Male mate choice in the stick insect *Clitarchus hookeri*: sexual vs. parthenogenetic females

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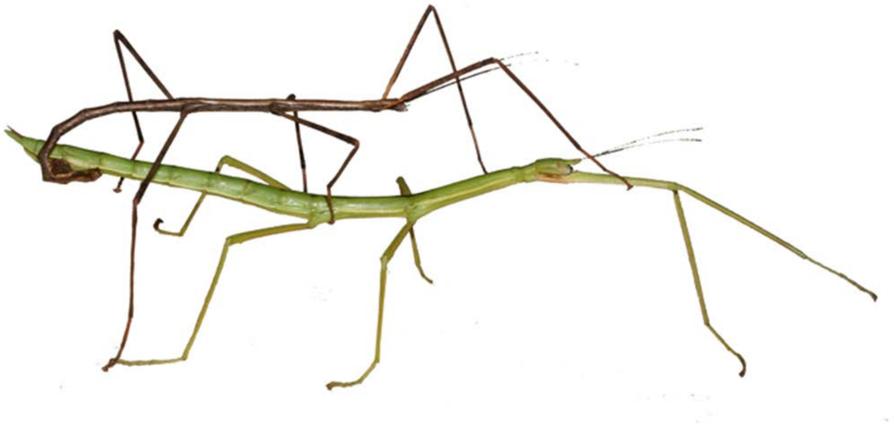

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Abstract

Learning about reproductive strategies in animals is an important step for understanding the evolution of species. New Zealand stick insect, *Clitarchus hookeri* include both sexual and parthenogenetic females, and parthenogenetic females occur in the distributional range where males are absent and have a limited capacity of sexual reproduction. Since *C. hookeri* exhibit a scramble competition mating system with distinctive sex roles where females and males co-occur, it is likely that parthenogenetic females do not exhibit traits that are related to the sex roles. Furthermore, due to limited capacity for parthenogenetic females to reproduce sexually, it is likely that *C. hookeri* males would benefit from discriminating between sexual and parthenogenetic females.

The main purpose of this thesis was to explore the unique reproductive features of *Clitarchus hookeri*. Specifically, I identified morphological and chemical traits that are likely to be under distinctive sex roles in scramble competition; revealed whether morphological and chemical traits seen in sexual females are also seen in parthenogenetic females; and observed whether males can discriminate between sexual and parthenogenetic females for their pre- and post-copulatory choices. As a result, C*. hookeri* exhibited sexual differentiation in terms of morphology and chemical signalling that are advantageous to their roles in scramble competition. However, sexual and parthenogenetic females overlapped in their phenotypic traits, and males failed to discriminate between sexual vs. parthenogenetic females both in pre- and post-copulatory choices. These results suggest the possibility of the maintenance of sexual traits in parthenogenetic females; and therefore males have failed to discriminate between females with a different reproductive mode.

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CHAPTER 1.

General Introduction

1.1. Male mate choice

Sexual selection favours the traits that maximise reproductive output of individuals. Under this concept, mate choice will develop when the individual increases its reproductive success by choosing to mate with partners that have particular qualities (Jones and Ratterman, 2009). In the traditional view of sex roles, males are indiscriminate and females are the choosy sex. This is due to greater gametic and parental investment by females, which results in lower reproductive capacity in females compared to males (Edward and Chapman, 2011, Bonduriansky, 2001, Birkhead and Pizzari, 2002). Therefore, females gain higher reproductive success from choosing to mate with high quality males due to their reproductive limitations.

On the other hand, males are considered to be the indiscriminate sex due to cheap production of gametes and general lack of parental investment (Birkhead and Pizzari, 2002). However, it is now recognized that sperm production is costly when it is produced as ejaculate, and males also become choosy when there is a high variation in female quality (e.g. fecundity and degree of sperm competition) and the benefit of choosing a female is higher than the cost of assessing (in relation to searching cost and female availability) (Bonduriansky, 2001, Edward and Chapman, 2011).

Variation in female quality in relation to fecundity is seen in many species (e.g. guppies *Poecilia reticulata*: Dosen& Montgomerie, 2004; long-tailed dance fly *Rhamphomyia longicauda*: Funk &Tallamy, 2000; salamander *Plethodon shermani*: Eddy et al., 2016), and is often positively correlated with female body size. Thus, males are likely to choose females that are larger in size. However, there are some instances where males choose smaller sized females (McCartney and Heller, 2008). In these situations, males may be choosing the youngest females who are more likely to be virgin. Males might gain higher paternity advantage by copulating with virgin females; so-called first male sperm precedence.

The benefit of choosing a female becomes higher when there is high female availability i.e. equal or female biased operational sex ratio (OSR). This circumstance is often rendered in monogamous, promiscuous and polyandrous mating systems, where sexually receptive females are equally or more abundant compared to sexually receptive males (Kvarnemo and Ahnesjii, 1996). Furthermore, female availability is also an important factor in determining the searching cost in males, as the searching cost increases when female abundance is scarce and males are unlikely to discriminate females if it incurs high searching cost (Bonduriansky, 2001).

1.2. Scramble competition and mate finding

Sexual selection favours mate searching ability on one sex when the species are under scramble competition mating system (Able, 1999, Kelly, 2014, Holwell *et al.*, 2007). In this mating system, the resources are distributed equally in time and space. Thus, individuals have equal access to mates. In most cases, males are searchers (Jones and Ratterman, 2009, Holwell *et al.*, 2007, Able, 1999, Kelly, 2014) and find partners by receiving signals (visual, acoustic, olfactory) released by females. This distinctive sex role results in morphological and functional difference between males and females, by favouring traits that relate to strong dispersal ability and developing specialized sensory systems in the searcher sex. As an example, male praying mantis *Pseudomantis albofimbriata* have significantly longer antennae length compared to females, and their antennae are covered with chemoreceptors that are absent in females (Holwell *et al.*, 2007). This antennal morphology and structure in males allows to locate females effectively by receiving chemical signals released by females. Furthermore, *P. albofimbriata* males preferred nutritionally good conditioned females more over poor conditioned females that had different chemical signals (Barry *et al.*, 2010). Therefore, the sensory modalities used for mate location can also be used in mate assessment.

1.3 Precopulatory and postcopulatory male choice

Male mate choice is the differential sexual response to females that vary in quality (e.g. fecundity, mating status) (Bonduriansky, 2001). Male choice can occur before, during and after copulation; these are called precopulatory and postcopulatory male choice respectively. Precopulatory male choice is exhibited as acceptance/rejection, frequency of copulation, or differential degree of investment in intrasexual competition (e.g. male-male combat, scramble competition) for females with varying quality (Bonduriansky, 2001). In precopulatory assessment, males make decisions based on the phenotypic quality of females, such as body size (Eddy *et al.*, 2016, Dosen and Montgomerie, 2004) and pheromone/cuticular hydrocarbon (CHC) profiles (Friberg, 2006, Barry *et al.*, 2010, Schwander and Crespi, 2013, Schneider and Elgar, 2010), that may be associated with fecundity or mating status.

In postcopulatory male choice, males allocate differential degree of resources depending on the quality of females (Bonduriansky, 2001). Examples of resources include level of parental care (Griggio *et al.*, 2003), quality and/or quantity of sperm (so-called strategic sperm allocation) (Friberg, 2006), duration of copulation (Alcock, 1994), and duration of mate guarding (Alcock, 1994, Myers, 2014). Although the exact mechanism is unknown, males are likely to make postcopulatory assessment by the physical contact during courtship, and this is considered to be more accurate in comparison to phenotypic assessment in the precopulatory choice (Edward and Chapman, 2011). This is due to the less likelihood of females to develop traits that signal their status in comparison to males (e.g. male peacock feather), since production of exaggerated phenotypic traits is associated with fecundity costs in females (Edward and Chapman, 2011, Bonduriansky, 2001).

1.4. Parthenogenesis

The predominant mode of reproduction for multicellular animals is sexual reproduction, which involves sperm and eggs that go through fertilization. However, some of animal species produce offspring from unfertilized eggs, which is called parthenogenesis (Dimijian, 2005, Schwander *et al.*, 2010). Parthenogenetic reproduction has many advantages over sexual reproduction (Maynard Smith, 1978, Bell, 1982) and has independently evolved in many stick insect lineages around the world (Scali, 2009, Schwander and Crespi, 2009, Schwander *et al.*, 2011, Ghiselli *et al.*, 2007, Burke *et al.*, 2015). The general rarity of parthenogenetic reproduction might be explained by a higher extinction rate for asexual lineages compared to their related sexual species. The prevalence of sexuality and maintenance of sexuality in parthenogenetic species are associated with advantages of sex, including avoidance of deleterious mutations and evolution of resistance against pathogens (Dimijian, 2005). Many parthenogenetic species are capable of both sexual and parthenogenetic reproduction (facultative parthenogenesis), but some are obligate parthenogens i.e. reproduction rely exclusively on parthenogenesis (e.g. Bdelloid rotifers: Dimijian, 2005; *Poecilimon intermedius*: Lehmann et al., 2007; *Acanthoxyla* spp.: Trewick et al., 2005).

There are some species that include both facultative and obligate parthenogens within species, including stick insects (Burke *et al.*, 2015, Schneider and Elgar, 2010, Kelly, 2014) and damselflies (Shreve and Johnson, 2014). The major difference between facultative and obligate parthenogens are sexual receptivity, where obligate parthenogens are reproductively isolated from males and do not produce male offspring. This is due to the fact that the traits that are historically used for sexual reproduction are likely to decay rapidly in parthenogenetic females since they are no longer useful and become fitness liability (Schwander *et al.*, 2013, Schwander *et al.*, 2010, van der Kooi and Schwander, 2014). Other examples of traits that are likely to decay in parthenogenetic females include courtship behaviours (Carson *et al.*, 1982, Lehmann *et al.*, 2011), communication mechanisms that are used between sexes to locate and attract mates (Lehmann *et al.*, 2011, Burke *et al.*, 2015, Schwander *et al.*, 2013), and sperm storage organ (i.e. spermatheca) (Lehmann *et al.*, 2011). The loss of these traits that are not required by parthenogenetic females will depend on both the cost of production to individuals and the variation within populations for their expression. Selection can only operate if variation exists, and the strength of selection will depend on the fitness gain by loss of the trait.

1.5. Insect-derived chemical signals in sexual communication

Communication via chemical signals, is of pivotal importance for most insects. Insect pheromones, involved in sexual and social communication, most likely evolved from compounds with a non-communication function. Most studies have focused on cuticular hydrocarbons (CHCs), although examples of pheromones exist that have arisen from defensive, secretions, hormones or dietary compounds (Stokl and Steiger, 2017).

Cuticular hydrocarbons are derived from the insect exoskeletons (Ingleby, 2015, Howard and Blomquist, 1982, Wicker-Thomas, 2013). Although the major functions for CHCs is to prevent water loss, they are also used in inter- and intra-specific communications, including defensive secretions against predators (Landolt and Phillips, 1997, Stokl and Steiger, 2017), mate location (Holwell *et al.*, 2007, Myers *et al.*, 2015), and reproductive isolation (Barry *et al.*, 2010, Friberg, 2006, Burke *et al.*, 2015, Schwander and Crespi, 2013). Furthermore, since they are used as a signal for identifying conspecifics or individuals from the same population, it is likely to be highly species- or population-specific (Wicker-Thomas, 2013, Ingleby, 2015).

Many insects sequester specific compounds from their food sources to use them as pheromones or its precursors (Reddy and Guerrero, 2004, Ingleby, 2015, Landolt and Phillips, 1997, Schwander and Crespi, 2013). As an example, various members of Arctiid moths sequester the chemical compounds called pyrrolizidine alkaloids (PAs) from their host plants and use them to attract mating partners (Reddy and Guerrero, 2004).

Pheromones may not only mediate attraction of mates but also be used as the phenotypic signals in mate choice (i.e. precopulatory choice), which allows assessing the quality of potential mates. Some qualities that are signalled from insect-derived volatiles include mating status (Friberg, 2006, Tabata *et al.*, 2017), age (Braga *et al.*, 2016), feeding condition (Barry *et al.*, 2010), and reproductive mode (Burke, 2016, Schwander *et al.*, 2013).

1.6. Stick insects

Stick insects belong to the order Phasmida (also known as Phasmatodea or Phasmatoptera), and there are about 3000 species described worldwide (Salmon, 1991, Bedford, 1978). They are primarily tropical and subtropical insects, but occur anywhere around the world (including Europe, North America, Australia, New Guinea, Africa, Southeast Asia) except for the Antarctic and Patagonia. Stick insects are experts of camouflage, and their cylindrical body and colouration resemble twigs or small sticks that make them difficult for predators to detect. They reproduce both sexually and parthenogenetically (e.g. *Timema* spp., *Bacillus* spp., *Extatosoma tiaratum*: Bedford, 1978; Salmon, 1991), and some females are facultative parthenogens while others are obligate parthenogens within species. It is unknown whether stick insects ubiquitously produce sexual pheromones or CHCs to attract mating partners, but it had been observed in some species (e.g. *Extatosoma tiaratum*: Burke et al., 2015; *Timema* spp.: Schwander & Crespi, 2013).

Another interesting feature of stick insects is reverse sexual dimorphism, where females are larger than males with different structure of sensory modalities (Kelly, 2014, Roy *et al.*, 2013, Myers *et al.*, 2015). The cause behind sexual dimorphism in stick insects can be the result of distinctive sex roles between males and females in scramble competition system, where lighter males with longer legs and elaborate antennae allow greater mobility and mate location.

Male stick insects are also well-known for a prolonged mate guarding. This allows males to avoid takeover of a female by other males and secure his insemination (Kelly, 2014, Myers *et al.*, 2015, Sivinski, 1978). Male stick insects mate guard females by mounting on the dorsal side of the female, and grasping on to her using claspers (modified abdominal segment). The duration of mate guarding can vary between species and female quality (Alcock, 1994), and one of the longest record observed in stick insects was 79days in Indian species *Necroscia sparaxes* (Sivinski, 1978).

In New Zealand, there are nine genera with 25 described species of stick insects (23 described species in Jewell and Brock (2002), two additional species described recently by Buckley *et al.* (2014)), and all are endemic to New Zealand Most of the species are common, and generally found throughout New Zealand. The species can be identified by looking at the structure of male claspers (genital organ that allows attachment on female during copulation) and egg morphology, that vary considerably between species (Salmon, 1991). Only one genus of the New Zealand stick insects are obligate parthenogens (genus *Acanthoxyla*) (Trewick *et al.*, 2005), although one male has been found recently in Scilly Isles, England (Brock *et al.*, 2018, Trewick and Morgan-Richards, 2018). Other genera are sexual or facultative parthenogens (e.g. *Clitarchus*, *Argosarchus*, *Micrarchus*). All species are foliage feeders and are able to feed on wide variety of plant species.

1.7. Study species: *Clitarchus hookeri*

Common mānuka (or tea tree) stick insect *Clitarchus hookeri* is endemic species to New Zealand. They are either bright green or brown coloured, and body size ranges from 8 to 11cm in females and 6 to 8cm in males (Salmon, 1991, Buckley *et al.*, 2014). There are two closely related species *C. tepaki* and *C. rakauwhakanekeneke* that can be differentiated from distributional range (*C. tepaki* occur in North Cape area and *C. rakauwhakanekeneke* in Poor Knights Island) and distinctive structure of male and female external genitalia (i.e. claspers and opercular organs) (Buckley *et al.*, 2014, Myers *et al.*, 2017).

1.7.1. Food sources

Clitarchus hookeri feed mainly on species of tea trees (Myrtaceae), *Leptospermum scoparium* (mānuka) and *Kunzea ericoides* (kānuka), where they also reproduce. Although all New Zealand stick insect species are known to feed on mānuka and kānuka, *C. hookeri* is the only species that is successfully able to carry its entire life on these leaves alone (Salmon, 1991). In addition to tea trees, *C. hookeri* are also known to feed on *Metrosideros* (pōhutukawa, white rātā), *Muehlenbeckia australis* (pōhuehue), *Cordyline australis* (cabbage tree) and *Coprosma* spp. (Myers, 2014, Trewick *et al.*, 2005, Salmon, 1991). As dispersal is limited for being apterous, individuals may complete their entire life cycle on only one species of plant.

The past studies have identified major secondary metabolites in mānuka and kānuka: mānuka is characterized by various types of sesquiterpenes, and kānuka is characterized by alpha-pinene (monoterpene) (Perry *et al.*, 1997, Douglas *et al.*, 2004, Porter and Wilkins, 1999). Many phytophagous insects sequester secondary metabolites from their host plants to incorporate them as sex pheromones or CHCs (Landolt and Phillips, 1997, Ingleby, 2015) that are further utilized as the basis for mate location and discrimination (Barry *et al.*, 2010, Holwell *et al.*, 2007, Friberg, 2006). Although it is still unknown whether *C. hookeri* also exploit host plant-derived volatiles as the basis of mate discrimination, it has been shown that males are capable of detecting air-born chemicals (Myers *et al.*, 2015).

1.7.2. Distribution and sex ratio

Clitarchus hookeri are found all over New Zealand and exhibit a pattern of geographical parthenogenesis i.e. parthenogenetic populations present in higher latitude compared to their sexual conspecifics (Morgan-Richards *et al.*, 2010, Buckley *et al.*, 2010). In North Island, sexual populations occur in the north-west, while the populations to the south and east are all female, except in Wellington where a few males are observed (Morgan-Richards *et al.*, 2010). This peculiar distribution of *C. hookeri* is considered to be the result of range expansion after Last Glacial Maximum (Morgan-Richards *et al.*, 2010, Buckley *et al.*, 2010).

Sex ratio is used to infer reproductive strategy: even sex ratio populations are sexual (Otaki, Lake Koripiko, Coromandel Peninsula, Great Barrier Island, and Auckland), whereas most southern populations have rare males, or no males and are parthenogenetic (Morgan-Richards *et al.*, 2010). Males have increased in frequency at one Wellington location over 10 years. No males were found at Te Whiti Riser, Lower

Hutt (Wellington), so it is assumed that the Lower Hutt population is parthenogenetic (personal obs.).

1.7.3. Sexual vs parthenogenetic females: sexual receptivity, fecundity, fertility and morphology

The females from sexual populations are capable of reproducing both sexually and parthenogenetically i.e. facultative parthenogenesis and equal numbers of males and females are produced from these females (Morgan-Richards *et al.*, 2010). On the other hand, females from unisexual populations and those in Wellington have limited capacity of sexual reproduction when crossed with males, indicating that most of them are obligate parthenogens (Morgan-Richards *et al.*, 2010). Thus, it will be energetically wasteful for males to copulate with these obligatory parthenogenetic females.

Both facultatively and obligatory parthenogenetic females are capable of producing eggs asexually every day (approximately every 17 hours: Stringer, 1970), and the egg hatching rates for unfertilized eggs are relatively similar between sexual and parthenogenetic females (Morgan-Richards *et al.*, 2010). Therefore, although sexual and parthenogenetic females differ in sexual receptivity, they have similar fecundity and fertility.

1.7.4. Mating system

In sexual populations, *Clitarchus hookeri* exhibit scramble competition mating system (in which individuals have equal accessibility to mates), and most of the males and females mate multiply i.e. promiscuous mating system (Myers, 2014, Myers *et al.*, 2015). However, their reproductive strategies vary depending on the operational sex ratio (OSR) and season. The mating season for *C. hookeri* is during summer and autumn (December-April) (Myers, 2014). Early in the season when OSR is male biased (as males develop faster than females), males are likely to guard females for extended periods of time (up to 10nights) by mounting on to female’s back. However, the duration of mate guarding decreases as the adult female density increases later in the season, and the average guarding period reduces to approximately one night (Myers *et al.*, 2015). Although it is unknown if the degree of male choosiness changes in response to change in OSR, it is likely that males are less choosy when females are scarce (due to costs associated with choice) but become more discriminative in later in the season when females are more abundant.

1.7.5. Males vs. females: scramble competition, sex roles, mate location

As it is seen in other stick insect species, *Clitarchus hookeri* also exhibits sexual dimorphism. This is due to the distinctive sex roles in scramble competition mating system, in which males are searchers and females are signallers. As male *C. hookeri* actively search for females during the reproductive season, the traits that enhances their mobility will be favoured (e.g. light body and long legs).

In addition, males probably locate mates by olfactory cues (pheromones) released from females. This was observed in an experiment where males were able to detect air-born chemicals effectively in Y-maze choice experiment (Myers *et al.*, 2015). Olfactory signals are detected by their antennae, and the structure differ significantly between males and females: males have higher density of basiconic and trichoid sensilla, which are used for detecting olfactory signals in insects (Myers *et al.*, 2015). Furthermore, the study by Myers and coworkers suggests that female *C. hookeri* may be producing sex pheromone to attract males, which would be advantageous for the individuals from sexual populations as they are under scramble competition mating system i.e. helps males to locate females. Conversely, although production of olfactory signals is advantageous for sexual populations, it may be wasteful for females from parthenogenetic populations (e.g. energetic cost) as they are not under scramble competition system. Thus, it is likely that selection will have favoured *C. hookeri* females from parthenogenetic populations with lower investment in chemical signals.

1.7.6. Mate choice

The factors that influence mate choice in *Clitarchus hookeri* (both males and females) have been investigated with laboratory and field studies.

Myers *et al.* (2016) demonstrated the structure of male external genitalia i.e. claspers as the basis for pre-copulatory choice in females: male *C. hookeri* have conspicuous teeth (three to five pairs) on their claspers, and removal of teeth led to active rejection by females. Females are able to sense and discriminate male claspers by the mechano-receptors called sensilla chaetica, which are present on females' operculum. On the other hand**,** males did not discriminate females by their external genitalia i.e. operculum, since males mated with females regardless of operculum being modified or not (Myers *et al.*, 2016).

In males, there seems to be limitation in precopulatory mate discrimination. According to Myers (2014), *C. hookeri* males failed to discriminate between conspecific and heterospecific females (*C. tepaki* and *C. rakauwhakanekeneke*). However, the duration of mate guarding after copulation was longer in conspecific females compared to *C. tepaki* or *C. rakauwhakanekeneke* females. This may be due to the fact that males assessment through genital contact during copulation has higher accuracy compared to phenotypic assessment in precopulatory choice, since females are less likely to invest on traits that have fecundity costs (Edward and Chapman, 2011). In another field study for male choice in *C. hookeri* (Myers *et al.*, 2015), authors observed a negative correlation between the number of mates and female weight. Males generally choose females that provides fecundity advantage, and fecundity is often positively correlated with body size (Edward and Chapman, 2011, Bonduriansky, 2001). However, the observation indicates that *C. hookeri* male choice did not follow this pattern, since males preferred lighter females over heavier ones (Myers *et al.*, 2015). This preference for lighter females may be associated with choice for younger females that are more likely to be virgin and reduces the risk of sperm competition (i.e. first sperm precedence).

1.7.7. Mating behaviours

Clitarchus hookeri are nocturnal species and reproductively active only during summer-autumn (December-April), since this is when individuals complete their final moult and become adults. In the field, males and females become active after sunset where females appear and hang on the branch edges (presumably to become conspicuous to males) and males start searching for females (Myers *et al.*, 2015). When males encountered female, male mounts on dorsal side of female and become attached on female's operculum via his legs or clasper.

There are different behavioural indicators of precopulatory mate choice between sexes in *C. hookeri*. For females, rejection of males can be observed when females start to move vigorously (shaking their body and moving around) and if the operculum stayed closed even though males attempting on copulation (Myers *et al.*, 2016). Acceptance can be observed if female was stationary and opens her operculum. On the other hand, there seems to be no active rejection by males (personal obs.). This may be due to the fact that females do not actively seek for males, and the attempt of copulation is initiated only by males. Instead, males exhibit precopulatory choice by mounting on top of particular females. If the male did not like that female, he would not mount on top of female or even though if he mounts on top, he quickly moves away.

As mentioned before (1.7.4. Mating system), males mate-guard females for one night on average when the sex ratio becomes 1:1 (Myers *et al.*, 2015). This guarding duration may be consistent with the duration of egg production in females since females lay one egg every 17hours (Stringer, 1970) as male stick insects are known to mate guard females until they lay eggs (Kelly, 2014). It is unknown whether males will guard females during the day-time, but as *C. hookeri* are inactive during day-time, males are likely to secure his paternity if he was able to guard female for overnight.

Being nocturnal, *C. hookeri* are sexually inactive during the day-time and they often stay still under branches. According to the observation by Myers *et al.* (2015), mating in *C. hookeri* is observed only during sunset to midnight (around 20:00-00:00h).

Therefore, sexually active time for *C. hookeri* can be considered between 20:00-00:00h.

1.7.8. Conclusion: known and unknown facts, and predictions

Clitarchus hookeri exhibit scramble competition mating system: males are searchers and females are signallers. Morphological difference between males and females further support this (longer antennae and presence of chemo-receptors in males). Their reproductive season is throughout summer and early autumn (December-April), and become sexually active after sunset. Males search for mates using their antennae and are known to move further than females during the reproductive season. As females are signallers in their role of scramble competition, females signal to males by becoming visually conspicuous (by hanging on the edge of branches) after sunset and possibly by producing sex pheromones.

Clitarchus hookeri have both sexual (males and females) and parthenogenetic (females only) populations, and females from sexual populations (north-western North Island) are capable of facultative parthenogenesis, whereas most of the rest of the populations (south-eastern North Island and South Island) exhibit obligate parthenogenesis. Females from parthenogenetic populations are most likely to be obligate parthenogens, as most of them fail to reproduce sexually after mating in captivity. Females that do not invest in the traits that are related to scramble competition mating system, as well as other traits that are required for sexual reproduction are likely to be at a selective advantage. In general, it will be energetically wasteful if males copulate with these parthenogenetic females.

Both sexes are known to exhibit mate choice at some extent; where females discriminate males by the structure of claspers and males discriminate females by their size (prefer lighter females), as the precopulatory assessment bases. However, males' precopulatory assessment seems to have limitation in accuracy as they failed to discriminate between conspecific and heterospecific females, as well as sexually receptive (sexual) from sexually unreceptive (parthenogenetic) females. It is likely that males can assess females more accurately during copulation as males mate guarded conspecific females compared to heterospecific females.

There are several questions to be answered:

- What are morphological and chemical traits that are related to sex roles in scramble competition mating system?
- Are there any other differences between sexual and parthenogenetic females apart from their reproductive mode? Do parthenogenetic females maintain the traits that are related to scramble competition system (e.g. chemical signalling) or other sexual traits (e.g. copulatory behaviours)?
- What are female quality factors associated with male choice apart from female body mass? Can males discriminate between sexual and parthenogenetic females? Do males choose the females with particular quality consistently in their precopulatory and postcopulatory choice?

CHAPTER 2.

Male mate choice in the stick insect *Clitarchus hookeri*: sexual vs. parthenogenetic females

2.1. Introduction and aims of the study

Males and females have distinctive roles in *Clitarchus hookeri* in relation to their scramble competition mating system i.e. males are searchers and females are signallers (see CHAPTER 1). These distinctive roles are seen in traits that relate to dispersal ability (e.g. longer legs, small body) and mate location (e.g. well-developed antennae) in males, and possibility of chemical signalling (e.g. cuticular hydrocarbons (CHCs) and sex pheromones) that are specific to females (quantitative and/or qualitative) and attract males (Myers, 2014, Myers *et al.*, 2016, Myers *et al.*, 2015). *Clitarhus hookeri* include both sexual (males and females) and parthenogenetic (all-female) populations and the selective pressure that is related to scramble competition is exerted only to those individuals from sexual populations. Therefore, females from parthenogenetic populations are likely to have different phenotypic (morphological and chemical) traits to that of sexual females as they are not under selection to signal. Due to lower sexual receptivity of parthenogenetic females, it would be wasteful for males to copulate with these females. Thus, the capacity for males to discriminate between sexual and parthenogenetic females will be favoured where they are in contact with each other.

In this chapter, the morphological and chemical traits in *Clitarchus hookeri* will be explored. (1) I will identify the differences between males and females in relation to scramble competition mating system. I predict that males will exhibit the traits that have strong dispersal ability and mate location, and sexual females will exhibit the traits that facilitate mate location by males. (2) I will compare the morphological and chemical traits that are related to sexual signalling and sexual reproduction of sexual and parthenogenetic females. I predict that morphological and chemical differences identified to be under sex roles will be reduced or lacking in parthenogenetic females.

(3) I will assess male choice between sexual and parthenogenetic females in *C. hookeri*. For this purpose, I will explore male precopulatory choice based on morphological and chemical differences identified in (1) and (2), and if they have different frequencies of guarding females overnight and changing partners between sexual and parthenogenetic females in postcopulatory choice. I predict that males will prefer sexual females over parthenogenetic ones as it will be selectively advantageous for the males to be able to discriminate between sexual and parthenogenetic females.

2.2. Materials and methods

2.2.1. Sample collection and sample size

Clitarchus hookeri were collected from sexual and parthenogenetic populations from North Island (in this study, the females from the populations include both males and females i.e. facultative parthenogens will be called sexual females; and the females from all-female populations i.e. obligate parthenogens will be called parthenogenetic females) (Figure 1). Males and sexual females were collected from Auckland, Whanganui and Paraparaumu, and parthenogenetic females from Palmerston North and Lower Hutt. These sampling areas were chosen based on the observation in Morgan-Richards, Trewick, and Stringer (2010) and personal observation. From each location, 15-20 females and (where available) 15-20 males, were collected.

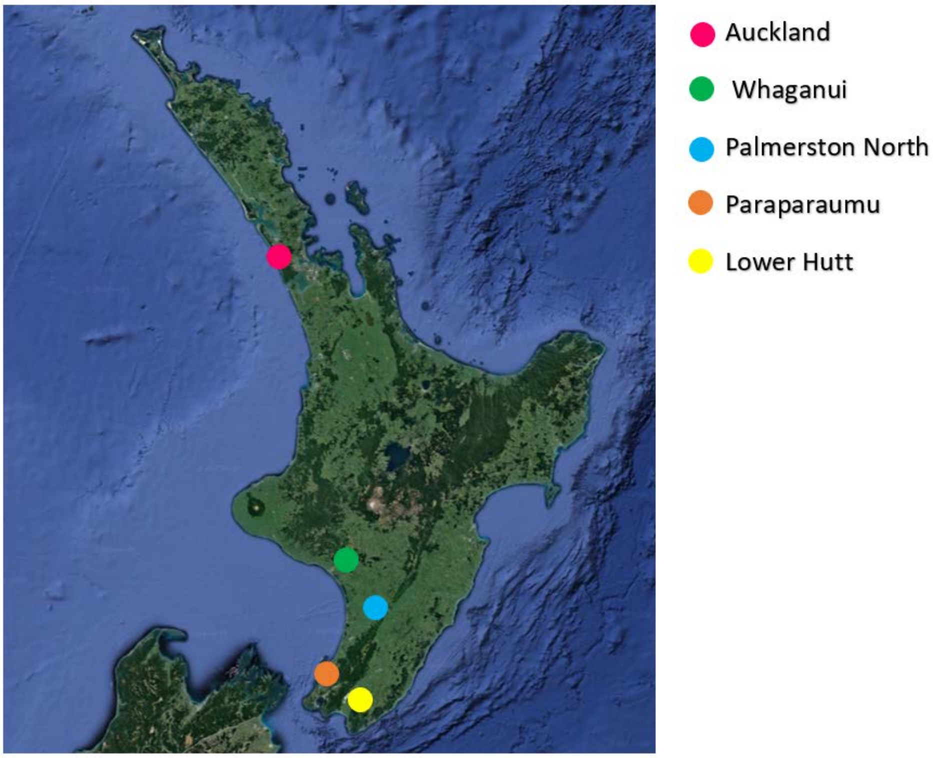


Figure 1. Sampling locations of *Clitarchus hookeri*.

Individuals were collected at the peak abundance time during the reproductive season: Auckland and Whanganui during late November to December 2017, Palmerston North during December 2017 to January 2018, and Lower Hutt and Paraparaumu during March to April 2018. However, the "age" of adult insects were kept relatively consistent for volatile collection (volatiles were collected about a week after final moult) and for male choice experiments (adult females that moulted to adult at similar timing were used).

For sexual females, only juvenile instars were collected to ensure that they were virgin. Males, sexual females and parthenogenetic females were kept in different tanks according to the populations they came from. Temperature was kept at 25°C with natural light for circadian rhythm. Branches of their food plant *Leptospermum scoparium* were provided with a plastic container filled with water, and the tank were sprayed with water every day to prevent desiccation.

2.2.2. Males vs. Females and Sexual vs. Parthenogenetic females

2.2.2.1. Morphological analysis

For analysing morphology (Figure 2), 16 morphological parameters were measured: the length (cm) of body, antennae, pronotum, mesonotum, metanotum, abdomen, operculum (female)/poculum (male), and fore-/mid-/hind-legs; the wdith (cm) of pronotum, mesonotum, metanotum, abdomen (measured at abdominal segment II and III) and operculum/poculum, and body mass (g).

These parameters were measured using electronic calipers (Q-1382, Dick Smith Electronics, Sydney, Australia) and scales (ED224S, Sartorius, Göttingen, Germany). All adult individuals were measured, but those individuals lacking both left and right legs or damaged antennae were removed (N=10). Live specimens were weighed and the maximum weight observed during the insect’s lifetime was used for the final analysis. Length and width of body and body parts were measured using frozen specimens after volatile collection (2.2.2.2.) and male choice experiment (2.2.3.).

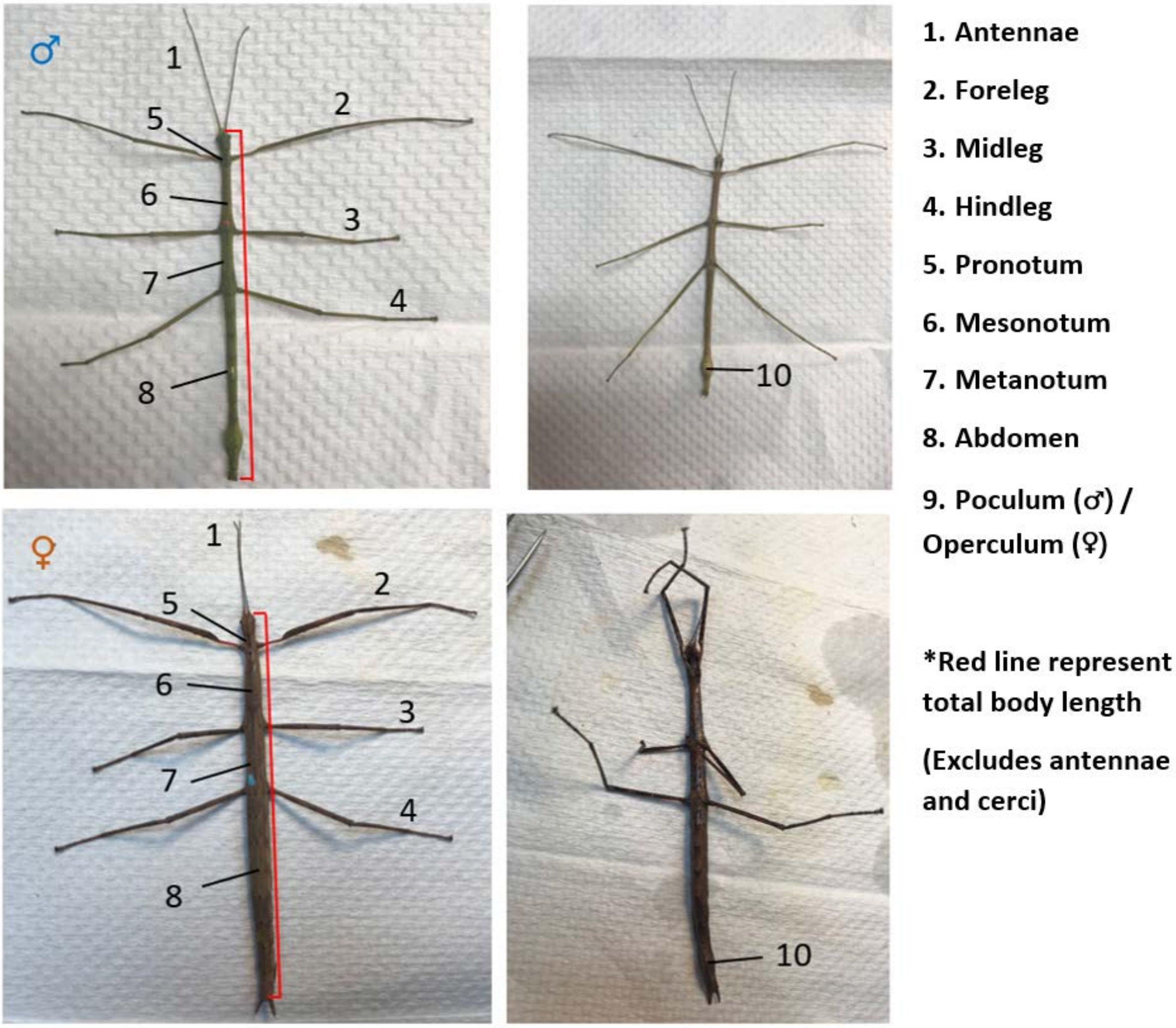


Figure 2. Dorsal (left) and ventral (right) views of male (top two) and female (bottom two) *Clitarchus hookeri*. Each number indicate the morphological variables that were measured and a red line indicates total body length.

Statistical analysis

All the statistical analyses were performed and figures were constructed using the software R studio (R version 3.4.2. Boston, MA, USA). 16 morphological parameters were analysed between males, sexual females, and parthenogenetic females (2.3.1.1.); and between females with different reproductive mode (sexual vs. parthenogenetic) and populations (Auckland, Whanganui, Paraparaumu, Lower Hutt, and Palmerston North females) (2.3.1.2.). Among females, populations analyses were performed to identify whether the morphological variation identified was derived from reproductive strategy or population. Principal component analysis (PCA) was used to visualize groupings and to identify morphological variation between sexes, reproductive mode, and populations. Then, correlation matrix was constructed to see which of the morphological parameters are correlated within all examined insects, males, sexual females, and parthenogenetic females (Appendix Table 1). Scatter plots were constructed for those morphological variables that have shown significant correlation. These analyses were done to identify the morphological variables that are proportionate/disproportionate among sexes and females with different reproductive strategies. Finally, one-way analysis of variance (one-way ANOVA) and Tukey's honestly significant difference were used to identify the morphological variation among females from different populations for those morphological variables that have shown contribution to the groupings.

2.2.2.2. Chemical analysis

Method for collecting volatiles from insects

Chemical volatiles from males and females from each population were sampled (N=6 for each sex per population) over ten hours (15:00h-01:00h), at two-hour intervals. Lights were on between 15-21:00h, and off between 21-01:00h. This continuous sampling from a lighted to dark period would detect a change in chemical profiles (quality and/or quantity) if there was a response to the change in photo-period in insects. This volatile collecting time frame is consistent with the sexually inactive to active time for *C. hookeri* (Myers *et al.*, 2015). Insect body weight was recorded before the volatile collection, then used for calculating the quantity of volatile emissions ($ng*gFw^{-1}h^{-1}$). All of the volatile collections were made between February-April 2018 in a temperature controlled room at 25 ^{o}C.

To collect volatiles (Figure 3), each individual was covered with a commercial polyethylene terephthalate (PET) foil bag (40cm), and a dynamic push-pull system (VAS, Renssealer, NY, US) was used to collect volatiles. During the collection, charcoal filtered air was pumped into PET bag at 0.85L/hour and pumped out 0.80L/hour through Teflon tubes to allow a slight overpressure and prevent contamination. Volatiles were trapped with a 20mg Hay SepQ adsorbent filter, which was placed at the tip of the Teflon tube for outgoing airflow. Sampled volatiles were eluted from the filter with 200µl of pentane containing nonyl acetate 10ng/µl as an internal standard.

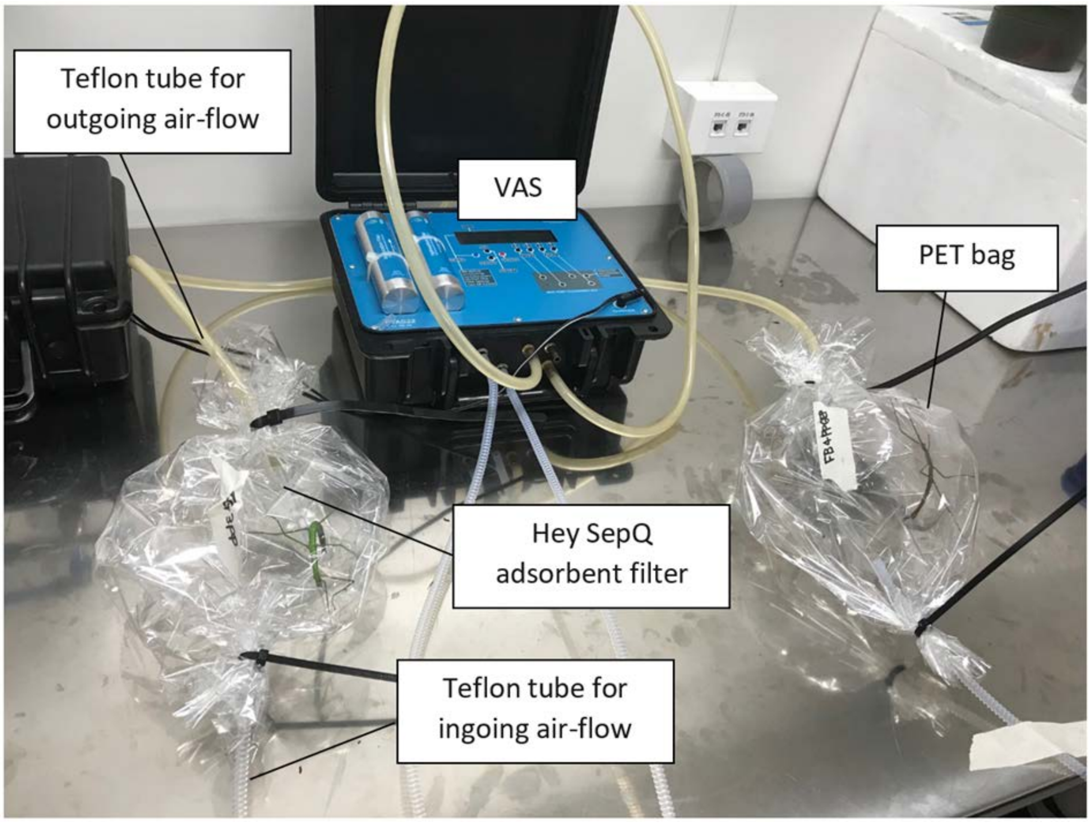


Figure 3. Schematic of volatile collection.

Samples were analysed using a Gas Chromatograph – Mass Spectrometer (GCMS-QP2010, Shimadzu Corporation, Kyoto, Japan), using a 30m7 x 0.32 mm DB-5 capillary columns. The temperature was programmed for 3min 50 °C then increased to 95 °C at 5 °C/min, 145°C at 15 °C/min, 180 °C at 10 °C/min, and finally 200°C at 10 °C/min. The samples were injected in split mode and sample running time was 23.83 minutes total.

Compounds were identified by comparing retention times and mass spectra to those in the NIST (National Institute of Standards and Technology) library 2005 and those compounds that had a similarity > 80% were used. Quantity of each compounds identified were calculated by comparing the peak height of the compound to peak height of the internal standard (ng*$gFw^{-1}h^{-1}$).

Statistical analysis

Chemical analyses were performed to identify the quantitative and qualitative differences between males vs. females, sexual vs. parthenogenetic, and females from different populations. As the quantity (ng*$gFw^{-1}h^{-1}$) of volatile emission was not normally distributed, Kruskal-Wallis test was used. To identify quantitative difference in volatile emission from sexually inactive to active time, whether the significant change in the quantity of volatile emission over 10 hours of volatile collection time (i.e. 15:00-01:00h) within males, sexual females, parthenogenetic females, and females from each populations were tested. In addition, whether the quantity of volatile emission significantly differed between sexes, between sexual and parthenogenetic females, and between the females from different population for each of the volatile collection time frame was also analyzed. This was to determine whether the time fame(s) when significant differentiation was observed was consistent with the time of sexual activity.

Finally, to see which of the compound group(s) (e.g. monoterpenes, sesquiterpenes) and compound(s) were emitted in the highest quantity, box plots were constructed to explore quantitative and qualitative difference among groups (i.e. between sexes, reproductive strategies, and populations). This allowed me to determine whether there was a difference in the chemical profiles among groups.

2.2.3. Male choice experiments

Open arena

Mate choice experiments (N=49) were conducted in an open arena (H39cm x W38cm x D20cm), by placing one male and two females i.e. parthenogenetic vs. sexual females, in the same tank. Sexual and parthenogenetic females were chosen randomly from different population samples from that of the male (to avoid male preference to the female from same population). Each insect was used only once to ensure that females were virgins and tests were independent. Two feeding grounds (a branch of *Leptospermum scoparium* in a plastic container of water) were provided at either end of the tank, to which a female attached, and a male was placed at the centre of the tank. Females were marked on their abdomen to identify reproductive mode. All insects were weighed before the observations as the body mass of females is used in male choice (Myers *et al.*, 2015). All of the male choice experiments were performed January-May 2018.

Males were given four hours (20:00-00:00h) to make a choice between the two females. During this time, the behaviours of male and females were observed, including the time when males and females become active, timing of pairing, and copulatory behaviours. I recorded which female that male made physical contact with (regardless of mating) (*1st*) and the second female (regardless of mating) (*2nd*; this was recorded only when males had changed partner from *1st* between 20:00-00:00h). *1st* was considered as precopulatory male choice. Then, they were left in the tank until 09:00 next morning (the insects were removed from the tank if the male did not stay attached on any female at 00:00h), and recorded whether male has stayed on the same female (overnight mate guarding: *OMG*); or changed partner from *1st* after mating during 20:00-00:00h or changed partner during 00:00-09:00h (mating was assumed if the males changed partner during 00:00-09:00h) (*switch*). These were considered as postcopulatory choice. Females were scored 1 point for each of the behavioural assays (*1st*, *2nd*, *OMG*, *switch*).

Statistical analysis

The score for each of the behavioural assays (i.e. *1st*, *2nd*, *OMG*, *switch*) was transformed into frequency as below (with example between qualities A vs. B females):

1st= (sum score of quality A or B females being *1st*) / (sum score of males made choice between quality A and B females)

2nd= (sum score of quality A or B females being *2nd*) / (sum score of males made choice between quality A and B females)

OMG= (sum score of quality A or B females being *OMG*) / (sum score of quality A or B females being *1st* and *2nd*)

Switch= (sum score of quality A or B females being *switch*) / (sum score of quality A or B females becoming *1st* and *2nd*)

These frequencies were calculated and compared between females with different qualities: reproductive mode (sexual vs. parthenogenetic), populations (i.e. Auckland, Whanganui, Paraparaumu, Lower Hutt, and Palmerston North), body mass (heavier vs. lighter), and total body length (longer vs. shorter).

A binomial test was used to evaluate whether the probability of *C. hookeri* males choosing the females with a particular quality differed significantly from the probability of males choosing two females in equal frequency (i.e. 0.5). This was calculated between parthenogenetic vs. sexual; heavy vs. light (in terms of body mass (g)); and long vs. short (in terms of total body length (cm)) females. The actual score was used to perform a binomial test for precopulatory choice (i.e. *1st*) and the frequency was used for postcopulatory choice (*OMG* and *switch*). The frequency was used in the latter as the scores of postcopulatory choice will be influenced by *1st* (e.g. if the females with a particular quality have a higher score on *1st* it will be likely that they will have a higher score on *OMG*).

2.3. Results

2.3.1. Morphological analysis

2.3.1.1. Males vs. females

Measuring stick insects demonstrated clear differences between the two sexes (Figure 4). Because many of the traits measured were associated with size I used principal component analyses to focus on uncorrelated components of that variation. The first two components of variation explained 87.6% of total variation - in the size and shape of my sample of *Clitarchus hookeri*. The segregation of morphological variables between sexes was evident on PC1, which accounted for 75% variation (Figure 4). Sexual and parthenogenetic females overlap in their morphology although my samples did have distinct means (details below 2.3.1.2.). The second component PC2 accounted for 12.6% of the total variation. The second component variation (PC2) did not separate males and females (Figure 4).

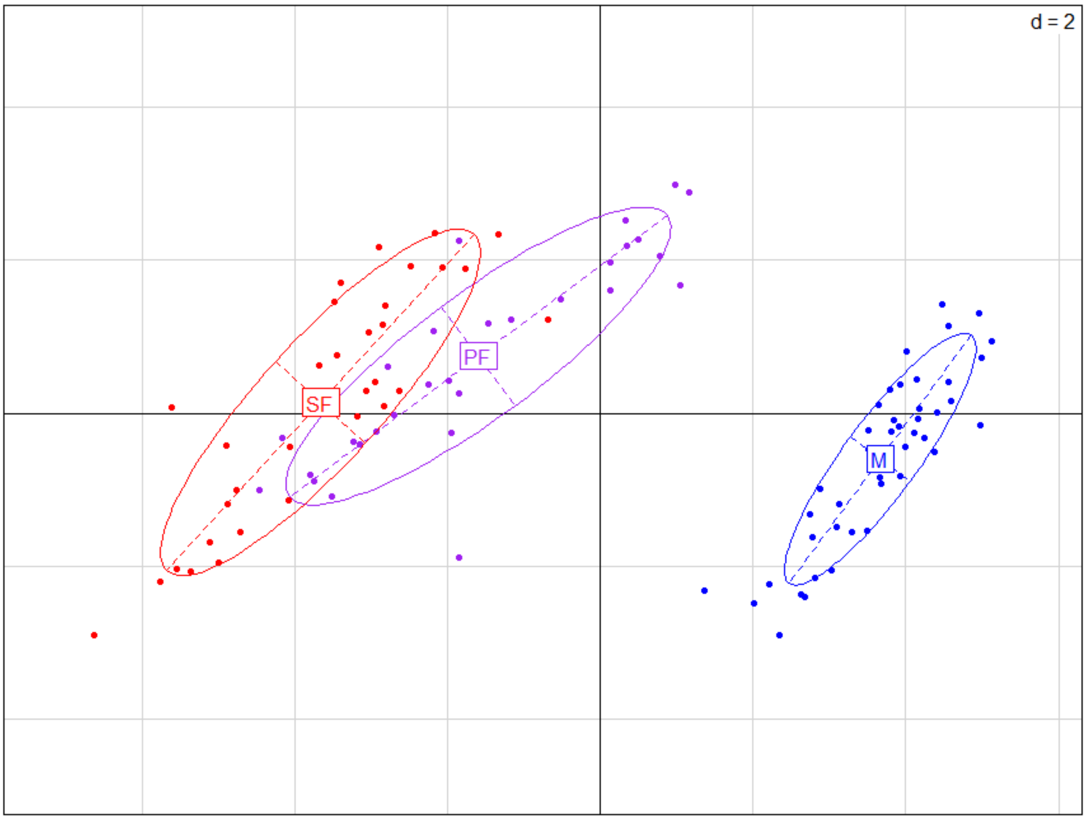


Figure 4. Morphological groupings of males (M, blue), sexual females (SF, red), and parthenogenetic females (PF, purple). Variation of principal components: PC1= 75% (x-axis), PC2= 12.6% (y-axis).

The body of adult female stick insects was always longer and fatter than males (Figure 5(a)-(c)). Body length was positively correlated with the length of antennae, fore-/mid-/hind-leg in males, sexual females, and parthenogenetic females (p-value < 0.05, correlation coefficient). However, the length of antennae and fore-/mid-/hind-legs, and width of poculum in males overlapped with the length of antennae and legs and width of operculum in females respectively (both sexual and parthenogenetic) (Figure 5(d)-(h)). These show that the length of antennae and fore-/mid-/hind-legs, and the width of poculum is disproportionately longer and wider in relation to the total body length in males, compared to that of females.

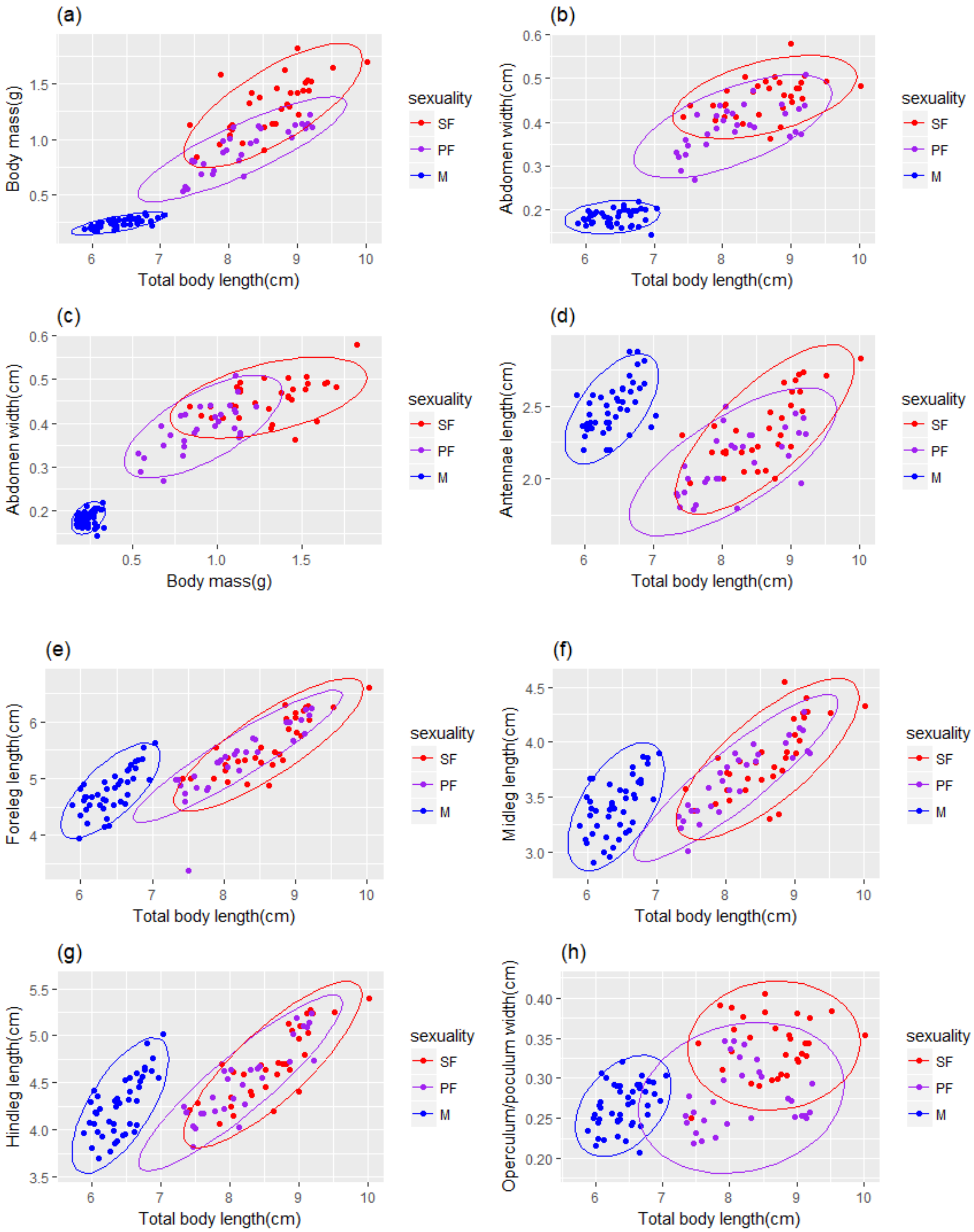


Figure 5. Female *Clitarchus hookeri* are longer (body length) and fatter (body mass, abdomen width) than conspecific males (a)-(c). However, the length of antennae (d), fore- (e), mid- (f), and hind- (g) legs, and the width of operculum/poculum (h) are disproportionately longer and wider in relation to total body length in males (M, blue), compared to that of sexual (SF, red) and parthenogenetic females (PF, purple).

The length and width of other morphological parts were longer and wider in females (both sexual and parthenogenetic) compared to males. Both body mass and total body length was positively correlated with the width of abdomen among all insects (including males and sexual/parthenogenetic females), sexual females, and parthenogenetic females (p-value < 0.05, correlation coefficient). However, this was not observed in males (p-value > 0.05). In addition, abdomen width was thinner in relation to body mass and total body length in males compared to that of sexual and parthenogenetic females (Figure 5(b) & (c)). These results show that males have disproportionately thinner abdomen width than females.

2.3.1.2. Sexual vs. parthenogenetic females

Size and shape variation of female *Clitarchus hookeri* was examined after excluding males from the analysis. The first two components explained 76% of total variation among females with different reproductive mode and populations. The segregation of the morphological variables between sexual and parthenogenetic females was observed in both PC1 (63.5% of variation) and PC2 (12.5%), with some overlap (Figure 6). This overlap is derived from Lower Hutt females that are morphologically similar to both Palmerston North (parthenogenetic) and Auckland (sexual) females, and some Whanganui (sexual) females were indistinguishable from Palmerston North (parthenogenetic) females (Figure 6).

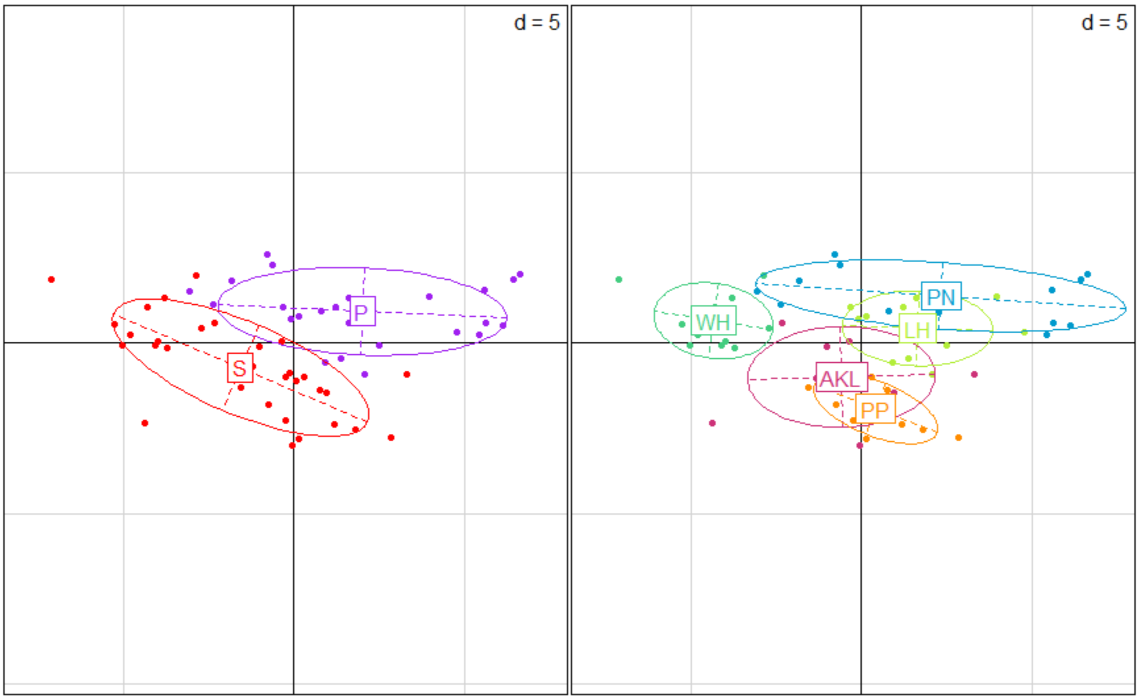


Figure 6. Morphological groupings of females with different reproductive mode (sexual (S, red) vs. parthenogenetic (P, purple); left figure) and between populations (Auckland (AKL, pink), Whanganui (WH, green), Paraparaumu (PP, orange), Lower Hutt (LH, yellow), and Palmerston North (PN, blue); right figure). Variation explained by principal components: PC1= 63.5% (x-axis), PC2= 12.5% (y-axis).

On average, females from sexual populations had longer (total body length) and fatter (body mass in relation to body length and abdomen width in relation to body length and mass) body (Figure 7(a), (c), & (e)). However, these relative sizes of morphological variables also have shown significant variation between populations (p-value < 0.05, one-way ANOVA) (Figure 7(b), (d), (f)), and many of the morphological variables did not show significant differentiation between the populations with different reproductive mode. For example, body mass in relation to total body length was significantly heavier in Whanganui and Auckland females (sexual) compared to that of Palmerston North and Lower Hutt females (parthenogenetic) (p-value < 0.05, Tukey's post-hoc test), but overlapped between Paraparaumu (sexual) and parthenogenetic females (p-value > 0.05). This morphological variation among populations prevented identification of traits that clearly separate sexual from parthenogenetic females.

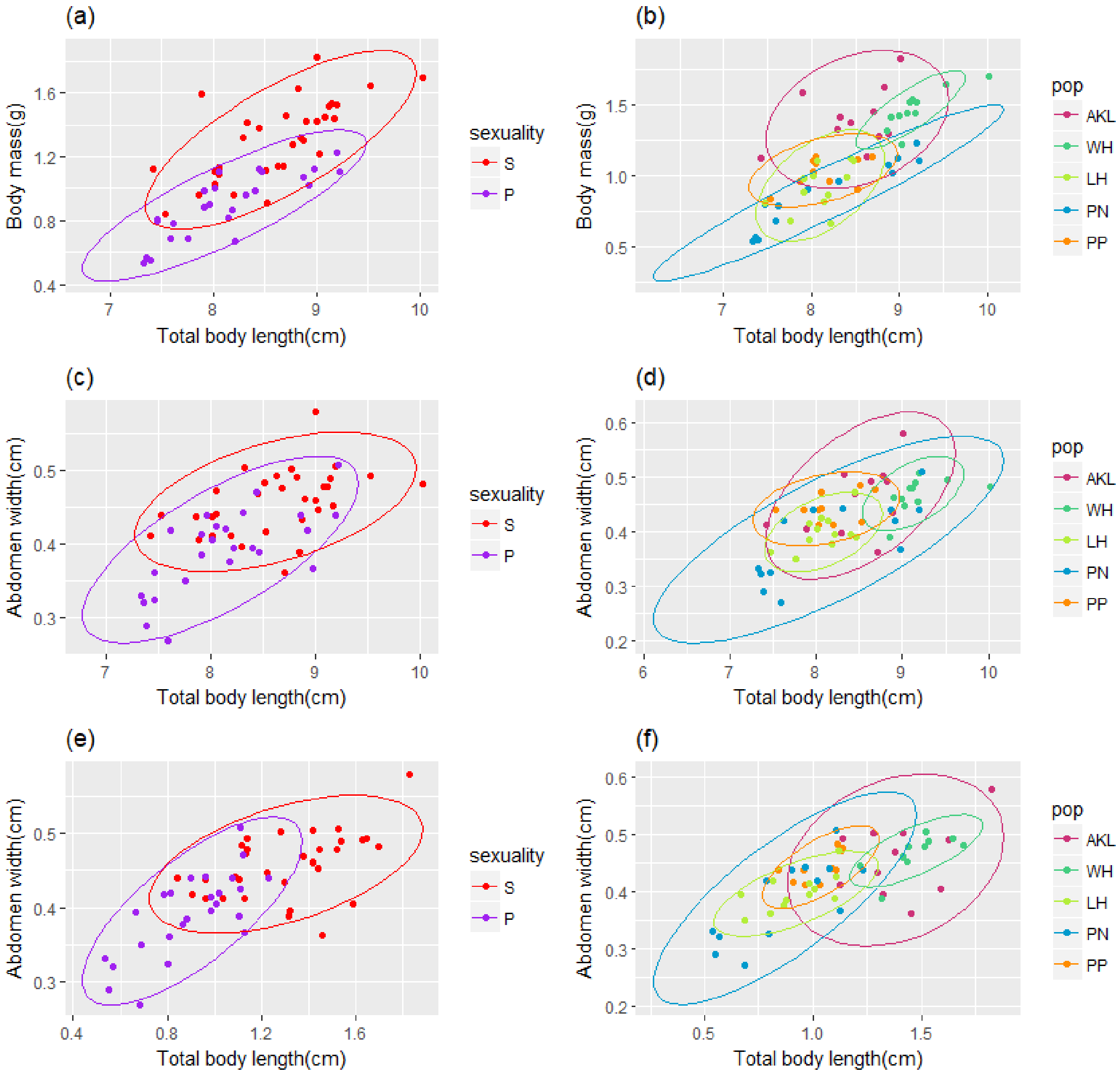


Figure 7. Sexual females (S, red) are generally longer and fatter than parthenogenetic females (P, purple) ((a), (c), (e)). However, significant morphological variation between populations have been observed (p-value < 0.05, one-way ANOVA) ((b), (d), (f)). Populations: Auckland= AKL, pink; Whanganui= WH, green; Lower Hutt= LH, yellow; Palmerston North= PN, blue; Paraparaumu=PP, orange.

2.3.2. Chemical analysis

24 volatile compounds had been identified from *C. hookeri* (Table 1) during the volatile collection time frames of 15-17:00h, 17-19:00h, 19-21:00h, 21-23:00h, and 23-01:00h. Compounds were identified by comparing to the NIST library '05 and all had > 80% retention time and mass spectra similarity to known volatile compounds. These 24 compounds were all terpenoids (monoterpenes, sesquiterpenes, and sesquiterpene derivatives). All individuals examined emitted greater quantities of sesquiterpenes than any other identified volatile group (i.e. monoterpenes, sesquiterpenes, and sesquiterpene derivatives) (Figure 8(b)-(d)).

Table 1. Retention time (RT), compound names (similarity > 80% with NIST library '05), volatile groups that each compound belongs to.

RT	compound	Chemical class
7.2	α-Phellandrene	monoterpenes
7.5	α-Pinene	monoterpenes
8.7	Sabinene	monoterpenes
8.8	β-Pinene	monoterpenes
9.34	β-Myrcene	monoterpenes
11.19	α-Ocimene	monoterpenes
11.5	γ-Terpinene	monoterpenes
17.15	α-Cubebene	sesquiterpenes
17.4	Ylangene	sesquiterpenes
17.54	Copaene	sesquiterpenes
17.63	β-Elemene	sesquiterpenes
17.73	γ-Elemene	sesquiterpenes
18.02	α-Gurjunene	sesquiterpenes
18.16	β-Caryophyllene	sesquiterpenes
18.4	Isoledene/Aromandrene	sesquiterpenes
18.5	α-Cubebene2/Isoledene	sesquiterpenes
18.6	α-Caryophyllene	sesquiterpenes
18.8	Cadina-1(10),4diene	sesquiterpenes
18.93	GermacreneD	sesquiterpenes
19.05	Eudesma-4(14),11-diene	sesquiterpenes
19.12	α-Seline	sesquiterpenes
19.24	Butylated Hydroxytoluene	sesquiterpenes
19.43	Calamenene	sesquiterpene derivatives
19.53(55)	Cadine-1,4-diene	sesquiterpenes

2.3.2.1. Males vs. females

Significant increase in the total quantity (ng*gFw^{-1}h^{-1}) of volatile emission overtime (i.e. 15-01:00h) was observed only in sexual (p-value < 0.05, Kruskal-Wallis test) and parthenogenetic (p-value < 0.05) females but not in males (p-value = 0.99), with the maximum emission observed at 21-23:00h for most of the females (Figure 8(a); explained more in 2.3.2.2.). Moreover, the quantity of volatile emission significantly differed between males and females (both sexual and parthenogenetic) at the volatile collection time frames 17-19:00h, 19-21:00h, 21-23:00, and 23-01:00h (p-value < 0.05, Kruskal-Wallis test), but not in the afternoon (15-17:00h; p-value > 0.05). Males and females do not show any differentiation in the quantity of volatile emission during 15-17:00h, but differentiation in the quantity of emissions became apparent as the night progresses (seen in Figure 8(a)). Only females increase their emission of volatiles after 17:00h. This increase in volatile emission is concordant with the timing of sexual activity in this species (Myers *et al.*, 2015).

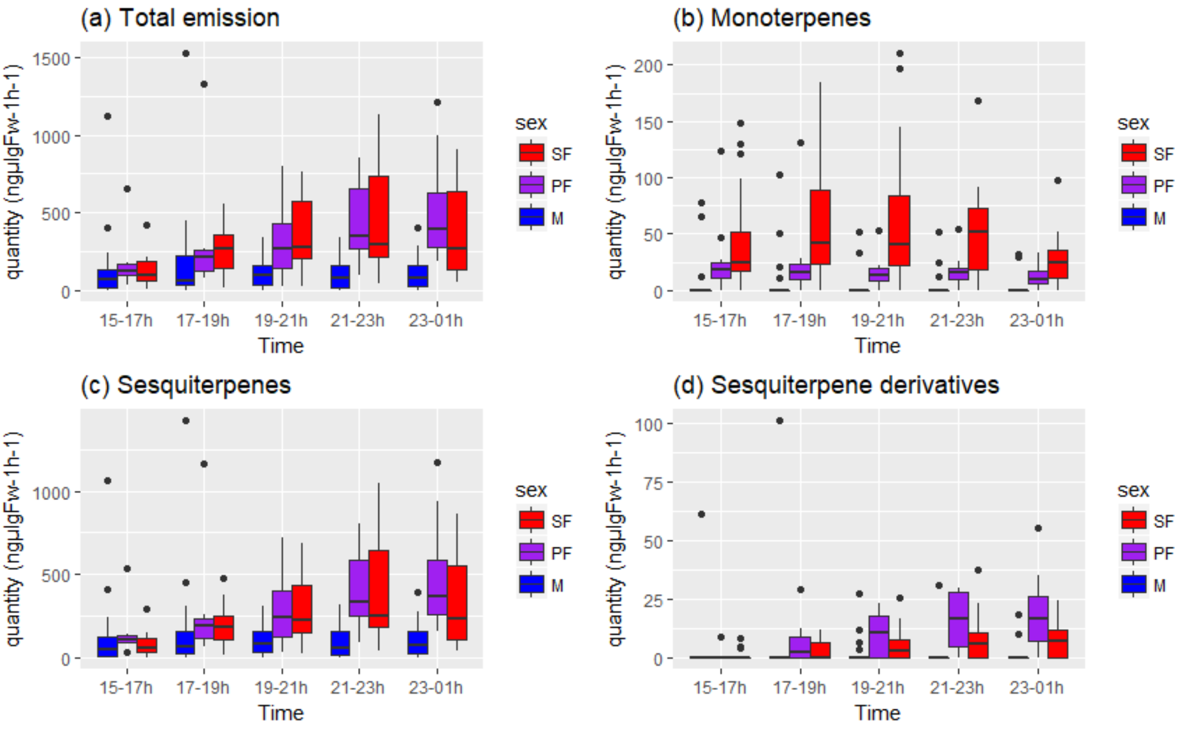


Figure 8. Higher quantity (ng*gFw^{-1}h^{-1}) of volatile emission in sexual (SF, red) and parthenogenetic (PF, purple) females compared to males (M, blue). Increase in the quantity of emission overtime (15-01:00h) in females but not in males. A significant difference in the quantity of emission observed between males and females (sexual and parthenogenetic) between 17-01:00h (p-value < 0.05, Kruskal-Wallis test).

Clitarchus hookeri release volatiles derived from their food plants. Among males, the highest quantity of volatile emission observed in Whanganui males, followed by Paraparaumu and Auckland males (Figure 9(a)). However, the highest emission of each of the compounds differed between populations: the highest quantity of alpha-caryophyllene was observed in Whanganui males (Figure 9(b)), eudesma-4(14), 11-diene in Auckland males (Figure 9(c)), and alpha-pinene in Paraparaumu males (Figure 9(d)). This show that populations of *C. hookeri* males differ in their chemical profiles.

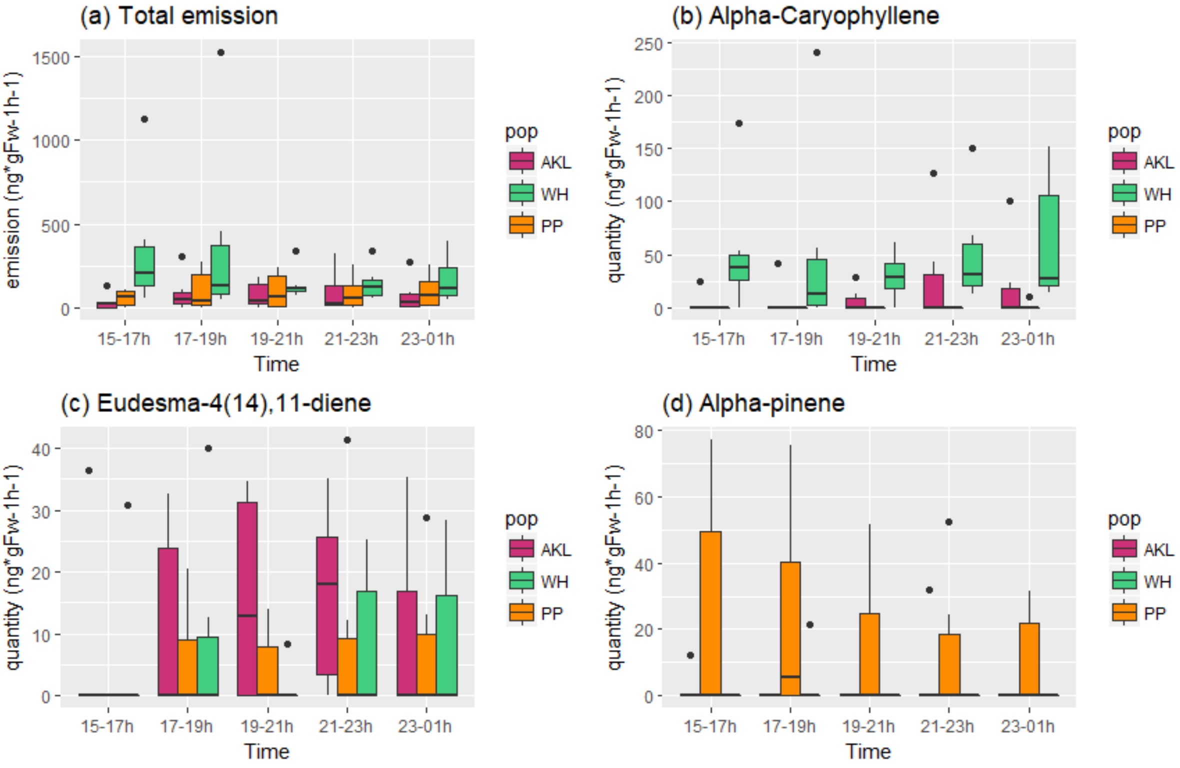
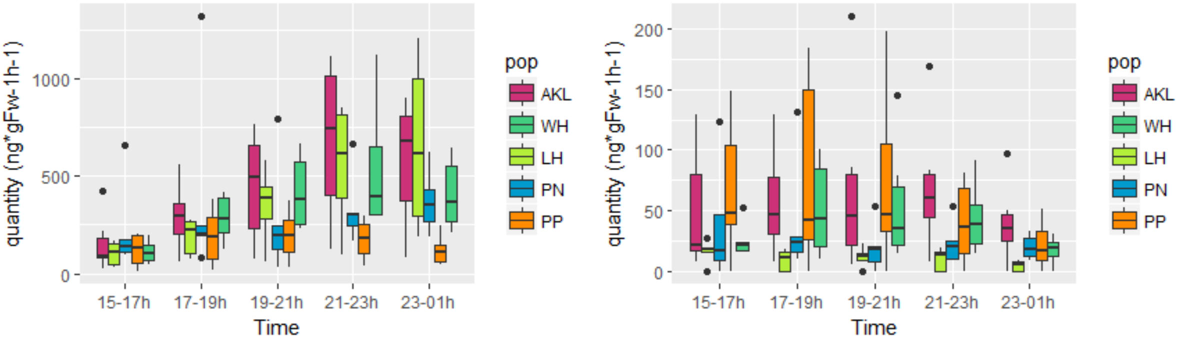


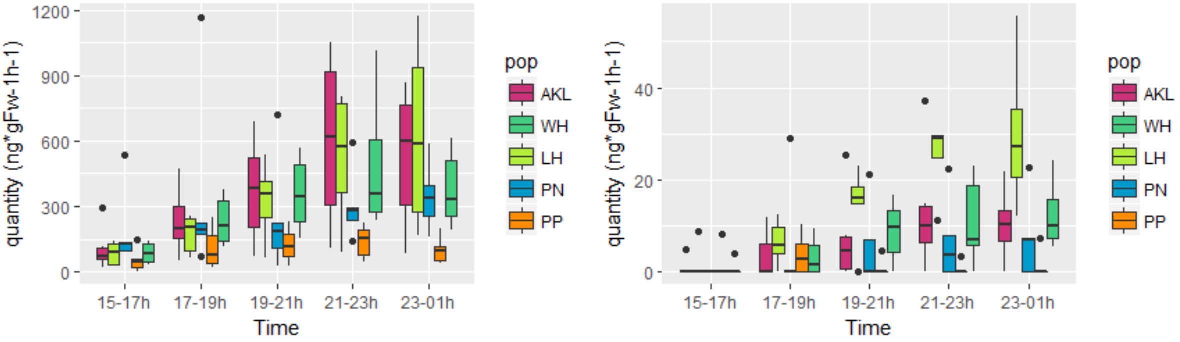
Figure 9. Variation in the quantity (ng*gFw^{-1}h^{-1}) of total emission (a) and emission of different compounds (b)-(d) in male *Clitarchus hookeri* from different populations (Auckland: AKL, pink; Whanganui: WH, green; Paraparaumu: PP, orange).

2.3.2.2. Sexual vs. Parthenogenetic females

All females examined increased the amount of volatiles emitted over the 10 hours measured (15-01:00h) (Figure 10). However, the change in the quantity of volatile emission was only significant for Lower Hutt and Whanganui females (p-value < 0.05, Kruskal-Wallis test). Thus, the degree of increasing volatile emission overtime differ between the females from different populations.


(a) Total emission

(b) Monoterpenes


(c) Sesquiterpenes

(d) Sesquiterpene derivatives

Figure 10. All examined females have shown an increase in the quantity (ng*gFw^{-1}h^{-1}) volatile emission overtime (15-01:00h). However, the quantity of volatile release varies between populations with the highest emission observed in Auckland (AKL) and Lower Hutt (LH) females (a). Increase in the quantity of emission overtime in monoterpenes only seen in Whanganui (WH) and Paraparaumu (PP) females (b), sesquiterpenes in Auckland, Whanganui, Lower Hutt, and Palmerston North (PN) females (c), sesquiterpene derivatives in Whanganui and Lower Hutt females (d).

The quantity of total emission ($ng*gFw^{-1}h^{-1}$) did not show significant differentiation between sexual and parthenogenetic females at any volatile collection time frames (p-value > 0.05, Kruskal-Wallis test; Figure 8(a)). Instead, the quantity of emission significantly varied between populations at 21-23:00h and 23-01:00h (p-value < 0.05, Kruskal-Wallis test), and Auckland and Lower Hutt females released the highest quantity of volatiles (Figure 10(a)). There was also variation in the time of maximum amount of volatile release observed between populations: Auckland and Whanganui at 21-23:00h Paraparaumu females at 17-19:00h, 23-01:00h in Lower Hutt and Palmerston North females (Figure 10(a)). These results indicate that sexual females do not necessarily release higher quantity of volatile than parthenogenetic females, and the time when the maximum volatile emission observed vary between the females sampled from different populations.

As mentioned above, Auckland and Lower Hutt females released the highest quantity of volatiles (Figure 10(a)). However, this does not mean that Auckland and Lower Hutt females released the highest quantity of every compounds that were identified. For example, the highest emission of isoledene/aromandrene was observed in Paraparaumu females (Figure 11(a)); and butylated hydroxytoluene in Palmerston females (Figure 11(b)). Moreover, not all the volatile groups (i.e. monoterpenes, sesquiterpenes, and sesquiterpene derivatives) have increased the quantity of emission overtime, and the volatile groups that have increased emission overtime varied between populations: increase in monoterpene emission seen only in Whanganui and Paraparaumu females (Figure 10(b)); sesquiterpenes in Auckland, Whanganui, Lower Hutt and Palmerston North females (Figure 10(c)); and sesquiterpene derivatives only in Lower Hutt and Whanganui females (Figure 10(d)). Variation in the emission of other compounds among populations in males and females are given in Appendix Figure 1 and 2. These results show that both males (stated in 2.3.2.1) and females from different populations have different chemical profiles although they were fed with same host plants.

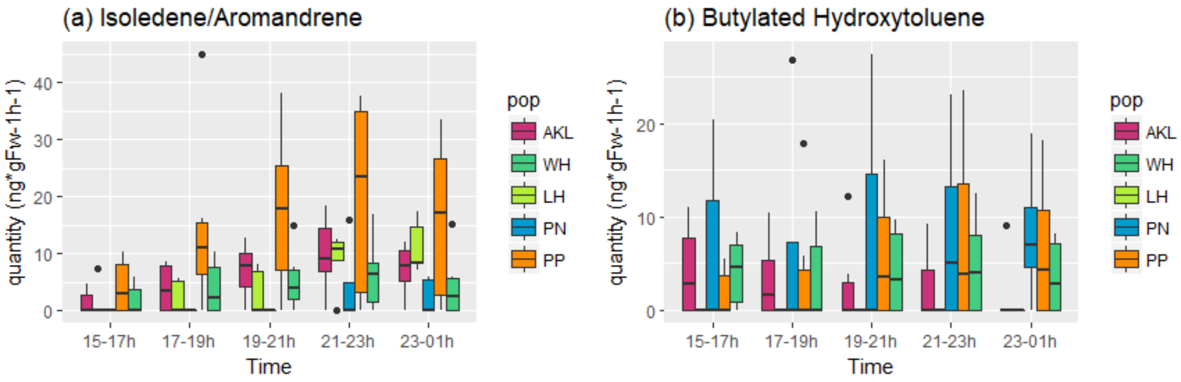


Figure 11. Although Auckland (AKL, pink) and Lower Hutt (LH, yellow) released the highest quantity (ng*gFw^{-1}h^{-1}) of volatiles (Figure 10(a)), (a) isoledene/aromandrene and (b) butylated hydroxytoluene were released the highest by Paraparaumu (PP, orange) and Palmerston North (PN, blue) females respectively.

2.3.3. Male mate choice

2.3.3.1. Mating behaviour

In the open arena male choice experiment, males and females were placed together at 20:00h. Both males and females stayed inactive when it is still light; and became active in complete dark (after 21:00h during January to March and almost as soon as placed in the open arena at 20:00h during April). After sunset and when completely dark, males started to move around the tank and/or feed on mānuka, but females were less active than males; they tended to stay on the branch and started to feed rather than moving from one branch to another. Typically, males made a choice of mating partner between 21:00-00:00h during January to March; and the time for making a choice became earlier as the time for sunset became earlier during April (sunset was earlier than 20:00h) and some males made a choice as soon as they placed into open arena at 20:00h.

Some males initiated mating as soon as they chose a female, while others took time until they initiate mating. In the latter case, females tend to move around (sometimes vigorously) before mating. This duration (i.e. from males making contact until the pair start mating) may be an indication of precopulatory female choice.

When males had attempted on mating, they curled their abdomen to direct their poculum towards the female abdomen. Then, phallus was everted from the poculum to insert into female's operculum. Females opened her operculum if she accepted the male, but did not if she reject. This can be considered as another indication of a precopulatory female choice. Males mated with females for about 15-30minutes (this is the duration of time when males kept his phallus inserted into female genitalia and females kept their operculum open).

Males from all three sample locations were observed to mate with sexual and parthenogenetic females, and parthenogenetic females did not reject males. Furthermore, parthenogenetic females have exhibited the same copulatory behaviours observed in sexual females.

2.3.3.2. Male choice

2.3.3.2.1. Precopulatory choice: first (*1st*) and second (*2nd*) choice

From 61 trials, males chose a mating partner for 49 times i.e. 80% of times males made a choice. This shows that most of the males initiated mating during the trials of January to May 2018. The probability of males choosing a second female (*2nd*) was not high; only 18% of males were observed to select second choice regardless of mating with *1st.*

Clitarchus hookeri males did not discriminate between sexual vs. parthenogenetic females for their first choice (*1st*) (p-value=0.39, Binomial test). Interestingly, males chose parthenogenetic females slightly more often than sexual females (sexual: 43%, parthenogenetic: 57%). Comparing populations, the frequency of Paraparaumu, Palmerston North, and Lower Hutt females being *1st* was above 50% (Figure 12(a)). For Auckland and Whanganui females, the frequency of being *1st* was lower: 28% and 45% respectively) (Figure 12(a)).

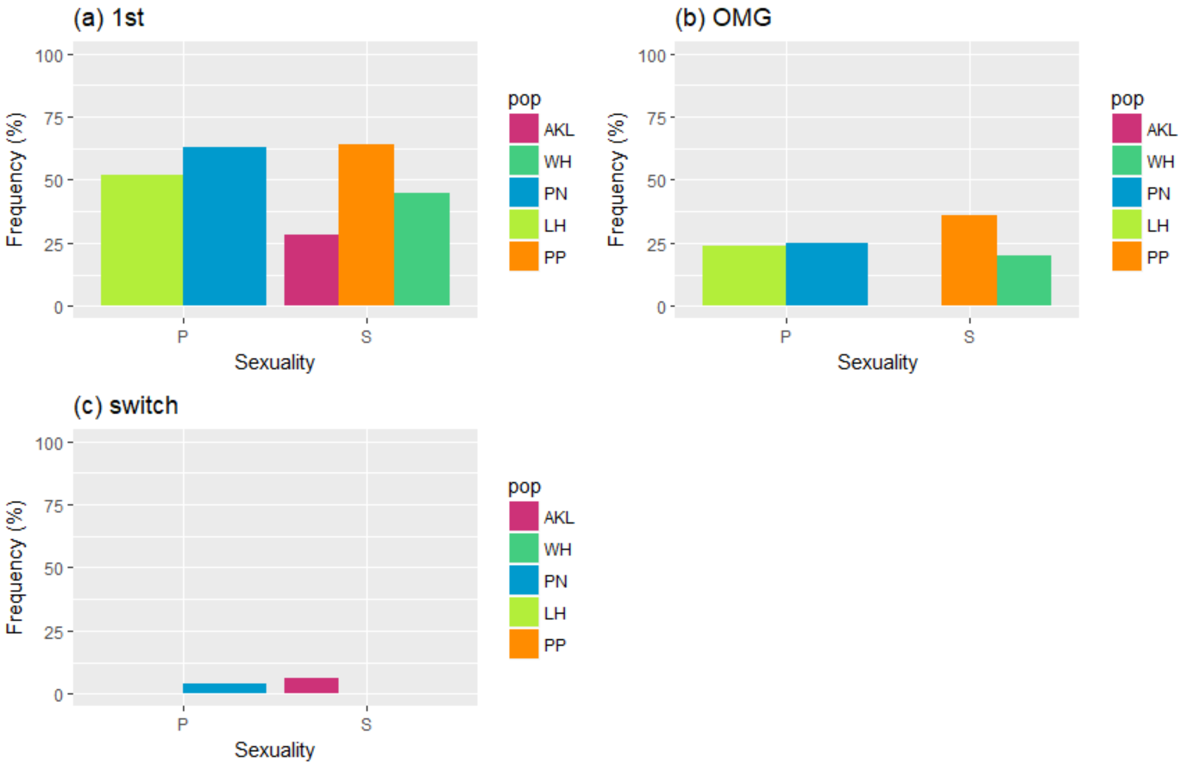


Figure 12. Frequencies of male mate choice factors (*1st*, *OMG*, and *switch*) between females from different populations (Auckland: AKL, pink; Whanganui: WH, green; Paraparaumu: PP, orange; Lower Hutt: LH, yellow; Palmerston North: PN, blue).

Male preference for morphological traits of females was also compared. Males preferred the females with lighter body mass (g) significantly more than heavier females as *1st* (p-value < 0.01, Binomial test). However, males did not discriminate females between longer vs. shorter total body length (p-value = 0.57, Binomial test).

2.3.3.2.2. Postcopulatory choice: overnight mate guarding (*OMG*) and probability of changing partner (*switch*)

More than half of the males did not mate guard females overnight; the probability of males mate-guarding 00:00-09:00h (*OMG*) was 41%. Males did not discriminate between sexual and parthenogenetic females in their postcopulatory choice (p-value > 0.05, Binomial test) and provided *OMG* to sexual (16%) and parthenogenetic females

(20%) in similar frequency. For between populations, there was more variation in the frequency of *OMG*: 0% in Auckland, 20% in Whanganui, 36% in Paraparaumu, 25% in Palmerston North, and 24% in Lower Hutt (Figure 12(b)). Males did not discriminate between lighter and heavier (p-value > 0.05, Binomial test) nor shorter and longer (p-value > 0.05) in their post-copulatory choice. However, males provided *OMG* slightly more frequently in females with shorter body length (41%) than the longer ones (27%). These results suggest a male preference for females after copulation may be based on population differences or size rather than reproductive mode.

The overall frequency of changing partner (*switch*) was low (4%). The two males who switched partners mated with both Auckland and Palmerston North females (Figure 12(c)).

2.4. Discussion

Sexual selection favours the traits that enhance mobility and mate searching ability in males when the species are under scramble competition mating system (Kelly, 2014, Holwell *et al.*, 2007). Males find their mating partners by receiving signals released from females; typically, chemical cues are used in insects (Holwell *et al.*, 2007, Lubanga *et al.*, 2014). These distinctive sex roles result in sexual dimorphism in phenotypic traits. Under these concepts, the morphological and chemical differentiation in male and female was explored in *Clitarchus hookeri*. This was analyzed to identify the traits that are under distinctive sex roles in scramble competition. However, *C. hookeri* include both sexual and parthenogenetic females. It is common that phenotypic traits that are involved in sexual reproduction are decayed in parthenogenetic lineages, which is seen in other stick insect species (*Timema* spp.: Schwander et al., 2013; *Extatosoma tiaratum*: Burke et al., 2015). Thus, the second objective of this study was to elucidate whether sexual traits seen in sexual females are also observed in parthenogenetic females. Finally, as parthenogenetic females are possibly investing less on sexual reproduction, males are likely to discriminate between sexual vs. parthenogenetic females in their mate choice. Therefore, I investigated whether males used the morphological and chemical differences in sexual and parthenogenetic females for their precopulatory and postcopulatory choice in open arena mate choice experiment.

I revealed that *C. hookeri* exhibit sexual differentiation of morphological and chemical traits that are advantageous to their roles in scramble competition. However, sexual and parthenogenetic females overlapped in terms of both morphology and chemical signalling. Parthenogenetic females showed traits that are related to sex roles in scramble competition even though they are no longer under the selective pressure of scramble competition mating system. Moreover, the morphological and chemical variation in *C. hookeri* females were attributed to between population variations rather than between reproductive mode (i.e. sexual vs. parthenogenetic) differences. Thus, these results do not support the expected decay in sexual traits in parthenogenetic females. Furthermore, males failed to discriminate between sexual vs. parthenogenetic females in both pre- and post-copulatory choices. Instead, males exhibited a significant preference towards females with lighter body mass compared to heavier ones in their precopulatory choice.

2.4.1. Males vs. Females: distinctive sex roles in scramble competition

2.4.1.1. Morphological differentiation

In *C. hookeri*, males and females have different roles in scramble competition mating system: males as searchers and females as signallers (Myers *et al.*, 2015). These distinctive sex roles result in morphological differentiation between the sexes. For example, as being a searcher, the traits that enhance mobility and mate location will be favoured in males (Kelly, 2014, Myers *et al.*, 2015, Holwell *et al.*, 2007, Lubanga *et al.*, 2014). My morphological analysis showed that *C. hookeri* males clearly possess smaller body (in terms of body length and mass) with disproportionately longer length of legs and antennae compared to that of females (of advantage to their sex role finding mates). Past study has shown that *C. hookeri* males tend to move around more than females during their reproductive season, and find females using olfactory cues (Myers *et al.*, 2015). Therefore, having smaller body size with longer legs will allow them to move around more effectively than females, and the antennae are disproportionately longer in males possibly to allow them to locate females using chemical signals.

The width of poculum was disproportionately wider in relation to body length in males compared to operculum in females (Figure 5(h)), and actual width was only slightly wider in females (males: 0.207-0.320cm, sexual females: 0.251-0.406, parthenogenetic females: 0.203-0.347cm). This disproportionately wider poculum compared to male body size possibly to match with the width of the female operculum. In *C. hookeri*, the past studies identified the coevolution of external genitalia in males (claspers) and females (operculum) in terms of the shape, and this functions as reproductive isolation between species (Myers, 2014, Buckley *et al.*, 2014). This coevolutionary interactions of male and female genitalia are widely common in animals (Brennan and Prum, 2015, Lupse *et al.*, 2016, Ramos *et al.*, 2005, Bedford *et al.*, 2004). As both poculum and operculum functions as a coverage of internal genitalia in *C. hookeri* (Myers *et al.*, 2016, Stringer, 1970), the width correspondence may have resulted from genital size coevolution between males and females, to allow copulation. Furthermore, as the genital contact during mating is suggested to be important in male choice for mate guarding in *C. hookeri* (Myers, 2014), it is possible that the operculum width is used during copulation for assessment of mate quality or compatibility.

My morphological analysis showed that *C. hookeri* females are longer (body length) and fatter (body mass, and abdomen width) with disproportionately shorter leg length compared to males. This fits my prediction that if *C. hookeri* females are signallers in their roles of scramble competition mating system, they do not require a body shape to enhance their mobility. Body size (length, width, and weight) distinguishes the two sexes in *C. hookeri*, and body size often positively correlated with fecundity (Bonduriansky, 2001, Edward and Chapman, 2011, Honěk, 1993). Therefore, *C. hookeri* females are larger than males not only because they have little need to search but because this provides fecundity advantage.

2.4.1.2. Chemical differentiation

Production of sex pheromones are used to attract males in many insects, and they are the important tool for discriminating sexes from a long distance (Stokl and Steiger, 2017, Reddy and Guerrero, 2004, Tabata *et al.*, 2017, Holwell *et al.*, 2007). However, my results found that none of the compounds released were specific to females. I found that both male and female *C. hookeri* produce volatile compounds that are derived from what they eat. Mānuka *Leptospermum scoparium* (from Palmerston North) was used to feed all the insects in this study. Every compounds that were identified from the insects belong to the chemical class of terpenoids (monoterpenes, sesquiterpenes, and sesquiterpene derivatives). All the individuals released the highest quantity ($ng*gFw^{-1}h^{-1}$) of sesquiterpenes among chemical groups (Figure 8) which is the major volatile compounds in mānuka (Douglas *et al.*, 2004, Van Vuuren *et al.*, 2014). Thus, it is unlikely that *C. hookeri* females release specific pheromones to attract males.

Instead of between sex differentiation, the chemical profiles of *C. hookeri* varied between populations for both males and females (Figure 9 & 11; Appendix Figure 1 & 2). As all individuals fed on the same host plants as adults, this variation may be due to different capacity of incorporating each compound into their CHCs between populations. However, I could not control for early exposure to different host plants. All stick insects were collected as late instars so each population would have fed on different populations of mānuka or kānuka in the first few months of their lives, and the chemical profiles of mānuka are known to diversify geographically (Douglas *et al.*, 2004, Van Vuuren *et al.*, 2014). Thus, the variation in chemical profiles between populations may be derived from both the diversity of chemical composition of their host plants and populations differences in physiology.

However, my results revealed that only females increase the quantity of volatile emissions during 17-01:00h, with maximum emission observed at 21-23:00h for most of females studied (Figure 8). Males, in contrast, showed no significant variation in the amount of volatiles they emitted over the night. Moreover, the timing of females' maximum emission was consistent with the timing when many males had made their choices in mate choice observations (2.3.3.1). As *C. hookeri* forage and mate on their host plants and their CHCs smell the same as their food, males have to locate females within an environment of same smell. However, many terpenoid compounds cannot be synthesized by plants at night due to their distinctive biosynthetic pathway that requires light for their synthesis (so-called methylerythritol phosphate pathway) (Arimura *et al.*, 2004, Gershenzon and Boland, 2005, Akhila, 2007, Dudareva *et al.*, 2005). Therefore, *C. hookeri* females are increasing their volatile emissions at night when the host plants cannot synthesize these particular chemicals, but when this species is sexually active, and they are using plant derived chemicals that the males must have receptors for.

2.4.2. Sexual vs. Parthenogenetic females: trait variation between reproductive strategies or between populations?

2.4.2.1. Morphological differentiation

Formerly adaptive traits can be selected against due to changes in selective pressure, if there are environmental shifts or changes in the life history of the population. Sexually selected traits in parthenogenetic lineages are traits that can be rapidly reduced or lost completely (Schwander *et al.*, 2013, Burke *et al.*, 2015, Tabata *et al.*, 2017, van der Kooi and Schwander, 2014). In *C. hookeri*, the historical range expansions after the last glacial maximum (LGM) had led to the evolution of parthenogenetic lineages, and all-female populations occur in the south-east range of New Zealand (Morgan-Richards *et al.*, 2010, Buckley *et al.*, 2010). As these females are no longer exposed to males, those traits that are under the selective pressures of scramble competition and sexual reproduction are expected to decay.

My morphological analyses have shown that sexual *C. hookeri* females have longer (body length) and fatter (body mass and abdomen width) body on average than parthenogenetic females (Figure 7(a), (c), (e)). Female body size is often positively correlated with fecundity (Bonduriansky, 2001, Edward and Chapman, 2011, Honěk, 1993), and sexual females mate multiple times with males (i.e. promiscuity) (Myers *et* *al.*, 2015). Thus, larger body size in sexual females compared to parthenogenetic females may be due to the stronger selective force operating on fecundity selection in sexual females in relation to their promiscuity.

However, although sexual females were generally larger than parthenogenetic females, the body size in females have shown significant variation between populations, and overlapped between sexual and parthenogenetic females. Examples include the high variation in the body length of Palmerston females that overlap with the females from every other populations (Figure 7(b)); and the body mass in relation to body length in Paraparaumu (sexual) that have shown significant differentiation with sexual females (Auckland and Whanganui) but not with parthenogenetic females (Palmerston North and Lower Hutt). These results show that female body size is likely to be influenced not only by their reproductive mode, but also between population variations.

One of the possible effect on the body size that have resulted in variation between populations is a latitudinal effect. Those females from the populations south to Palmerston North (i.e. Lower Hutt and Paraparaumu) tend to have smaller body size (body length and body mass) than that of the females from the populations north to Palmerston North (Figure 13). This tendency is also seen in males (Figure 14). This latitudinal trend of increasing body size in lower latitude is called converse Bergmann's rule and seen in other species of insects (Blanckenhorn and Demont, 2004, Whitman, 2008). The possible cause of this latitudinal cline is mediated by seasonal limitation for developmental time in higher latitude, and those insect species exhibit large body size and long developmental time tend to exhibit converse Bergmann's rule (Whitman, 2008, Blanckenhorn and Demont, 2004). In *C. hookeri*, their body size are relatively large (males: 5.8-7cm, females: 7.3-10cm) and they take few months to complete their final moult (those insects caught at the end of November 2017 as fourth or fifth instars became adults during late December or early January 2018). Furthermore, although reproductive season for *C. hookeri* is during summer i.e. December to April (Myers *et al.*, 2015) and many adults and late instars (fourth-sixth) were spotted from Auckland, Whanganui and Palmerston North during December, no adults or late instars were spotted from Paraparaumu and Lower Hutt (only first-third instars) in December (personal obs.). Late instars and adults were seen in Paraparaumu and Lower Hutt during March-April. This may be inferring that Lower Hutt and Paraparaumu females are limited in available developmental time (March to April?) compared to Auckland, Whanganui, and Palmerston North females, which further results in smaller body size. However, additional observations are required for the possibility of latitudinal variation in available developmental time between populations with inclusion of the morphological analyses for the individuals from South Island to elucidate whether there really is a latitudinal effect in their morphology.

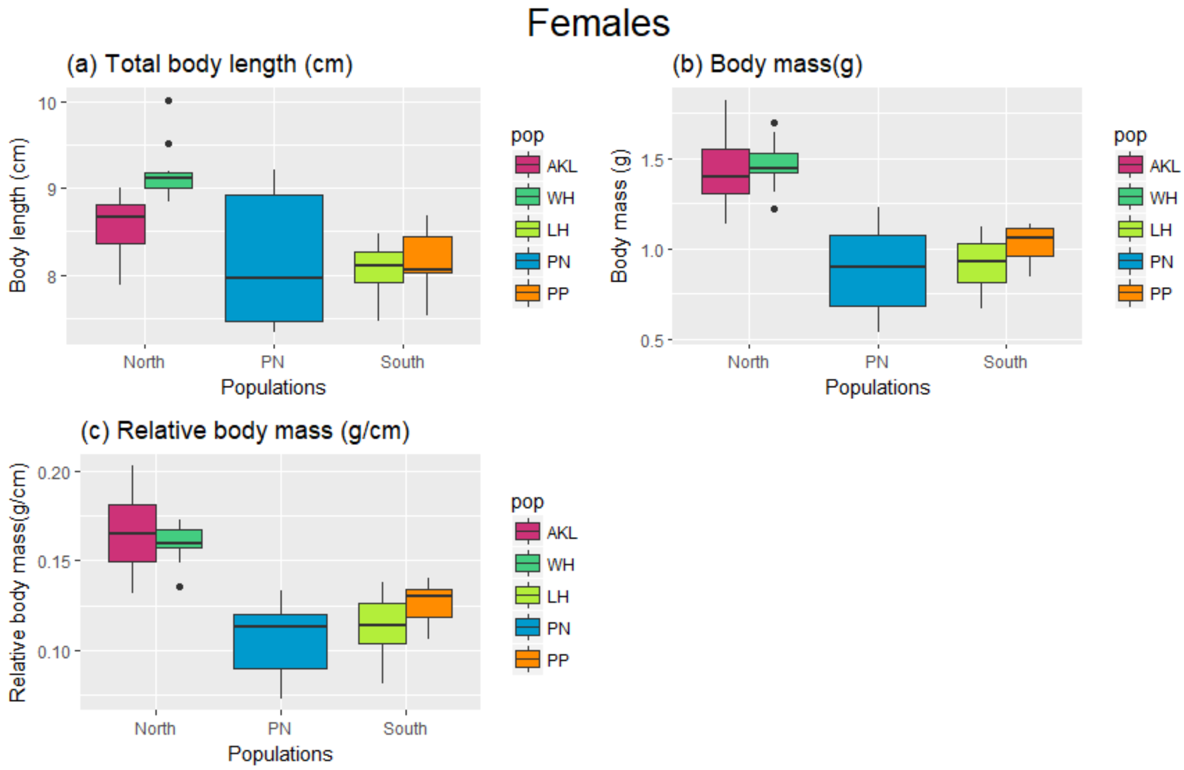


Figure 13. Showing the possibility of the converse Bergmann's rule in female *C. hookeri*. Populations north to Palmerston North (PN) (i.e. AKL: Auckland and WH: Whanganui) are generally larger and south to Palmerston North (i.e. PP: Paraparaumu and LH: Lower Hutt) are generally are smaller.

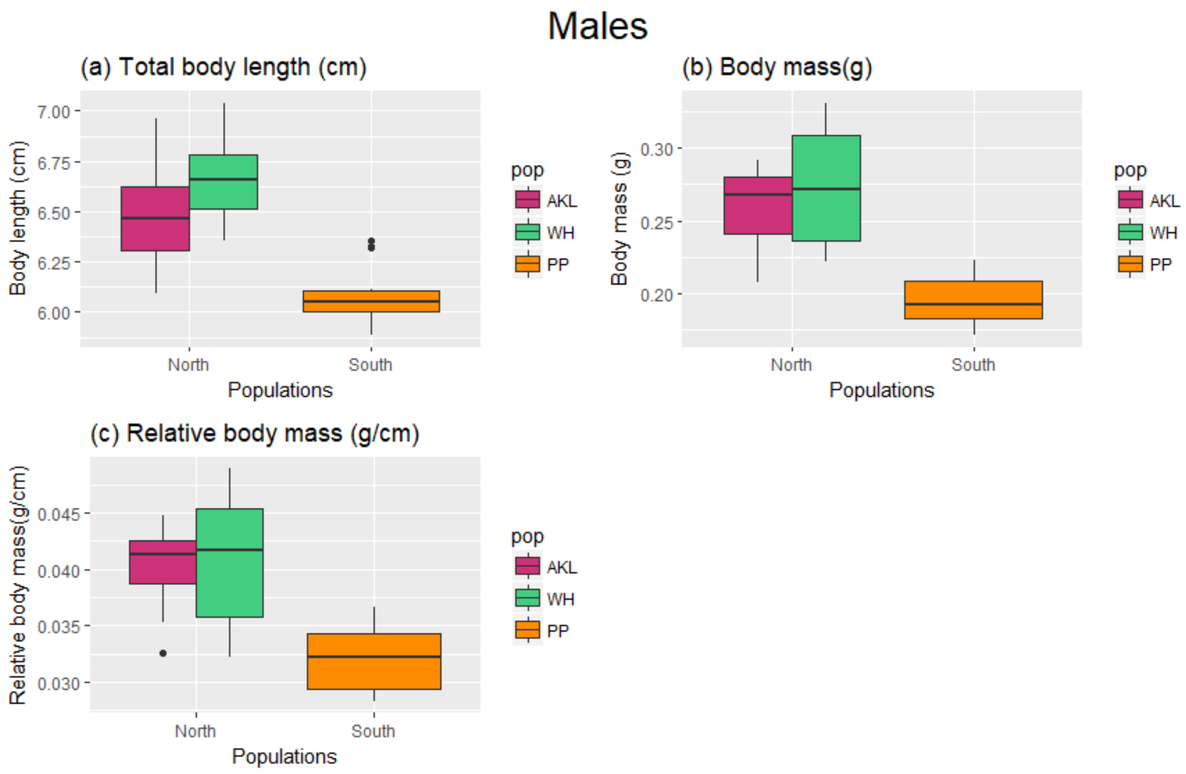


Figure 14. Showing the possibility of the converse Bergmann's rule in male *C. hookeri*. North and south on x-axis refers to the populations that are north (AKL: Auckland and WH: Whanganui) and south (PP: Paraparaumu) to Palmerston North respectively.

2.4.2.2. Chemical differentiation

The communication system that males and females use for their mate location and attraction is one of the traits that has been seen to decay rapidly in parthenogenetic lineages. For example, in the obligate parthenogenetic bush-crickets *Poecilimon intermedius*, the auditory structure that was used to receive male calls had been reduced (Lehmann *et al.*, 2007). As in for stick insects, chemical profiles of parthenogenetic lineages have altered in *Timema* (Schwander *et al.*, 2013) and *Extatosoma tiaratum* (Burke *et al.*, 2015).

In the case of *C. hookeri*, as the increase in the quantity of volatile emission was observed only in females and the change in volatile emission had occurred when the species are sexually active; this change in volatile release in *C. hookeri* females is likely to be related their role in scramble competition mating system i.e. signallers. However, both sexual and parthenogenetic females increased the quantity of volatile emission over 10 hours of volatile collection. Moreover, the degree of increase in the quantity of volatile emission varied among populations. One sample from a parthenogenetic population (Lower Hutt), had a greater degree of increase in volatile emission than some sexual populations (Auckland and Paraparaumu). Thus, these results show that the mechanism of sexual signalling is still maintained in parthenogenetic females, and the intensity of signalling varied between populations rather than between reproductive strategies.

The fact that parthenogenetic females have maintained their quantity of emission suggests that parthenogenetic females are still maintaining their sexual traits. This possibility was also seen in their morphology (e.g. relative body mass) that overlapped with sexual females. The reason behind the maintenance of sexual traits in parthenogenetic females may be due to their peculiar pattern of geographical distribution. *Clitarchus hookeri* exhibit geographical parthenogenesis with (mostly) northern sexual and southern parthenogenetic populations (Buckley *et al.*, 2010, Morgan-Richards *et al.*, 2010). The range expansion of *C. hookeri* is likely to have occurred < 2,5000 years ago after LGM which is when parthenogenetic lineages are inferred to had evolved (Buckley *et al.*, 2010). If correct, the relatively recent evolution of the parthenogenetic lineages in *C. hookeri* might not provide time for the loss of sexual traits. Parthenogenetic lineages may take millions of years to generate phenotypic expressed variation of sexual traits and their loss, especially when the traits are under drift rather than selection (van der Kooi and Schwander, 2014, Schwander *et al.*, 2013). Furthermore, some of the sexual populations in *C. hookeri* occur in the southern range (e.g. Wellington and Paraparaumu) and the populations from southern area has low genetic diversity (Morgan-Richards *et al.*, 2010); thus, it is possible that parthenogenetic females may not be isolated from males completely in *C. hookeri*, and the selective force is not strong enough to allow decay in sexual traits in parthenogenetic females. Therefore, relatively recent origin of parthenogenetic lineages and possibility of lack in complete isolation from males may have resulted in the maintenance of sexual traits in parthenogenetic females in *C. hookeri*.

2.4.3. Male mate choice in *Clitarchus hookeri*: sexual vs. parthenogenetic females

Onset of sexual activity according to the photoperiod is common in insects (Tabata *et al.*, 2017, Charlton and Cardé, 1982). This is also seen in *C. hookeri*, as both males and females became active and males always chose their partner after sunset during the observations of January-May 2018. The males chose partner at 21-00:00h during January to March and the timing of when males made choice became earlier as the sunset became earlier during April to May (sunset before 20:00h and some males made choice as soon as they are placed into the arena). Furthermore, this onset of sexual activity according to photoperiod is also seen in the pattern of volatile emission in females (discussed in 2.4.2.2). Altogether, these results show that sexual activity in *C. hookeri* corresponds with the photoperiod.

Male mate choice evolves when there is a high variation in female quality and the reproductive output of individuals increase from choosing to mate with females that have particular quality (Bonduriansky, 2001, Edward and Chapman, 2011). In the case of *C. hookeri*, it will be advantageous for males if they can discriminate between sexual and parthenogenetic females due to the possibility of parthenogenetic females investing less in sexual reproduction (Morgan-Richards *et al.*, 2010). One of the possible traits that may be decayed in parthenogenetic females is courtship behaviours (Carson *et al.*, 1982, Lehmann *et al.*, 2011, Schwander *et al.*, 2013). However, as parthenogenetic females have successfully copulated and did not actively reject males, the courtship behaviours are still kept in the parthenogenetic females in *C. hookeri*.

2.4.3.1. Precopulatory choice: the first female that males have chosen to mate

Male *Clitarchus hookeri* did not discriminate between sexual and parthenogenetic females in their precopulatory choice; therefore did not support my prediction. This may be due to lack in phenotypic differentiation between sexual and parthenogenetic females both in terms of morphology and chemical signalling (discussed in 2.4.2.).

Instead, the results have shown significant preference towards the females with lighter body compared to the heavier ones. This male preference to lighter females was also observed in past study and this is considered to be preference towards virgin females (Myers *et al.*, 2015). However, although parthenogenetic females generally have lighter body mass (discussed in 2.4.2.1), the significant preference for parthenogenetic females was not observed. The males showed some preference towards Paraparaumu (sexual), Palmerston North, and Lower Hutt (parthenogenetic) in relatively equal frequencies compared to Auckland or Whanganui females (sexual) (Figure 12(a)). This may be due to the fact that Paraparaumu females have lighter body mass compared to the females from other sexual populations (i.e. Auckland and Whanganui). Thus, these results confirm that males choose females based on body mass rather than reproductive mode in females.

The next question is, how do males assess female body mass? One of the possible assessment base can be total body length of females. However, as males did not discriminate between longer and shorter females, it is unlikely that *C. hookeri* males assess female body mass by their body length. Furthermore, the morphological analysis showed that those females that have longer body length do not necessarily have heavier body mass (e.g. Palmerston North females) and vice versa (e.g. Lower Hutt and Paraparaumu females are heavier but shorter in comparison to Palmerston North females) (Figure 7(b)).

Alternatively, males may be assessing female body mass by their abdomen size. Female abdomen size and weight increase during their adulthood in *C. hookeri* and newly moulted adult females have smaller abdomen size with lighter body mass (personal obs.). Thus, as young female adults have lighter body with thinner abdomen, it is possible that males choose females based on the size of abdomen. However, I cannot exclude the possibility that chemical signalling is also involved as population-level variation in chemical profiles were detected (2.3.2.).

2.4.3.2. Post-copulatory choice: frequency of mate-guarding overnight (*OMG*) and frequency of changing partner after copulation (*switch*)

The benefit of mate-guarding in males is to repel rivals and to ensure their own insemination is completed (Alcock, 1994). Past study has identified that male *C. hookeri* mate guarded one-night on average when operational sex ratio (OSR) is 1:1 (Myers *et al.*, 2015), and this mate-guarding duration is consistent with the cycle of female egg laying (Stringer, 1970).

In this study, the frequency of males mate guarding over-night (00:00-09:00h) was examined under conditions where rivals were absent. The results have shown that overall, less than half of males provided mate guarding overnight. *Clitarchus hookeri* males adjust the duration of mate guarding according to OSR and the duration were longer when the OSR was male-biased (maximum 10 nights) (Myers *et al.*, 2015). Thus, the reason why more than half of the males did not provide mate guarding in this study may be associated with the experimental design where males were exposed to two females without any competitors.

The benefit of mate guarding will also increases if there is a high variation in female quality (Alcock, 1994). The main focus of the female quality in this study was different reproductive mode i.e. sexual vs. parthenogenetic in female *C. hookeri*. Past study has demonstrated that males were able to discriminate between conspecific and heterospecific females possibly through the genital contact during copulation (Myers, 2014). In this study, it has been elucidated that the actual size of poculum and operculum width is likely to be under genital size coevolution between males and females (discussed in 2.4.1.1.). Although most of the sexual females had similar/wider operculum compared to males (males: 0.207-0.320cm, sexual females: 0.251-0.406), more than a half of parthenogenetic females had thinner operculum width (0.203-0.347cm). Thus, it is possible that males cannot copulate and/or do not provide mate-guarding for those parthenogenetic females that had smaller operculum than poculum. However, as the results show, males did not discriminate between sexual and parthenogenetic females in their postcopulatory choice, and copulated and provided overnight mate-guarding to females with operculum width at any size range (from the thinnest in Palmerston North to the widest in Whanganui). Therefore, it is unlikely that male *C. hookeri* discriminate females by the width of operculum at least between sexual and parthenogenetic females.

Although males have preferred lighter females significantly more than heavier females, males did not discriminate between lighter and heavier females in their postcopulatory choice (frequency of *OMG*: 35% in lighter females, 33% in heavier females). Instead, males have provided overnight mate guarding slightly more frequently in females with shorter body length (41%) compared to the longer ones (27%). However, as past study has shown that there was no correlation between female body length and duration of mate-guarding (but negative correlation between body mass and duration of mate guarding: Myers et al., 2015), the higher frequency of *OMG* in shorter females may be coincidence. Alternatively, lower frequency of male mate-guarding the females with longer body length may be associated with the cost of mate guarding; where males may have increased risk of being detected by predators due to increase in apparent size (Alcock, 1994, Cooper and Vitt, 2002) and most of the predators for stick insects are birds i.e. visual predators (Gibbs, 2010, Haw *et al.*, 2001). Thus, it is possible that lower frequency of males mate-guarding longer females may be associated with the predation costs. However, the costs associated with male mate-guarding needs more observations.

Only 4% of males had changed their partner after copulation (*switch*). The difference between *2nd* and *switch* is the former was recorded if the males had changed his partner from *1st* regardless of mating or no mating, and the latter was recorded when the males had changed his partner after mating with *1st* or *2nd*. As the frequency of *2nd* (18%) was higher than *switch* (4%), it can be considered that males are less likely to seek for another female after mating. Thus, although male *C. hookeri* are able to mate multiply during their lifecycle (Myers, 2014), this may be showing that males have a limited capacity in mating more than once per night.

Lastly, *switch* was observed only in females from Auckland and Palmerston North (Figure 12(c)). Furthermore, although the females from Whanganui, Paraparaumu, Lower Hutt and Palmerston North females have been mate-guarded overnight, none of the males provide mate-guarding for Auckland females (Figure 12(b)). These results show that Auckland females are the least favoured females among the focal populations in this study. The possible reason behind this may be associated with male preference towards the mates that are from geographically adjacent population, which is common in animals (Schwander and Crespi, 2009, Tregenza *et al.*, 2000, Wong *et al.*, 2004, Nosil and Crespi, 2006, Jennings *et al.*, 2011). Auckland is the most geographically distant population (> 500km away from other populations) compared to the distance between other focal populations (100-200km). However, the mechanisms of the discrimination is unknown as none of the traits (morphological and chemical profiles) were specific to Auckland. The possibility of preference towards mate from adjacent populations over foreign will be discussed more in detail in the next chapter (CHAPTER 3).

2.4.4. Conclusion

In conclusion, morphological and chemical traits that are under distinctive sex roles in scramble competition in *Clitarchus hookeri* were identified. These include the body shape in males that enhances their mobility and mate location; and although both male and female *C. hookeri* smell like their food plant, only females increased volatile emission at night when the plants cannot synthesize the compounds. However, clear distinction of phenotypic traits between sexual and parthenogenetic females were not found (both morphology and chemical signalling). Instead, it is likely that high variations observed in female morphology and chemical profiles are influenced not only by their reproductive strategy but also by other evolutionary and ecological factors; including incomplete isolation of parthenogenetic females from males, latitudinal difference in available developmental time, and chemical profiles of their local host plants. Furthermore, male choice experiments have shown that male *C. hookeri* did not discriminate between sexual and parthenogenetic females, which is likely to be the result of the maintenance in sexual traits in parthenogenetic females. Alternatively, males have shown preference towards lighter females in their precopulatory choice.

CHAPTER 3.

Additional analysis

3.1. Introduction and aims

In this chapter, additional analyses were performed to further explore the chemical ecology and male choice in *Clitarchus hookeri*.

In the previous chapter, males failed to discriminate between sexual and parthenogenetic females in the open arena male choice experiment. One possibility behind this is a lack of sex-specific pheromone production by female *C. hookeri*. Thus, the purpose of this chapter is to confirm that *C. hookeri* females are not producing sex pheromones, by exposing males only to the smells using Y-maze olfactometer. Male *Clitarchus hookeri* in a Y-maze do detect conspecifics when given a choice of female or nothing (Myers *et al.*, 2015), suggesting positive response to chemical volatiles. As parthenogenetic females possibly are maintaining sexual traits (discussed in CHAPTER 2), males will be exposed to sexual vs. parthenogenetic females, as well as males vs. females. I predict that male *C. hookeri* cannot discriminate between sexual and parthenogenetic females nor between males and females when they are exposed only to their smells, since it is unlikely that *C. hookeri* females are producing sex-specific pheromones. If all females are producing sex pheromones, males will not discriminate between sexual and parthenogenetic females.

Male choice experiments also showed that Auckland females were least preferred by males in the postcopulatory choice. As Auckland was the most distantly located among the focal populations, this has raised the possibility of male preference for the females from local populations over non-local. This preference towards local mating partners over foreign is widely common among animals, and mate-recognition between populations can occur both before (Schwander and Crespi, 2013, Wong *et al.*, 2004, Tregenza *et al.*, 2000, Schwander and Crespi, 2009) and during or after (Jennings *et al.*, 2011, Nosil and Crespi, 2006) mating. Thus, the second purpose of this study is to elucidate whether male *C. hookeri* has a preference towards females from local populations over foreign both in their pre- and post-copulatory choices. I predict that males will choose females from local populations over non-local both in pre- and post-copulatory choices due to their preference towards local females.

3.2. Materials and methods

3.2.1. Y-maze choice test

Y-maze olfactometer (Figure 15) was used to explore different male response between males vs. females (N=15) and sexual vs. parthenogenetic females (N=15) using chemical signals. The purpose of the former choice assay is due to the possibility of parthenogenetic females maintaining sexual signalling. Y-maze olfactometer avoids smells being diffused throughout Y-maze using airflow that will be directed towards a particular destination. Male was placed at the entrance of bottom tube, and the airflow was directed towards the point where the maze splits into two arms, which allows a male to make a choice when he walks upwards to this point. Female or male was placed at each end of the arms, with their visual being occluded using mesh. Those males and females that were placed in the arms of Y-maze were given ten minutes to get acclimatized to the environment. The airflow was produced using the push-function (0.85L/hour) in VAS (Renssealer, NY, US), with Teflon tube connected to VAS and each and of the arms of the Y- maze where female or male was accommodated.

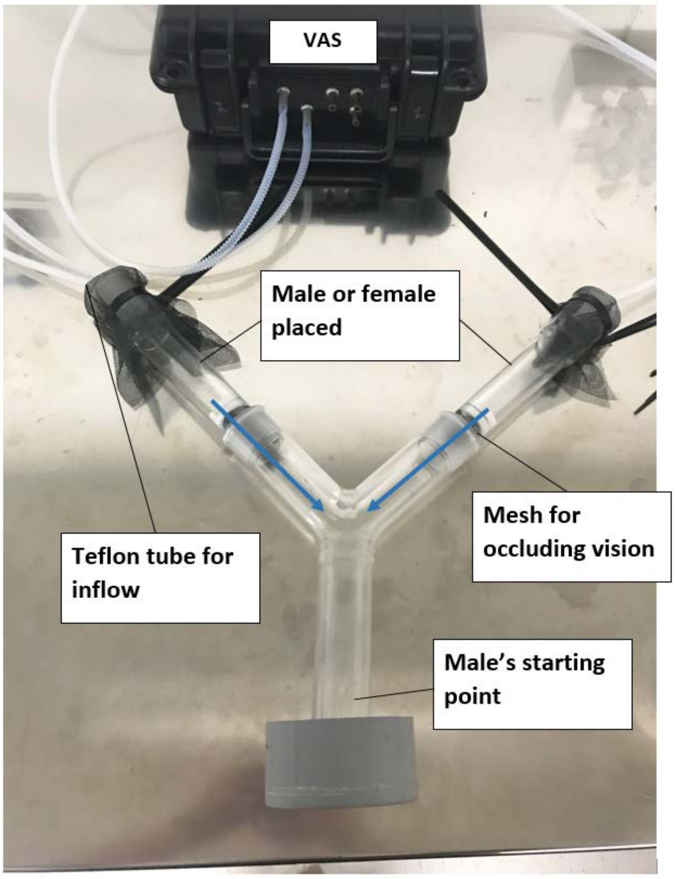


Figure 15. Schematic of y-maze olfactometer.

For the test, those individuals that were used in male choice observations (2.3.3.) were used, i.e. non-virgins. The tests were performed between 20:00-00:00h (lights off) and males were removed from the Y-maze if he did not make a choice within 20 minutes after being placed in the maze.

A binomial test was employed to test the probability of male *C. hookeri* choosing males or females, and sexual or parthenogenetic females differ significantly from the probability of males choosing them in equal frequency (i.e. 0.5).

3.2.2. Open arena male choice

In CHAPTER 2 open arena male choice, the choice was between sexual vs. parthenogenetic females that were both from different populations to males. In this study, a male was exposed to two females, but one of the two females were from the same population as the male (the female from a different population to male was either sexual or parthenogenetic). Then, whether male discriminates between the females from same vs. different populations in their precopulatory and postcopulatory choices were explored. Other procedures and statistical analysis followed the protocols in 2.2.3.

3.3. Results

3.3.1. Y-maze choice test

The y-maze choice tests showed that out of 15 trials, males chose a female nine times and chose a male six times. With a choice of two females, males chose sexual or parthenogenetic females, eight and seven times respectively. This shows that male *C. hookeri* did not discriminate between males and females (p-value = 0.6, Binomial test). In addition, *C. hookeri* males did not discriminate between sexual and parthenogenetic females (p-value = 1, Binomial test) when they were exposed only to the smells. Thus, these results support my hypothesis that female *C. hookeri* do not produce sex-specific pheromones to attract males.

3.3.2. Male choice between females from same vs. different population

3.3.2.1. Precopulatory choice

The results of mate choice experiments showed that male *C. hookeri* did not discriminate between the females from the same vs. different populations for their first choice (*1st*) (p-value= 0.54, Binomial test). However, males chose the females from different population as *1st* slightly more frequently (58%) than the females from the same population (42%) (Figure 16(a)). These results provide no support for my prediction that male *C. hookeri* would prefer females from the same populations more frequently than non-local females.

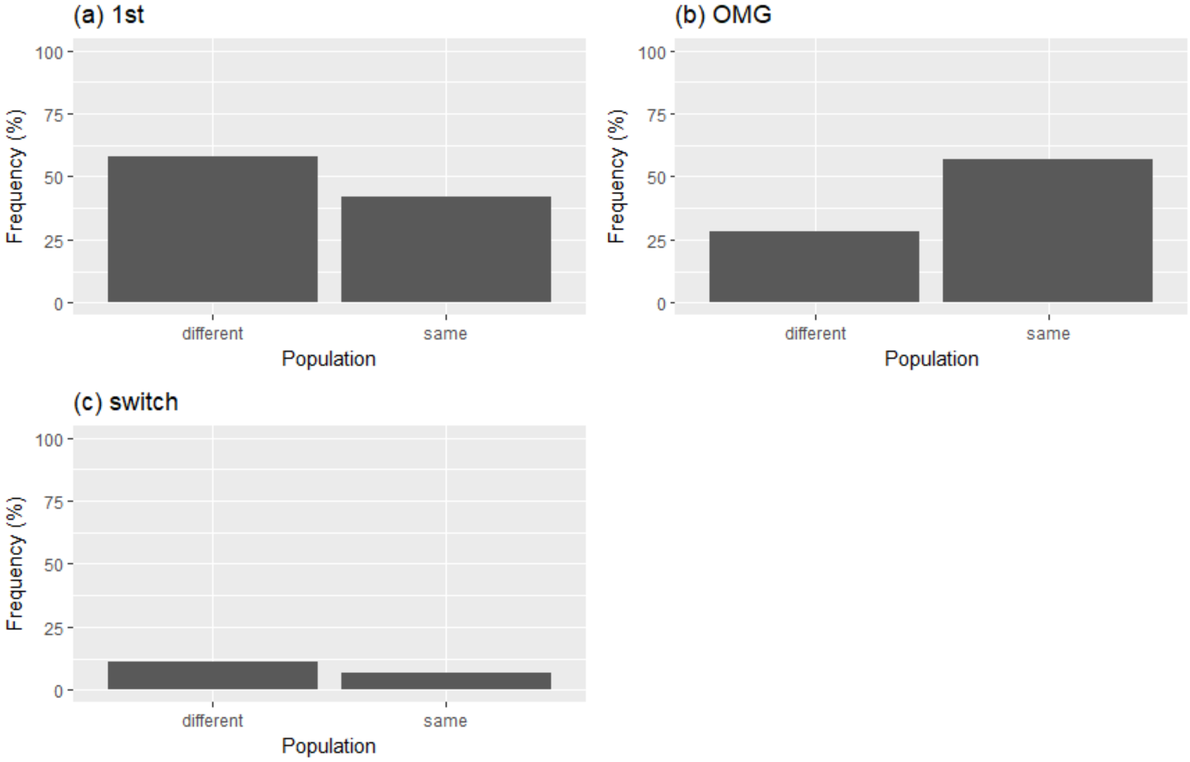


Figure 16. Male *Clitarchus hookeri* preferred a female from different population slightly more frequently in precopulatory choice (a), but preferred a female from the same population significantly more frequently in postcopulatory choice (b). No difference observed in the frequency of changing partner after mating (c).

3.3.2.2. Post-copulatory male choice

The frequency of overnight mate-guarding (*OMG*) was 54%. Males provided *OMG* significantly more frequently for the females from the same populations (57%) compared to the ones from different populations (28%) (p-value < 0.05, Binomial test) (Figure 16(b)). There was no difference in the frequency of *switch* between females from same vs. different populations (p-value > 0.05, Binomial test) (Figure 16(c)).

These results show that male *C. hookeri* prefer females from the same populations in their postcopulatory choice, which supports my prediction.

3.4. Discussion

3.4.1. Y-maze choice test: production of sex-specific pheromones?

Insect pheromones are particular smells produced and released that induce a specific response of another sex (Reddy and Guerrero, 2004). In *C. hookeri*, past study using a y-maze has shown male locate females using olfactory cues when they were exposed to a female in one arm and kept empty on another (Myers *et al.*, 2015). Thus, this raised the possibility of females releasing sex pheromones. However, my Y-maze choice test showed that male *C. hookeri* did not discriminate between males and females nor between sexual and parthenogenetic females when they were exposed only to their smells. These results suggest that male *C. hookeri* cannot discriminate between sexes or females with different reproductive mode when they are exposed only to the smells. This finding supports the evidence presented in my earlier chapter that *C. hookeri* females do not release sex-specific pheromones.

3.4.2. Male preference to local females over non-locals?

Preference to the mating partners from the local populations over non-locals is common in animals. This preference is due to reproductive isolation between populations resulted from reduced contemporary gene flow (Wong *et al.*, 2004). Discrimination of mates between populations often involves phenotypic characteristics, including morphology (Wong *et al.*, 2004), chemical profiles (Schwander and Crespi, 2009, Schwander and Crespi, 2013), and courtship songs (Tregenza *et al.*, 2000), but it can also occur during or after mating (Jennings *et al.*, 2011, Nosil and Crespi, 2006).

In *C. hookeri*, both males and females showed high morphological and chemical diversity between populations (CHAPETR 2); thus, it is likely that males discriminate females between same and different populations based on this variation. However, the results that males did not discriminate between the females from the same and different populations in their precopulatory choice. Previous study has shown male *C. hookeri* could not discriminate between conspecific and heterospecific females in their precopulatory choice (Myers, 2014). Thus, although males discriminate females by their body mass (discussed in CHAPTER 2), it is likely that males do not use other phenotypic characteristics at all for their precopulatory mate recognition.

My results showed males preferred the females from their own to different ones in their postcopulatory choice, as males mate-guarded local females more frequently over different ones. Thus, this fits my expectations that males prefer the females from local population over non-locals. Past study has shown that males successfully discriminate between conspecific and heterospecific females in their postcopulatory choice, presumably through copulation (Myers, 2014). Thus, males probably discriminated females during copulation in this study as well. This explains why males discriminated females from the same vs. different populations not in their precopulatory choice but in postcopulatory choice. Furthermore, my previous chapter has raised the probability of coevolution in genitalia size between males and females. The size of poculum and operculum vary between populations, and the pattern of between population size variation is similar in males and females (e.g. both Whanganui males and females had the widest, and both Paraparaumu males and females had the thinnest poculum and operculum) (Figure 17). Thus, the mechanisms of mate recognition can be based on the width of operculum during copulation.

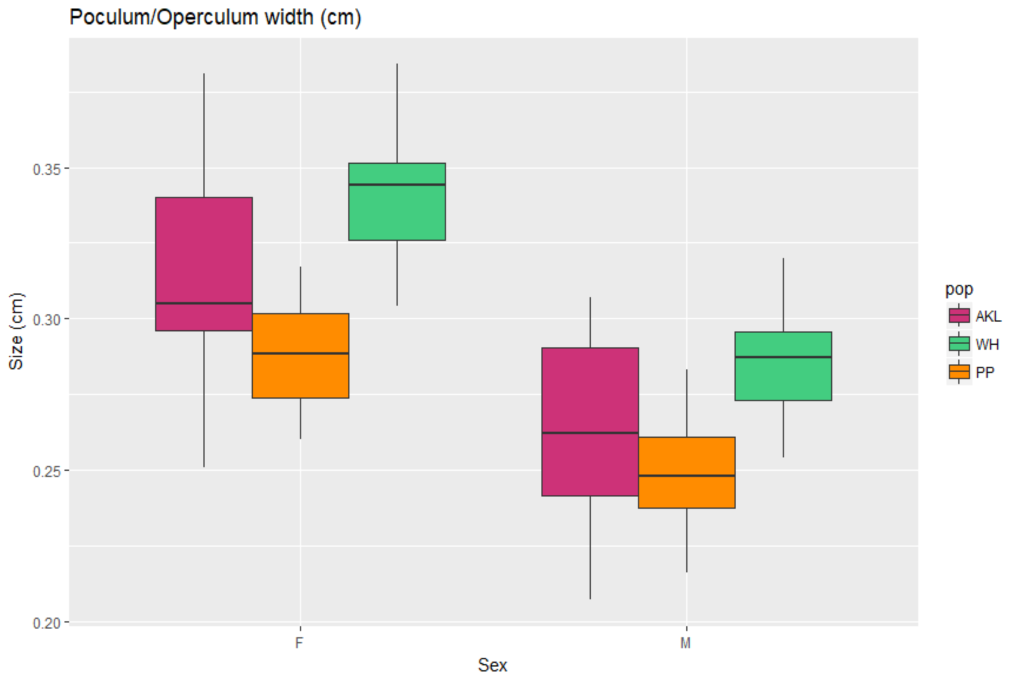


Figure 17. Males (M) and females (F) showing the similar pattern of size variation in poculum and operculum between populations (Auckland: AKL, pink; Whanganui: WH, green; Paraparaumu: PP, orange).

3.4.3. Conclusion

These additional analyses showed that *C. hookeri* females are unlikely to produce sex-specific pheromones. Although females increase the release of chemical volatiles at night when adult stick insects are sexually active, they produce the same compounds as males. Males can detect these compounds but cannot discriminate between sexes. Only the quantity of volatiles can provide males with information about potential mating opportunity.

In addition, males preferred the females from local over foreign populations in their postcopulatory choice, and mate discrimination may occur during copulation. However, as the females and males used in this study were all non-virgins, it is possible that the results have been influenced by female mating status.

CHAPTER 4.

General Discussion

This thesis explored intriguing reproductive strategies in *Clitarchus hookeri* by analysing their phenotypic traits (morphology and chemical profiles) and observing their reproductive behaviours. Prior to my study, there have been several studies about their reproductive mode (sexual or parthenogenetic), historical range expansion, and genetic diversity (Morgan-Richards *et al.*, 2010, Buckley *et al.*, 2010). The reproductive features of *C. hookeri* have also been investigated, including mating system (i.e. scramble competition and promiscuity), mechanisms of reproductive isolation, and mate choice based on genital structure (Myers, 2014, Myers *et al.*, 2017, Myers *et al.*, 2016, Myers *et al.*, 2015). The purpose of this study was to investigate further about their two reproductive strategies based on the past studies. Specifically, I explored (1) morphological and chemical traits that are likely to be under distinctive sex roles in scramble competition mating system; (2) whether morphological and chemical traits seen in sexual females are also seen in parthenogenetic females; and (3) whether males can discriminate between sexual and parthenogenetic females for their pre- and post-copulatory choices.

4.1. Sex roles in scramble competition mating system

Sexual selection favours the traits that enhance access to mates and thus increase reproductive output of individuals. In the case of *Clitarchus hookeri*, distinct sex roles in scramble competition mating system had resulted in phenotypic (morphology and chemical signalling) differentiation between males and females. I documented sexual size dimorphism in *C. hookeri*, a common pattern in insects (Honěk, 1993). Males have a smaller body (body length and mass) with disproportionately longer legs and antennae compared to females. This body shape in males allows them to move and locate females effectively, which will be advantageous for being a searcher.

Female *C. hookeri* are larger and fatter, possibly related to fecundity as body size of females is often positively correlated with number of eggs produced (Fairbairn, 1997, Myers *et al.*, 2015). *Clitarchus hookeri* females are signallers in their role of scramble competition. Past study has inferred the possibility of *C. hookeri* females releasing sex pheromones since males successfully located females using olfactory cues (Myers *et al.*, 2015). However, none of the volatiles I identified was specific to females in my results. The compounds identified suggested that *C. hookeri* are releasing volatiles based on what they feed. Furthermore, males could not discriminate between males and females when they were exposed only to their smells (CHAPTER 3). Thus, it is unlikely that female *C. hookeri* are releasing sex-specific pheromones. Instead, females increased their quantity of volatile emission at night when the species are in sexually activity, and their host plants cannot synthesize the volatile compounds. This increase in emission was not observed in males. Therefore, the increase in volatile emission *C. hookeri* females is likely to be related to their role as being signallers.

4.2. Sexual vs. parthenogenetic females: Decay in sexual traits?

The traits that are historically used for sexual reproduction are likely to decay rapidly in parthenogenetic females, and this has been documented in many insects (Schwander *et al.*, 2013, Schwander *et al.*, 2010, Burke *et al.*, 2015, Lehmann *et al.*, 2007, Tabata *et al.*, 2017). For example, alternation in pheromone signals in parthenogenetic females has been observed in stick insects *Extatosoma tiaratum* (Burke *et al.*, 2015) and *Timema* spp. (Schwander *et al.*, 2013). In *C. hookeri*, females include both sexual and parthenogenetic, and parthenogenetic females are not under scramble competition as their populations do not include males. Thus, because parthenogenetic females are released from the selective force of sexual reproduction, it is likely that they do not exhibit the traits that are seen in sexual females. However, my results showed parthenogenetic females overlapped with sexual females in both morphological and chemical traits. Those traits that overlapped include body mass in relation to body length, the width of operculum, and chemical signalling. Moreover, the results showed a high variation in morphological and chemical traits between populations rather than different reproductive mode. These variations possibly have derived from various evolutionary and ecological factors: parthenogenetic females maintaining sexual traits due to incomplete isolation from males; a latitudinal difference in available developmental time (i.e. the converse Bergmann's rule); and the variation in chemical composition of their host plants. These show that phenotypic traits in female *C. hookeri* is not simply a distinction between sexual and parthenogenetic, but may also have influenced from various evolutionary and ecological factors.

4.3. Male mate choice: sexual vs. parthenogenetic females

Sexual selection also favours the evolution of male mate choice when there is a high variation in female quality (Bonduriansky, 2001, Edward and Chapman, 2011). In this study, the main focus of the female quality was a different reproductive mode in females i.e. sexual vs. parthenogenetic. It is advantageous for males if they could discriminate between sexual and parthenogenetic females, as parthenogenetic females are likely to be investing less on sexual traits. However, my results showed that male *C. hookeri* did not discriminate between sexual and parthenogenetic females both in their pre- and post-copulatory choices. This is possibly due to the fact that parthenogenetic females are still maintaining sexual traits (as mentioned above). Instead, males chose females with a lighter body mass in their precopulatory choice. This was also observed in a past study and may be associated with the male preference towards virgin females (Myers *et al.*, 2015). Auckland females were least favoured by males and Auckland is the most geographically distant population among the examined populations; so during postcopulatory choice, it is possible that males prefer local females over non-local. A trend towards postcopulatory male preference for local females was also seen in the additional analysis (CHAPTER3), but more observations are required.

4.4. Limitations and future studies

For the first time the chemical volatiles released by *Clitarchus hookeri* have been identified. Evidence that volatiles are used by females for attracting mates comes from the sex differences and timing of release documented here. Mate choice experiments suggest that males have limited ability to differentiate females and thus this study has shown interesting reproductive features of *C. hookeri*, although many questions remained unanswered.

Firstly, my results for morphological and chemical analyses show variation among populations both in male and female *C. hookeri*. The morphological analyses raised the possibility of *C. hookeri* exhibiting converse Bergmann's rule (discussed in 2.4.2.1.). This may be one of the reasons that prevented morphological distinction between sexual and parthenogenetic females. Thus, additional morphological analyses are required, especially the ones from South Island, to confirm that whether *C. hookeri* are exhibiting the distribution pattern of the converse Bergmann's rule. My chemical analyses revealed a variation among populations. The reason behind this may be due to the variation in the chemical composition of local host plants. Examined insects were each exposed to their local host plants in their early developmental stage. Some insects retain the chemical compounds that they sequestered from their host plants from juvenile to adult stage (Reddy and Guerrero, 2004). Therefore, the reason for the variation in chemical profiles between populations may be derived from the retention of chemical compounds from the early developmental stage in insects, and control of the host plants from juvenile stage may be required to prevent the variation in chemical profiles of *C. hookeri.*

Males, I observed, did not discriminate between sexual and parthenogenetic females either in pre- or post-copulatory choices. This result fits with my evidence that parthenogenetic females do not differ significantly from sexual females in size or smells. Instead, male choice in *C. hookeri* may be based on between populations differences and on body mass differences. Collecting volatiles at different stages of an individual's life (immature, mature, virgin, pos-mating, etc) might establish what cues males use to determine female's size (or age). My focus was not on the population level mate discrimination but this direction could be productive. Altogether, future work should be based on between populations, and life stages for analysing morphology and chemical profiles, as well as female mate choice in *C. hookeri*.

Appendices

Appendix Table 1. Correlation coefficients between morphological traits of *Clitarchus hookeri*. Morphological parameters: Body = total body length, mass = body mass, Lan = length of antennae, Lf = length of foreleg, Lm = length of midleg, Lh = length of hindleg, Labd = length of abdomen, Wabd = width of abdomen, L pro = length of pronotum, Wpro = width of pronotum, Lmeso = length of mesonotum, Wmeso = width of mesonotum, Lmeta = length of metanotum, Wmeta = width of metanotum, Lop.po = length of operculum (female)/poculum (male), Wop.po = width of operculum/poculum

(a) ALL individuals (N=107)

```
       Body   mass   Lan    Lf     Lm     Lh     Labd   Wabd   Lpro   Wpro   Lmeso  Wmeso  Lmeta  Wmeta  Lop.po Wop.po
Body          0.0000 0.0990 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
mass   0.0000        0.0910 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Lan    0.0990 0.0910        0.0525 0.0056 0.0014 0.0354 0.0011 0.0069 0.0304 0.3078 0.0000 0.1317 0.0000 0.0053 0.0149
Lf     0.0000 0.0000 0.0525        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Lm     0.0000 0.0000 0.0056 0.0000        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Lh     0.0000 0.0000 0.0014 0.0000 0.0000        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Labd   0.0000 0.0000 0.0354 0.0000 0.0000 0.0000        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Wabd   0.0000 0.0000 0.0011 0.0000 0.0000 0.0000 0.0000        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Lpro   0.0000 0.0000 0.0069 0.0000 0.0000 0.0000 0.0000 0.0000        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Wpro   0.0000 0.0000 0.0304 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
Lmeso  0.0000 0.0000 0.3078 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000        0.0000 0.0000 0.0000 0.0000 0.0000
Wmeso  0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000        0.0000 0.0000 0.0000 0.0000
Lmeta  0.0000 0.0000 0.1317 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000        0.0000 0.0000 0.0000
Wmeta  0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000        0.0000 0.0000
Lop.po 0.0000 0.0000 0.0053 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000        0.0000
Wop.po 0.0000 0.0000 0.0149 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000
```

Appendix Table 1. continued.

(b) Males (N=45)

```
       Body   mass   Lan    Lf     Lm     Lh     Labd   Wabd   Lpro   Wpro   Lmeso  Wmeso  Lmeta  Wmeta  Lop.po Wop.po
Body          0.0000 0.0069 0.0000 0.0000 0.0000 0.0000 0.3548 0.4040 0.0212 0.0000 0.1522 0.0000 0.1837 0.0092 0.0159
mass   0.0000        0.0724 0.0016 0.0865 0.0011 0.0000 0.0677 0.6820 0.0083 0.0000 0.6955 0.0000 0.4070 0.2739 0.0127
Lan    0.0069 0.0724        0.1395 0.1229 0.2086 0.0327 0.8992 0.2539 0.5602 0.0242 0.8684 0.0243 0.6463 0.0836 0.1246
Lf     0.0000 0.0016 0.1395        0.0000 0.0000 0.0000 0.1362 0.2460 0.0417 0.0000 0.4004 0.0000 0.6738 0.0360 0.0634
Lm     0.0000 0.0865 0.1229 0.0000        0.0000 0.0002 0.1601 0.1056 0.2462 0.0000 0.5407 0.0026 0.4370 0.4665 0.0149
Lh     0.0000 0.0011 0.2086 0.0000 0.0000        0.0000 0.0834 0.3335 0.1344 0.0000 0.6018 0.0000 0.9703 0.3513 0.0206
Labd   0.0000 0.0000 0.0327 0.0000 0.0002 0.0000        0.3070 0.1094 0.0627 0.0000 0.1526 0.0000 0.6119 0.0142 0.0024
Wabd   0.3548 0.0677 0.8992 0.1362 0.1601 0.0834 0.3070        0.9571 0.1844 0.3707 0.3536 0.7915 0.0922 0.5651 0.0266
Lpro   0.4040 0.6820 0.2539 0.2460 0.1056 0.3335 0.1094 0.9571        0.6776 0.6154 0.1003 0.3787 0.6363 0.9992 0.2058
Wpro   0.0212 0.0083 0.5602 0.0417 0.2462 0.1344 0.0627 0.1844 0.6776        0.1193 0.2030 0.2556 0.4433 0.0305 0.0448
Lmeso  0.0000 0.0000 0.0242 0.0000 0.0000 0.0000 0.0000 0.3707 0.6154 0.1193        0.3207 0.0000 0.1698 0.1414 0.0514
Wmeso  0.1522 0.6955 0.8684 0.4004 0.5407 0.6018 0.1526 0.3536 0.1003 0.2030 0.3207        0.2698 0.1869 0.8893 0.7127
Lmeta  0.0000 0.0000 0.0243 0.0000 0.0026 0.0000 0.0000 0.7915 0.3787 0.2556 0.0000 0.2698        0.0653 0.0742 0.0530
Wmeta  0.1837 0.4070 0.6463 0.6738 0.4370 0.9703 0.6119 0.0922 0.6363 0.4433 0.1698 0.1869 0.0653        0.6537 0.0054
Lop.po 0.0092 0.2739 0.0836 0.0360 0.4665 0.3513 0.0142 0.5651 0.9992 0.0305 0.1414 0.8893 0.0742 0.6537        0.5631
Wop.po 0.0159 0.0127 0.1246 0.0634 0.0149 0.0206 0.0024 0.0266 0.2058 0.0448 0.0514 0.7127 0.0530 0.0054 0.5631
```

(c) Sexual females (N=32)

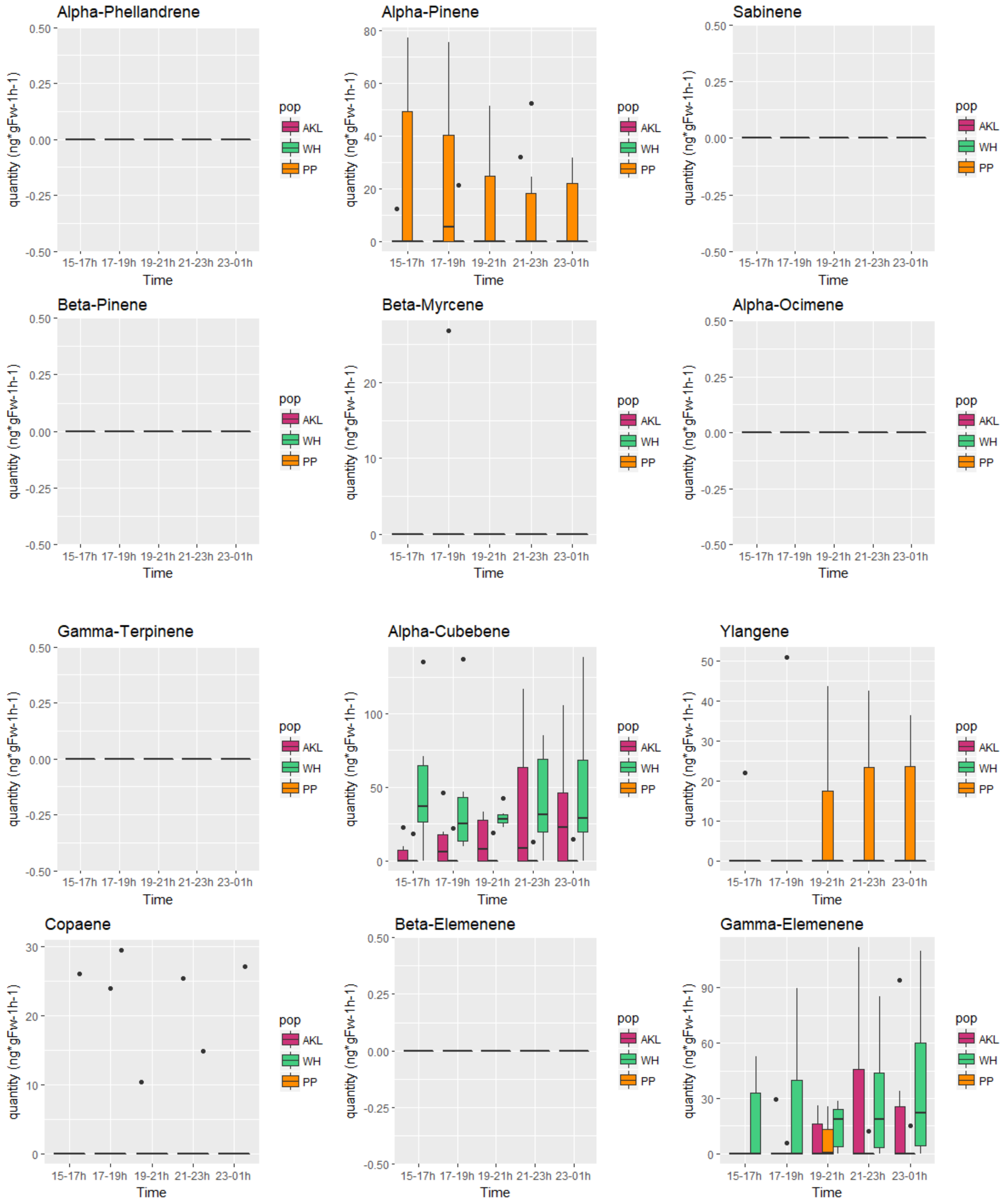
```
       Body   mass   Lan    Lf     Lm     Lh     Labd   Wabd   Lpro   Wpro   Lmeso  Wmeso  Lmeta  Wmeta  Lop.po Wop.po
Body          0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0103 0.8165 0.0028 0.0000 0.2121 0.0000 0.2277 0.0000 0.3943
mass   0.0000        0.0005 0.0000 0.0002 0.0000 0.0101 0.0081 0.4302 0.0268 0.0000 0.8529 0.0000 0.6527 0.0001 0.4940
Lan    0.0000 0.0005        0.0000 0.0000 0.0000 0.0010 0.5906 0.3044 0.0246 0.0000 0.1237 0.0000 0.6107 0.0000 0.6381
Lf     0.0000 0.0000 0.0000        0.0000 0.0000 0.0000 0.2220 0.4560 0.0061 0.0000 0.4006 0.0000 0.8885 0.0000 0.4098
Lm     0.0000 0.0002 0.0000 0.0000        0.0000 0.0008 0.4845 0.1042 0.1070 0.0000 0.4424 0.0000 0.8076 0.0000 0.5141
Lh     0.0000 0.0000 0.0000 0.0000 0.0000        0.0000 0.1136 0.6829 0.0470 0.0000 0.3236 0.0000 0.4955 0.0000 0.5708
Labd   0.0000 0.0101 0.0010 0.0000 0.0008 0.0000        0.1009 0.9856 0.0128 0.0000 0.1411 0.0000 0.3012 0.0000 0.3070
Wabd   0.0103 0.0081 0.5906 0.2220 0.4845 0.1136 0.1009        0.6551 0.1093 0.0745 0.1478 0.0488 0.0430 0.0496 0.2267
Lpro   0.8165 0.4302 0.3044 0.4560 0.1042 0.6829 0.9856 0.6551        0.7465 0.7802 0.5814 0.2854 0.8837 0.0393 0.2771
Wpro   0.0028 0.0268 0.0246 0.0061 0.1070 0.0470 0.0128 0.1093 0.7465        0.0021 0.5763 0.0008 0.2408 0.0005 0.3016
Lmeso  0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0745 0.7802 0.0021        0.1394 0.0000 0.4777 0.0000 0.6085
Wmeso  0.2121 0.8529 0.1237 0.4006 0.4424 0.3236 0.1411 0.1478 0.5814 0.5763 0.1394        0.2956 0.4999 0.6751 0.8576
Lmeta  0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0488 0.2854 0.0008 0.0000 0.2956        0.3015 0.0000 0.6816
Wmeta  0.2277 0.6527 0.6107 0.8885 0.8076 0.4955 0.3012 0.0430 0.8837 0.2408 0.4777 0.4999 0.3015        0.6632 0.8007
Lop.po 0.0000 0.0001 0.0000 0.0000 0.0000 0.0000 0.0000 0.0496 0.0393 0.0005 0.0000 0.6751 0.0000 0.6632        0.1943
Wop.po 0.3943 0.4940 0.6381 0.4098 0.5141 0.5708 0.3070 0.2267 0.2771 0.3016 0.6085 0.8576 0.6816 0.8007 0.1943
```

Appendix Table 1. continued.

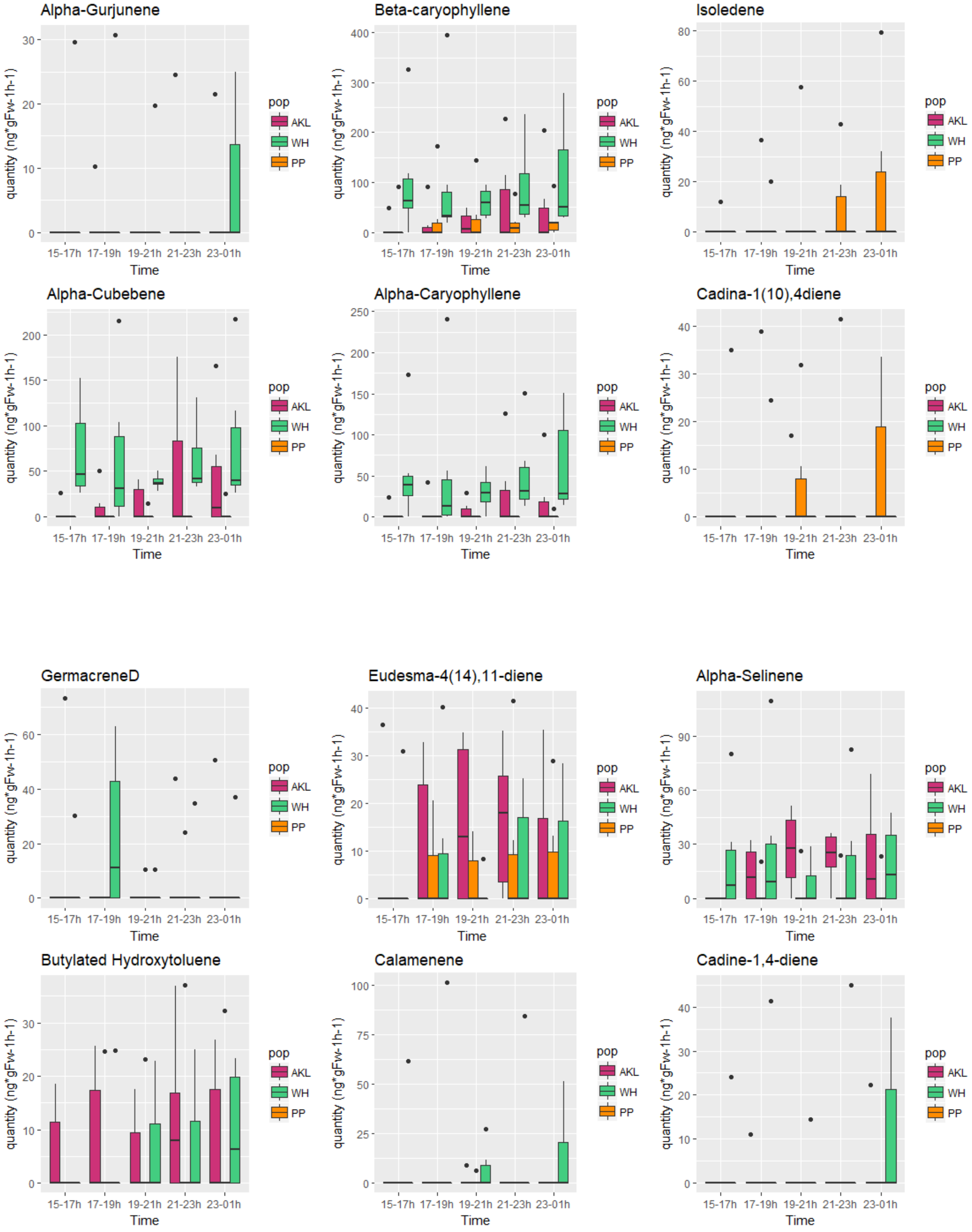
(d) Parthenogenetic females (N=30)

```
       Body   mass   Lan    Lf     Lm     Lh     Labd   Wabd   Lpro   Wpro   Lmeso  Wmeso  Lmeta  Wmeta  Lop.po Wop.po
Body          0.0000 0.0002 0.0000 0.0000 0.0000 0.0000 0.0003 0.0000 0.0147 0.0000 0.0844 0.0000 0.0054 0.0015 0.4775
mass   0.0000        0.0000 0.0000 0.0000 0.0000 0.0000 0.0000 0.0010 0.0138 0.0000 0.0294 0.0000 0.0008 0.0003 0.2280
Lan    0.0002 0.0000        0.0012 0.0001 0.0014 0.0000 0.0002 0.0067 0.0553 0.0129 0.1567 0.0046 0.0330 0.0008 0.0379
Lf     0.0000 0.0000 0.0012        0.0000 0.0000 0.0000 0.0013 0.0000 0.0120 0.0000 0.2565 0.0000 0.0170 0.0040 0.1896
Lm     0.0000 0.0000 0.0001 0.0000        0.0000 0.0000 0.0012 0.0000 0.0093 0.0000 0.3925 0.0000 0.0766 0.0001 0.1285
Lh     0.0000 0.0000 0.0014 0.0000 0.0000        0.0000 0.0101 0.0002 0.0358 0.0000 0.2615 0.0000 0.0598 0.0072 0.7937
Labd   0.0000 0.0000 0.0000 0.0000 0.0000 0.0000        0.0001 0.0000 0.0046 0.0000 0.0335 0.0000 0.0017 0.0001 0.1423
Wabd   0.0003 0.0000 0.0002 0.0013 0.0012 0.0101 0.0001        0.0090 0.0148 0.0015 0.0181 0.0008 0.0000 0.0022 0.0264
Lpro   0.0000 0.0010 0.0067 0.0000 0.0000 0.0002 0.0000 0.0090        0.0170 0.0069 0.6017 0.0023 0.2480 0.0239 0.1024
Wpro   0.0147 0.0138 0.0553 0.0120 0.0093 0.0358 0.0046 0.0148 0.0170        0.0202 0.0001 0.0001 0.0009 0.0000 0.0047
Lmeso  0.0000 0.0000 0.0129 0.0000 0.0000 0.0000 0.0000 0.0015 0.0069 0.0202        0.0448 0.0000 0.0079 0.0022 0.9103
Wmeso  0.0844 0.0294 0.1567 0.2565 0.3925 0.2615 0.0335 0.0181 0.6017 0.0001 0.0448        0.0013 0.0000 0.0042 0.0883
Lmeta  0.0000 0.0000 0.0046 0.0000 0.0000 0.0000 0.0000 0.0008 0.0023 0.0001 0.0000 0.0013        0.0004 0.0000 0.4107
Wmeta  0.0054 0.0008 0.0330 0.0170 0.0766 0.0598 0.0017 0.0000 0.2480 0.0009 0.0079 0.0000 0.0004        0.0022 0.1569
Lop.po 0.0015 0.0003 0.0008 0.0040 0.0001 0.0072 0.0001 0.0022 0.0239 0.0000 0.0022 0.0042 0.0000 0.0022        0.0001
Wop.po 0.4775 0.2280 0.0379 0.1896 0.1285 0.7937 0.1423 0.0264 0.1024 0.0047 0.9103 0.0883 0.4107 0.1569 0.0001
```

Volatile emission in males

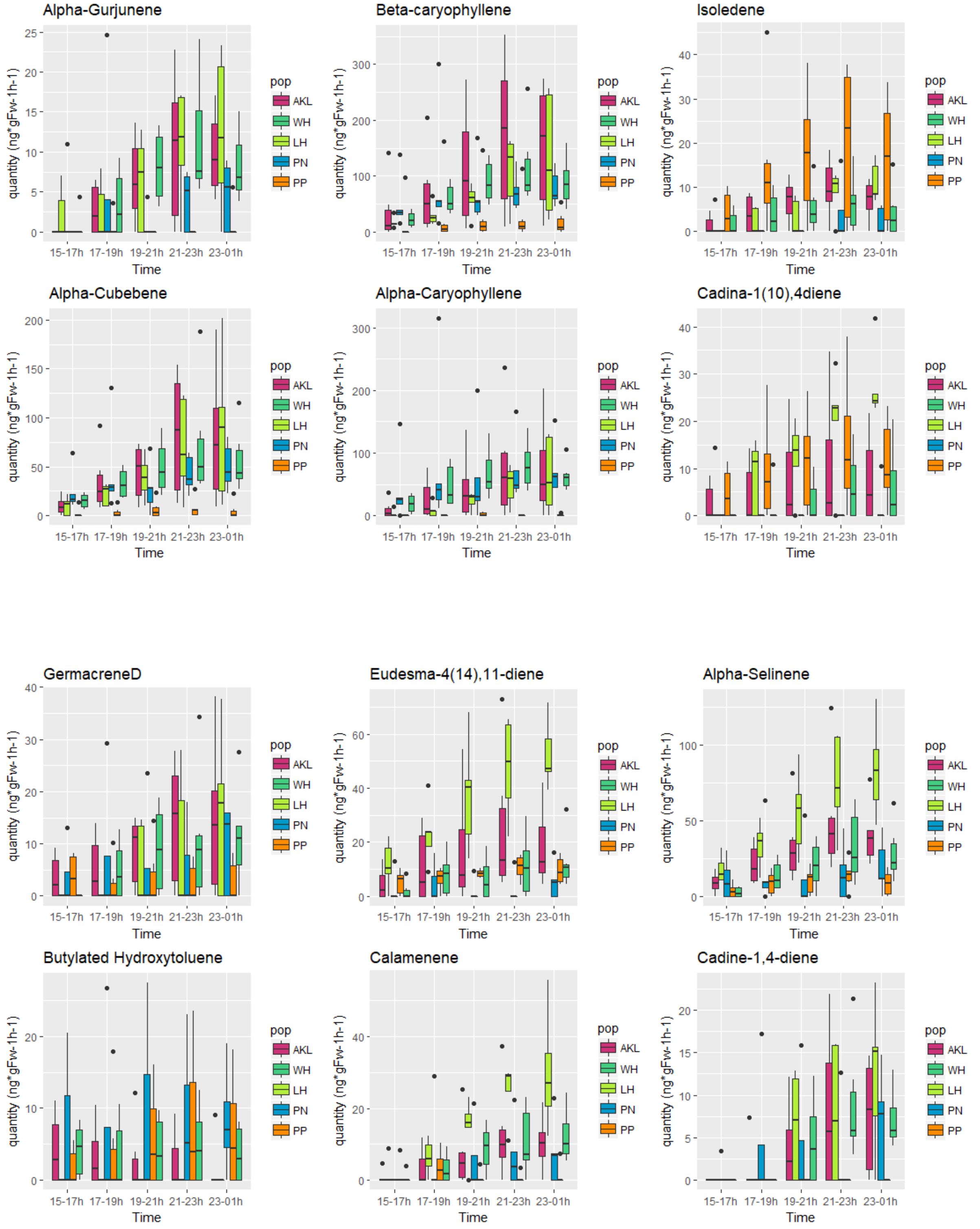


Appendix Figure 1. Variation in chemical profiles among populations in male *Clitarchus hookeri*. Populations: Auckland (AKL, pink), Whanganui (WH, green), and Paraparaumu (PP, orange).



Appendix Figure 1 continued.

Appendix Figure 2. Variation in chemical profiles between populations in female *Clitarchus hookeri*. Populations: Auckland (AKL, pink), Whanganui (WH, green), Lower Hutt (LH, yellow), Palmerston North (PN, blue), and Paraparaumu (PP, orange).



Appendix Figure 2 Continued.

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