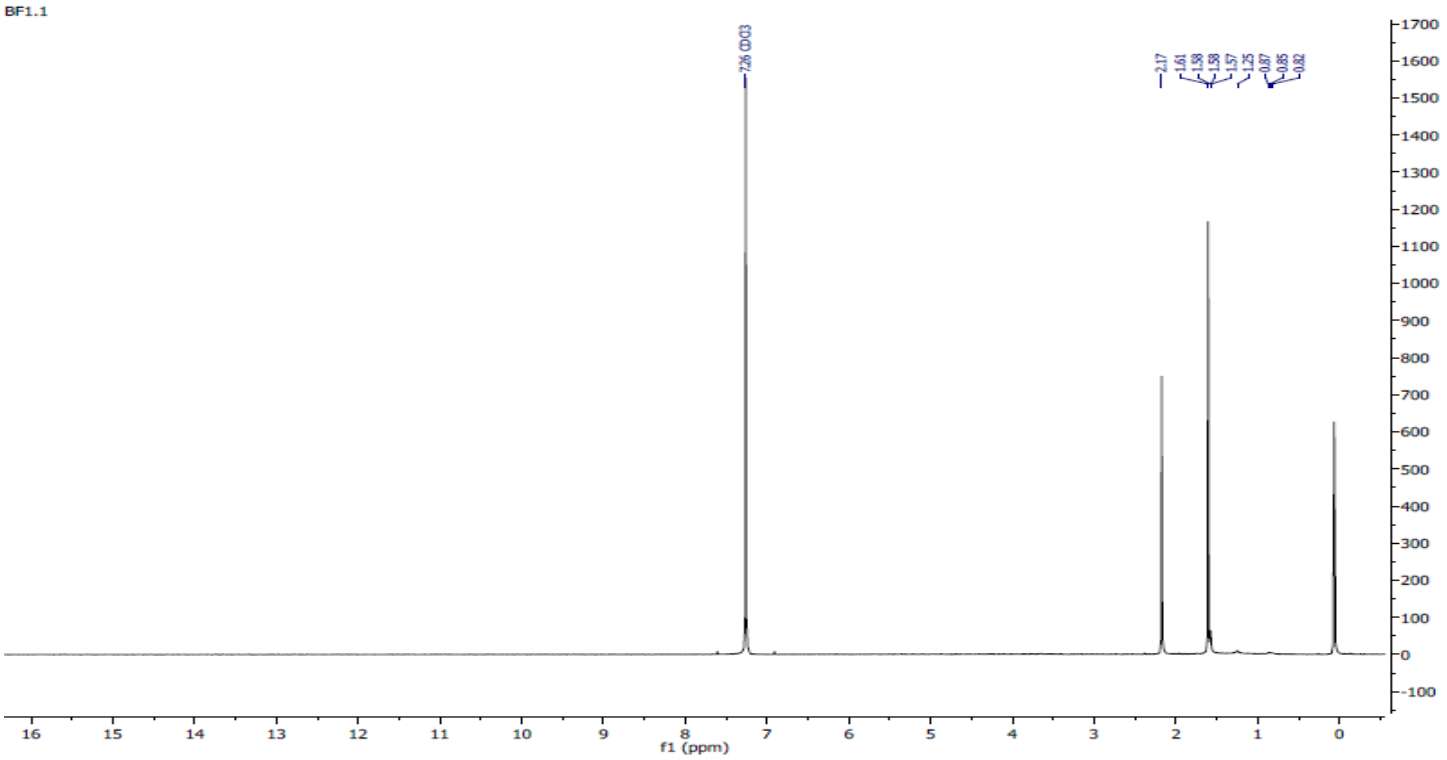
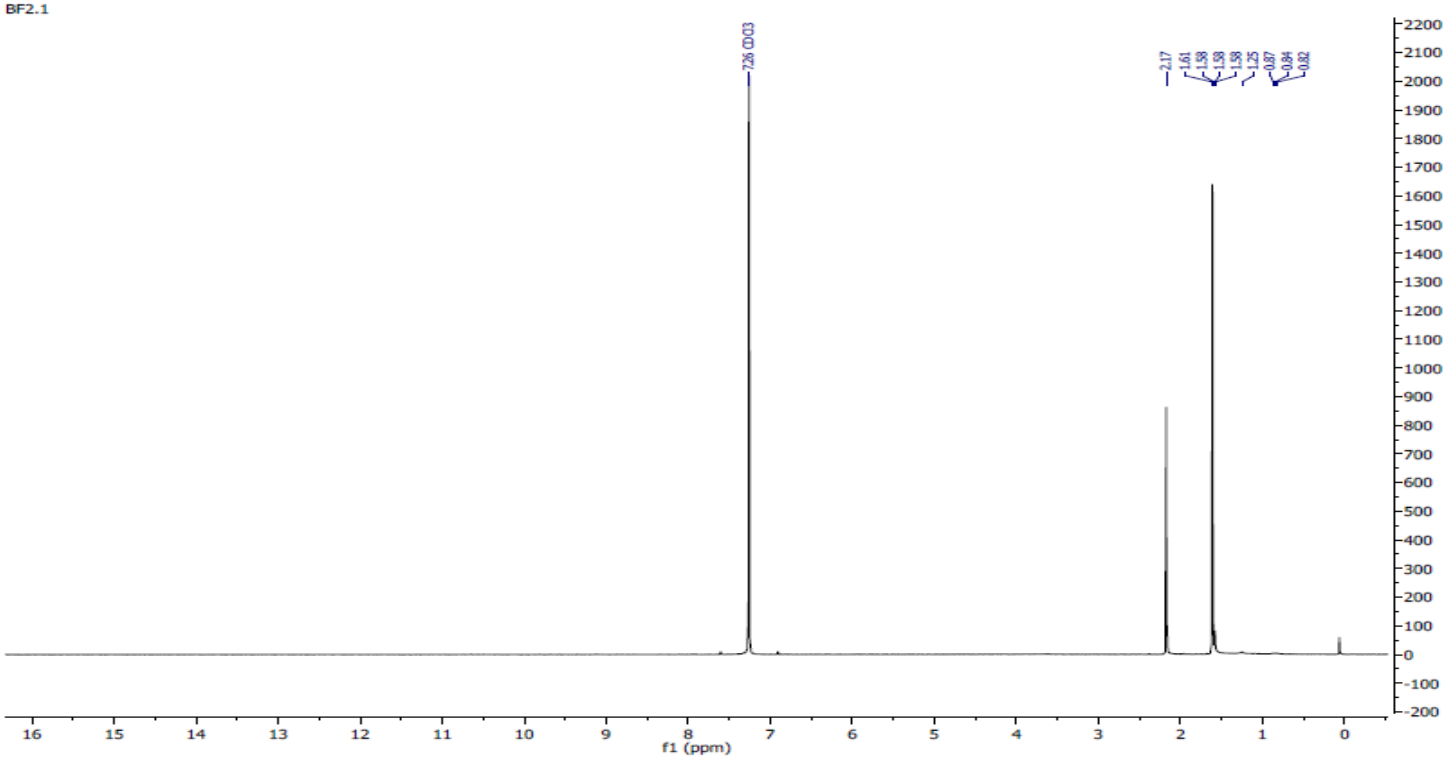
Appendix

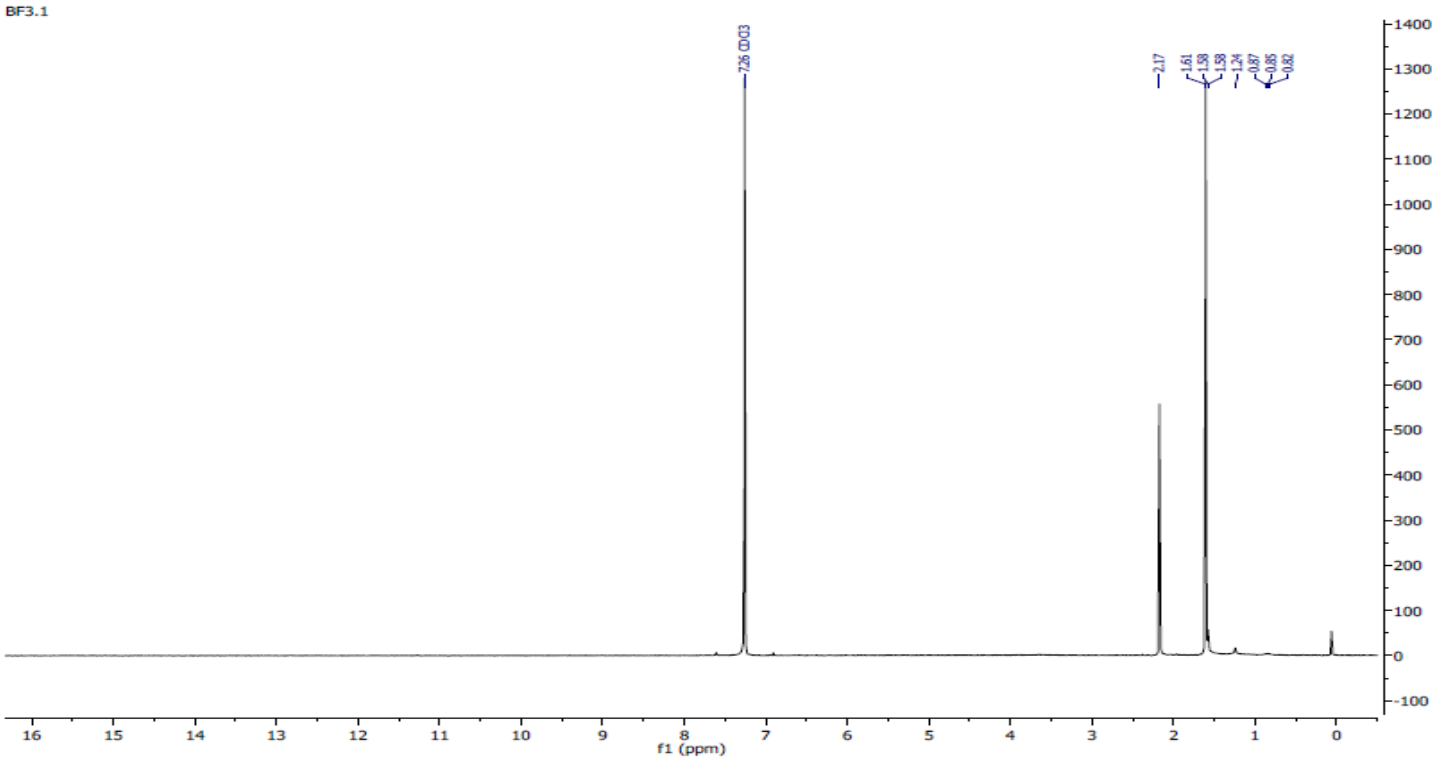
Nuclear Magnetic Resonance (NMR) spectra of the geographically and reproductively isolated cryptic species of *Eccritotarsus* spp. (Hemiptera: Miridae). The NMR spectra provided show four replicates for both sexes, and this was done for both populations. *E.catarinensis*$_{female}$ replicates are shown as: Bf1.1, Bf2.1, Bf3.1 & Bf4.1, *E.catarinensis*$_{male}$ replicates are shown as: Bm1.1, Bm2.1, Bm3.1 & Bm4.1, *E.eichhorniae*$_{female}$ are shown as: Pf1.1, Pf2.1, Pf3.1 & Pf4.1 and *E.eichhorniae*$_{male}$ are shown as: Pm1.1, Pm2.1, Pm3.1 & Pm4.1 as depicted below. The Brazilian population remains *Eccritotarsus catarinensis* while the Peruvian population is *Eccritotarsus eichhorniae*.



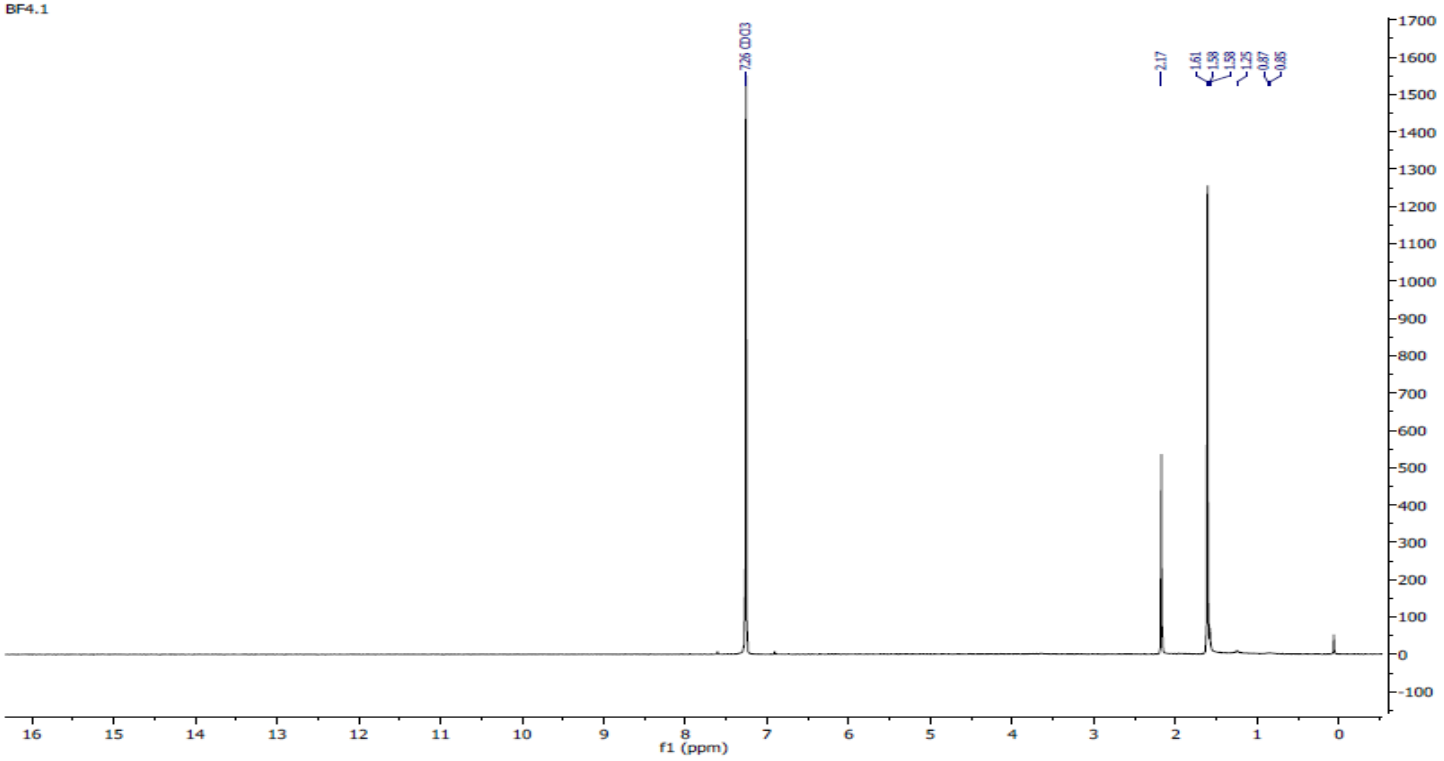
BF2.1


7.26 CDCl3
2.17
1.61
1.58
1.58
1.58
1.25
0.87
0.84
0.82
f1 (ppm)

BF3.1


7.26 CDCl3
2.17
1.61
1.58
1.58
1.24
0.87
0.85
0.82
f1 (ppm)

BF4.1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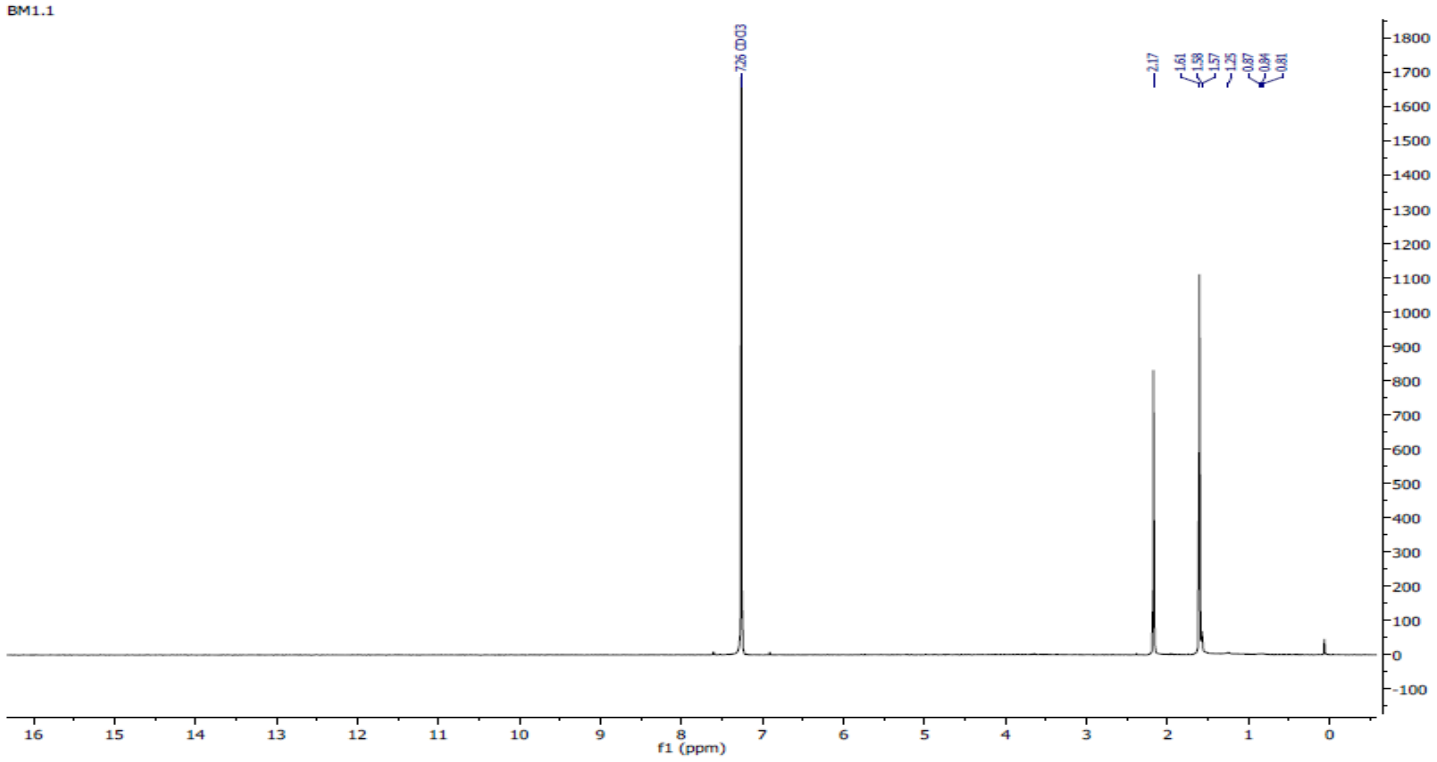
2.17
1.61
1.58
1.58
1.25
0.87
0.85
f1 (ppm)

BM1.1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1.57
1.25
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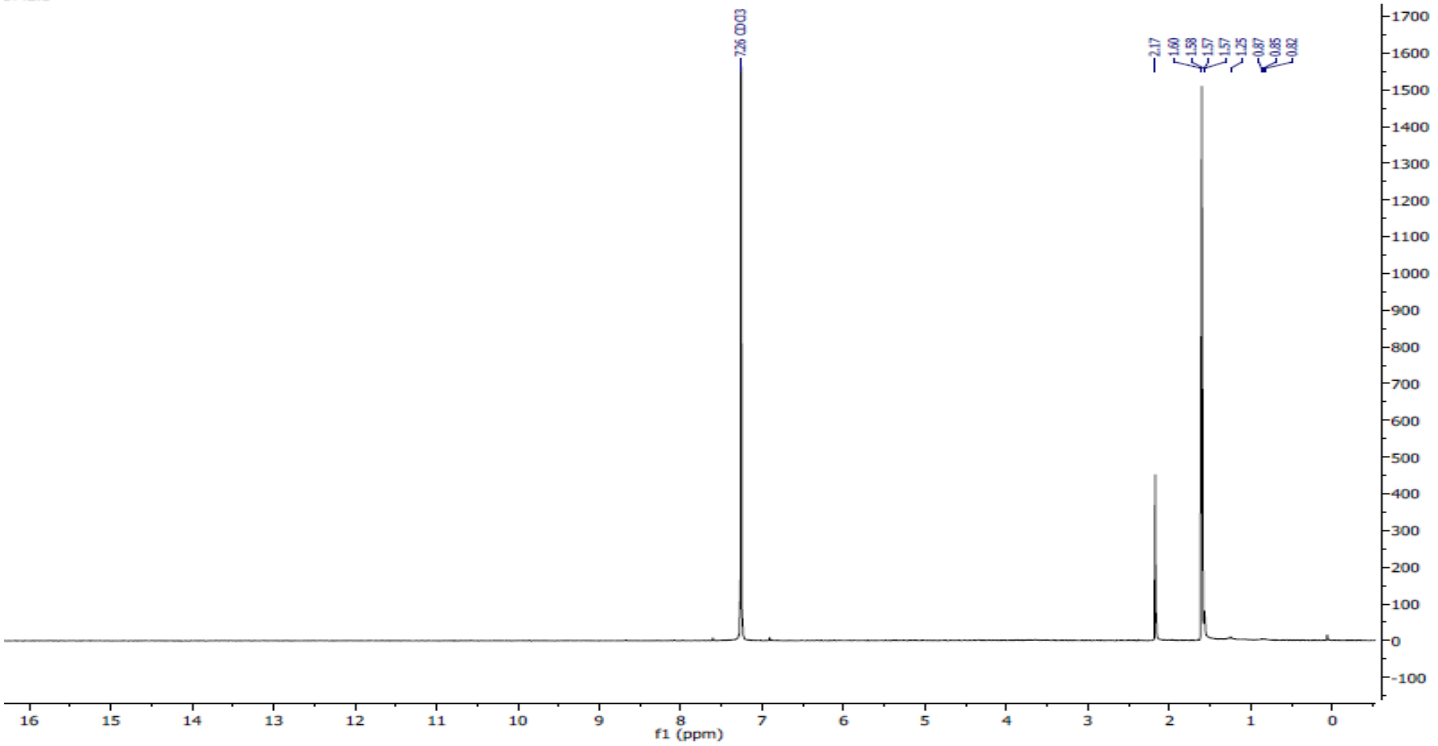
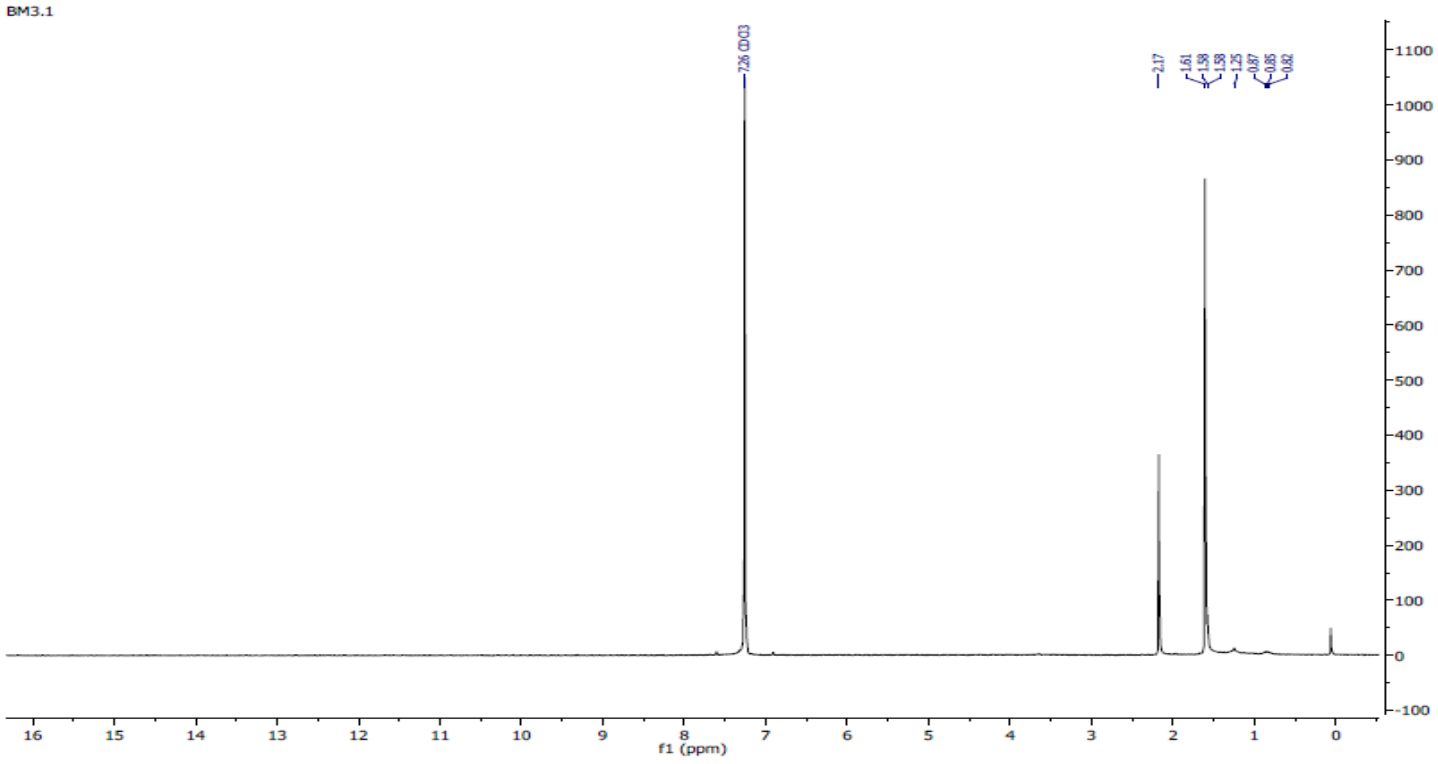
0.84
0.81
f1 (ppm)

BM2.1


7.26 CDCl3
2.17
1.60
1.58
1.57
1.57
1.25
0.87
0.85
0.82
f1 (ppm)

BM3.1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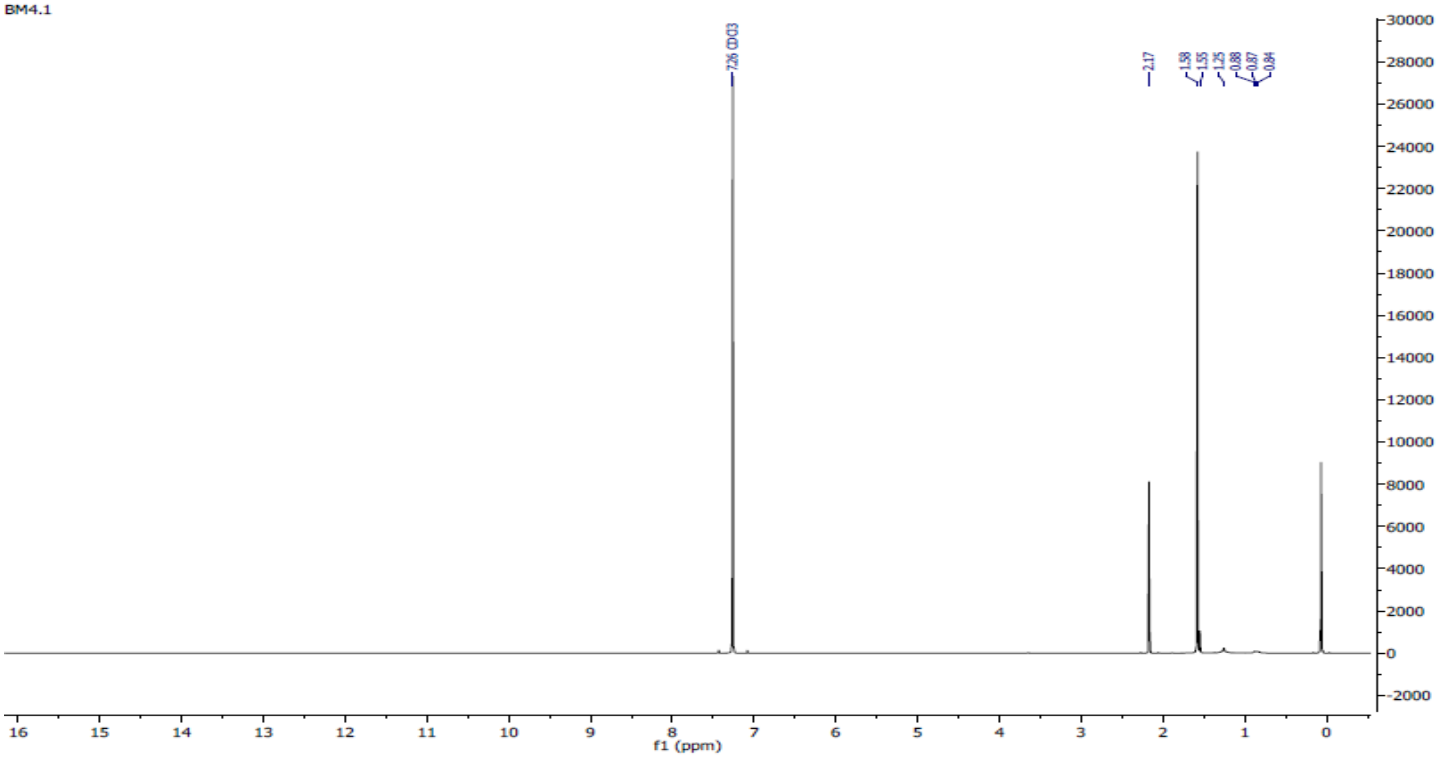
2.17
1.61
1.58
1.58
1.25
0.87
0.85
0.82
f1 (ppm)

BM4.1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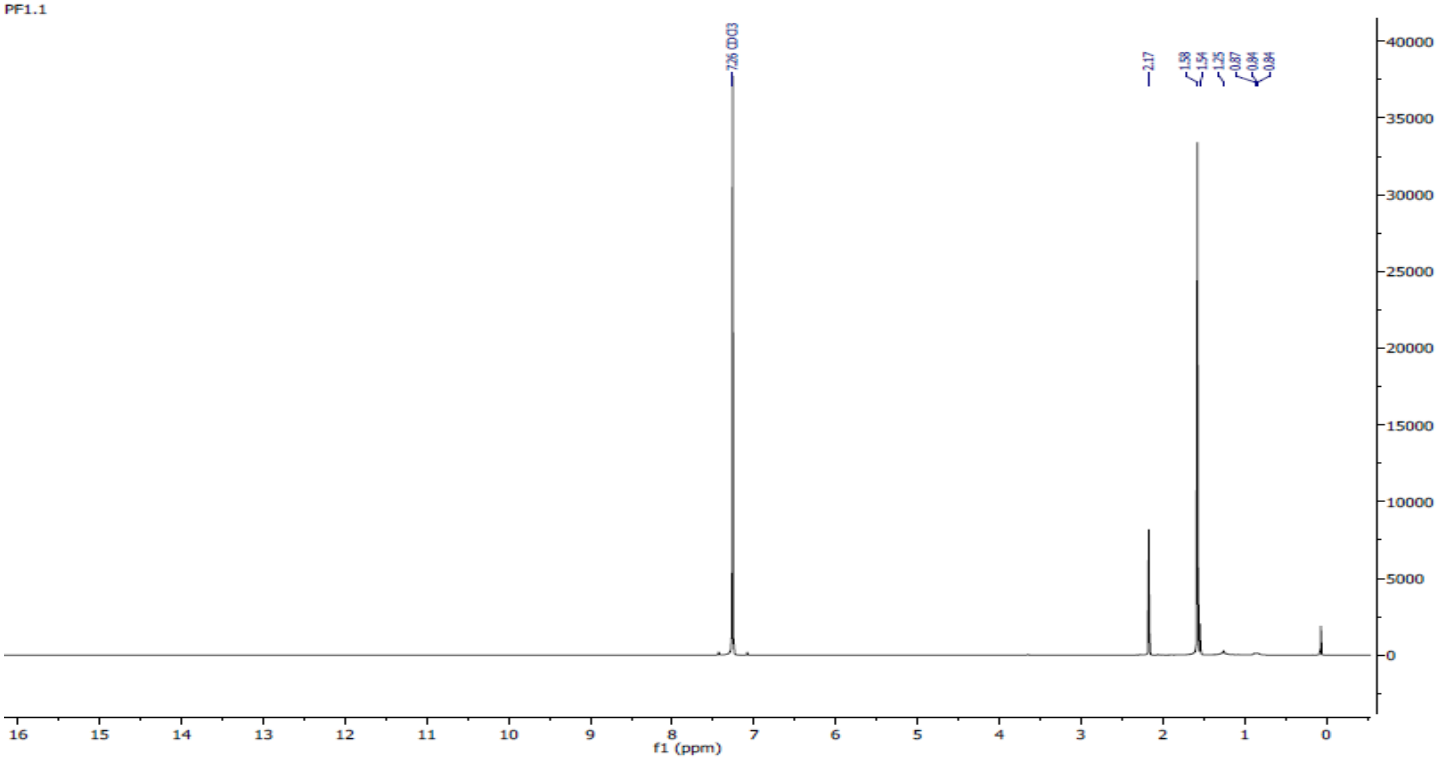
0.87
0.84
f1 (ppm)

PF1.1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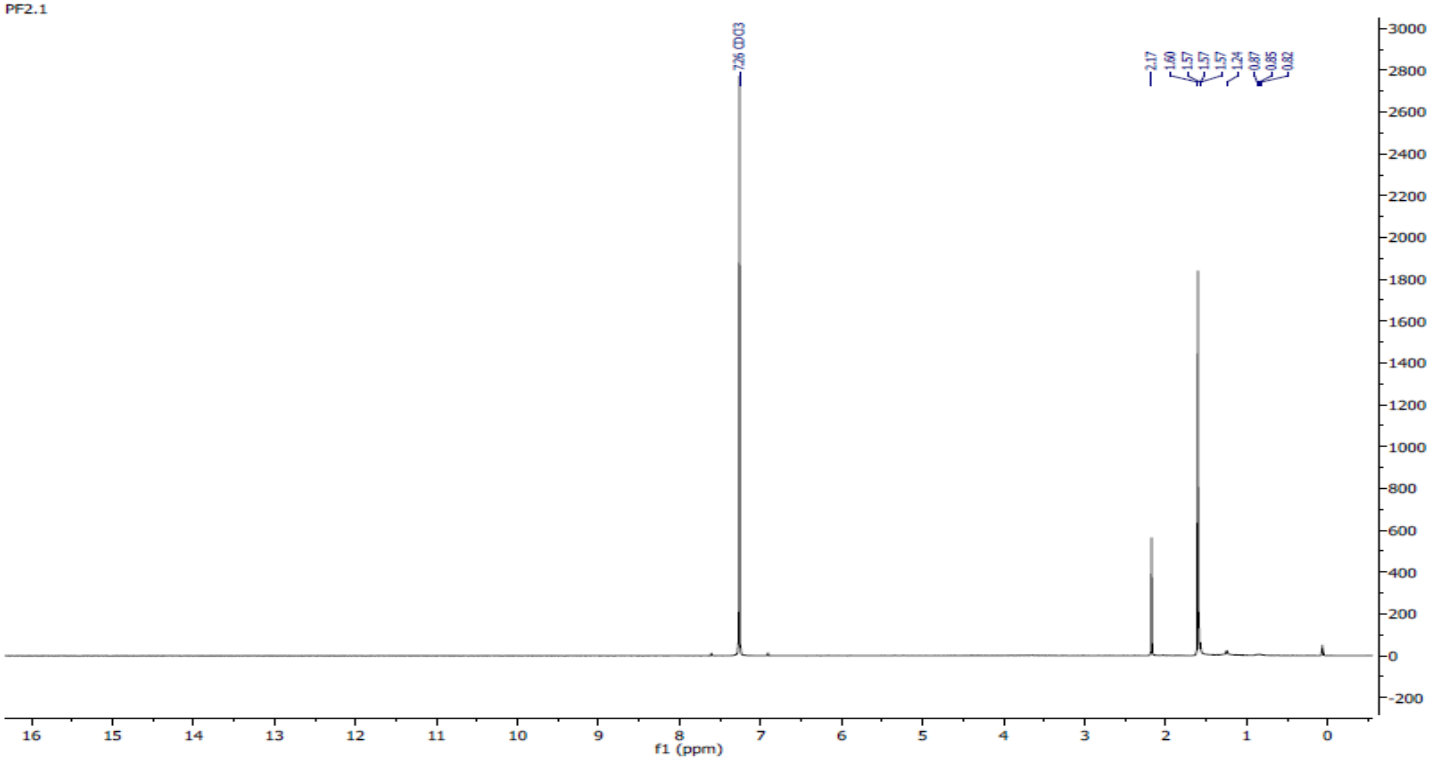
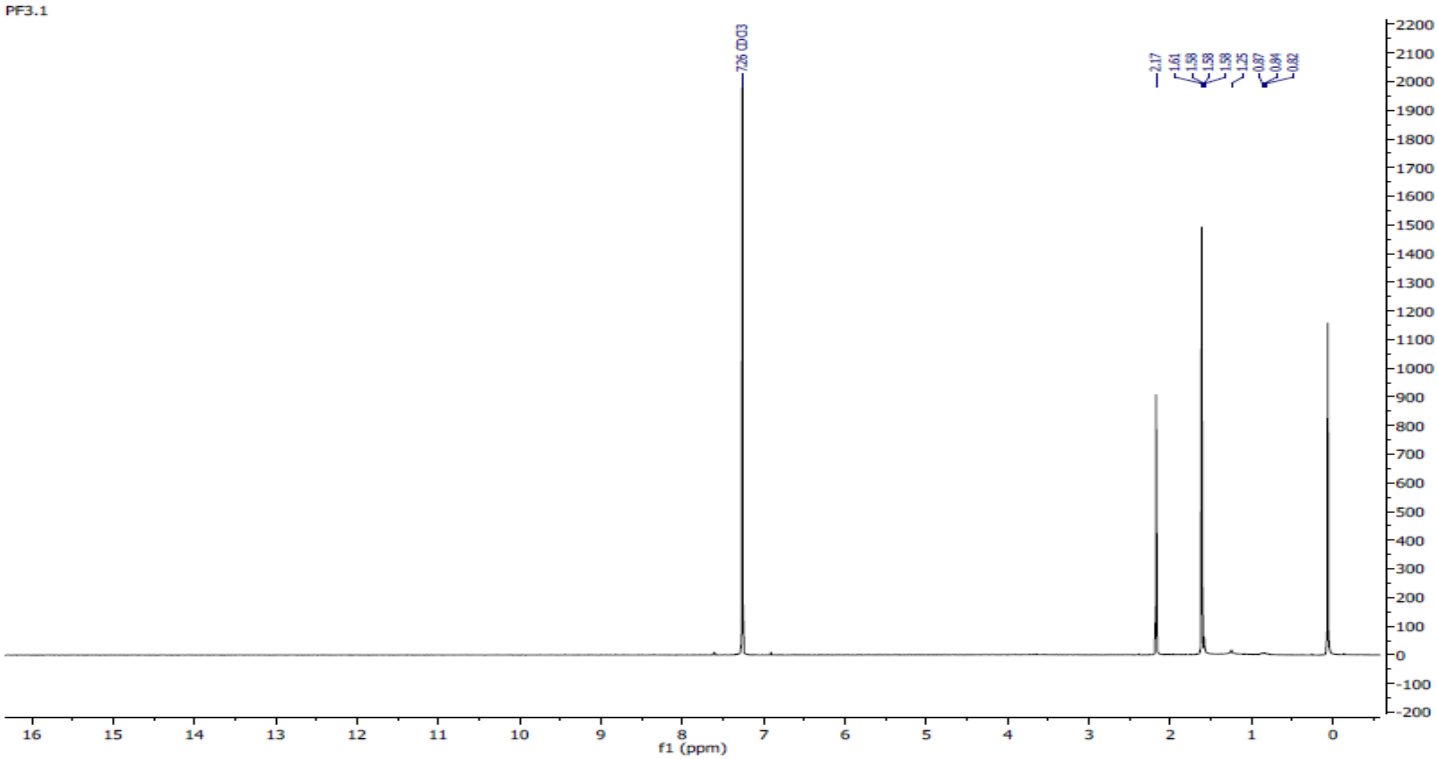
1.58
1.54
1.25
0.87
0.84
0.84
f1 (ppm)

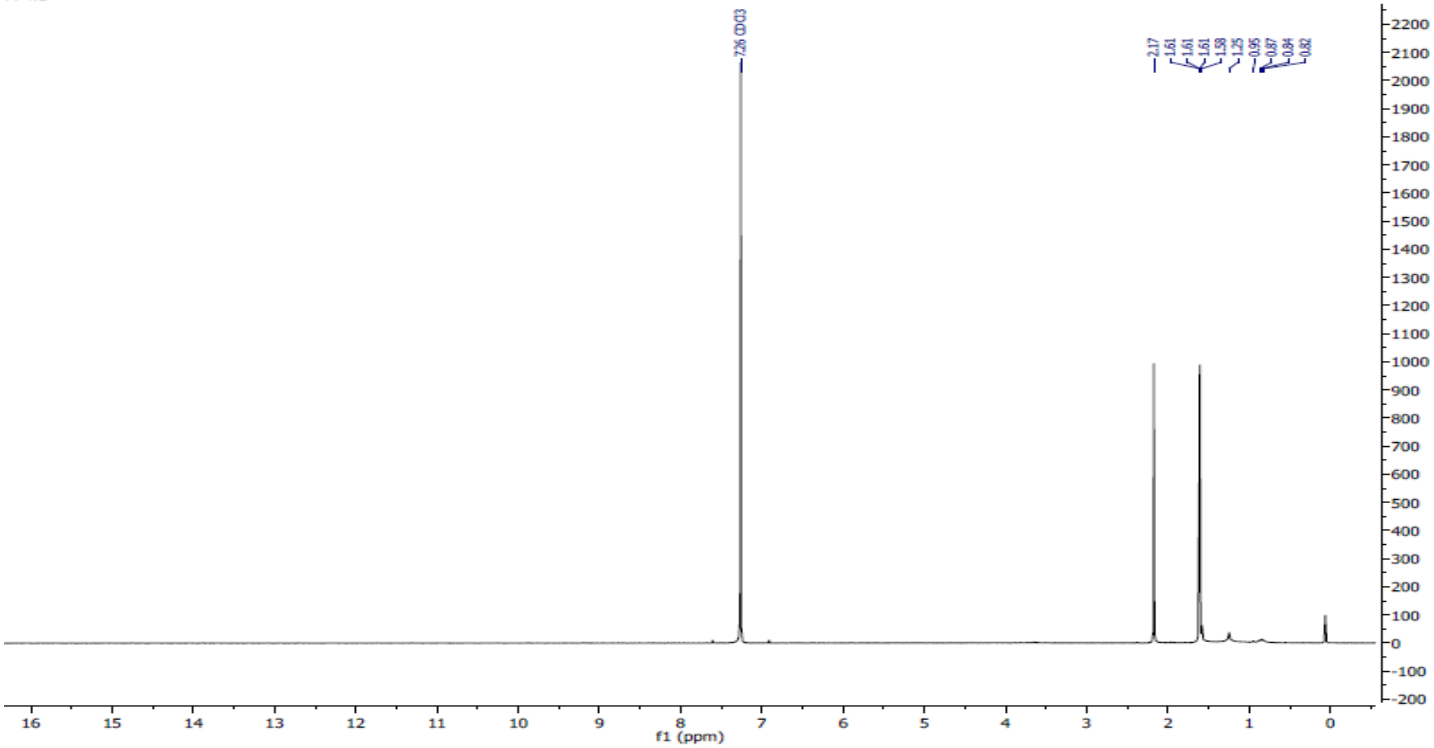
PF2.1


7.26 CDCl3
2.17
1.60
1.57
1.57
1.57
1.24
0.87
0.85
0.82
f1 (ppm)

PF3.1


7.26 CDCl3
2.17
1.61
1.58
1.58
1.58
1.25
0.87
0.84
0.82
f1 (ppm)

PF4.1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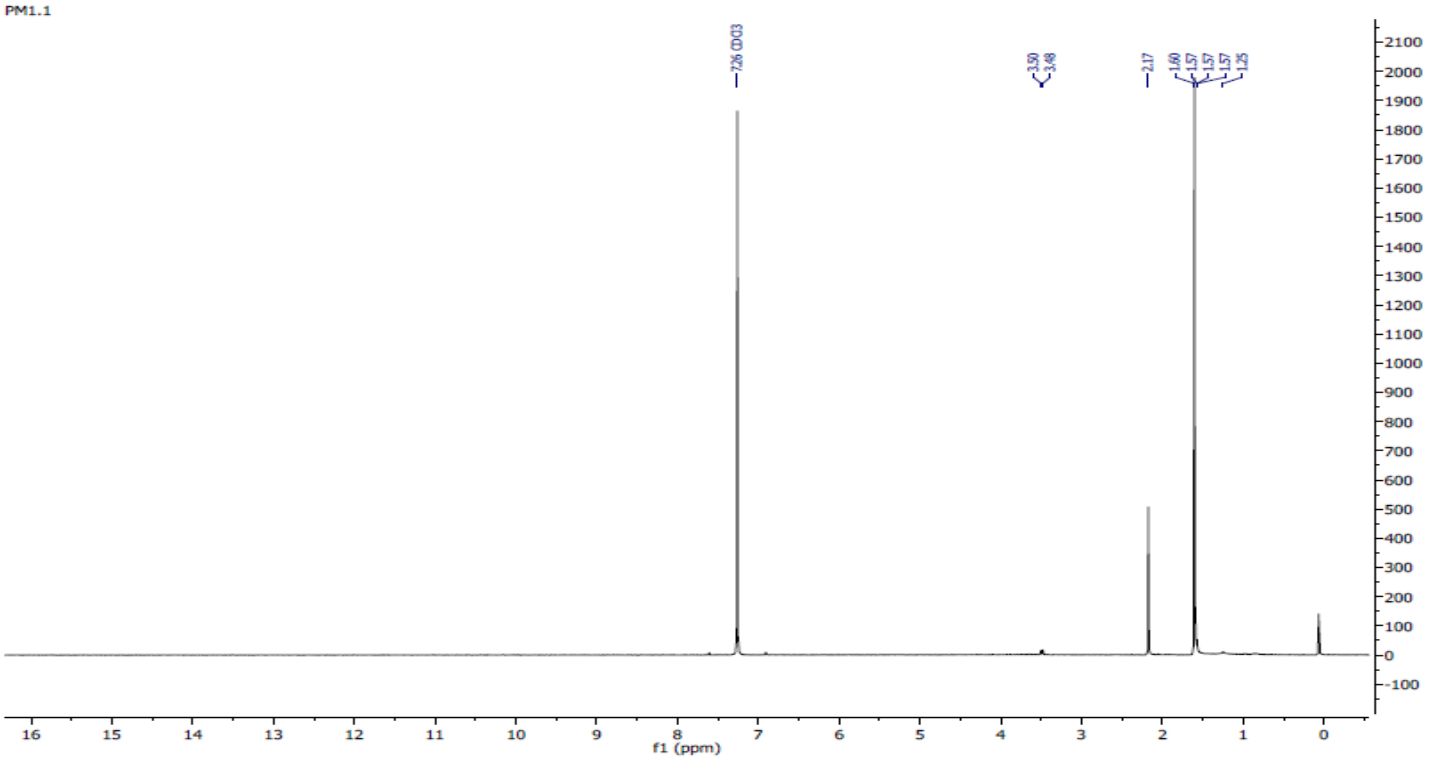
2.17
1.61
1.61
1.61
1.58
1.25
0.95
0.87
0.84
0.82
f1 (ppm)

PM1.1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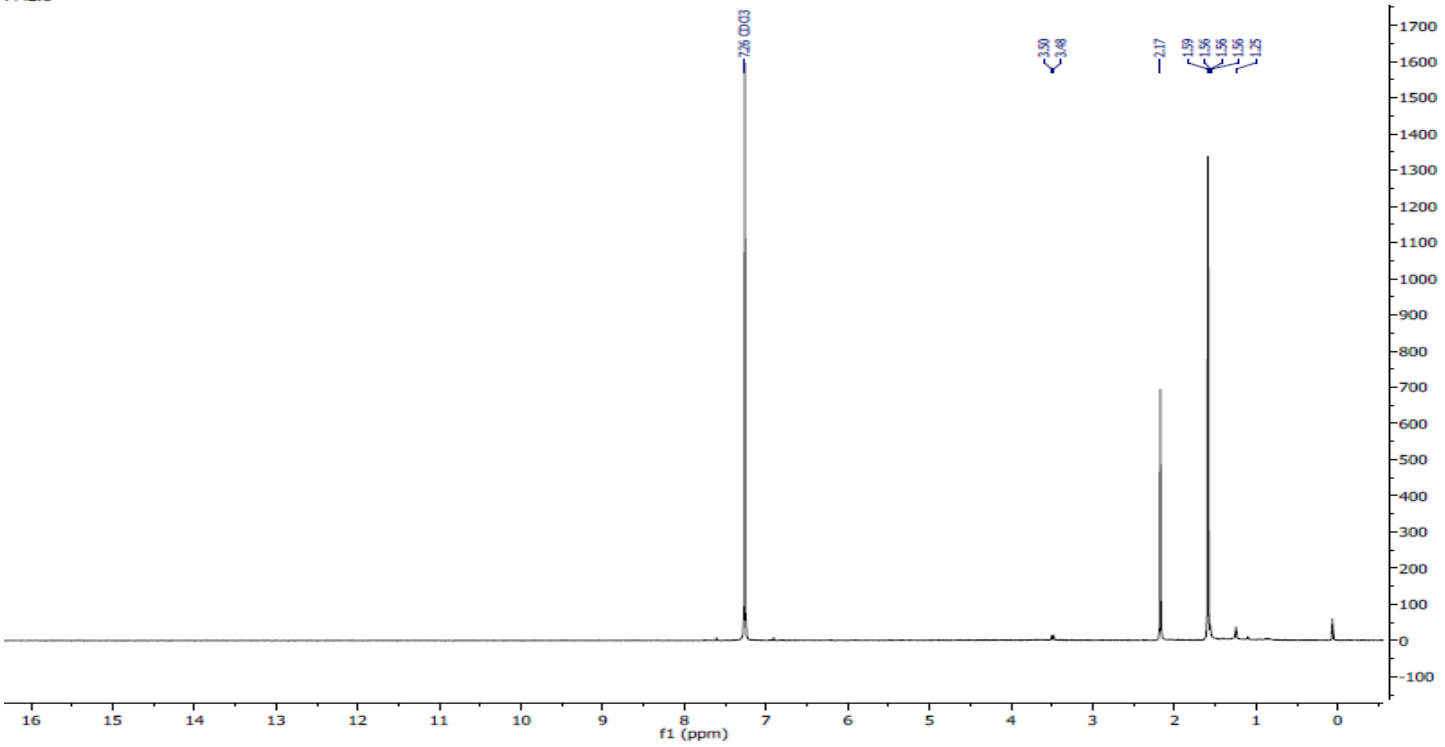
3.48
2.17
1.60
1.57
1.57
1.57
1.25
f1 (ppm)

PM2.1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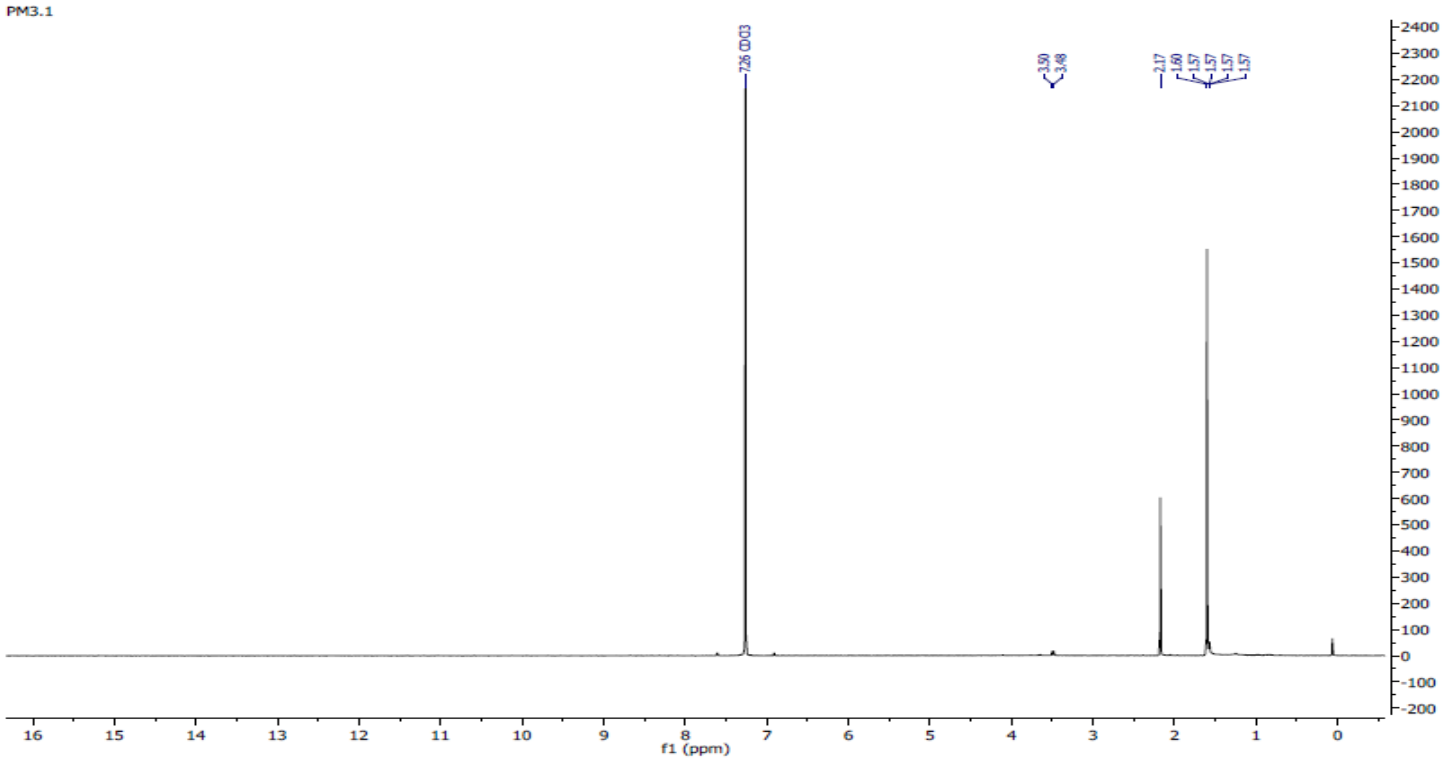
3.48
2.17
1.59
1.56
1.56
1.56
1.25
f1 (ppm)

PM3.1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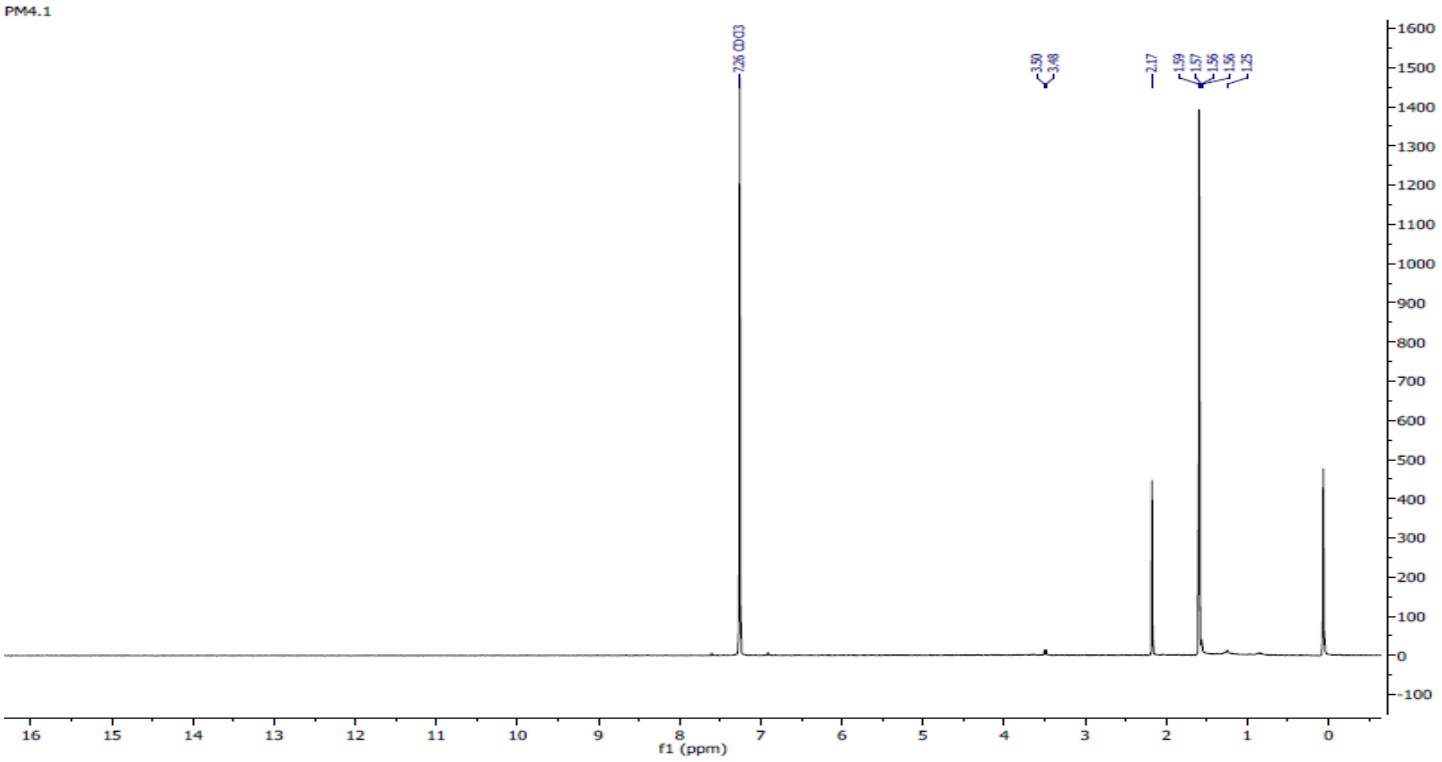
3.48
2.17
1.60
1.57
1.57
1.57
1.57
f1 (ppm)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7.26 CDCl3
3.50
3.48
2.17
1.59
1.57
1.56
1.56
1.25
f1 (ppm)