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Sexual conflict and genital evolution in moths



Rebecca Marie Bennik

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degree of Doctor of Philosophy in Biological Sciences.

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Abstract

Male genitalia are one of the fastest evolving and most divergent structures in sexually reproducing organisms. Theories behind genital evolution include the lock and key hypothesis (species isolation), pleiotropy and sexual selection, however, recent research has proposed sexual selection to be the most likely driver of rapid and divergent evolution of genitalia. One facet of such selection - sexual conflict - may drive genital evolution through opposing selection on male and female reproductive strategies as genital adaptations that allow males a competitive advantage may consequently reduce female fitness leading to an evolutionary arms race via sexually antagonistic coevolution. Lichen tuft moths of the genus *Izatha* (Lepidoptera: Xyloryctidae) and New Zealand *Glaucocharis* (Lepidoptera: Crambidae) were excellent candidates for examining the evolution of male genitalia and exploring sexual conflict driving genital evolution. By constructing a molecular phylogeny for both genera, the evolutionary relationships between species, patterns of male genital evolution and estimated ancestral state reconstructions of the male genitalia could be determined. Both New Zealand *Glaucocharis* and *Izatha* show variation in a number of structures associated with the genital capsule and phallus, and closely related species were more likely to share similar traits.

In New Zealand *Glaucocharis*, the external apical spur on the phallus has the potential to cause harm to part of the female reproductive tract with which it most likely makes contact. In most species of *Izatha*, males also have sclerotised phallic teeth that make

contact with the female ductus bursae during copulation and may cause scarring, while some *Izatha* males also possess sclerotised spines (deciduous cornuti) attached to the internal vesica wall of the phallus which are ejected into the female reproductive tract during mating.

The coevolution of these male and female structures, controlling for phylogeny, was tested and revealed a significant positive correlation between the size of the external apical spur and the thickness of the female ductus bursae wall among species of New Zealand *Glaucocharis*, providing evidence of the potential sexually antagonistic coevolution of these two traits. In *Izatha*, there was a significant correlation between the size of the external phallic teeth and the thickness of the female ductus bursae wall, and between the shape of the phallus and area of scarring. A narrower phallus is associated with larger phallic teeth and larger teeth caused a larger area of scarring in females. The male deciduous cornuti and the female signum also revealed significant positive correlations between the presence, number, area and length of the deciduous cornuti and the area of the female signum. Previous research has suggested the signum evolved as a counter-adaptation to the hard spermatophores developed by males to increase the female refractory period to remating. In the present study it was found that females of *Izatha* species in which males possess deciduous cornuti have a larger signum, but females of some other species that do not possess these structures had a signum present, although reduced in size. Therefore, the evolution of a female trait, the signum, may be driving the evolution of deciduous cornuti in male *Izatha* via sexually antagonistic coevolution and deciduous cornuti may have evolved to counteract the

effect of the signum. Therefore, sexually antagonistic coevolution appears to be occurring between these two sets of male and female traits in *Izatha*.

In order to put these findings into context, background information about mating system dynamics help us to gain a better understanding of the type and level of sexual selection involved within a species. Three species of *Izatha* exhibited protandry, male-biased sex ratios and female-biased sexual size dimorphism. Male mating status showed a significant negative correlation with body size, with smaller males more likely to have mated. In one species, *I. peroneanella*, males present later in the breeding season were also more likely to have mated. However, these two factors may be strongly linked, as males that are smaller are also flying later in the season. The later emergence of females and potential scramble competition between males for mates may explain these patterns.

These studies have contributed to the understanding of species relationships and the evolution of male genitalia in two genera of New Zealand moths. The comparison of key male and female traits among New Zealand *Glaucocharis* and *Izatha* have revealed the coevolution of structures, with sexually antagonistic coevolution appearing to be the driver in both sets of traits within *Izatha*. Examining the seasonal variation of three common species of *Izatha* has revealed primary characteristics within the mating system of these species and potential determinants of mating success. This provides the foundations for further investigation of the mating system and evolution of genitalia within *Glaucocharis* and *Izatha*, as well as other Lepidoptera.

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1.

Introduction

1. Introduction

1.1 GENITAL EVOLUTION

The morphology of genitalia is often species-specific with more divergence in closely related species than any other morphological characters (Eberhard 1985). This also makes genitalia key structures in identifying species and many animal species can be defined by the morphology of their male genitalia (Eberhard 2010a). Therefore genital evolution appears to be a rapid and divergent process (Arnqvist 1997a; Hosken & Stockley 2004; Eberhard 2010a); rapid in respect to changes in other morphological traits, and “divergent” in terms of the variation that can then present itself among closely related species (Eberhard 2010a). There are three main theories behind genital evolution - species isolation (or the Lock and Key hypothesis), pleiotropy, and sexual selection hypotheses, which will be discussed in turn in the following sections.

1.1.1 *Species/mechanical isolation (Lock and key Hypothesis)*

Pre-Darwin, diverse genitalic features between species were proposed as a process of species isolation, the “Lock and key hypothesis” (Dufour 1848; in Arnqvist 1997a), as it was suggested that unique genitalic characteristics avoided the possibility of heterospecific matings by mechanical default (Eberhard 1985). Essentially, males and females of a species had a lock and key system, in which females may evolve complicated or complimentary genitalia in order to exclude heterospecific males from successful copulation, but permit conspecifics with the right “fit” (Eberhard 2010a). This

mechanism may play a role in species isolation in some instances, as Sota & Kubota (1998) claim may be relevant for certain species of Carabid beetles. Simmons et al. (2009) also highlight that the lock and key mechanism may apply to certain genitalic structures within a species, whilst others are under different adaptive pressures. However, other prior research, including recent publications (Andrade et al. 2009; Gilligan & Wenzel 2008; Mutanen et al. 2006; Ohno et al. 2003), have rejected the lock and key hypothesis in favour of models of sexual selection. One of the main arguments against the generality of this hypothesis is the fact that in many animal taxa displaying diverse “keys”, concomitant diverse “locks” do not exist (Ohno et al. 2003; Eberhard & Ramirez 2004; Briceno et al. 2007). One sex (the male) displays a large variation in genitalic structures between closely related species; whereas, contrasting female genitalia are relatively uniform (Shapiro & Porter 1989; Eberhard 2010a). The existence of mirror image genitalic dimorphism also contradicts the proposition of exact ‘fits’ (Huber & Gonzalez 2001; Holwell & Herberstein 2010). In addition, Arnqvist’s (1997a) intra-specific analysis of water striders found no correlation between the relationship of male and female morphology and male mating success. The evidence of exact mechanical fits or “locks” that may inhibit insemination success in heterospecific mating attempts, so far, are relatively scarce (Eberhard 2010a).

Eberhard (1985) compared taxa geographically isolated by small neighbouring islands around New Zealand and the Galapagos and found that compared to more sympatric species, genitalic structures of these more isolated species were no more or less elaborate, providing further evidence against the lock and key hypothesis as a good

basis for genital evolution. If the avoidance of heterospecific matings were the primary pressure shaping genitalia, we would expect isolated taxa to be under less pressure, resulting in less genitalic elaboration.

Furthermore, hybridization is evident in many animal taxa, including birds (Barilani et al. 2007; Chan et al. 2006; Dietzan et al. 2007), mammals (Andersone et al. 2002; Lancaster et al. 2010), fish (Hayden et al. 2010), molluscs (Ribi & Porter, 1995) and insects (Goropashnaya et al. 2004; Morgan-Richards et al. 2009) showing that the generalization of mechanical isolation inherent to the lock and key hypothesis does not hold for many species, with other physiological cues utilized to distinguish between conspecifics for avoidance of heterospecific matings (Campbell & Hauber 2009; Detto et al. 2006; Leonard & Hedrick 2009; Symonds et al. 2009; Zimmerman et al. 2009).

1.1.2 The Pleiotropy Hypothesis

The pleiotropy hypothesis was first proposed by Mayr (1963). According to this theory, variation in genitalia evolves via pleiotropic gene effects that code for both genitalic and general morphological characters, correlating genital morphology phenotypically with other non-genital traits (Mayr 1963; Eberhard 1985; Arnqvist 1997a). Therefore, genitalic variation is selectively neutral; genitalic structures are not directly selected for and may accumulate over a period of time (Arnqvist 1997a). It is difficult to disprove that genitalic and non-genitalic traits may be - at least in part - genetically linked to non-genitalic traits (Arnqvist & Thornhill 1998), with indepth quantitative genetics needed to

further test this hypothesis (House & Simmons 2003). Eberhard (2010) argues that this hypothesis does not explain why pleiotropic effects could occur more profoundly in genitalia as opposed to other morphological structures, or why there appears to be a lack of pleiotropic effects present in species with external fertilization.

Recent publications have suggested that pleiotropic effects may contribute to variation in genitalic morphology (Arnqvist & Thornhill 1998; House & Simmons 2005). Morrow et al. (2003) investigated the origin of harmful structures within the fruitfly *Drosophila melanogaster* and the bruchid beetle *Callosobruchus maculatus*, in which post-mating harm has been observed, as well as the tenebrionid beetle *Tribolium castaneum*, in which it has not been documented. The authors experimentally induced varying treatments of post-mating harm by ablating a mesothoracic leg, an antenna, a wing, or by puncturing the abdomen or thorax (Morrow et al. 2003). Their results showed that females in which harm was induced did not delay remating or increase reproductive rate, but to the contrary, females remated sooner and laid fewer eggs. This provides support for the pleiotropic harm hypothesis, where harmful male structures may be a byproduct of pleiotropic effects encoding non-genitalic structures, in which females may not have responded with counter-adaptive strategies or traits to minimize harm incurred and thus may not necessarily compromise male interest (Morrow et al. 2003).

1.2 SEXUAL SELECTION

Recent comparative and experimental research has proposed sexual selection to be the most likely general driver behind the rapid and divergent evolution of genitalia (Arnqvist

1998; Arnqvist & Danielsson 1999; House & Simmons 2003; Simmons & Garcia-Gonzalez 2011). Darwin (1859) first made the distinction between natural and sexual selection - Natural selection favours traits that increase an organism's individual viability, whilst sexual selection favours traits that enhance the individual's reproductive success. Therefore, an individual may increase its overall fitness by ensuring its own reproductive success. To summarise the physiological difference between male and female sexual reproduction, males of most animal taxa produce a large number of smaller gametes to be transferred during reproduction, whilst females tend to produce fewer costly and relatively large gametes. This phenomenon is known as anisogamy. This causes an uneven cost ratio between males and females, where males can "cheaply" and quickly produce a large number of gametes, whereas the production of female gametes are much more "expensive" (ie. in terms of energetic costs) with a slower turnover, favouring a higher mating frequency in males as opposed to females. Under these circumstances, the male has an interest in mating with as many females as possible, in contrast to the female who would benefit more from careful consideration of the quality, rather than the quantity of males that are to receive access to her more costly gametes. One important consequence of anisogamy is that it has led to the intense competition among male gametes for the fertilization of female gametes, establishing the key conditions for sexual selection (Kodric-Brown & Brown 1987). This difference in reproductive investment between the sexes may result in the evolution of different strategies for ensuring the highest individual success in the process of reproduction, which places selective pressure on the morphology of the structures that are used to transfer gametes.

Different processes of sexual selection are evident in a wide variety of animal taxa, and there is a plethora of research on the subject. However, in the following sections I will focus on cases that involve arthropods, mainly insects, to avoid being overloaded by the vast amount of research on the topics and for the sake of the relevance to my research. There are three primary suggestions for how sexual selection may have an effect on genitalic morphology. These include male-male conflict or sperm competition, cryptic female choice, and sexual conflict.

1.2.1 Sperm Competition (Male-male conflict)

In mating systems in which females are polyandrous, males will have selective pressures favouring traits that allow them an advantage over other males in their ability to optimally position their gametes (sperm) spatially and temporally within the female reproductive tract and/or in reducing the ability of their mate engaging in subsequent matings (Simmons 2001). This strategy encompasses sexual selection via sperm competition. In the 1970's and 80's, sexual selection during or post-copulation was largely focused on the biology of the male, with the concept of sperm competition originally proposed by Parker (1970). Parker (1970) suggested that internal fertilization may have arisen as a selective strategy for males to be able to position their gametes closer to the site of fertilization than rival males within the female. Hence, sperm competition is defined as the competition between the sperm of two or more males for the fertilization of a female's ova (Simmons 2001) and may manifest itself in a number of

ways, such as via genital structures that displace or remove a previous male's ejaculate (Simmons & Siva-Jothy 1998). Such competition may place adaptive pressure on genitalic structures that are more efficient in the placement of sperm or, in the case of non-monogamous taxa, the removal of a previous male's sperm.

The use of male genitalic structures for the removal of previous male's sperm has been shown in much pioneering research in odonates (Waage 1979, 1986; Robinson & Novak 1997; Cordoba-Aguilar 2002; Cordero Rivera et al. 2004; Hayashi et al. 2005; Tsuchiya & Hayashi 2008) as well as many other insects including Coleoptera (Gack & Peschke 1994; Haubruge et al. 1999; Takami 2007; Takami & Sota 2007; Sumitomo et al. 2011), Orthoptera (Ono et al. 1989; Von Helversen & Von Helversen 1991), Hemiptera (Arnqvist 1988; Rubenstein 1989); Diptera (Hosken & Ward 2000), and Dermaptera (Kamimura 2000; Lieshout & Elgar 2010).

Males of the rove beetle *Aleochara curtula* use their spermatophore to remove previous male's sperm (Gack & Peshcke 1994). It is the sclerotised teeth in a valve structure of the female's reproductive tract that ultimately bursts the spermatophore, but the male relies on a flagellum on the tip of the endophallus to position the spermatophore in a way that also moves previous males' sperm. This places direct pressure on these primary reproductive structures to aid in sperm competition. Male tree crickets, *Trujjalia hibinonis*, use the phallus to remove previously stored sperm (Ono et al. 1989). During copulation, sperm is ejaculated into the anterior region of the female's spermatheca,

forcing previous ejaculates posteriorly and onto the shaft of the phallus. By dying seminal products, it was shown that 88% of prior ejaculates were displaced during copulation. Sumimoto et al. (2011) recently recorded the removal of previously placed spermatophores within male silphid beetles, *Silpha perforata*. Mating pairs were frozen during copulation and the male phallis morphology was examined. The apical part of the phallus was endowed with many spines that play a role in sperm displacement.

Insects that use genitalic structures other than the phallus for sperm displacement include the bush cricket *Metaplastes ornatus*. Males of this species use a modified subgenital plate to evert the female reproductive tract. The female is then able to consume the nutritious sperm package left by previous mates. This proves to be a very effective way of discarding a rival's sperm, as 85% of the previous male's sperm is removed prior to the transfer of a new spermatophore (Von Helversen & Von Helversen 1991).

Calopterygid damselflies in particular, have been widely studied for the investigation of the mechanical processes of sperm competition. The male phallus of many species within the family Calopterygidae is terminated with a specialized recurved head and spiny lateral processes used in sperm removal prior to depositing their own ejaculates (Waage 1979; Hayashi et al. 2005; Tsuchiya & Hayashi 2008). The relative success of sperm removal, however, is dependent on the shape and length of such structures. Narrower lateral processes are more effective at removing sperm compared to wider ones, which may not be able to adequately reach sperm storage sites. In species with

wider lateral processes, other mechanisms may be utilized for sperm removal. There also appears to be a trend in species with wider lateral structures exhibiting monogamy, which obviously places much less emphasis on the maintenance of structures efficient at displacing sperm (Hayashi et al. 2005). Cordero Rivera et al. (2004) found evidence of this by comparing two populations of the calopterygid damselfly *Calopteryx haemorrhoidalis*. They found that even though males of both populations were proficient at removing sperm, they used different mechanisms to achieve this. Italian populations directly physically remove sperm from the spermathecae, whereas Spanish populations stimulate the female to promote sperm dumping. This was tested on three further species within the same family, with similar results, demonstrating geographic variation in sperm removal mechanisms and genitalia within species, providing evidence of postmating sexual selection acting on intraspecific evolution of genitalia. This shows that in some species sexual selection may be placing pressure on these male structures for sperm competition, whereas in others they are devices to influence cryptic female choice. Arnaud et al. (2001) also proposed that different pressures may be working in congruence. They found that some structures of the male genitalia in red flour beetles (*Tribolium castaneum*) function in the displacement of previous males' sperm, whilst others possibly influence cryptic female choice by stimulating areas of the female reproductive tract.

The above cases highlight that many insect species from numerous orders have genitalic structures that aid in the removal or displacement of rival males' sperm. This competition in the positioning of male gametes to ensure fertilization places pressure on

males of species in which multiple mating occurs and on individuals to elaborate these structures to gain an advantage over others. In this respect sexual selection on male genitalia for the use in sperm competition is almost undeniable and hard to refute. The trick is being able to provide evidence and disentangle it from other selective processes that may also have an influence on the evolution of genitalic structures.

1.2.2 *Cryptic female choice*

Another mechanism of sexual selection influencing genital morphology is through control of sperm utilization and fertilization by the female via cryptic female choice. In its most general sense, cryptic female choice (CFC) proposes that females actively select males that possess a certain phenotype to sire their offspring (Eberhard 1996). It was not until the early 80s that the possibility of female post-copulatory choice was first proposed by Thornhill's (1983) conceptualization of cryptic female choice with findings of increased rates of oviposition post-mating among certain females of the scorpionfly *Harpobittacus nigriceps*. Females preferred to mate with larger males and males that provided a larger nuptial arthropod prey item, in which larger males presented larger gifts. Thus, females were able to regulate oviposition rate in accordance with the characteristics of their male partners. Eberhard (1985) further suggested that male genitalic structures may be used as courtship devices, which are selected for by females, during or post-copulation. Certain male genitalia may be selected for by the female due to a more effective stimulatory ability or if they fit better with the female's genitalia. Many female reproductive processes such as sperm transport, egg maturation, and the

inclination to remate, amongst others may be initiated by favourable stimuli that occur during copulation (Eberhard 2010b).

As a consequence, such female cryptic choice may have an effect on the evolution of male genitalia. This may occur as suggested by the type of stimulation the female receives from male genitalic structures or on the mechanical fit between male and female genitalia. Females may be able to use discretion in choice based on a male's ability to hold on to or reach particular areas within her reproductive tract. Not to be confused with the mechanical fit basis of the lock and key hypothesis, what is distinguishable is that the selection pressures are not for the purpose of species isolation, but are as a result of female choice (Eberhard 1985).

However, there is limited empirical evidence in support of cryptic female choice, seemingly due to the difficulty in teasing apart sexually selective processes due to sperm competition, female choice, or male manipulation of females (Danielsson & Askenmo 1999).

Much prior research on the topic has only tentatively been able to demonstrate cryptic female choice as playing a part in the selective pressures on male genitalia specifically, and most of these propositions arise from the premise that in some polyandrous species male paternity appears to be under female control. Work among the water strider genus *Gerris* provided evidence that females may play a role in mediating paternity (Arnqvist &

Danielsson 1999; Danielsson & Askenmo 1999). The male genitalia of one species, *G. lateralis*, is unable to reach or penetrate sperm storage sites within the female (Anderson 1982, in Arnqvist & Danielsson 1999), suggesting these males have little control over previous ejaculates stored, and sperm utilized is likely to be determined by the female (Arnqvist & Danielsson 1999). Indeed, House & Simmons (2003) suggested that within the scarab beetle, *Onthophagus taurus*, male stimulation of the female may influence female's choice of paternity or fertilization success.

As previously mentioned, damselfly males often possess structures at the distal end of the endophallus used in sperm removal (Waage 1979). However, in the species *Calopteryx haemorrhoidalis*, despite morphological similarities of the male genitalia to other species, sperm ejection is under the control of the female's sensory system. Like the genital tract of other female odonates, *C. haemorrhoidalis* has two sclerotised lateral cuticular plates lined with sensilla, which are used for sperm ejection during the egg laying process (Siva-Jothy 1987; Corboda-Aguilar 1999). As an egg passes through the genital tract, the plates are forced apart, which stimulates the release of sperm for fertilization (Siva-Jothy 1987). Male *C. haemorrhoidalis* appear to be unable to adequately mechanically remove rival males sperm, but stimulation of the female's genital plates incites the ejection of sperm (Corboda-Aguilar 1999). Therefore, female genital morphology may subsequently have an effect on the evolution of the male genitalic structures within this species.

The Eucalyptus beetle, *Gonipterus scutellatus* exhibits a huge variation in copulation duration, lasting from an hour through to 24 hours (Carbone & Rivera 1998). In this species, it was shown that the male genitalia were unable to reach the female spermatheca, so prolonged copulation did not appear to be advantageous for the removal of previously deposited sperm. Copulation duration also did not affect the amount of ejaculate transferred. Furthermore, the order in which males mated did not correlate with the percentage of eggs fertilized, even within multiple clutches of the same female. Therefore, males able to remain in copulation for longer periods of time may be preferred by females, and more likely to fertilise a higher proportion of her eggs. Andres & Cordero Rivera (2000) found similar results in the damselfly *Ceriagrion tenellum*. In these instances copulation duration may have evolved under sexual selection by cryptic female choice, as the female appears to be the one that determines which ejaculates will be utilized. Such propositions about the influence of copulation duration on female choice are speculative at best and without direct evidence the selective pressures for such behavior cannot be definitively determined.

Recent research has provided more direct evidence of CFC during or post-copulation. Briceno & Eberhard (2009a) experimentally altered two male genitalic structures of the tsetse fly, *Glossina pallidipes*, involved in tactile stimulation of the female abdomen during copulation. They found that males that had a stimulatory disadvantage due to removal or manipulation appeared to be selected against via CFC. Females mated with altered males showed a decrease in ovulation and sperm storage, whilst remating increased compared with those who mated with control males. Even though it is difficult

to test the occurrence of CFC and distinguish between the adaptive and/or sexual selective processes that may be at work in the evolution of genitalia among certain taxa, such recent attempts at providing more direct evidence (Briceno & Eberhard 2009a; Briceno & Eberhard 2009b) as well as the use of microscale laser ablative manipulation of genitalic structures (Polak & Rashed 2010) may be able to provide more exciting insights into the influence of male-male conflict and female choice on genitalic variation. It does appear, however, that selective pressure such as sperm competition may be influencing CFC or vice versa, as structures designed to displace sperm may put pressure on females to develop ways of maintaining some control in the ultimate choice of sperm. As has also been suggested, such structures may have the dual function of influencing the female through mechanical stimulation of her reproductive tract.

1.2.3 *Sexual Conflict*

One of the early contributors to the theory behind of sexual conflict was Trivers' (1972) chapter published in *Sexual selection and the descent of man*. He noted that the sexes commonly differ in the amount of investment provided to the offspring, resulting in different sex roles and consequently conflict in interests. It was Parker's (1979) publication on sexual conflict, however, that really brought this theory into the spotlight. He recognized that traditional sexual selection models do not explain circumstances in which adaptations in males decrease the fitness of the female. Furthermore, during mating, differences in genetic interests between the sexes may result in conflict. In this respect he stated that "selection can act in opposing directions between the sexes" and

that sexual conflict may occur whenever there is a difference in the “evolutionary interests of individuals of the two sexes” (Parker 1979).

Sexual conflict can occur across many traits such as in infanticide (Arnqvist & Rowe 2005) and mate cannibalism (Parker 1979; Arnqvist & Rowe 2005). However, two main forms of conflict relate to mating/fertilization and parental investment (Parker 2006). It is sexual conflict in regards to mating and fertilization that is to be the focus of the following paragraphs.

As outlined in the previous section, anisogamy refers to the occurrence of disparity in the size and costs of the production of gametes between males and females. As such, the two sexes often try to maximize their reproductive success in different ways (Arnqvist & Rowe 2005). This may cause conflict between the sexes as males benefit from mating with, or more importantly transferring as many of their gametes to, as many females as possible, as their gametes are generally relatively inexpensive to produce. In contrast, female gametes are usually much larger and are more costly to produce inspiring females to choose their mates wisely on the basis of males that may produce the best offspring viability. In this sense, males generally benefit from an increase in mating frequency, whereas females may not. As a consequence, females may often resist male mating attempts. However, resistance to mating may be a great expense to the female through increased energy expenditure, susceptibility to predation, or loss of opportunities, suggesting that resistance to mating may carry huge costs that are not outweighed by any such benefits of multiple matings (Arnqvist &

Rowe 2005). Females may accrue other costs of superfluous matings, which includes increased risk of predation and decreased foraging efficiency (Rowe et al. 1994).

1.2.4 Female resistance and male persistence

If interactions between the sexes often involve conflict over whether or not to mate, with females commonly resisting copulation attempts, then males would benefit from traits that were able to circumvent female resistance behavior.

Overcoming female resistance is not always easy, although males of many taxa have developed strategies to try to avoid female resistance, defeat, or harass females into mating (Arnqvist & Rowe 2005). Male fruitflies, *Drosophila melanogaster* and *D. simulans*, try to avoid female resistance by mating with teneral females. Teneral females are newly emerged from pupae and are still soft-bodied and in which the wings have not yet unfolded. Males patrol hatching sites and attempt to mate with newly emerged females, as they are less capable of resisting in their fragile state (Seeley & Dukas 2011).

Males of the common bedbug, *Cimex lectularius* (family Cimicidae) mate at a high rate and in a way that is harmful to the female. Females of this species need to feed regularly in order to produce eggs, and during feeding the female may increase their body volume by as much as 300%. Males exploit the situation by attempting to mate with post fed females more frequently (Reinhardt et al. 2009), as engorged females are slow-moving, so have a greatly reduced ability to resist (Siva-Jothy 2006; Reinhardt et al. 2009).

Male water striders, *Gerris gracilicornis*, use intimidation tactics to try to overcome female resistance. Water striders mate on the water's surface, and the typical male mounting position is on top of the female. Females are then at a greater risk of predation from underwater predators during copulation. Within this species, males use their legs to produce ripples on the water to try and coerce females to mate even though it draws the attention of predators. Once in copula, the male ceases to produce the vibrations. However, when females resist, males will not decrease the frequency of vibrations and in some cases increase the frequency, essentially intimidating females into allowing them to mate (Han & Jablonski 2010). The authors suggest that this male strategy may be a counter-adaptation to the female's morphological shield that protects her genitalia from male coercive intromission attempts.

Males of many different taxa often possess secondary sexual characters such as grasping structures that are required for mating (Moreno-Garcia & Cordero 2007). In some species, however, these devices are not required, but may give the male an advantage over others by successfully holding onto the female in the company of rival males and due to female resistance. Male diving beetles within the subfamily Dytiscinae have adhesive discs on their tarsi which they use to attach themselves to the female's elytra, irrespective of premating courtship, and allow them to remain attached during female resistance. Females display dorsal modifications of the elytra, which make it difficult for males to remain attached. Therefore, sexual conflict has been suggested as an explanation for this variation in morphology (Bergsten et al. 2001; Miller 2003).

Although cases of male strategies in overcoming female resistance provide solutions to conflict over mating, the evidence that sexual conflict is the main process driving these traits is not compelling, as the pressure on these male traits may in fact be working through cryptic female choice of males that are successful at over-riding resistance. Indeed, key to the establishment of the occurrence of sexual conflict are the direct costs of mating to the female or “female preference” (Chapman 2006) in polyandrous systems, in which the male reaps the most benefit from multiple matings. An early model of sexual selection by female choice was established by Fisher (Arnqvist & Rowe 2005), and according to this model, attributes that may appear to generate sexual conflict, may in fact not be sexually antagonistic. Traits that confer direct benefits to the male but incur costs to their female partner may still provide indirect benefits to the female via the production of “superior” sons which suggests that female resistance may in fact represent a form of choice (Parker 2006).

However, if males evolve harmful traits to overcome female resistance, then logically females may try to reduce the amount of harm incurred. Hence, sexual conflict may drive sexual evolution through opposing selection on male and female reproductive strategies. More directly, it may influence genital evolution by placing selection on structures that overcome resistance or allow males a competitive advantage. Genital adaptations that allow males a competitive advantage may consequently reduce female fitness, causing intense conflict. As a result, females may respond with counter-adaptations to reduce the harm imposed by male adaptations (Arnqvist & Rowe 2002a, 2005; Ronn et al. 2007).

1.2.5 Primary sexual characters and sexual conflict

The previous section outlines behavioural and secondary sexual characters that are employed by males to gain an advantage. Focusing more directly on sexual conflict in terms of primary sexual characteristics such as internal genitalia, harm or damage to the female may occur during or post-copulation as a direct result of male genitalic structures. Thus far this has provided the most convincing support for the evolution of genitalia due to sexual conflict.

One of the classic systems relating to harm incurred from male genitalia includes the unconventional mating system of cimicid bed bugs and mirid plant bugs within the genus *Coridromius*. Bugs within these families exhibit obligate traumatic insemination, in which the male uses his intromittent organ (paramere) to pierce the female abdomen to directly transfer the spermatophore into her haemocoel (Carayon 1966; Morrow & Arnqvist 2003; Tataranic et al. 2006); even in the presence of the female's functional genital tract (Siva-Jothy 2006). This form of traumatic insemination is suggested to have emerged as a response to either mating resistance in females or as a means to influence sperm precedence in the context of sperm competition (Arnqvist & Rowe 2005).

Irrespective of the origin of this mode of reproduction, this mating tactic causes physical damage to the female (Carayon 1966; Morrow & Arnqvist 2003; Tataranic et al. 2006) and may reduce longevity and lifetime fecundity under certain conditions (Siva-Jothy 2006).

Research into the mating system of the bean weevil, *Callosobruchus maculatus*, revealed that the tip of the male's intromittant organ (aedeagus) is endowed with numerous sclerotised spines (Crudgington & Siva-Jothy 2000). These spines are everted into the

main region of the female reproductive tract during copulation and puncture the cuticular lining. Once mated, females produce melanised haemocyte plugs in order to repair damage incurred from the spines (Crudgington & Siva-Jothy 2000). Females of this species repeatedly kick males towards the end of mating, so Crudington & Siva-Jothy ablated the legs of some females to compare this treatment with the mean copulation duration of a treatment control (ablation of non-kicking legs) and control group (unmanipulated). They found that females unable to kick effectively with their hindlegs copulated significantly longer than both the treatment control and unmanipulated females. As a result, mate kicking reduces the amount of time in copula, and as a function may reduce the amount of damage incurred by the male's sclerotised structures. To investigate life history costs due to copulation frequency, the authors then measured the longevity of single and double-mated females. Females mated twice died significantly sooner than singly mated females, demonstrating that remating results in a reduction in longevity, and that earlier death rates associated with remating may be due to genital damage obtained during mating (Crudgington & Siva-Jothy 2000). Ronn et al. (2007) went on to describe the varying complexity of sclerotised spines that exist on the male aedeagus of several other species within this genus of seed beetle which cause damage to the female reproductive tract during copulation. Hence, they have become a good model system for the study of harmful male genitalic structures.

1.3 SEXUALLY ANTAGONISTIC COEVOLUTION

According to sexual conflict theory, male genitalia can be used as devices with which to manipulate females in order to increase their reproductive success. However, as a

consequence the female's reproductive success may be negatively influenced. Sexually Antagonistic Coevolution (SAC) is the process whereby females coevolve with males to counteract the negative effects produced by males, leading to an arms race between the sexes (Holland & Rice 1998; Chapman et al. 2003; Arnqvist & Rowe 2005). As the previous section highlights, sexual conflict appears to be operating in a number of arthropods including waterstriders (Han & Jablonski 2010), fruitflies (Seeley & Dukas 2011), diving beetles (Bergsten et al. 2001; Miller 2003), seed beetles (Crudgington & Siva-Jothy 2000), bed bugs (Morrow & Arnqvist 2003; Siva-Jothy 2006; Reinhardt et al. 2009), plant bugs (Tatarnic et al. 2006) as well as cases which have been recorded in dung flies (Allen & Simmons, 1996), scorpionflies (Thornhill & Sauer 1991), houseflies (Andres & Arnqvist 2001), and bruchid beetles (Eady et al. 2007); and in some of these systems there is evidence that sexual antagonism is operating to produce a coevolutionary arms race between the sexes (Arnqvist & Rowe 2002a, 2005; Ronn et al. 2007).

Many male water striders use their forelegs anteriorly, and genital segments, posteriorly, to grasp onto and maintain a grip onto females during copulation (Arnqvist & Rowe, 2002b). As mentioned previously, females are generally reluctant to mate (Han & Jablonski 2010) and often engage in a pre-mating struggle with males (Arnqvist & Rowe 2002b). Female water striders possess a pair of posteriolateral spines on the abdomen and they use these structures to disengage the male abdomen during pre-mating struggles and obstruct the male's access to the tip of their abdomen, making it challenging for the male to establish a strong grip. They also use their forelegs to

displace the male's forelegs which they use to grip onto the female's pronotum (Arnqvist 1997b). Arnqvist & Rowe (2002b) compared male grasping structures and female anti-grasping characters in 15 species of water striders for evidence of SAC. They found that elaboration of these male grasping structures were correlated by the elaboration of female anti-grasping structures, supporting a case of sexually antagonistic coevolution. Perry & Rowe (2012) found similar inter-population results for the water strider *Gerris incognitus*, in which the extent of exaggeration of male and female traits within this species were also highly positively correlated, further suggesting an arms race between the sexes.

As mentioned in the previous section, there appears to be sexual conflict among many species of diving beetles. Males exhibit variation in the modification of structures on their pro- and mesotarsi often in the form of specially modified setae that function like suction cups to latch onto and adhere to females (Miller 2003; Green et al. 2013). In accordance, females display variation in the dorsal morphology of the elytra, including highly longitudinally sulcated or granulated surfaces (Miller 2003), which may impede male attachment pre-copulation. Phylogenetic assessment of these male and female traits showed cladistic and inter-specific correlations between the coarseness of the modified dorsal surface of female elytra with the modification of male setae, supporting a case of sexually antagonistic coevolution (Miller 2003; Bergsten & Miller 2007).

Karlsson Green et al. (2013) went further to test the adhesive power of male suction cups on morphs within two diving beetle species - *Dytiscus lapponicus* and *Graphoderus zonatus*. The females of these species display two types of morphs within localized

populations; one morph with smooth elytra, and the other with a rough surface. Males had reduced adhesive strength on female morphs with rough surfaces compared with their smooth morph counterparts.

Cases of sexual antagonistic coevolution are also evident in bed bugs of the family Cimicidae and plant bugs in the genus *Coridromius*. As highlighted in the previous section on sexual conflict, males pierce the body wall of females with an intromittent organ known as the paramere to directly transfer ejaculatory products into the female's haemocoel, which causes injury to the female (Carayon 1966; Morrow & Arnqvist 2003; Tataric & Cassis 2010) and increases the female's exposure to infection (Reinhardt et al. 2003). However, in the region where the male's paramere punctures the abdominal wall, females of many species have evolved paragenital structures - modified unilateral sacs called spermaleges, which Carayon (1966) originally proposed as a counter-adaptation to sexual conflict. The spermalege consists of the ectospermalege and the mesospermalege. In the bed bug *Cimex lectularius*, the ectospermalege is an indentation and thickening of the cuticle on the right side of the abdomen; and the mesospermalege is a sac-like receptacle underlying the ectospermalege that is permeated with haemocytoid cells and is located within the inner abdominal wall (Carayon 1966). In this species, Morrow & Arnqvist (2003) experimentally pierced females with infected needles either in the area of the spermalege on the right or in the opposite position between the fifth and sixth sternite on the left side of the abdomen. Females that were pierced in the spermalege simulating conditions of natural copulation received no significant effects on lifespan or lifetime fecundity. However, females pierced outside of

the spermalege did suffer a large decrease in longevity and lifetime reproductive success (Morrow & Arnqvist 2003). This lends support to the proposition of the adaptation of the spermalege in response to an antagonistic male trait, in order to reduce the costs incurred by this harmful mode of reproduction. Plant bugs within the genus *Coridromius* made excellent study species for investigating the role of SAC in traumatic insemination and the evolution of paragenital structures. Criticism of the occurrence of sexual conflict and the possibility of SAC in traumatically inseminating taxa has often been based on the lack of variation of the male intromittent organ (Hosken & Stockley 2004; Eberhard 2006). However, males and females within *Coridromius* show a diversity of modifications in both male and female genital structures. Male species of *Coridromius* possess a diverse range of modifications in the length, curvature, angle and thickness of the tip, and the amount of twisting that occurs along the axis of the intromittent organ (Tatarnic & Cassis 2010). Similarly, females show variation in the modification of the areas in which the male's intromittent organ makes contact. In some species, males inseminate by piercing the intersegmental membrane that connects abdominal segments, whereas in other species, females have developed hardened cuticular indentations and/or "copulatory tubes formed by invagination of the abdominal cuticle" – spermalege - that function as a guide for the male's intromittent organ and inhibit the amount of potential damage from these structures (Tatarnic & Cassis 2010). On the tergites and laterotergites of the abdomen there are also varying degrees of swelling and desclerotisation (Tatarnic & Cassis 2010), which is thought to allow for the inflation of the mesospermalege (sac-like receptacle) whilst sperm is deposited (Tatarnic et al. 2006). In addition, plate-like modifications of the metepimeron and the formation of copulatory tubes on the hemelytron of the thorax have evolved and appear to play a

role in mating; the latter as a copulatory guide for the male's intromittent organ (Tatarnic & Cassis 2010). Similar to previous research undertaken on diving beetles, Tatarnic & Cassis (2010) took a macroevolutionary approach to examine the occurrence of SAC in this genus. By developing a phylogenetic reconstruction of these primary structures involved in reproduction within *Coridromius*, they showed that the male intromittent organ displays variation between species and correlates strongly with the complexity of the female spermatheca, providing further evidence of the SAC of traumatic insemination and paragenital evolution.

Seed beetles within the family Bruchidae, likewise, have harmful intromittent structures in the form of sclerotized spines on the tip of the phallus, and as noted in the previous section, these puncture and cause scarring to the female reproductive tract during copulation (Crudgington & Siva-Jothy 2000; Ronn et al. 2007). Ronn et al. (2007) used comparative and experimental methods to investigate the occurrence of sexual conflict and SAC in several species of these seed beetles. They found that the elaboration and level of harmfulness of the male genitalia was highly correlated with the reinforcement of the part of the female reproductive tract with which the spines make contact, suggesting a female counter-adaptation to minimize the amount of damage from the spines. They then went on to test the prediction that coevolution between the sexes should be balanced, in the respect that an increase in the harmfulness of the male structures should not necessarily be related to an increase in harm incurred by the female. If the coevolutionary balance is in equilibria, male offensive and female defensive traits could obscure or negate the degree of negative consequences, such as a

decrease in female lifetime or reproductive fitness (Ronn et al. 2007). This is evident in the results that neither the level of harmfulness of male genitalia nor the reinforcement of the female reproductive tract was individually significantly linked to reproductive or lifetime fitness in females. However, when male and female traits were combined in a multivariate analysis they did show that the degree of female reinforcement relative to the male harmfulness did relate to female fitness; such that when males have more spines but females less defence in terms of reproductive tract reinforcement than would be expected, harm was greater. The authors offer explanations behind this pattern that may occur within taxa exhibiting SAC (Ronn et al. 2007).

Even though there is a large amount of research on the topic of sexual selection in regard to genital evolution, sexual conflict and sexually antagonistic coevolution, there was a relative lack of investigation of these phenomena within the Lepidoptera until recently (Galicia et al. 2008; Sanchez et al 2011). The following sections give a breakdown of the basic male and female genital structures in Lepidoptera and an overview on the current research into the evolution of some of these structures to date.

1.4 MALE LEPIDOPTERAN GENITALIA

In a general sense, the genitalia of male Lepidoptera can broadly be defined and separated into the genital capsule and the intromittent organ – the aedeagus or phallus.

1.4.1 *Genital capsule*

The male genital capsule consists of structures derived from the ninth and tenth segments of the abdomen (Ever 1924; Klots 1970). The foundation of the genital capsule is made up of the tegumen, which comprises the dorsal aspect and the vinculum, which is ventral (Ever 1924; Klots 1970). The main structures of the genital capsule include the uncus, gnathos, transtilla, valvae, costa, and the juxta; however, different lepidopteran taxa vary in the presence and elaboration of these characters (see Kristensen 1998) (Figure 1). The uncus (Gosse 1883, in: Ever, 1924) and gnathos (Pierce 1914, in: Ever, 1924) arise from the tegumen. The uncus is the dorsal most aspect, with the gnathos ventrad of the uncus, arising from the tegumen close to the base of the uncus. The anal tube is located between these two structures (Ever 1924; Klots 1970). The transtilla (Pierce 1914, in: Ever 1924; Klots 1970) is a transverse band dorsal of the central diaphragm of the genital capsule and the valvae (Burmeister 1832, in: Ever 1924) are a pair of clasping organs ventral to this, which are connected to the posterior region of the vinculum (Ever 1924; Klots 1970). The valvae are sometimes simple, but in certain taxa they can be more complex, possessing a number of elements or regions; one such structure is the costa (Klots 1970). Interior to this region is the anellus, which is a “cone-like tube” in which the phallus protrudes (Ever 1924). The juxta is a plate that articulates with the ventral surface of this structure and processes of the juxta are proposed to provide support and/or guidance of the phallus (Ever 1924; Klots 1970).

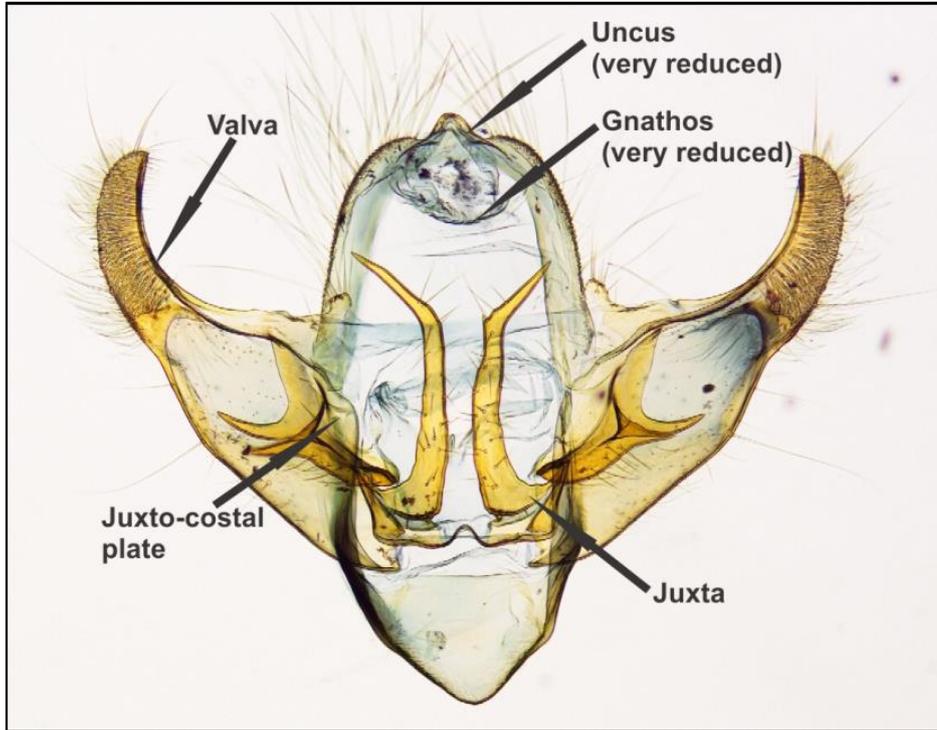


Figure 1. *Izatha taingo* male genital capsule, with main structures labeled.

1.4.2 *Phallus and Cornuti*

As the previous sections have discussed, male genitalia are often complex organs that contain many different structures, which may play various roles during copulation (Eberhard 1985). However, the functionality of many male genitalic structures is still unknown or very poorly understood (Eberhard 1985; Powell 2009; Werner & Simmons 2008; Cordero 2010). The organ male Lepidoptera use primarily for the transfer of sperm is the sclerotised structure known as the phallus or aedeagus. The phallus contains a membranous tube known as the vesica or endophallus and is connected to the ductus ejaculatorius (Figure 2), which functions in the transport of sperm (Klots 1970; Powell 2009). During copulation the vesica is everted and in some species is endowed with a singular or multiple sclerotised structures known as cornuti (Klots 1970; Powell 2009). It is the female's ductus bursae and corpus bursa into which the vesica is fully everted to deposit the spermatophore. In some species, there are spines attached to the vesica wall that detach with the transfer of the spermatophore (Figure 2) and remain in the female's bursa post-copulation, which are termed deciduous cornuti (Klots 1970; Powell 2009). Numerous publications have described the male genitalia of species within different families of Lepidoptera, and a growing number have recorded the occurrence of cornuti among certain taxa within this order (Gaskin 1985; Pogue 2004; Rawlins & Miller 2008; Yakovlev 2009). Cordero (2010) proposes that sclerotised spines that are permanently attached and do not break off during copulation be referred to as non-deciduous cornuti. An earlier publication refers to these structures as acanthae (Richards & Richards 1979), and therefore not true cornuti, so there has been some debate over whether these cornuti-like processes that are not detachable should be

classed as cornuti. Nevertheless, for simplicity, these structures will from this point on be referred to as non-deciduous cornuti. Cordero (2010) has discussed a number of hypotheses about the function of both deciduous and non-deciduous cornuti and the selective pressures influencing them.

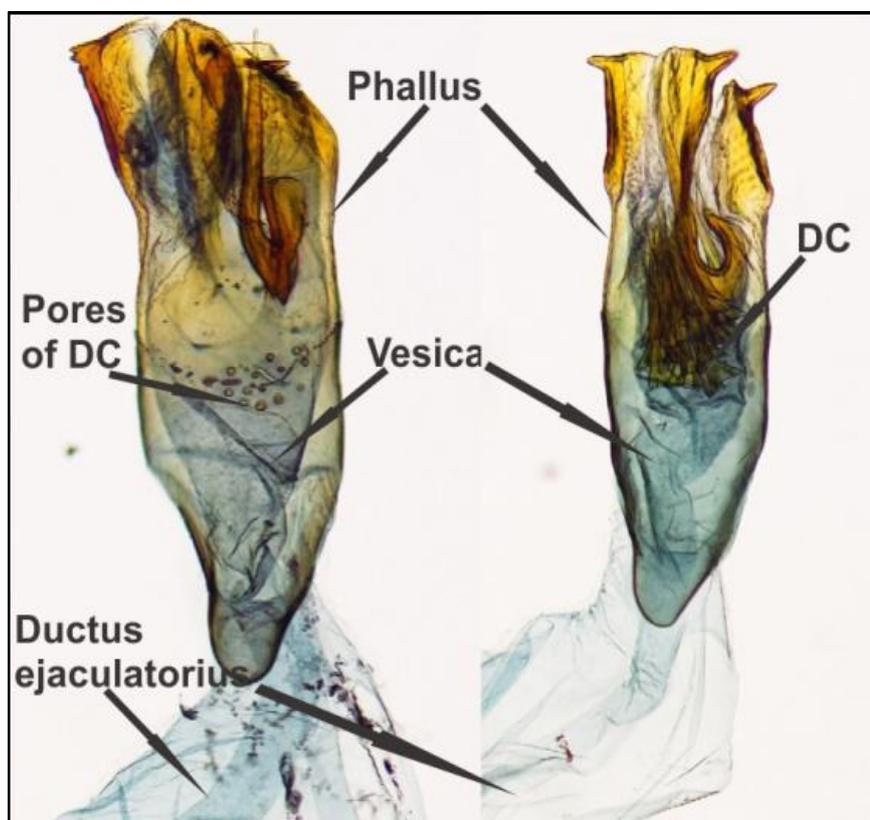


Figure 2. Phallus of *Izatha epiphanes* on the left and *Izatha mesoschista* to the right with basic structures labeled. DC = deciduous cornuti.

1.4.3 Hypotheses behind the function of non-deciduous cornuti

Numerous hypotheses have been suggested for the role of non-deciduous cornuti – cornuti that remain attached to the vesica (i.e. endophallus). Under the influence of natural selection, some propositions include that they aid the male by guiding the insertion of the vesica into the female reproductive tract, allowing males to hold on to the female's genitalia and maintain adequate positioning for spermatophore transfer or operate in a lock and key scenario (Cordero 2010). Another is that they assist in transferring the spermatophore into the female bursa (Callahan 1958; Callahan & Chapin 1960). Tuxen (1970) surmised that much like deciduous cornuti, non-deciduous cornuti were involved in the rupturing of the spermatophore.

Cordero (2010) further postulated that non-deciduous cornuti may also be under sexual selection as organs that allow the male to hold on the female. This is to avoid being usurped from the female by rival males, or interruption of copulation from the female. In addition, they may have evolved to coerce the female by puncturing her reproductive tract or protect the vesica from damage by the female's signa (sclerotised structure found in the bursa of some species of Lepidoptera). A few known studies have tested some of these hypotheses.

Two studies lead by Callahan (Callahan 1958; Callahan & Shapin 1960), as described in the previous hypotheses for non-deciduous cornuti, demonstrated that in male noctuids *Heliothis zea* and *Pseudaletia unipuncta* these structures were used to assist in the

transfer of the spermatophore. Cordero (2010) argues that this explanation may not be applicable to many other species of Lepidoptera, as placement of the spermatophore in these species is quite complicated and relies on female structures that do not exist in many other species (Klots 1970).

Cordero (2010) also suggests conditions under which different hypotheses for the function of non-deciduous cornuti may be rejected or apply to certain species but not others. Therefore, it is important to establish life history traits and morphological discrepancies between different taxa that may be driving the evolution of non-deciduous cornuti.

1.4.4 Hypotheses behind the function of deciduous cornuti

There are four hypotheses for the function of deciduous cornuti (cornuti that fully detach from the vesica) and their role in reproduction as suggested by Cordero (2010). The first hypothesis infers that cornuti are under natural selection and postulates that they may be used to assist females in breaking up the spermatophore at the conclusion of copulation; by females contracting the corpus bursae post-copulation, cornuti may be used to puncture the spermatophore. The following hypotheses suggest that cornuti are under sexual selection. One suggests that they may be employed in the process of sperm competition by being utilized to interfere with the positioning of subsequent males spermatophores within the female reproductive tract, or by breaking off rival's spermatophores before sperm have any opportunity to migrate to the spermatheca.

Another hypothesis proposes that cornuti developed to stimulate the female at the cessation of copulation, in order to coax females to utilize their spermatophore. In contrast, the final hypothesis proposes that cornuti cause post-copulatory damage, which functions in reducing the probability of or latency period at which a female may remate (Cordero 2010).

It is important to note that hypotheses may not be mutually exclusive, and cornuti, both deciduous and non-deciduous, may have multiple functions (Cordero 2010) or be under different selection for certain species. However, the role or roles that these structures play during copulation is still largely unknown, which is inhibiting insights into the evolutionary origins, maintenance and diversification of cornuti (Cordero 2010).

Cordero (2010) confers that a number of questions need to be addressed in order to begin to understand how deciduous cornuti function:

- 1) To establish whether there is individual variation in the number of deciduous cornuti as has been found within four species of tortricids in the genus *Pelochrista* (Wright 2008).
- 2) Whether the female participates in the deployment of cornuti or if it is completely under the control of the male.
- 3) Are the complete sets of cornuti ejected during the first copulation or only some released at each or in only some matings? And how may this affect reproduction, e.g. copulation duration, amount of ejaculate transferred, number of offspring produced in multiple matings etc.

- 4) The time during copulation in which the cornuti are deployed.
- 5) Whereabouts and in what position they remain within the female reproductive tract.
- 6) Do cornuti puncture the spermatophore or the female reproductive tract.

Cordero (2010) advocates the need for observational research, experimental manipulation of these structures, and comparative studies to help elucidate the mechanisms behind the development and functional maintenance of cornuti and suggests ways of testing hypotheses using these methods. However, evaluating cases of sexual conflict and evidence of sexually antagonistic coevolution of lepidopteran genitalia requires a sound knowledge of the female reproductive structures between and within different groups.

1.5 FEMALE LEPIDOPTERAN GENITALIA

There are three major divisions of Lepidoptera – the Zeugloptera, the Monotrysia, and the Dytrisia. In the Zeugloptera there is no distinct external female genital opening, the genitalia are connected to the exterior with the posterior region of the digestive tract – the anus. In Monotrysia, there are one or two genital openings at the distal end of the eighth sternite, close to the anus (Klots 1970). The Dytrisia, which are the basis of my further discussion of the female genitalia, have two distinct genital openings. The aperture used in oviposition is still located close to the anus, but the orifice for insemination is located further away and more anteriorly, on the ventral surface of the seventh or eighth sternite (Klots 1970; Holloway et al. 2001). It is the internal

reproductive structures that are directly connected to the latter genital opening that will be discussed further in this section.

Therefore, excluding the ovipositor opening, the invaginated genital chamber involved during insemination internally opens into the ostium. The ostium opens up into the ductus bursae, which may be expanded into a funnel-shaped tube, known as the antrum, which progresses to the more inner region of the ductus bursae (Holloway et al. 2001) (Figure 3). The ductus bursae is often a tubular structure, but can vary in complexity between different taxa (Kristensen 1998). The ductus bursae then opens up into a sac-like structure, the corpus bursae, in which the male's spermatophore is deposited during copulation (Drummond 1984; Holloway et al. 2001). In some species, the inner wall of the corpus bursae may bear a sclerotized structure known as the signum (Drummond 1984; Holloway et al. 2001; Hoare 2010) (Figure 3); which vary in shape within different taxa (see Sanchez et al. 2011).

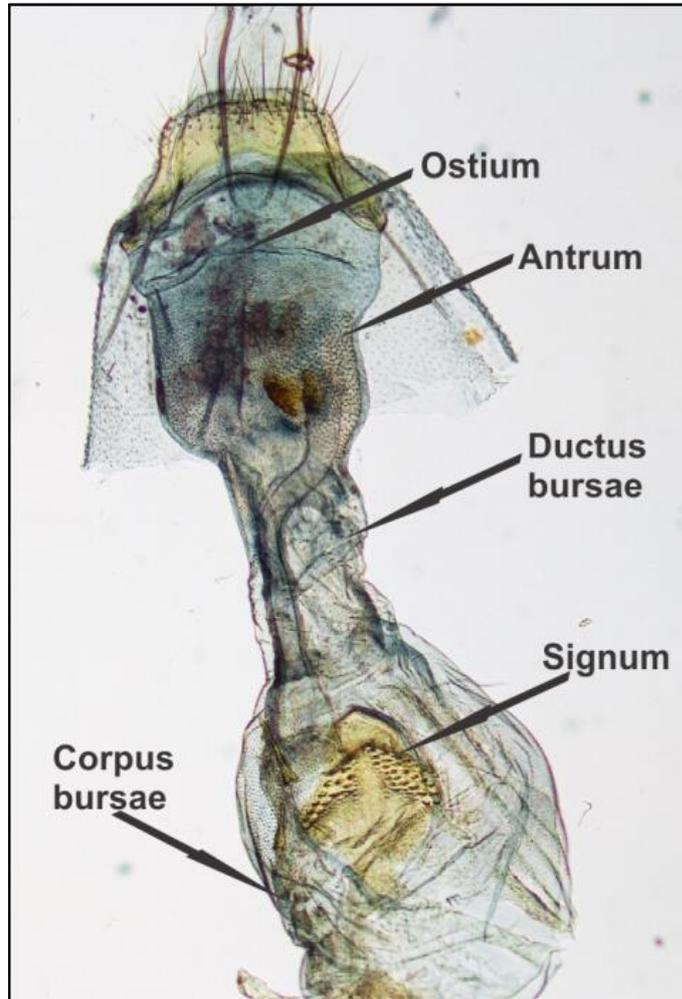


Figure 3. Female reproductive tract of *Izatha haumu* with main structures labeled.

1.5.1 *Hypotheses on the function of the signum*

Cordero (2005) proposed three hypotheses as to the evolutionary origin and function of signa (singular = signum) in Lepidoptera. The first hypothesis suggests that signa are a naturally selected adaptation in order to increase oviposition rate. An adaptive trait that could speed up the digestion of the spermatophore would be a benefit to the female as it may increase the rate at which oviposition stimulants from the spermatophore are released, and/or the rate at which the female may obtain valuable nutrients or defensive substances for use in the provisioning of eggs. A primary advantage of an increase in the rate of oviposition is the potentially increased chance of the availability and utilization of larval food resources under dynamic environmental conditions. However, an increase in oviposition rate may affect male fitness in polyandrous mating systems, as the female latency period to remating will be shorter (Cordero 2005). This leads into the second hypothesis, that signa are a sexually antagonistic counteradaptation to male control over the refractory period before a female may remate.

Under this scenario, the evolution of polyandry may in part be driven by the benefit that females may gain from mating with multiple and potentially younger males, which may have a “fitter” composition of sperm in their ejaculates (Siva-Jothy 2000) in order to acquire additional nutritional or hormonal substances contained in the spermatophore (Karlsson 1995; Torres-Vila et al. 2004; Sanchez et al. 2011). As a consequence, a shorter latency period to remating should be selected for in females (Galicía et al. 2008; Sanchez et al. 2011). However, this provides a disadvantage to the male, as it may result in the female consuming the resources transferred with the ejaculate before utilizing or

completely depleting their last mate's sperm (Kaitala & Wiklund 1994). In this case, males would benefit from promoting a female post-mating latency period long enough for their mate to use all their sperm (Galicia et al. 2008; Sanchez et al. 2011), and Drummond (1984) proposed that this would select for spermatophores with a higher chitin content, which is therefore tougher and harder for the female to tear or digest. In turn, females may counter-adapt by evolving structures capable of tearing tougher spermatophores, leading to sexually antagonistic coevolution as a driver in the evolution of signa (Cordero 2005).

The third hypothesis suggests that signa originated from bypassing the disadvantage of cryptic female choice (CFC) of males with tougher spermatophores for which the offspring may acquire a better ability to manipulate female remating refractory periods and reduce the probability of sperm competition (Cordero 2005). However, Cordero (2005) also posits that in this case, CFC may not necessarily be the driving factor in the origin of the signa, but may be responsible for further maintenance or elaboration of these structures, or a synthesis of both SAC and CFC may be responsible for their evolution.

Galicia et al. (2008) provided further hypotheses behind the potential function of signa. Among several propositions are that the signum is a structure that "locks" with corresponding structures of the male phallus or is an organ protecting the corpus bursae from potential damage from male phallic structures, such as cornuti. Another suggests

that they operate as sense organ, determining the presence and/or the size of a spermatophore deposited during copulation. Galicia et al. (2008) also elaborated on previous hypotheses first postulated by Hinton in 1964. These include that the signum is used to stimulate males to ejaculate, hold/position the spermatophore correctly, break up the spermatophore or involved in “filtering particles of degraded spermatophore that could block the ductus seminalis”, which could inhibit the migration of sperm into the spermatheca (Galicia et al. 2008). Galicia et al. (2008) then tested these hypotheses among four butterfly species and surmised that in these species the signa functions in the breaking up of the spermatophore, which supported the theory of sexual coevolution as a driver in the evolution of these structures.

With evidence pointing toward the sexually antagonistic coevolution of tough spermatophores and the signa, Sanchez et al. (2011) made the following assumptions: that signa should evolve in species that exhibit polyandrous mating systems; and if a monandrous mating system is secondarily selected for, selection would also favour a thinner and easier to break membrane on the spermatophore; which could subsequently select for a reduction or loss of signa. By employing comparative phylogenetic analysis, the authors then tested the prediction that signa should be present in polyandrous species and that monandry promotes loss or reduction of the signum. The results supported the plesiomorphic evolution of signa in polyandrous taxa and the lack of these structures in all but two monandrous groups. Therefore, there is mounting evidence that SAC is occurring between a male trait and counter-adaptive female genital structure between certain groups of Lepidoptera. However, further

investigation into the origin and evolution of other genital characters and examination of other lepidopteran groups with novel genital structures is warranted.

1.6 IZATHA

One particular group that may be an appropriate model as an insight into lepidopteran genital evolution and the co-evolutionary effects of offensive and defensive traits, are moth species within the genus *Izatha*. Formerly placed within the family Oecophoridae, recent research has found the genus more closely related to the xyloryctid assemblage (Kaila, 2004; Kaila et al. 2011; Heikkila et al. 2013), positioning them in the family Xyloryctidae. The genus is comprised of 40 species, 15 of which have been recently described, and all are endemic to New Zealand (Hoare 2010). Most species are found inhabiting forests within the North Island, some throughout both islands, while others are confined to the South and offshore islands. The caterpillars (larvae) of this genus are often found in rotting wood; however, they have also been associated with bracken fungi and lichen. The adults are commonly termed “lichen tuft moths” due to their often-camouflaged colouration and tufts of raised scales that mimic bark, moss or lichen whilst the moths rest on tree trunks. As well as their beautiful and unusual appearance, *Izatha* are fascinating in the respect that there appears to be a higher radiation of species within the North Island of New Zealand compared with the South, which is an unusual pattern for larger genera of Lepidoptera within New Zealand (Hoare, 2010). The species within this genus also display interesting genitalic structures, especially the males. There is a large variation in the size, shape and complexity of the male genital capsule and phallus within this genus. The phallus (aedeagus) of some species is often

adorned with strong sclerotised ridges armed with backward pointing teeth, and possible damage from these structures has been identified within the female genital tract (Hoare 2010). Males of one group of species within the genus have also been found to possess a varying number of detachable spines (deciduous cornuti) attached to the inner vesica (ie. endophallus) of the phallus, which are all ejected into the female reproductive tract within a single mating (Hoare 2010). Little is known about the functionality of these structures, but sexual conflict may be important in the form of a male's use of these structures to dissuade or delay females from remating, as females within the genus have been known to mate more than once. Females of species where males possess larger teeth on the phallus appear to have their genital tract reinforced and in species where males have deciduous cornuti females also possess a signum, a sclerotised region of the corpus bursae, which is the part of the female reproductive tract that receives the spermatophore. These female structures may function to minimize the amount of damage that may be inflicted by these male adaptations (Hoare 2010).

1.7 GLAUCOCHARIS

Another group of moths that are interesting in terms of evolutionary and genitalic diversification are species within the genus *Glaucocharis* (Family Crambidae). There are 143 species of *Glaucocharis* (Lepidoptera: Crambidae: Crambinae) located worldwide (Li & Li, 2012), including 18 species endemic to New Zealand. New Zealand *Glaucocharis* were originally assigned to the genus *Pareromene*. However, the holotype of *Glaucocharis stella* was discovered in the British Museum collection and found to be

conspecific with species of *Pareromene*. As the *Glaucocharis* holotype was described earlier, “Rules of priority” govern that the older genus name takes precedence (Gaskin, 1985). Most New Zealand Crambinae are grass feeders, whereas *Glaucocharis* are largely moss feeders, often found in dense forest or sub-alpine areas (Gaskin, 1985).

Glaucocharis lack deciduous cornuti but some species have a number of non-deciduous or “fixed” cornuti attached to the inner vesica of the phallus. In the species in which cornuti are present, they range from a number of microscopic or small elongate to large singular structures. Some species within the genus also have sclerotised protrusions – spurs at the apex of the phallus, as well as variation in the length of the gnathos and uncus, fusion of the valval costa, and the presence of an extra process on the valval costa of the genital capsule (Gaskin, 1971; Gaskin, 1985).

1.8 RESEARCH OBJECTIVES

My aim was to employ comparative approaches to determine evolutionary relationships, and patterns of genital evolution in two moth genera – New Zealand *Glaucocharis* and *Izatha*, which is endemic to New Zealand. In addition, I tested whether an arms race through SAC could be responsible for the diverse genital morphology displayed within these genera, and to gather more indepth data on the ecology, seasonal variation and mating patterns among a few common species of *Izatha* to further research into the mechanisms behind the extraordinary genital diversity within this genus. Four studies are presented to support the overall thesis aims.

In chapter two I describe the molecular phylogenetic relationships and patterns of male genital evolution in New Zealand *Glaucocharis* (Lepidoptera: Crambidae). The prediction of sexual conflict and SAC of a potentially harmful male and resistant female genital structure is then tested and discussed.

In chapter three the molecular phylogenetic relationships and patterns of male genital evolution of the endemic *Izatha* (Lepidoptera: Xyloryctidae) are presented along with a discussion on the potential processes behind the current distribution of species within this genus.

Chapter four tests the prediction of sexual conflict as a driver of SAC in two sets of male and female genital structures within *Izatha*: male deciduous cornuti and the female signum; and harmful external teeth of the phallus and the reinforcement of the female reproductive tract.

In chapter five I describe the seasonal variation in three common species of *Izatha* – *I. peronenaella*, *I. churtoni*, and *I. epiphanes*. The occurrence of protandry, sexual size dimorphism, sex ratios and mating success in regards to time and body size is investigated to gain a better understanding of the mating systems in these species. The findings are discussed in an evolutionary context in order to understand the potential natural and sexual selection drivers of these phenomena.

In chapter six I summarise the main findings of the research undertaken in this thesis and suggestions for areas of further research.

2.

Molecular phylogenetics, genital evolution and sexual coevolution of New Zealand *Glaucoccharis* (Lepidoptera: Crambidae)

2. Molecular phylogenetics, genital evolution and sexual coevolution of New Zealand *Glaucocharis* (Lepidoptera: Crambidae)

2.1 ABSTRACT

Male genitalia are amongst the fastest evolving structures in the animal kingdom.

Hypotheses to explain rapid genital evolution include species isolation (the lock and key mechanism), pleiotropy, and sexual selection. More recent research supports sexual selection as one of the main drivers of the rapid and divergent evolution of genitalia.

Sexual selection may influence genital evolution through a number of distinct mechanisms: male competition for fertilization (sperm competition), female preference (cryptic female choice), or conflict between the sexes, which may lead to an arms race between the sexes via sexually antagonistic coevolution (SAC). New Zealand species of moths in the genus *Glaucocharis* Meyrick, 1938 display large variation of male genital traits, making them ideal candidates for the examination of the processes driving genital evolution. Here, the phylogenetic relationships of New Zealand *Glaucocharis* were determined along with the ancestral character states and direction of evolution of key male genital traits. The relationship of a potentially harmful male structure - the apical spur on the phallus – and the part of the female reproductive tract with which it makes contact was examined further to assess the potential for sexually antagonistic coevolution to have driven its diversity in this genus. There was a significant correlation between the area and length of the male apical spur (genital spine) and thickness of the female ductus bursae (reproductive tract) supporting the coevolution of these two

structures. Due to the potentially harmful nature of the apical spur and the expected corresponding thickening of the female ductus bursae, sexual conflict seems to be the most likely scenario behind the evolution of these genital traits in this genus, however further investigation is warranted to distinguish from other types of sexual selection, such as cryptic female choice. Additionally, the molecular phylogeny reveals there are three main clades of New Zealand species of *Glaucocharis* and they display a number of apomorphic male genitalic traits. The ancestry, direction of evolution, potential origins and functionality of male genitalic traits are discussed.

2.2 INTRODUCTION

It is well documented that male genitalia are among the fastest evolving morphological structures and frequently used in species delimitation and identification (Eberhard, 1985; Hosken & Stockley, 2004; Eberhard, 2010). The rapid and divergent evolution of male genitalia was originally suggested to relate to the process of species isolation, often referred to as the “lock and key hypothesis” (Dufour 1848; in Arnqvist 1997a). In this case males and females of a species possess a lock and key system, in which females may evolve distinct complementary genitalia in order to exclude heterospecific males from successful copulation, but permit copulation with conspecifics that produce the exact corresponding fit (Eberhard 2010b). Another hypothesis suggested that the divergent evolution of male genitalia may be a neutral pleiotropic side effect of evolutionary divergence in other traits related to speciation (Mayr, 1963; Eberhard 1985; Arnqvist 1997a). Although evidence from some systems supports these two older hypotheses (Arnqvist & Thornhill 1998; Sota & Kubota 1998; Morrow et al. 2003; House

& Simmons 2005; Holwell 2008), more recent evidence suggests that the large diversification and complexity of male traits is largely a result of sexual selection (Arnqvist 1998; Arnqvist & Danielsson 1999; Arnqvist et al. 2000; Hosken & Stockley, 2004).

There are three main processes under which genitalia may be under sexual selection (Simmons, 2001; Hosken & Stockley, 2004). Cryptic female choice, which incorporates the “sexy sons” (fisherian selection) and “good genes” hypotheses, proposes that male genitalia may be under selection through female choice of males possessing certain traits in order to gain indirect genetic benefits (Eberhard, 1985; 1996). In polyandrous mating systems, males may also avoid sperm competition through selective pressure for genital structures that may displace or remove rival male’s sperm to attempt to ensure paternity (Parker, 1970; Simmons, 2001). Conflict between the sexes may also have an effect on genital morphology. According to sexual conflict theory, male genitalia can be used as devices with which to manipulate females in order to increase their reproductive success, however, as a consequence female fitness may be negatively influenced (Parker, 1979; Arnqvist & Rowe, 2005). This conflict of fitness interests between the sexes may lead to sexually antagonistic coevolution (Chapman & Partridge 1996; Rice 1996). In this scenario, females may evolve to counteract the negative effects produced by the male, leading to an arms race between the sexes (Holland & Rice 1998; Chapman et al. 2003; Arnqvist & Rowe 2005).

A prime example of sexual conflict is evident in water striders. Some male water striders use their forelegs and genitalic structures to clasp onto and remain attached to females during copulation (Arnqvist & Rowe, 2002a). However, females often display mating resistance by engaging in a pre-mating struggle with males (Arnqvist & Rowe 2002a; Han & Jablonski 2010) by using posterolateral abdominal spines and forelegs to hinder male attachment (Arnqvist 1997b). Inter- and intra-specific studies of male clasping and female anti-grasping structures in water striders have shown a high level of correlation between the levels of elaboration of these male and female morphological traits (Arnqvist & Rowe 2002b; Perry & Rowe 2012) providing evidence of sexually antagonistic coevolution.

More primary genital structures have also been shown to play a role in sexual conflict in terms of traumatic matings that may produce physical damage to the female. In cimicid bed bugs and plant bugs of the genus *Coridromius*, traumatic insemination involves males puncturing the female abdominal wall with specialized intromittent organs and depositing sperm directly into the haemocoel (Carayon 1966; Morrow & Arnqvist 2003; Tataric et al. 2006); irrespective of the female's fully functional reproductive tract (Siva-Jothy 2006). This type of reproductive strategy causes physical injury to the female (Carayon 1966; Morrow & Arnqvist 2003; Tataric et al. 2006) showing reduced female longevity and lifetime fecundity in some circumstances (Siva-Jothy 2006). Females of these species however, have coevolved structures called spermaleges localized at the area of male penetration in order to minimize the amount of damage incurred by the

male's intromittent organ (Morrow & Arnqvist 2003; Tataric et al. 2006), suggesting the involvement of sexual antagonistic coevolution.

Other traumatic mating organs include sclerotised structures such as genital or phallic spines, which are present in a number of insect taxa and in many cases these genital spines cause injury to the female reproductive tract symptomatic of sexual conflict (Blanckenhorn et al. 2002; Baer & Boomsma 2006; Flay et al. 2013; Flowers & Eberhard 2006; Ronn et al. 2007; Kamimura 2007, 2010; Okuzaki et al. 2012). However, there are also instances of the apparent coevolution of male and female genitalia in which no direct costs to the female are obvious (Yassin & Orgogozo 2013).

One group of insects that are suitable for the examination of inter-generic genital evolution and sexual coevolution of genital traits are New Zealand moths of the genus *Glaucocharis* Meyrick, 1938. New Zealand *Glaucocharis* are often associated with mosses and ferns, and primarily inhabit subalpine forest (Gaskin, 1971), with two species – *G. helioctypa* and *G. epiphaea* often located among alpine and subalpine mossy wetlands (Gaskin, 1985). Males of many species within this genus possess a potentially harmful sclerotized structure – an apical spur present on the external surface of the phallus - and display wide intra-specific variation in other primary and secondary genital structures. There are currently 143 species of *Glaucocharis* (Lepidoptera: Crambidae) known worldwide (Li & Li, 2012); there are representatives found throughout Asia and Australasia (Li & Li, 2012) including 18 species native to New Zealand (Gaskin, 1985). Previous genital morphological examination of *Glaucocharis* species has revealed

variation in a number of male genitalic characters (Gaskin, 1971). There is divergence in the relative growth of the uncus, differential growth patterns of the gnathos, presence and development of cornuti, and variation in the shape and complexity of the juxta, valva and valval costa. Female genitalia also show disparity in the posterior segments of the abdomen, antrum, and development of the ductus and corpus bursae (Gaskin, 1985). The signum, which is a sclerotised structure present on the inner wall of the female corpus bursae – the reproductive structure that receives the spermatophore - of many groups of Lepidoptera (Cordero, 2005; Galicia et al. 2008) is absent in all but one species of New Zealand *Glaucocharis* - *G. interrupta*, in which there is a small star shaped signum present (Gaskin, 1971). Gaskin (1985) described New Zealand species as displaying primitive features and along with New Caledonian members, were postulated to be of a more unspecialized Gondwanan origin. In the New Zealand *Glaucocharis* there is notable differentiation of the uncus, gnathos, and valvae - structures used to grip onto the female abdomen at the onset of copulation (Common, 1975; De Jong, 1977; Fanger & Naumann, 1998; Mikkola, 2008). Between species there is variation in the length of the uncus and gnathos; the presence on the valval costa of a distinct singular apical prong (fused) or alternatively a distinct division, displaying the separation of the apical prong into two projections (separation); and presence of an extra process on the valval costa in some species. There is also variation in the presence of sclerotised structures of the phallus. As mentioned, some species have an apical spur (spine) at external tip of the phallus; some have a large sub-apical cornutus attached to the inner vesica (ie. endophallus); and others have rows of smaller cornuti. Cornuti are characterized as sclerotised spines or structures that are attached to the inner wall of the phallus – the vesica - that is everted during copulation.

In the present study a molecular phylogeny of New Zealand species of *Glaucocharis* will be constructed in order to explore the evolution of genital traits in the group. The phylogenetic information will then be used to apply phylogenetically controlled comparative methods to test whether a male and female genitalic traits show evidence of coevolution, as the position of the apical phallic spur of some males would most likely make contact with the soft tissue female ductus bursae. The apical spur is quite a large and sharp structure that appears like the kind of genital trait that may cause harm to the female during copulation. Therefore, it is predicted that this apical spur has evolved through a process of sexually antagonistic coevolution.

2.3 METHODS

2.3.1. Taxon sampling and data collection

Sixteen of the eighteen endemic species of New Zealand *Glaucocharis* were included in the phylogenetic analyses (two rarer species were not included due to collection limitations) (Table 1), in addition to four outgroup species from related crambid genera - *Gadira* Walker, 1866, *Orocrambus* Purdie, 1884, *Tawhitia* Philpott, 1931 to root the tree. A species of *Culladia* Moore, 1886 – *Culladia strophaea* was also included in the outgroup taxa, however, it must be noted that it is currently a crambid of unknown affinities (Hoare 2001). Further specimens were acquired for dissection and histological preparation. Most of the specimens were collected over the 2012/2013 summer season by the first author R.M. Bennik (see Appendix One), with contributions from R.J.B. Hoare, A.W. Emmerson, N. Hudson, and B.H. Patrick. Further samples were sourced from the New Zealand Arthropod Collection (NZAC) at Landcare Research, Auckland,

New Zealand. Field caught specimens were collected at night using a 125 Watt mercury vapour light, with a few specimens collected resting on plants during the day. For the phylogenetic analyses, freshly collected specimens were preserved in 95% ethanol and stored at -20°C. When fresh material was not available, pre-set specimens less than 10 years old were used. All other specimens were pinned and dried out for microscope slide preparation or fixed in 10% formaldehyde for histological sectioning of the female reproductive tract.

Table 1. Species groups and list of species of New Zealand *Glaucocharis* based on Gaskin (1985). Species included in the phylogenetic and ancestral state analyses are in bold.

Group name:	Glaucocharis species:
<i>epiphaea</i>	<i>epiphaea</i> <i>pyrsophanes</i> <i>holanthes</i> <i>parorma</i>
<i>auriscriptella</i>	<i>harmonica</i> <i>bipunctella</i> <i>auriscriptella</i> <i>stella</i> <i>metallifera</i> <i>microdora</i> <i>interrupta</i> <i>lepidella</i> <i>helioclypa</i> <i>selenaea</i>
<i>elaina</i>	<i>planetopa</i> <i>elaina</i>
<i>chrysochyta</i>	<i>chrysochyta</i> <i>leucoxantha</i>

2.3.2. DNA extraction and sequencing

Three legs from each adult specimen were used for DNA extraction. All tissue samples were dried and crushed up using microtube pestles, with DNA extracted and purified using Qiagen's DNeasyTM extraction kit following manufacturer's instructions.

The LCO (1490) and HCO (2198) primers were used for the amplification of the cytochrome oxidase subunit I mitochondrial gene (CO1) and 28S_Ff and 28S_Dd primers for the ribosomal 28S gene. A hemi-nested PCR was done to amplify the nuclear gene, Elongation factor 1-alpha (Ef1 α). The first sets of primers used were For1deg/Cho10mod and For1deg/Cho10Rev1 for the second PCR (Table 2).

Table 2. PCR primers used in the present study. Amplification product length follows sequence trimming.

Gene region	Primer direction	Primer name	Primer sequence (5'-3')	Anneal. temp (°c)	Sequence length (bp)	Reference
COI	Forward	LCO1490	GCTCAACAAATCATAAAGATATT GG	50	596	Folmer et al. 1994
COI	Reverse	HCO2198	TAAACTTCAGGGTGACCAAAAAA TCA	50	596	Folmer et al. 1994
28S	Forward	28S_Ff	TTACACACTCCTTAGCGGAT	45	591	Hillis & Dixon 1991
28s	Reverse	28S_Dd	GGGACCCGTCTTGAAACAC	45	591	Hillis & Dixon 1991
Ef-1 α	Forward	For1deg	GYATCGACAARCGTACSATYG	55	906	Danforth & Ji 1998
Ef-1 α *	Reverse*	Cho10mod	ACRGCVACKGTYTGHCKCATGTC	55	N/A*	Danforth & Ji 1998
Ef-1 α	Reverse	Cho10Rev1	AGCATCDCCAGAYTTGATRGC	55	906	McKenna et al. 2009

PCR amplification was conducted in 20 µl volume reactions comprised of: 12.8 µl milliQ H₂O, 2 µl of 10x PCR buffer, 2 µl dNTPs, 0.5 µl of each primer (10 mM), 0.8 µl BSA, 0.2 µl Taq DNA Gold Polymerase, 0.2 µl DMSO, and 1 µl DNA template. The standard thermal cycling profile for CO1 and 28S was an initial cycle at 95°C for 5 minutes, 94°C for 30 seconds, annealing at 45°C (28S) and 50°C (CO1) for 45 seconds, extension/elongation at 72°C for 2 minutes for 40 cycles, then final elongation at 72°C for 7 minutes. For the Ef1 α gene, the same protocol was used with the following differences: the initial PCR annealing temperature was 55°C for 38 cycles and 57°C for 35 cycles for the second amplification.

Product clean-up was conducted in 10 µl volumes: 5.6 milliQ H₂O, 2 µl of 5x sequencing buffer, 1 µl of each primer (3 mM), 0.4 µl of big dye terminator mix and 1 µl of diluted PCR template. Most often, PCR product was diluted by a 5(milliQ H₂O):1(PCR product) ratio, occasionally 3(milliQ H₂O):1(PCR product) for less purified product. The standard thermal cycling profile was the same for all three genes: an initial cycle at 96°C for 1 minute; 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 75 seconds; and a final extension at 10°C for at least 10 minutes.

Prior to sequencing, 10 µl of x-Terminator and 45 µl of SAM solution were added to the sequencing PCR product. It was then vortexed for 30 minutes @ 2000rpm and centrifuged for 2 minutes @ 1000rpm.

2.3.3. *Phylogenetic analyses*

Sequences were initially edited and aligned using Geneious 6.0.5 (Drummond et al.). Model selection was performed in Jmodeltest 2.1.4 (Darriba et al. 2012), using the AIC method (Akaike, 1978). Garli 2.0 (Zwickl, 2006) was used for maximum likelihood tree searches and bootstrapping, with bootstrap consensus trees constructed in PAUP*4.0b10 (Swofford 2002) using the output from Garli. Bayesian trees were executed in MrBayes v3.2 (Ronquist et al. 2012). MrBayes was run using the best fit AIC model (which was $\gamma\tau+\iota+\gamma$ for each gene) – with each gene partitioned – 28S, CO1, and EF1 α . Six individual MCMC runs were conducted each using 4 chains for 5 million generations, with every 1000 generations sampled. The first 1000 (20%) samples of each of the analyses were discarded as burn-in, following inspection in Tracer1.6 (Rambaut et al. 2014), utilizing the remaining 4000 samples to calculate the posterior probabilities for nodal support.

2.3.4. *Genital characters*

Male genital characters were assessed from prepared slides of specimens collected during the 2012/2013 summer season and existing specimens from the New Zealand arthropod collection (NZAC). The dissection of genitalia was performed using techniques modified from Hoare (2005). Once the genitalia were separated from the abdomen, the phallus was removed from the genital capsule and both prepared onto microscope slides along with the abdomen.

The genital structures presented in the current study were those showing the greatest amount of variation among *Glaucocharis* species. These include three genital capsule and phallic structures. For the genital capsule, the length of the uncus and gnathos, the separation or fusion of the valval costa and the presence of an extra process on the valval costa were examined. For the phallus, the presence of an apical spur, a large singular cornutus, or other smaller cornuti were further investigated. Example species displaying various male genital traits, and conspecific female reproductive tracts, highlighting ostial pouches – a term tentatively given to paired sac-like structures arising from the ostium - are displayed in Figure 1.

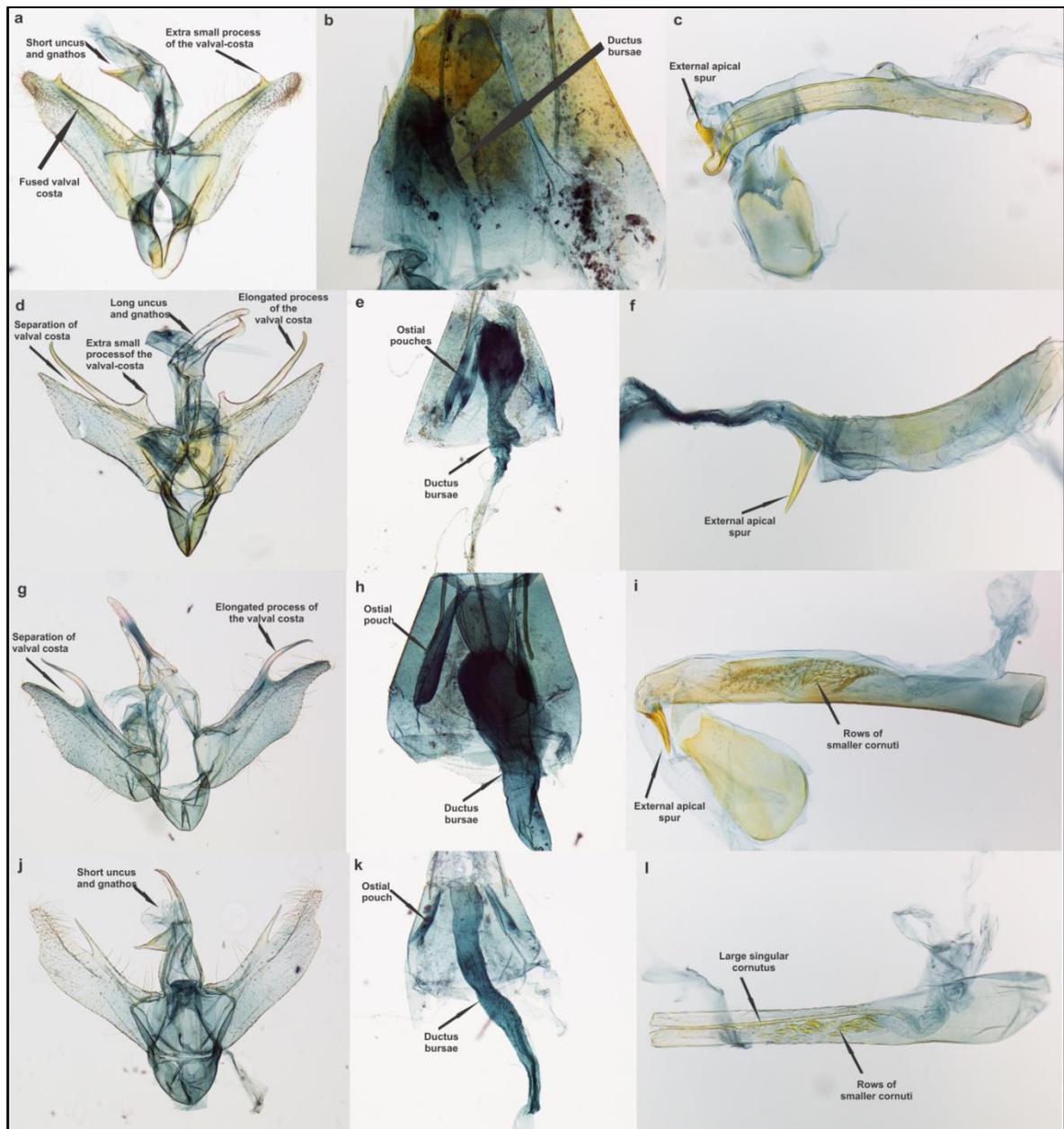


Figure 1. Images displaying various genital structures from representative species of New Zealand *Glaucocharis*. a) genital capsule of *G. holanthes* b) female reproductive tract (showing ostium and ductus bursae) of *G. holanthes* c) phallus of *G. holanthes*; d) genital capsule of *G. elaina* e) female reproductive tract (showing ostium and ductus bursae) of *G. elaina* f) phallus of *G. elaina*; g) genital capsule of *G. interrupta* h) female reproductive tract (showing ostium and ductus bursae) of *G. interrupta* i) phallus of *G. interrupta*; j) genital capsule of *G. leucoantha* k) female reproductive tract (showing ostium and ductus bursae) of *G. leucoantha* l) phallus of *G. leucoantha*.

2.3.5. *Ancestral state reconstruction*

An ancestral state reconstruction of male genitalic characters was performed in Mesquite 2.75 (Maddison & Maddison, 2011) and mapped onto a cladogram, with within species clades compressed, based on the topology of the optimal ML tree. The Mk1 model (Lewis, 2001) was used to calculate the maximum likelihood reconstruction probabilities (Maddison & Maddison, 2006).

2.3.6 *Male phallic shape variation and the external apical spur*

Nine species of New Zealand *Glaucocharis*, five that possess an external apical spur on the phallus and four in which this structure is absent, were used to investigate the coevolution of the elaboration of this character and the reinforcement/thickness of the female ductus bursae wall. Digital images of at least three specimens of each species of the male phallus of New Zealand *Glaucocharis* from microscope preparations were taken using a Leica microscope and compiled with NIS- Elements and Helicon Focus Pro imaging software. These images were then imported into tpsDig2 (Rohlf 2010) and an equal number of landmarks were then applied to each image to produce quantitative coordinates of phallic shape variation in order to employ geometric morphometric analyses (Adams et al. 2004). Elliptic Fourier Analysis (EFA) (Lestrel 1997) was conducted in PAST (Hammer et al. 2001) to quantitatively describe phallic shape variation by converting the digitized outlines produced in tpsDig2 into a series of harmonics, each of which is comprised of four Fourier coefficients, which collectively define the shape of a structure – which in this case, the shape of the phallus. Analyses were made invariant to

rotation, size and starting position and size was removed from the resulting dataset in order for the Fourier coefficients to be calculated exclusively on shape variation. Many previous studies of insect genitalia have used EFA and found it a very precise for analyzing shape variation (Arnqvist 1998; Holwell 2008; Holwell et al. 2010; Rowe & Arnqvist 2012). The dataset produced from the EFA was then reduced in PAST using a Principal Components Analysis (PCA) to produce a more compact series of variables that described shape variation among the nine species of New Zealand *Glaucocharis*. The factor loadings for the first four PCA scores were included as they collectively explained a total of 89% of the cumulative percentage variance.

For quantification of the external apical spur of the phallus, digital images of three specimens of each of the nine species taken using an Olympus SZX16 microscope with camera attached and measurements of the average area and length of the external apical spur of the phallus were conducted in cell^D software. All values were then averaged for each species.

2.3.7 *Thickness of the female ductus bursae*

The ductus bursae is part of the reproductive tract in female Lepidoptera with which the male phallus of many species commonly makes primary contact during copulation (Fanger & Naumann 1998; Sihvonen 2007; Mikkola 2008). To measure the thickness of the ductus bursae, fresh female abdomens were fixed in 10% formaldehyde and prepared for histological sectioning and staining. Three specimens of nine species of

New Zealand *Glaucocharis*, representative of each of the three major phylogenetic clades – *G. auriscriptella*, *G. chrysochyta*, *G. elaina*, *G. helioctypa*, *G. holanthes*, *G. interrupta*, *G. lepidella*, *G. leucoxantha*, and *G. selenaea* - were sectioned and prepared onto slides. Multiple measurements of the width of the connective tissue of the ductus bursae of each specimen was taken at several different points along the ductus bursae and averaged to get a mean measurement of the thickness in millimetres. The mean thickness of each specimen within a species was then averaged to get an overall measurement of the thickness of the ductus bursae within species.

2.3.8 Comparative analyses

All phylogenetic comparative analyses were undertaken in Rstudio (R 3.0.2) using Caper (Orme et al. 2012), APE (Paradis et al. 2004) and GEIGER (Harmon et al. 2008) packages. A consensus tree for the nine species of *Glaucocharis* included in the analyses (Figure 5) was developed in PAUP*4.0b10 (Swofford 2002). Initially, Pagel's λ was calculated (Pagel 1997; 1999) for the variables: average thickness of the female ductus bursae, average area of the external phallic apical spur, length of the external phallic apical spur and first four PCA scores. Pagel's λ provides a value between 0 and 1, where a value of 0 signifies a lack of association between a trait or set of traits and phylogeny (i.e. closely related species tend not to share similar trait values) and a value of 1 signifies that species' traits covary precisely as would be predicted by a Brownian motion pattern of evolution (i.e. closely-related species would share highly similar trait values) (Symonds & Elgar 2013). The level of phylogenetic signal for each trait was then measured by testing whether the calculated λ value for each trait differed significantly from a λ value of 0. Phylogenetic

generalized least squares (PGLS) regression was then applied to test the relationships between the thickness of the female reproductive tract and the area and length of the apical spur of the phallus, and PC's describing phallic shape. PGLS analyses produce a test of relationships between traits, but also account for phylogeny.

2.4 RESULTS

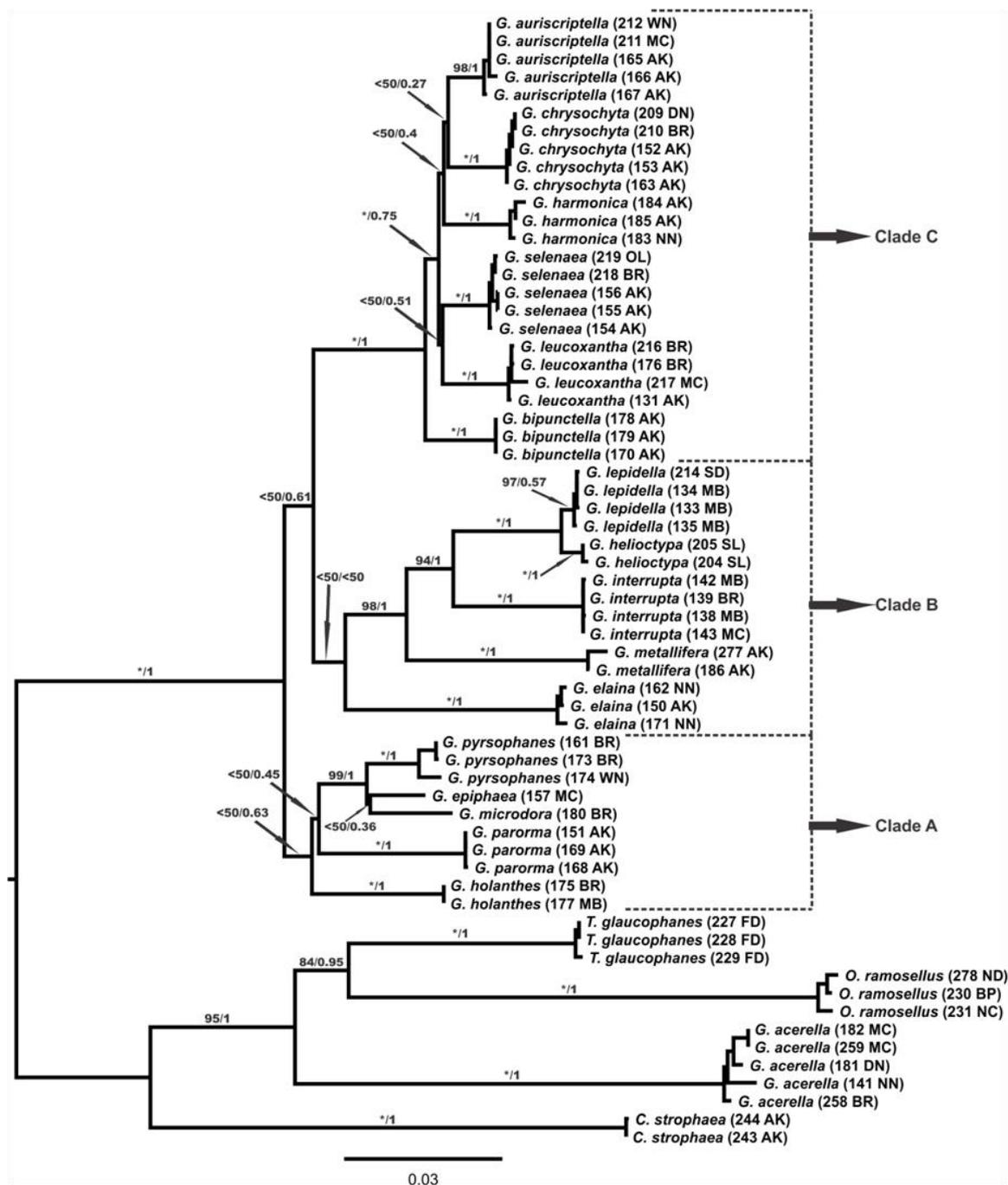


Figure 2. Best scoring maximum likelihood phylogeny of *Glaucocharis* and related outgroups (20 sp., 5 genera) combined data set (28S, COI, and EF1 α). Nodes are labeled with 1000 bootstrap replicates performed in PAUP*4.0 and MrBayes posterior probability values respectively (* denotes bootstrap value of 100). Numbers next to species name represent specimen number and regional code within New Zealand, following Crosby et al. (1998). ND = Northland; AK = Auckland; BP = Bay of Plenty; WN = Wellington; SD = Marlborough Sounds; MB = Marlborough; NN = Nelson; BR = Buller; NC = North Canterbury; MC = Mid Canterbury; OL = Otago lakes; DN = Dunedin; SL = Southland.

2.4.1 Phylogenetic relationships

The final alignment contained 591 base pairs (bp) from the 28S ribosomal gene, 596 bp from the COI mitochondrial gene, and 906 bp from the EF1 α nuclear gene. The topologies of the optimal trees constructed via Bayesian and maximum likelihood inference were almost identical, and the best ML tree is presented in Figure 2. The optimal Bayesian and ML trees only differed at a few poorly resolved nodes.

Based on both the ML and Bayesian inference, the monophyly of New Zealand *Glauccharis* is strongly supported (100% bootstrap/1 posterior probability, figure 2). The monophyly of all species is highly supported with a bootstrap percentage of 97 or higher and/or posterior probabilities of 1.

The monophyly of Gaskin's (1985) *epiphaea*-group (Table 1) is not supported, with the inclusion of the species *G. microdora* of his *auriscriptella*-group (<50 bootstrap support, 0.63 posterior probability) (Figure 2, Table 1). The monophyly of the other groups are also not supported. Some species within Gaskin's *auriscriptella*-group (Figure 2) form a monophyletic clade with the two species of the *chrysochyta*- group (100/1), while *G. metallifera*, *G. interrupta*, *G. helioctypa*, and *G. lepidella* placed within the *auriscriptella*-group are monophyletic (98/1) and group tentatively with *G. elaina* of the *elaina*-group. (Figure 2, Table 1).

New Zealand *Glaucoccharis* are divided into 3 main clades – clade A, B and C (Figure 2). At the root of the tree, *G. holanthes*, *G. parorma*, *G. microdora*, *G. epiphaea*, and *G. pyrsophanes* form a clade - clade A (Figure 2). The support for the positioning of clade A, however, is quite low, <50/0.63 (Figure 2). There is higher support for the monophyly of *G. microdora*, *G. epiphaea*, and *G. pyrsophanes* within this clade, 99/1, whereas support for the position of *G. parorma* (<50/0.45) and *G. holanthes* (<50/0.63) is low (Figure 2), which may explain the low nodal support for clade A as a whole. Clade A was recovered as sister group to all other New Zealand *Glaucoccharis* (Figure 2).

Glaucoccharis elaina, *G. metallifera*, *G. interrupta*, *G. helioctypa*, and *G. lepidella* form the next major clade - clade B (Figure 2). Like clade A, support for the positioning of clade B is low, <50/<50 (Figure 2). There is very strong support for the monophyly of all but *G. elaina* within the clade, 98/1 (Figure 2). All species within clade B are a sister group to clade C.

Clade C is the third major clade of New Zealand *Glaucoccharis*. The clade includes *G. bipunctella*, *G. leucoxantha*, *G. selenaea*, *G. harmonica*, *G. chrysochyta* and *G. auriscriptella*. Support for the positioning of clade C is very strong – 100/1 (Figure 2). There is also strong support for *G. bipunctella* as a sister species to all other species within this clade – 100/0.75, however, support for the positioning of other species groups is low - <50/0.51, <50/0.4, and <50/0.27 respectively (Figure 2).

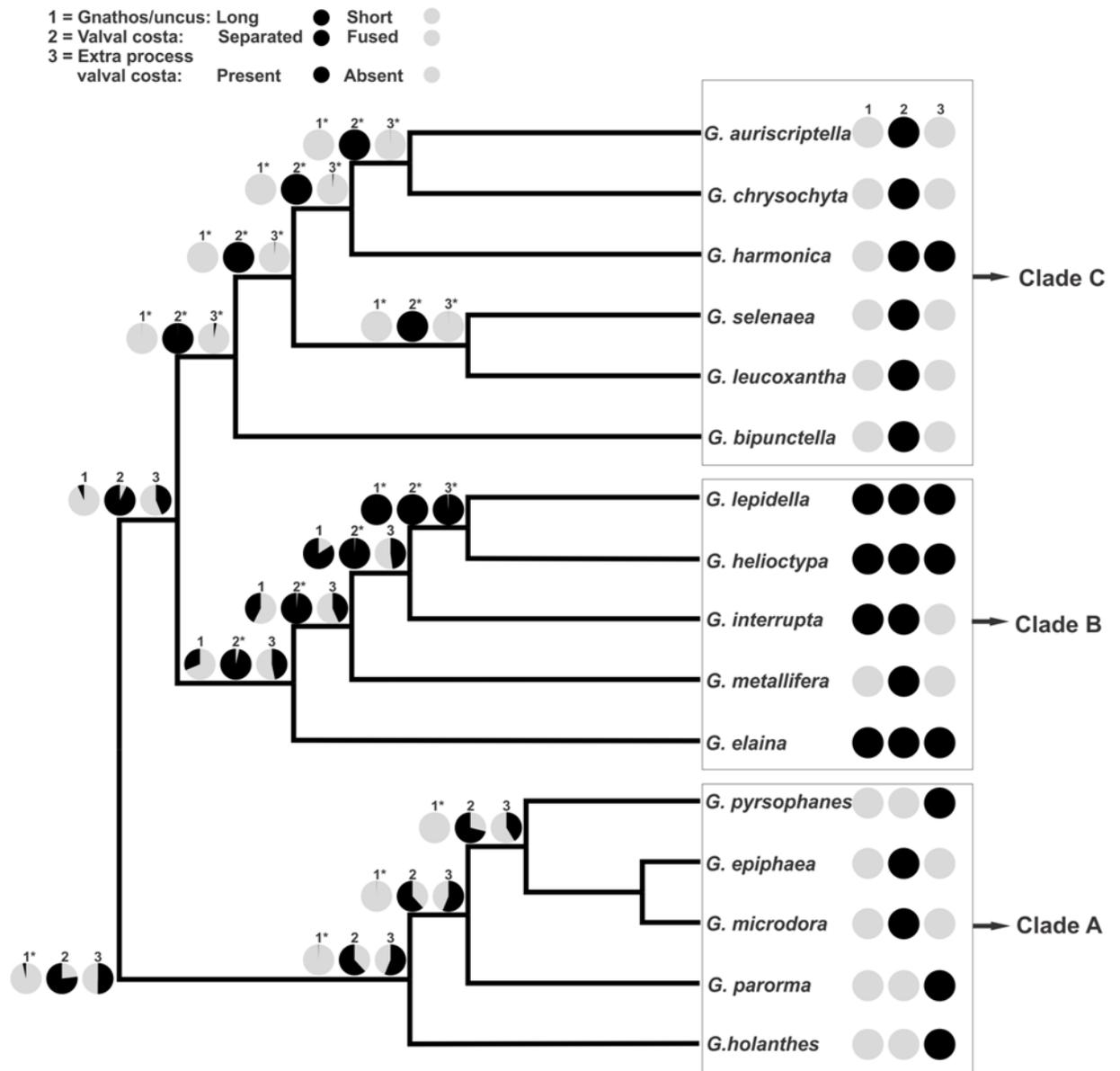


Figure 3. Cladogram of phylogenetic relationships of New Zealand *Glaucocharis* based on the optimal ML tree. Pie charts at nodes represent ancestral state probabilities for three male genital capsule characters.

1 represents the length of the gnathos and uncus; 2 represents the separation or fusion of the valval costa; 3 represents presence or absence of an extra process on the valval costa. Circles next to species name denote character states of that species. * = significant result.

2.4.2 Ancestral states and direction of evolution of male genitalic characters

Figure 3 shows a cladogram adapted from the best scoring maximum likelihood tree with ancestral state probabilities of three male genital capsule structures – length of the uncus and gnathos, development of the valval costa, and the presence of an extra process on the valval costa based on the MK1 model - in addition to species character states. Figure 4 shows the cladogram of New Zealand *Glaucocharis* with ancestral state reconstructions and associated probabilities of three male phallic characters – apical spur, large singular sub-apical cornutus, and other cornuti as well as species character states.

2.4.3 Length of the uncus and gnathos

A short uncus and gnathos is the ancestral state in New Zealand *Glaucocharis* (short = 0.965*). There is significant support for the ancestry of a short uncus and gnathos in clade A (short = 0.997*). A longer uncus and gnathos is evident in *G. elaina*, and all but *G. metallifera* in the corresponding clade – clade B (short = 0.687), with a significant probability for *G. helioctypa* and *G. lepidella* (long = 0.999*). All species within clade C have a short uncus and gnathos, with equivalent significant support of ancestry (short = 0.999*) (Figure 3).

2.4.4 Fusion or separation of the valval costa

The ancestral state of the valval costa of New Zealand *Glaucocharis* appears to be fusion of this structure, however, support for this is non-significant (fused = 0.231; separated =

0.769). In clade A, all but *G. epiphaea* and *G. microdora* have a fused valval costa (fused = 0.381; separated = 0.619). There is a separation of the valval costa for all species within clade B (separated = 0.964*) and clade C (separated = 0.998*) (Figure 3).

2.4.5 *Extra process on the valval costa*

The presence or absence of an extra process on the valval costa of the genital capsule is fairly evenly split for New Zealand *Glaucocharis* (absence = 0.497; presence = 0.50294695), suggesting multiple gains or losses, or multiple ancestry of clades of New Zealand *Glaucocharis*. The acquisition of this structure has occurred independently in all three clades; once in three species of clade A (presence = 0.565); twice in clade B – in *G. elaina* (presence = 1.0), and *G. lepidella/G. helioctypa* (presence = 0.992*); and once in *G. harmonica* of clade C (presence = 1.0) (Figure 3).

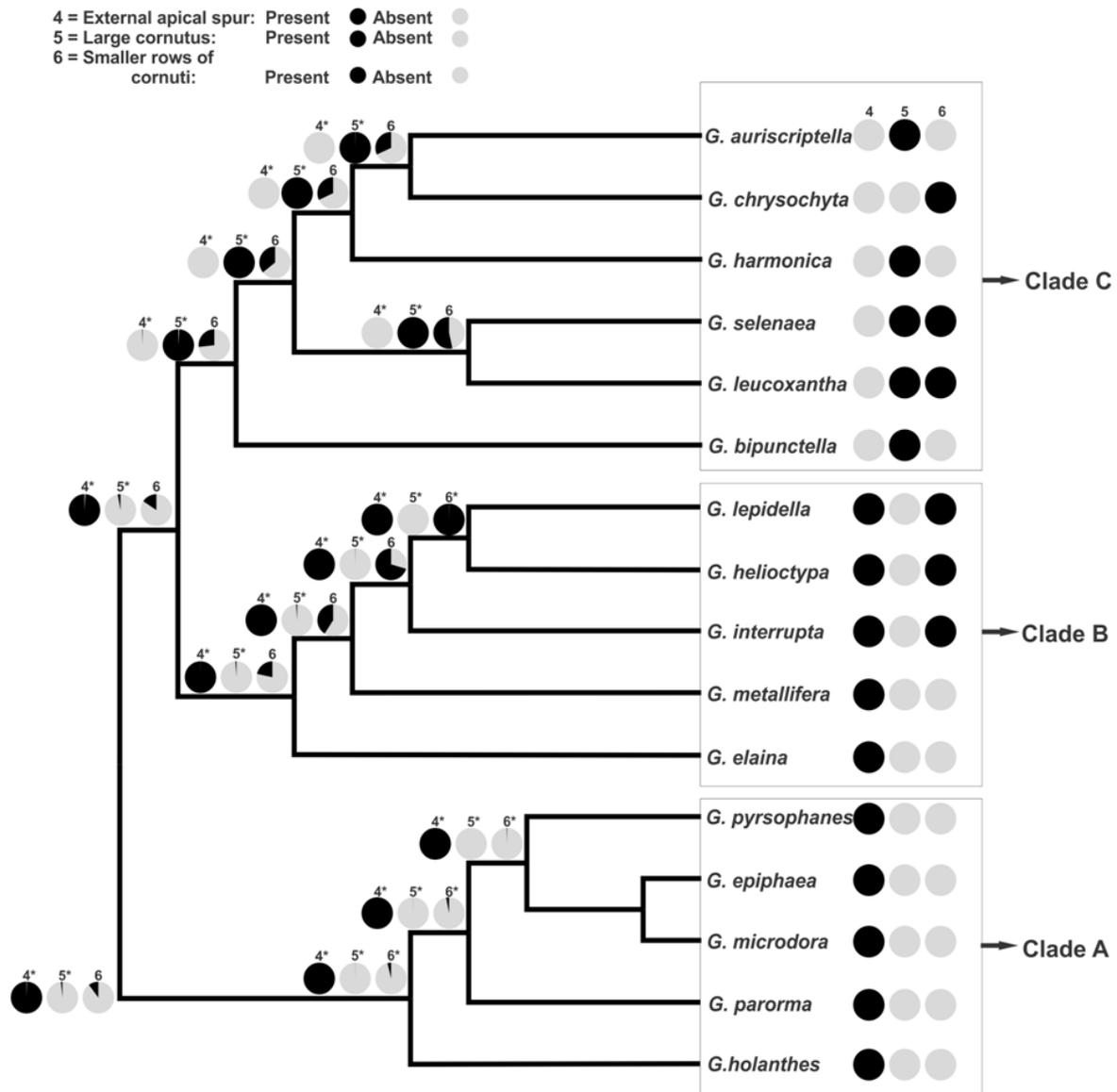


Figure 4. Cladogram of evolutionary relationships of New Zealand *Glaucocharis* based on the optimal ML tree. Pie charts at nodes represent ancestral state probabilities for three male phallic characters. 4 represents the presence or absence of an apical spur; 5 represents the presence of a singular large sub-apical cornutus; 6 represents presence or absence of other cornuti. Circles next to species name denote character states of that species. * = significant result.

2.4.6 *External apical spur*

There is significant support for the ancestry of the presence of an apical spur on the phallus of New Zealand *Glaucocharis* (presence = 0.995*). The presence of this structure is significant within clade A (presence = 0.999*) and clade B (presence = 0.998*) with it present in all species of both clades. However, this structure is absent from all species within clade C (absence = 0.996*) (Figure 4).

2.4.7 *Large singular cornutus*

The ancestral state is the lack of a large singular cornutus in New Zealand *Glaucocharis* (absence = 0.985). This structure is absent within clade A (absence = 0.999*) and clade B (absence = 0.990*), but is present within all but *G. chrysochyta* of clade C (presence = 0.991*) (Figure 4).

2.4.8 *Smaller sets of fixed cornuti*

There is strong support for the ancestry of the absence of smaller fixed cornuti within New Zealand *Glaucocharis* (absence = 0.898). Fixed cornuti are absent in clade A (absence = 0.963*), but present in *G. interrupta*, *G. helioctypa*, and *G. lepidella* of clade B (presence = 0.704) and *G. leucoxantha*, *G. selenaea*, and *G. chrysochyta* of clade C, although ancestry for the presence of smaller sets of cornuti for clade C as a group is not so strong (absence = 0.732; presence = 0.268) (Figure 4).

2.4.9 Coevolution of a male and female genitalic trait

There was a significant amount of phylogenetic signal in all but two traits examined (Table 3). Therefore, both the area and length of the external apical spur on the phallus, the thickness of the female ductus bursae wall and shape variation of the phallus – with the exclusion of PC 3 and PC 4 - showed high levels of similarity between closely related species.

Table 3. Measure of phylogenetic signal in the thickness of the female ductus bursae, area and length of the male external apical spur, and PCA scores of phallus shape variation of New Zealand *Glaucocharis* using the ML phylogeny developed for analysis fig. 1. Significant Pagel's λ values are denoted by an asterix.

Trait	λ value
Mean thickness of the ductus bursae	0.97*
Mean area of external apical spur	1.0*
Mean length of external apical spur	1.0*
PCA 1 scores	0.99*
PCA 2 scores	0.58*
PCA 3 scores	0
PCA 4 scores	0

Figure 5a shows the relative area of the apical spur on the male phallus and figure 5b the thickness of the female ductus bursae wall. The comparison suggests that the area of the apical spur and the reinforcement of the ductus bursae wall have coevolved (Figure 5a & 5b).

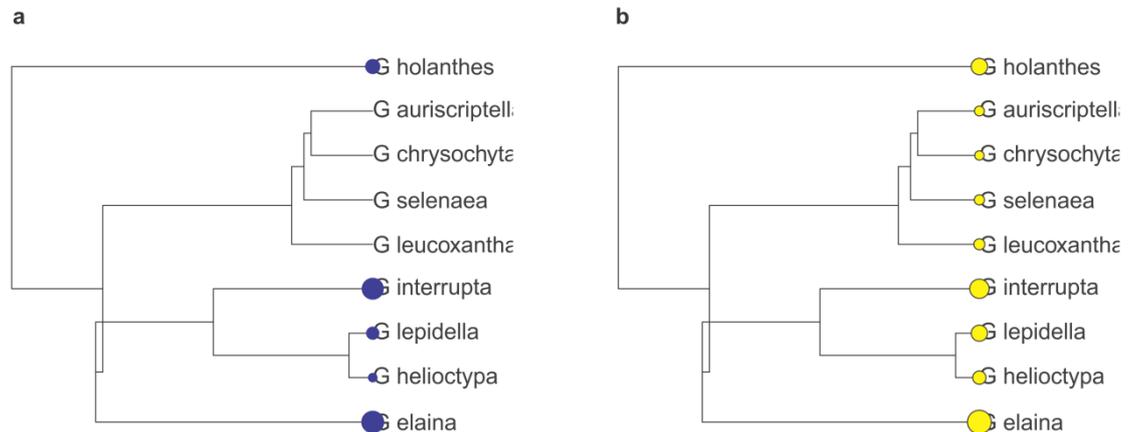


Figure 5. Phylogeny developed for PGLS analyses of 9 species of *Glaucocharis*, with size of the coloured circles proportional to a) Average area of apical spur on the male phallus and b) Average thickness of the female ductus bursae wall mapped onto the ML tree.

In the PGLS analyses of the 9 species of New Zealand *Glaucocharis* examined, the average thickness of the ductus bursae wall in females showed a significant correlation with the average area and length of the external apical spur on the phallus in males, but not the shape of the phallus (Figure 6; Table 4). In species with a phallic apical spur, females had thicker connective tissue of the ductus bursae wall and this also correlated with the relative size of the apical spur (Figure 6; Table 4).

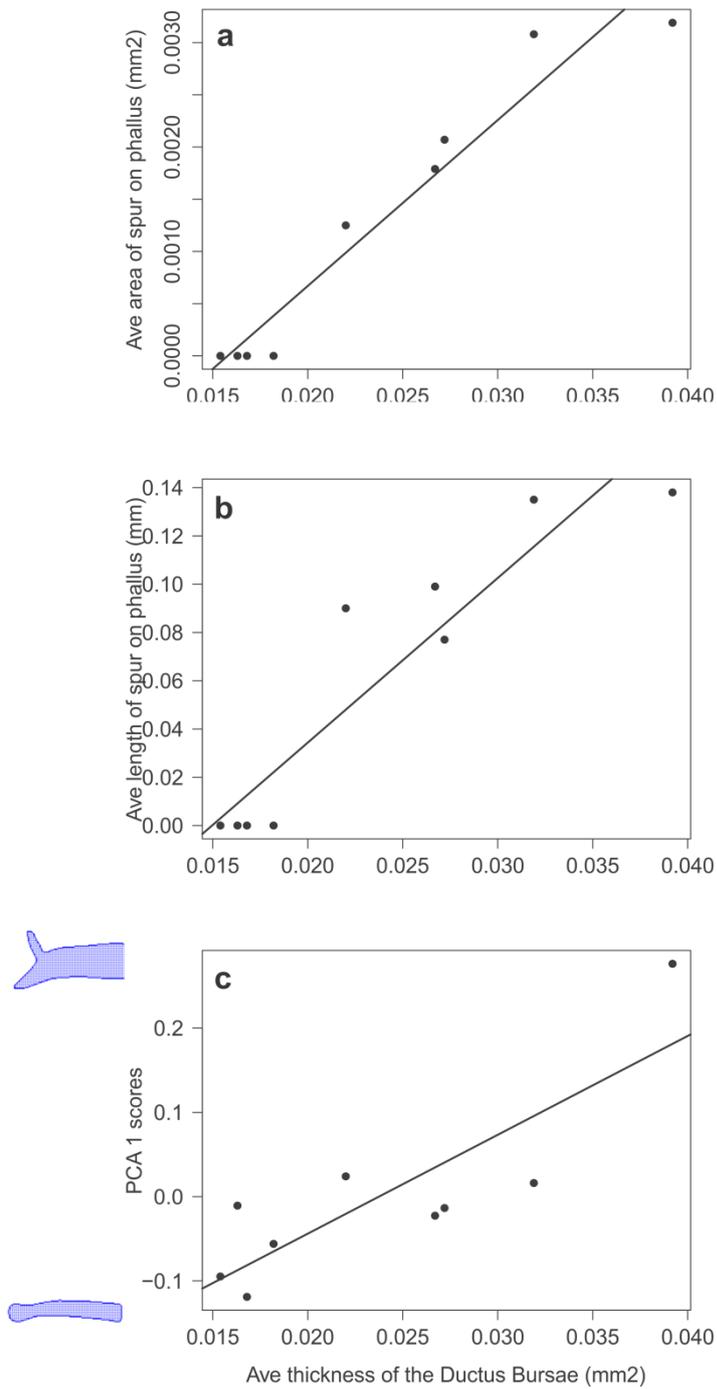


Figure 6. Relationship between the average area and length of the spur, and PC 1 for the phallus shape and the average thickness of the female ductus bursae in 9 species of *Glaucocharis*. PC 1 graph also shows the maximum and minimum EFA deformation shapes that correspond to the PCA 1 scores.

Table 4. PGLS regression of the relationship between average thickness of the ductus bursae wall and the average area and length of the external apical spur, and first 4 PCA scores in 9 species of New Zealand *Glaucocharis*. Significant values are in bold.

PGLS Regressions	Average thickness of the female ductus bursae wall		
	β (\pm s.e.)	R^2	<i>p</i> -value
Intercept	-0.002 (\pm 0.001)	0.896	0.006
Ave area of spur (mm ²)	0.145 (\pm 0.019)		<0.001
Intercept	-0.068 (\pm 0.031)	0.770	0.065
Ave length of spur (mm)	5.256 (\pm 1.086)		0.002
Intercept	-0.176 (\pm 0.150)	0.203	0.281
PC 1	7.001 (\pm 5.247)		0.224
Intercept	-0.085 (\pm 0.081)	0.220	0.330
PC 2	4.248 (\pm 3.022)		0.203
Intercept	-0.023 (\pm 0.037)	0.040	0.548
PC 3	0.814 (\pm 1.516)		0.608
Intercept	0.015 (\pm 0.039)	0.034	0.714
PC 4	-0.794 (\pm 1.603)		0.636

2.5 DISCUSSION

The genital morphology was highly variable among species of New Zealand *Glaucocharis* and the evolution of individual male traits showed intriguing patterns. Traits of the external apical spur on the phallus, phallic shape variation (PC 1 & 2), and thickness of the female ductus bursae wall had strong phylogenetic signal, and there was a strong positive correlation between the size of the apical phallic spur and the thickness of the female ductus bursae wall, providing evidence of the coevolution of these two traits within New Zealand *Glaucocharis*.

2.5.1 *Coevolution of male and female structure*

There was a strong correlation between the area and length of the external apical spur on the male phallus and thickness of the female reproductive tract, while accounting for phylogeny, whereas there was no significant correlation between overall shape variation of the phallus and thickness of the reproductive tract. In species with larger and longer male phallic spines, females possessed more connective tissue within the ductus bursae, suggesting that these two morphological traits have coevolved. This pattern is consistent with the hypotheses of sexually antagonistic coevolution. The apical spur may have arisen as a tool to overcome female resistance during mating or delay females remating post-copulation. The addition of experimental data would help decipher the costs that the male apical spur might pose to the female. During dissections, there was no evidence of scarring of the reproductive tract in female *Glaucocharis*. Therefore there is no direct indication of a mating cost to females. Nevertheless, sexual conflict may still be a factor in the evolution of these male structures as a lack of scarring is solely not a good

indication of the absence or previous occurrence of sexual antagonism. Ronn et al. (2007) found that scarring per se provided an insufficient measure of the net cost of mating to females among several species of seed beetles. An explanation for these results was that in addition to a reinforcement of the female reproductive tract, females may have developed other mechanisms to help handle physical injury from male structures, which may include an immune system better equipped to deal with any physical trauma (Ronn et al. 2007) or other behavioural or anatomical traits that may work in association with more directly observable structures.

If females are at an advanced state in the coevolutionary arms race, counter-adaptations of defensive structures or mechanisms may simply already be evident, obscuring the initial harm or antagonism imposed by males. Furthermore, the evolution of increasingly harmful male structures may not directly relate to an elevation in the amount of harm imposed on the female as selection for increased female resistance to the development of more harmful male traits may combat the imbalance between the sexes (Ronn et al. 2007).

Indeed, the dynamic nature of sexually antagonistic coevolutionary processes may conceal the presence or costs of sexual conflict (Hardling et al. 2001; Hardling & Smith 2005; Rowe & Day 2006). If the coevolution of the sexes is in a state of equilibrium, then adaptation in one sex should be balanced by a corresponding counter-adaptation in the other (Rice 1996; Holland & Rice 1998; Hardling & Smith 2005). The increased thickness

of the female reproductive tract in New Zealand *Glaucocharis* may in fact protect the female from potential damage by the sclerotised apical male spur. Accordingly, there is support for sexually antagonistic coevolution playing a role in the evolution of the male apical phallic spur and the thickness of the female ductus bursae wall, but, without further examination alternative mechanisms such as pleiotropic harm cannot be ruled out. However, in this case females have not usually evolved counter adaptations to counteract potential harm (Morrow et al. 2003).

Alternatively, for species of *Glaucocharis* in which males possess an apical spur, females may show a preference for males with this trait, as it may accrue indirect benefits for sons with a superior ability to maintain a hold or stimulate the female during copulation. It is notoriously difficult to disentangle the influence of sexual conflict and CFC on genital structures (Cordero & Eberhard 2003, 2005; Arnqvist & Rowe 2005). In both instances, at some stage during the coevolution of corresponding male and female traits, an increased elaboration of the male trait may result; but also counteradaptation by the female, either as a way to de-escalate the amount of costs imposed, or as a means for females to exert preference for males that are able to overcome resistance/maintain a tighter grip during copulation as the result of exaggeration of the male trait (Rice & Holland 1999; Cordero & Eberhard 2005). As there is no direct evidence of harm to female *Glaucocharis* from this structure, it is hard to make any conclusive statements as to the selective origin of the apical phallic spur. Being able to quantitatively define the relative costs and benefits of females mating with males of various phenotypes of the apical phallic spur i.e. shorter to longer, would provide a clearer understanding of its

origin. Mating females singly and multiply with various phenotypes would help decipher such direct and indirect costs and benefits, in addition to whether the female is driven by direct resistance or whether they receive indirect benefits from mating with males which have a superior potential to harm the female's reproductive tract.

2.5.2 *Ancestral states and direction of genital evolution within New Zealand*

Glaucocharis.

Uncus and gnathos

In copulating pairs of Lepidoptera, the uncus has been observed to grip the female dorsally and the gnathos ventrally (Common 1975; de Jong 1978; Sihvonen 2007) thus acting as external aids for positioning of internal male genitalia (Mikkola 2008). Patterns among New Zealand *Glaucocharis* show variation in the length of these structures, which closely match cladistic relationships. Clades A and C possess a reduction of the uncus and gnathos, whereas in species from clade B, with the exception of *G. metallifera*, they remain elongated. There are no obvious corresponding grooves on the female abdomen in *Glaucocharis* with which they make contact, therefore the length of these structures may not necessarily indicate a lock and key mechanism with the female, but may be a reflection of sexual selection pressures. Sexual conflict would select for male traits that could maintain a tight connection at the onset of copulation to avoid rejection from the female. Cryptic female choice would select for structures that are sufficient in stimulating the female to permit copulation to progress or influence the

uptake and utilization of the spermatophore by the female. Similarly, in polyandrous mating systems, male competition would also select for traits that allowed for the maintenance of a tighter connection with the female during copulation to avoid physical interruption from rival males. In this respect, female resistance and male competition could drive the evolution of structures efficient in obtaining a strong grip both at the pre-copulatory stage and during copulation or cryptic female choice may drive the adaptation of these structures in providing a superior stimulatory function. The reduction of the uncus and gnathos in certain species could also be offset by the elaboration of other genital capsule structures such as the valvae, which are also known to provide a lateral gripping function (de Jong 1978; Fanger & Naumann 1998; Mikkola 2008). However, there is no trend that species with a shorter uncus and gnathos have distinct elaboration of the valvae, so sexual selective pressures are a more likely scenario in the development of these structures.

Fusion/separation and extra process of the valval costa

As previously mentioned, the valvae of the male genital capsule laterally grip onto the female pre-copulation. In New Zealand *Glaucocharis*, three species within clade A display a fusion of the valval costa, whereas the other two species within this clade and all species within clade B and C show a separation of the valval costa. Structures derived from the valvae in some gelechiid moths are also shown to grip onto internal parts of the female reproductive tract, such as the ductus bursae (Ponomarenko 2009). In species where there is a separation of the valval costa, the extended structures that have developed may play an internal role during copulation. The females in species that

have developed a separation and subsequently elongated process, have internal pouches that appear connected to and extend laterally from the ostium (ostial pouches); whereas in species where males do not possess these structures, these female pouches are absent or greatly reduced (Fig. 1). Therefore, these male structures may lock into the pouches during copulation to provide further stability. Furthermore, length of the elongated valval process corresponds to the length of these conspecific female pouches.

In addition to the elongated structure produced from a separation of the valval costa, four species (*G. elaina*, *G. harmonica*, *G. helioctypa*, *G. lepidella*) also have a smaller extra process present on the valval costa; the presence of a small extra process also occurs in three species of which the fusion of the valval costa has been retained (*G. holanthes*, *G. parorma*, *G. pyrsophanes*). These smaller processes may potentially play either an internal or external grasping role during copulation and further allow males a tighter grip on females to avoid interruption via male competition or female resistance.

Large singular sub-apical cornutus and smaller sets of fixed cornuti

Within the Diptychophorini tribe, which includes *Glaucocharis*, Gaskin (1985) proposed that the primitive state was the presence of one or more rows of microscopic cornuti, with a lack of cornuti being a secondary adaptation. However, this does not appear to be the case in New Zealand *Glaucocharis*. There is an absence of any cornuti within clade A at the root of the phylogenetic tree, which is a sister clade to all other species of New Zealand *Glaucocharis*. Within clade B, there is a complete lack of the presence of a large

singular cornutus, but a varying number of smaller sets of fixed cornuti are present in three species – *G. interrupta*, *G. helioctypa* and *G. lepidella* within this clade. The presence of a large singular cornutus occurs in all but *G. chrysochyta* of the more derived clade C. The presence of smaller rows of cornuti is also prevalent in this clade, with the exceptions of *G. harmonica* and *G. auriscriptella*. In light of this, it appears that the ancestral condition of New Zealand *Glaucocharis* is the absence of cornuti, followed by various divergences in the number and size of these structures. Rows of smaller sets of fixed cornuti appear to have arisen first, with the acquisition of a large singular cornutus occurring in one clade. However, these conclusions are based on the assumption that the New Zealand species are monophyletic. Without further analyses including the two missing New Zealand members and species from the rest of the globe multiple origins and radiations cannot be ruled out.

In relation to hypotheses behind the evolution of fixed cornuti, it is unlikely that any of these structures in New Zealand *Glaucocharis* function as a lock and key mechanism, as there are no obvious corresponding structures in females with which these male organs may “lock”. Fanger & Naumann (1998) observed in a species of zygaenid moth, that both the prephallus and the vesica (i.e. the endophallus) were tightly locked against the female ductus bursae, which has also been observed in other species of Lepidoptera (Sihvonen 2007; Mikkola 2008). However, the position of these internal structures can vary depending on the species and potentially the size and shape of the phallus; as in some species the phallus enters the ostium bursae at the beginning of the reproductive

tract, but does not make contact with the ductus bursae (Callahan & Chapin 1960, in: Mikkola 2008).

The shape and position of large singular cornuti displayed in (Figure 1l) when the vesica is everted suggests that it could potentially enter the corpus bursae in species such as *G. leucoxantha* and *G. selenaea*, as the cornutus is attached closer to the apex of the everted vesica. These long, slender cornuti may surpass the ductus bursae and seemingly aid in the transfer or breaking up of the spermatophore. It is doubtful that it functions as a holding device, but under sexual selection may be used to stimulate the female during or post-copulation (CFC) or potentially pierce the female to delay remating (sexual conflict), however, this latter scenario appears unlikely as the tip of the cornutus appears relatively blunt. As there is only one species of New Zealand *Glaucocharis* that possesses a small signum (*G. interrupta*), the hypothesis of protecting the vesica from these sclerotized structures becomes irrelevant.

The large sub-apical cornuti are shorter and sharper than the cornuti described in the previous paragraph. In contrast to the longer, slender and blunt type, they are attached to the base of the vesica, and would therefore be positioned closer to the sclerotised phallus during the eversion of the vesica. Subsequently, they would most likely make contact along the ductus bursae or towards the junction of the ductus bursae and the corpus bursae in species such as *G. auriscriptella*, *G. bipunctella* and *G. harmonica*. It is unlikely they would reach far into the corpus bursae in comparison to the former type. Comparatively, these large, spined cornuti most likely originated under natural selection

to aid the insertion of the vesica into the corpus bursae; or under sexual selection to provide anchorage to avoid displacement from rival males (sperm competition) or the female during copulation, to stimulate the female to favour successful insemination (CFC), or as an antagonistic tool (sexual conflict) to decrease the female's propensity or refractory period to remating.

The positioning of the rows of smaller cornuti in the vesica suggests they may make contact with the ductus bursae and/or conceivably enter the corpus bursae (Figures 1i & 1l). Therefore, they may have potentially developed under natural selection to assist in the breaking up of the spermatophore. As they also have the capacity to pierce the connective tissue, they may have evolved as a sexual conflict device to extend the female refractory period to remating, or to stimulate the female into utilizing the current male's spermatophore. Unfortunately, without functional morphological examination or experimentation we cannot adequately distinguish which particular role or roles each distinct type of cornuti provides during copulation, however, the shape and location allows inferences into the potential functionality.

Apical external spur of the phallus

The presence of an apical spur on the male phallus (Figures 1c, 1f & 1i) occurs exclusively in all species comprising clades A and B (Figure 5), showing a presence of this structure in ancestral New Zealand *Glaucocharis*. It is absent in all species of the derived sister clade C, however, in which it is has perhaps been secondarily lost. The spur is unlikely to

enter the corpus bursae and is inferred to make contact with the female ductus bursae independently or in conjunction with the vesica of the phallus. Previous hypotheses on the function of phallic spines include the premise that they are used to firmly anchor the phallus in the female reproductive tract to avoid rejection (Eberhard 1985) and/or disruption from rival males (Blanckenhorn et al. 2002; Okuzaki 2012; Flay et al. 2013); or alternatively delay or deter females from remating (Johnstone & Keller 2000; Kamimura 2007).

Regardless of the original function of phallic spines, if they do or have the potential to cause physical injury, which would result in costs to the female such as infection and healing to the wounded site and risk of increased vulnerability to sexually transmitted diseases (Lange et al. 2013), female counteradaptations to circumvent or minimize damage should be expected.

2.5.3 Do molecular phylogenetic relationships match proposed morphological species groupings?

The molecular phylogeny of New Zealand *Glaucocharis* is partly in agreement with Gaskin's (1985) classification, with some discrepancies. The *epiphaea*-group forms a monophyletic clade – *epiphaea* clade, but with the inclusion of *G. microdora*, which was assigned to the *auriscriptella*-group by Gaskin (1985). The monophyly of Gaskin's (1985) *auriscriptella*-group is not supported, as some species of this group form a clade – the *bipunctella* clade (clade C, Figure 3) - with the two species of his *chrysochyta*-group, while the remaining species form a separate monophyletic clade – clade B. The only

member of Gaskin's (1985) *elaina*-group (*G. elaina*) included in the analyses appears to group with species within clade B, but this is unresolved, suggesting that *G. elaina* may be more closely related to other non-native *Glaucocharis* or represent its own separate clade.

Resolution of the positioning of the clades within New Zealand *Glaucocharis* is incomplete, suggesting multiple immigrations that may have occurred around similar time periods. The genus is not endemic to New Zealand and has related species in Australasia, the South Pacific, Indo-Malaysian regions and China. Gaskin (1971) suggested a close phylogenetic relatedness between New Guinean species of *Glaucocharis* with New Zealand species of clade C, such as *G. bipunctella*, *G. harmonica* and *G. auriscriptella* based on the structure of the gnathos, valvae and cornuti of the phallus. There are genital traits that appear to be distinct to particular clades, further suggesting multiple lineage origins. The addition of evolutionary time scaling would help reveal potential migration and recent speciation events, as short branch lengths and low nodal support for the monophyletic species groups within clade C suggests a potential rapid radiation.

2.5.4 Summary

The molecular phylogeny of New Zealand *Glaucocharis* is in partial agreement with previous morphological species groupings by Gaskin (1985). New Zealand representatives of the genus are divided into three main clades, with a potential rapid

radiation occurring among members of the *bipunctella* group incorporating clade C. The inclusion of species of *Glaucocharis* from Australia and the South Pacific may provide additional information as the potential origin of and ancestral genital states within the New Zealand fauna as clades within the molecular phylogeny of New Zealand *Glaucocharis* display apomorphic traits.

Applying proposed hypotheses of the evolution of cornuti to the different forms displayed in New Zealand *Glaucocharis* provides insight into the origin and function of these structures. The shape and position of attachment of cornuti indicate the mechanisms behind the evolution of each distinct cornuti type. Observations of mating pairs in copula and direct experimentation would help make the selective forces behind the evolution of these structures more clear, but the current review provides a good framework to build upon. Further research into the mating systems and copulatory mechanisms of other male and female genital structures is warranted to help elucidate the function of specific structures, including the likely interaction between the elongated process of the male valval costa and the internal female ostial pouches evident in species of New Zealand *Glaucocharis*.

Closer examination of the relationship of a potential harmful structure and the corresponding region of the female reproductive tract revealed a strong correlation suggestive of the sexual coevolution of these structures. However, additional research is needed to distinguish exactly which sexually selective mechanism, such as sexual antagonism or female choice is driving this coevolution.

3.

**Phylogenetic systematic, genital
character evolution and
biogeography of the New Zealand
moth genus *Izatha* (Lepidoptera:
Xyloryctidae)**

3. Phylogenetic systematics, genital character evolution and biogeography of the New Zealand moth genus *Izatha* (Lepidoptera: Xyloryctidae)

3.1 ABSTRACT

Male genitalia are known to be one of the most rapidly evolving structures, with a large amount of divergence evident in some animal taxa. Theories behind the evolution of genitalia include the lock and key hypothesis (species isolation), pleiotropy, with most recent research providing mechanisms of sexual selection as the best explanation of the rapid and divergent evolution of genitalia. Species of moths within the genus *Izatha* Walker, 1864 are ideal candidates for investigating the processes driving genital evolution as they display a large variation of male traits. In addition, *Izatha* are also interesting in the respect that there is a higher radiation of species in the North Island of New Zealand; an unusual pattern within New Zealand Lepidoptera. This may have relevance in examining patterns of genital evolution within the genus, as highly similar male genital traits may be a good indicator of recent speciation. In order to examine male genital evolution in *Izatha* a molecular phylogeny of the genus was reconstructed using one mitochondrial and two nuclear genes, to examine the evolutionary relationships, estimations of ancestral male genital states and patterns of male genital

evolution. The genus was divided into two well-supported clades and two poorly supported clades at the root of the phylogeny. Incomplete genetic resolution within two species groups may be explained by recent speciation events. *Izatha* display a number of apomorphic phallic traits including deciduous, compound and fish hook cornuti. There has also been a reduction of the unculus and gnathos, but an elaboration of another grasping structure, the juxta, in the genus. Male genital characters were a good indication of species relationships, as more closely related species share more similar traits. However, caution must be taken when relying solely on morphological characters as the previous assumption of monophyly of a group of species within the genus based on the presence of deciduous cornuti has been refuted. Ancestral character state reconstruction indicates these structures have arisen on more than one occasion. The potential processes behind the current distributions of *Izatha* and the ancestry, direction of evolution, and inferences about the type of selection behind the origin of and functionality of male genitalic traits are also discussed.

3.2 INTRODUCTION

Many systematists utilize male genitalic characters in drawing up species descriptions and determining species boundaries. Often, closely related species, substantially similar in general morphology may exhibit a large disparity in genitalic structures, and it is well documented that the evolution of genitalia is a relatively rapid process (Eberhard, 1985; Arnqvist 1997a; Hosken & Stockley 2004; Eberhard 2010b).

Initial theories to explain the rapid and divergent evolution of genitalia included the lock and key hypothesis (Eberhard, 1985; Shapiro & Porter, 1989) and pleiotropy, in which genital traits are indirectly affected by a variety of genes and mutations (Mayr, 1963; Eberhard 1985; Arnqvist 1997a). However, more recent hypotheses propose sexual selection as the main driver of genital evolution (Arnqvist 1998, Arnqvist & Danielsson 1999, Arnqvist et al. 2000, Eberhard 2004b).

Male genital structures may be under sexual selective pressure due to cryptic female choice of males that can sufficiently stimulate the female or physically coerce her to use their sperm (Eberhard, 1985; Briceno & Eberhard, 2009; Eberhard, 2010). They may also be under pressure to adapt structures that may preferentially position the spermatophore or in non-monogamous systems, remove or displace a rival male's sperm under intense competition - sperm competition (Simmons & Siva-Jothy 1998). Additionally male genitalia may develop in a way to counteract female resistance – sexual conflict. With the presence of anisogamy – unequal size and production costs of gametes - in sexually reproducing animals, the evolutionary interests of males and females are hardly ever identical (Arnqvist & Rowe 2005). As a consequence, there may be opposing selection of male and female reproductive strategies (Parker 1979) and genital adaptations that allow males a competitive advantage may consequently reduce female fitness (Arnqvist & Rowe 2005). Therefore, there is a variety of sexual selection mechanisms that may influence the evolution of male genitalia.

The New Zealand endemic moth genus *Izatha* (Lepidoptera: Oecophoridae) is an ideal candidate for the examination of genital evolution as the male genitalia show some striking diversification within the genus (Hoare, 2010). Though it is currently placed within the Oecophoridae, recent research has proposed a revised placement of *Izatha* within the closely related Xyloryctidae (Kaila, 2004; Kaila et al. 2011; Heikkilä et al. 2013). Hoare (2005) noted that *Hierodoris*, also placed within Oecophoridae, more closely resemble the xyloryctid assemblage and proposed a number of genera be included in a wider *Hierodoris* group. This, as redefined by Kaila et al. (2011) includes *Izatha* and other New Zealand endemic genera - *Hierodoris*, *Gymnobathra*, *Lathicrossa*, *Thamnosara*, and *Tinearupa*.

A recent taxonomic review of *Izatha* described the morphological systematics of the genus and revealed 15 new species, bringing the total number of species to 40 (Hoare, 2010). Hoare (2010) divided the genus into seven informal species-groups based on an intuitive assessment of 12 morphological characters, including seven from the male genitalia. However, an explicit phylogenetic analysis to support these species groupings was not included in the generic revision.

Some of the unusual and distinctive genital structures among *Izatha* species include the elaboration of sclerotized teeth on the outside of the phallus and the development of deciduous, “compound” and “fish hook” cornuti. Deciduous cornuti (DC) are spines that are attached to the internal vesica (endophallus) wall of the phallus; the vesica is everted and the cornuti shed in the female reproductive tract during copulation.

“Compound” and “fish hook” cornuti are terms coined for fixed or non-deciduous cornuti present in species of *Izatha* by Hoare (2010) in his revision of the genus and unlike DC remain attached to the vesica wall. Compound cornuti refer to a number of closely connected small spine or leaf-like sclerotized structures (present in a few species) derived from the vesica, and hence categorized as cornuti. The fish hook cornutus is a single, occasionally double, large fixed cornutus near the base of the everted vesica, sometimes fairly straight, but often with a strongly curved base, much like the shape of a fish hook (Hoare, 2010). Several hypotheses behind the function of both deciduous and fixed cornuti have been proposed. In regards to DC, one hypothesis is that they evolved under natural selection to aid in breaking up the spermatophore at the cessation of copulation. Other hypotheses propose they are under sexual selection to stimulate females post-copulation, deter females from remating through potential damage from the contraction of the corpus bursae with the spines present, or prevent adequate positioning of subsequent males spermatophores (Cordero 2010). Hypotheses for fixed or “non-deciduous” cornuti include natural selective processes such that they are structures that may facilitate the insertion of the vesica or transfer of the spermatophore into the female’s corpus bursae; aid in the breaking up of or positioning of the spermatophore; or operate as a kind of lock and key mechanism between conspecific males and females. Intersexual selective hypotheses suggest they are organs for avoidance of displacement during copulation from rival males or the female; for stimulating the female (i.e. courtship or seductive device); for penetrating the female reproductive tract (i.e. physical/antagonistic coercion); or to protect the vesica from damage caused by female structures such as the sclerotised signum (Cordero 2010).

In addition to species-specific development of varying cornuti types, there is distinct variation in the elongation of the juxtal arms and the presence of one or more flanges of the juxto-costal plate of the genital capsule within the genus (see Hoare 2010: figs 210-221). The valvae of the genital capsule of Lepidoptera are known to be the primary structures for grasping the external 8th sternite of the female during copulation (Fanger & Naumann 1998; Powell 2009). The function of other structures such as the juxta and corresponding flanges, which are internally inserted during copulation, are not clear, however, previous propositions are that they provide guidance and support of the phallus (Ever 1924; Klots 1970). Many previous studies into the evolution of genital characters have focused on secondary external structures that play a role in sexual selection in overcoming female resistance (Arnqvist & Rowe 2002a, 2002b; Miller 2003; Perry & Rowe 2012). However, primary internal genital structures that manipulate to ensure paternity may be under even greater evolutionary pressure (Eberhard 1985).

Another striking feature of *Izatha* is that there are more species of this genus inhabiting the North Island than the South Island. This is an uncommon pattern for larger genera of Lepidoptera within New Zealand (Gaskin, 1975; Dugdale, 1988; Dugdale, 1994), where the usual pattern is a higher diversity within the South Island. If species within the North Island exhibit more homogeneous genital morphology indicative of rapid speciation of this may help explain current distributions. *Izatha* larvae feed primarily on decaying wood, especially certain native plants, but some species have been found in association with lichens and fungi (Hoare, 2010). More widespread and common species, such as *I. peroneanella*, *I. churtoni*, *I. mesoschista*, and *I. austera* have been most often extracted

or reared from New Zealand native plants such as tutu (*Coriaria arborea*) and *Coprosma* sp., but these and other species have also been found feeding in the decaying wood of other unidentified plants. Therefore, some species within the genus appear to have a high level of generality in terms of their host plants and may not be restricted to highly localized habitats, but may be constrained by microclimate or historical processes. The majority of *Izatha* are forest species, some are found in high altitude alpine habitats, while others such as *I. psychra* and *I. gekkonella* are confined to dry shrublands of the South Island. Two are restricted to northern offshore islands: *I. dulcior* to the Poor Knights and *I. quinquejacula* to the Three Kings Islands, and a further two: *I. oleariae* and *I. spheniscella* inhabit the Subantarctic Snares Islands (Hoare 2010).

Here, a molecular phylogeny of *Izatha* was used to test the robustness of Hoare's (2010) species groups and provide insight into the phylogenetic relationships, genital evolution and biogeographic patterns within *Izatha*. Information collected from phylogenetic groupings of taxa, especially those exhibiting a rapid radiation provides valuable insight into the processes and patterns behind the diversification of species (Kodandaramaiah et al. 2010) and the evolution of genitalia. Mapping characters such as morphological, ecological, behavioural, or life history traits onto molecular phylogenies can tell us how many times each state has evolved, what states ancestors had and if different characters have evolved independently or not.

The present study aims to address the following questions: 1) Do molecular phylogenetic relationships match proposed morphological species groupings? 2) What were the

probable ancestral states and direction of genital evolution within *Izatha*? 3) Have derived states evolved more than once? 4) What are the evolutionary or biogeographic processes behind the current distribution and radiation of species within the genus?

3.3 METHODS

3.3.1 Taxon sampling and data collection

Due to a lack of recent material of certain rare species, 31 out of the 40 known *Izatha*, with representatives from each morphological group, were sequenced for this study (Table 1). Where possible at least three specimens for each species were used, however, for rarer species only one or two specimens could be included. Several other species from the closely related genera *Gymnobathra*, *Hierodoris*, and *Lathicrossa* were included as outgroups. As previously mentioned, species within the genera *Gymnobathra*, *Hierodoris*, and *Lathicrossa* are considered part of the xyloryctid assemblage and are included in a wider *Hierodoris* group along with *Izatha* (Hoare, 2005; Kaila et al. 2011). Overall, 36 species were included in the phylogenetic analyses. Most specimens were collected by R.M. Bennik (see Appendix Two), R.J.B. Hoare and T.R. Buckley, as well as contributions from N. Hudson and A.W. Emmerson. Further samples were sourced from the New Zealand Arthropod Collection (NZAC) at Landcare Research, Auckland, New Zealand. Most specimens were collected at night using a 125 watt mercury vapour light, some reared from dead wood, and a few collected resting on tree trunks during the day. Wherever possible, freshly collected specimens were preserved in 95% ethanol and stored at -20°C. When fresh material was not available, dried pinned specimens less than 10 years old were utilized.

Table 1. Species groups, complexes and list of species of *Izatha* based on Hoare (2010). Species included in the phylogenetic and ancestral state analyses are in bold.

Hoare (2010) groups	Species complex	Species		
<i>attactella</i>		<i>I. attactella</i>		
		<i>I. blepharidota</i>		
		<i>I. voluptuosa</i>		
		<i>I. austera</i>		
		<i>I. psychra</i>		
<i>mira</i>		<i>I. copiosella</i>		
		<i>I. walkerae</i>		
		<i>I. florida</i>		
		<i>I. mira</i>		
<i>apodoxa</i>		<i>I. notodoxa</i>		
		<i>I. katadiktya</i>		
		<i>I. apodoxa</i>		
		<i>I. acmonias</i>		
		<i>I. lignyarcha</i>		
		<i>I. picarella</i>		
<i>balanophora</i>	<i>epiphanes</i>	<i>I. metadelta</i>		
		<i>I. balanophora</i>		
		<i>I. churtoni</i>		
		<i>I. dulcior</i>		
		<i>I. epiphanes</i>		
	<i>peroneanella</i>	<i>I. mesoschista</i>		
		<i>I. haumu</i>		
		<i>I. quinquejacula</i>		
		<i>I. heroica</i>		
		<i>I. hudsoni</i>		
		<i>I. huttonii</i>		
		<i>I. peroneanella</i>		
		<i>I. taingo</i>		
		<i>oleariae</i>		<i>I. oleariae</i>
				<i>I. spheniscella</i>
	<i>I. prasophyta</i>			
<i>caustopa</i>		<i>I. caustopa</i>		
		<i>I. dasydisca</i>		
		<i>I. manubriata</i>		
<i>convulsella</i>		<i>I. convulsella</i>		
		<i>I. gekkonella</i>		
		<i>I. gibbsi</i>		
		<i>I. minimira</i>		
		<i>I. rigescens</i>		
		<i>I. phaeoptila</i>		

3.3.2 DNA extraction and sequencing

Up to three legs from adult specimens were used for DNA extraction. All tissue samples were crushed using microtube pestles, with DNA extracted and purified using Qiagen's DNeasy™ extraction kit following the manufacturer's instructions.

The LCO (1490) and HCO (2198) primers were used for the amplification of a region of the cytochrome oxidase subunit I mitochondrial gene (COI). Partial sequences of the Isocitrate dehydrogenase (IDH) and Carbamoylphosphate synthase domain protein nuclear gene (CAD) were amplified using primers designed in Geneious 6.0.5 (Drummond et al.). These were designed specifically for *Izatha* based on *Izatha peroneanella* and *Izatha austera* sequences (Kaila et al., 2011) obtained in GenBank - Accession numbers: JF818960 and JF818681 respectively (Table 2).

Table 2. PCR primers used in the present study. Sequence length is post trim.

Gene region	Primer direction	Primer name	Primer sequence (5'-3')	Anneal. temp (°c)	Amplified region length (bp)	Reference
COI	Forward	LCO1490	GCTCAACAAATCATAAAGAT ATTGG	50	583	Folmer et al. 1994
COI	Reverse	HCO2198	TAAACTTCAGGGTGACCAAA AAATCA	50	583	Folmer et al. 1994
IDH	Forward	IDH-Iza-F	AAGTGCGCCACCATCACCCC	55	431	This study
IDH	Reverse	IDH-Iza-R2	GCCTGCAGCCTCAAAGTGG	55	431	This study
CAD	Forward	CAD-Iza-F	AACTCAGAGAACCCACAGAC AAACG	55	494	This study
CAD	Reverse	CAD-Iza-R2	ACACCATCGGGGTGCTCAAT GT	55	494	This study

PCR amplification was conducted in 20 µl volume reactions comprised of: 12.8 µl milliQ H₂O, 2 µl of 10x PCR buffer, 2 µl dNTPs, 0.5 µl of each primer (10 mM), 0.8 µl BSA, 0.2 µl Taq DNA Gold Polymerase, 0.2 µl DMSO, and 1 µl DNA template. The standard thermal cycling profile for CO1 was an initial cycle at 95°C for 5 minutes; 94°C for 30 seconds, annealing at 50°C (CO1) and 55°C (CAD & IDH) for 45 seconds, extension/elongation at 72°C for 2 minutes for 40 cycles; then final elongation at 72°C for 7 minutes.

Product clean-up was conducted in 10 µl volumes: 5.6 milliQ H₂O, 2 µl of 5x sequencing buffer, 1 µl of each primer (3 mM), 0.4 µl of big dye terminator mix and 1 µl of diluted PCR template. Most often, PCR product was diluted by a 5(milliQ H₂O):1(PCR product) ratio, occasionally 3(milliQ H₂O):1(PCR product) for less purified product. The standard thermal cycling profile was the same for all three genes: an initial cycle at 96°C for 1 minute; 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 75 seconds; and a final extension at 10°C for at least 10 minutes.

Prior to sequencing, 10 µl of x-Terminator and 45 µl of SAM solution were added to the sequencing PCR product. It was then vortexed for 30 minutes @ 2000rpm and centrifuged for 2 minutes @ 1000rpm.

3.3.3. Phylogenetic analyses

Sequences were initially edited and aligned using Geneious 6.0.5. Model selection was performed in jModelTest 2.1.4 (Darriba et al. 2012), using the AIC method (Akaike,

1978). Garli 2.0 (Zwickl, 2006) was used for maximum likelihood tree searches and bootstrapping, with bootstrap consensus trees constructed in PAUP*4.0b10 (Swofford 2002) using the output from Garli. Bayesian trees were estimated in MrBayes v3.2 (Ronquist et al. 2012). MrBayes was run using the best fit AIC model, which was GTR+I+G for each of the three genes, with each gene partitioned – CO1, IDH and CAD. Six individual MCMC runs were conducted each using 4 chains for 5 million generations, with every 1000 generations sampled. The first 1000 (20%) samples from each of the analyses were discarded as burn-in, following inspection in Tracer1.6 (Rambaut et al. 2014), utilizing the remaining 4000 samples to calculate the posterior probabilities for nodal support.

3.3.4. *Genital characters*

Male genital characters were assessed by examining previously prepared slides of collection specimens, as well as new preparations of specimens collected during this research project. Dissections were performed using techniques modified from Hoare (2005). Once the genitalia were extracted from the abdomen, the phallus was separated from the genital capsule and both prepared onto microscope slides along with the abdomen. The genital characters mapped onto the phylogeny were chosen based on high variation among *Izatha* species. Therefore, six key male *Izatha* genital characters – 3 cornuti types, 1 phallic, and 2 genital capsule structures were used for ancestral state reconstruction and evolutionary patterns. For cornuti, the presence and absence of deciduous and fish hook cornuti, as well as the presence and number of compound cornuti were included.

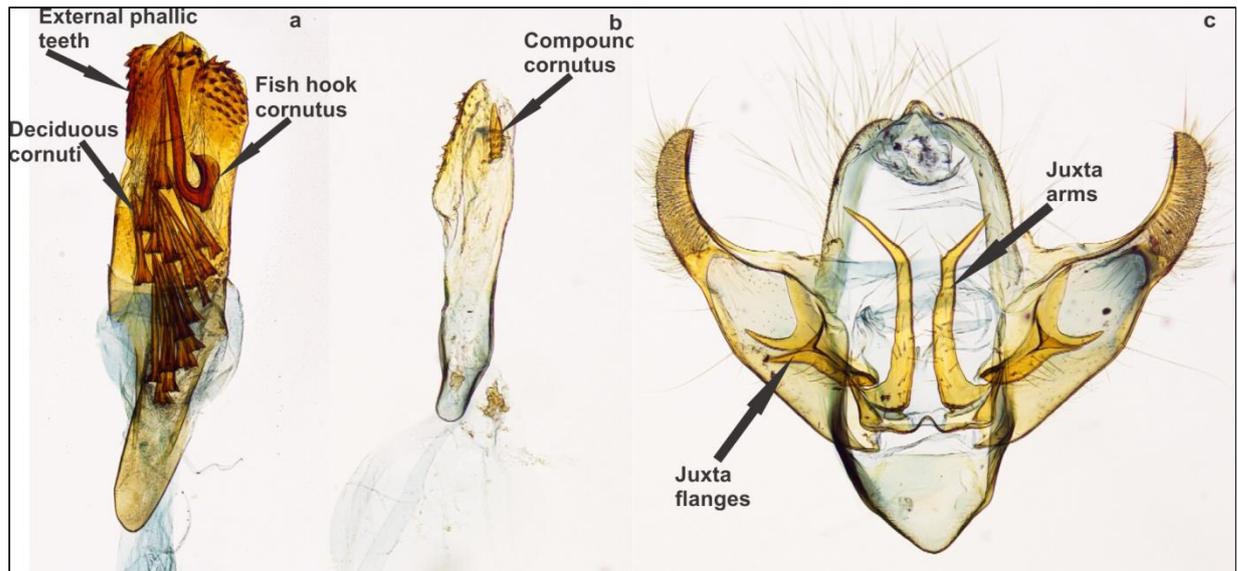


Figure 1. Images showing the various structures used in ancestral state reconstruction: a) *Izatha epiphanes* phallus displaying external phallic teeth, deciduous cornuti and fish hook cornutus; b) *Izatha convulsella* phallus with compound cornutus; and c) *I. taingo* showing the arms and flanges of the juxta.

Other genitalic characters used in the analysis included the average relative size of teeth of the phallus, the presence and number of flanges of the juxto-costal plate, and the presence and length of juxtal arms, which are all primary genitalic characters that are inserted into the female during copulation. In defining the size of teeth on the phallus, species possessing teeth with an average area of $2.0E-4 \text{ mm}^2$ or greater were categorised as having large teeth. The length of the juxtal arms of the genital capsule of each species was based on the length of the arms in comparison to the length of the valvae. Juxtal arms with an average ratio 0.5 or less than the length of the valva were categorised as short. Juxtal arms with a ratio of 0.5 or above were categorised as long.

Measurements of the phallic teeth and juxtal arms were conducted in cell[^]D imaging software.

3.3.5. Ancestral state reconstruction

Ancestral state reconstructions of all male genitalic characters were performed in Mesquite 2.75 (Maddison & Maddison, 2011). Character states and probabilities were then mapped on to a cladogram, with individual specimens of species clades compressed to denote the position of the species, based on the topology of the optimal ML tree. The Mk1 model (Lewis, 2001) was used to calculate the maximum likelihood reconstruction probabilities (Maddison & Maddison, 2006). The genitalic characters presented were chosen based on key characters used to define the genus and species, as well as those of functional interest.

3.4 RESULTS

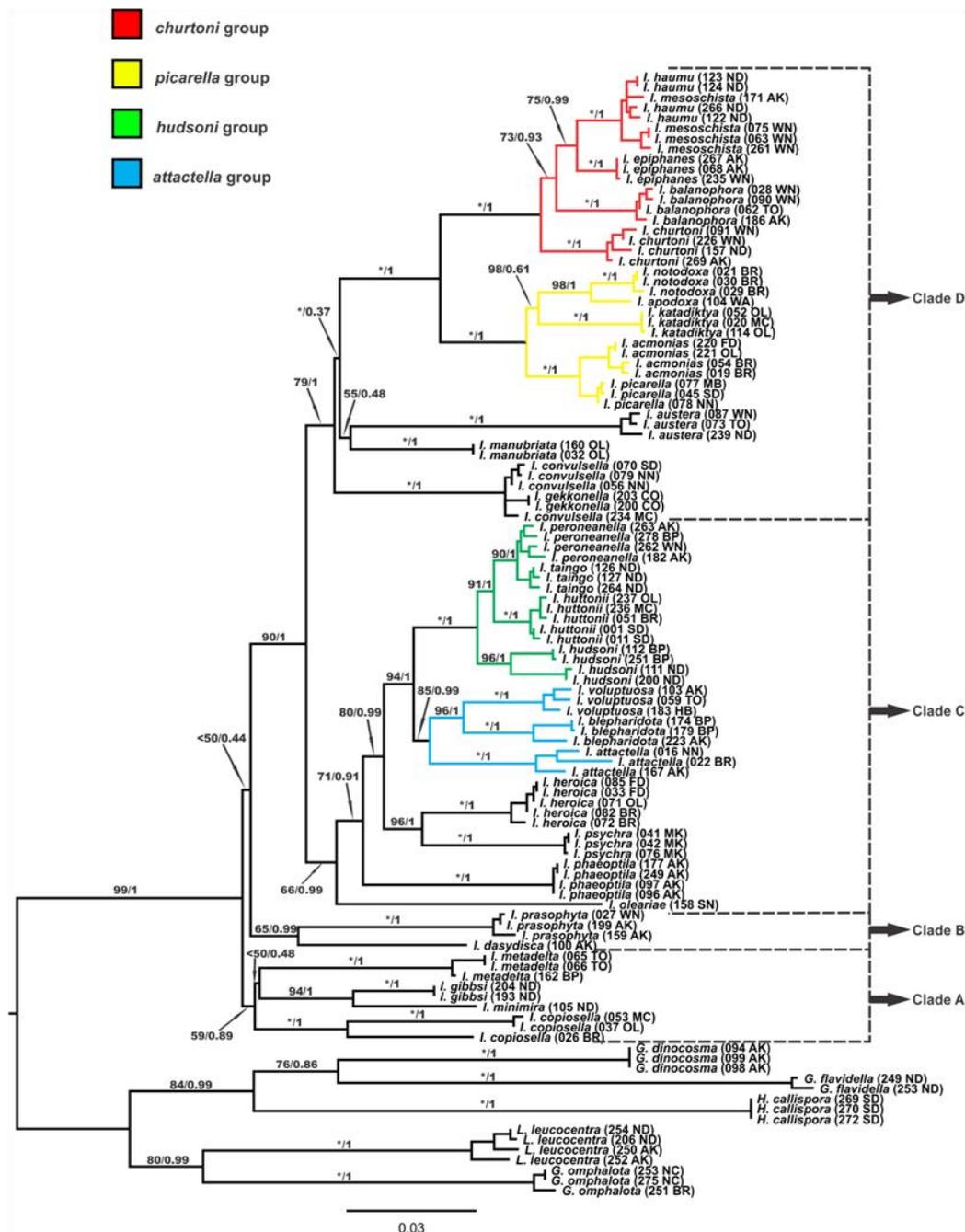


Figure 2. Best scoring maximum likelihood phylogeny of *Izatha* and closely related outgroups (36 *sp.*, 4 genera) combined data set (COI, IDH, and CAD). Nodes are labeled with 1000 bootstrap replicates and posterior probability values (* denotes a bootstrap value of 100). Four main clades (A-D) are represented by dashed lines. Numbers next to species name represent specimen number and regional code within New Zealand, following Crosby et al. (1998). ND = Northland; AK = Auckland; BP = Bay of Plenty; TO = Taupo; HB = Hawkes Bay; WN = Wellington; WA = Wairarapa; SD = Marlborough Sounds; MB = Marlborough; NN = Nelson; BR = Buller; NC = North Canterbury; MC = Mid Canterbury; MK = Mackenzie country; FD = Fiordland; OL = Otago lakes; CO = Central Otago; SN = Snares Island. Distinct morphological groups within major clades are colour coded.

3.4.1 Phylogenetic relationships

The final alignment contained 583 base pairs from the COI mitochondrial gene, and 494 bp and 431 bp from the CAD and IDH nuclear genes. The topologies of the optimal trees constructed via Bayesian and maximum likelihood inference were almost identical, and the best ML tree is presented in Figure 2. Based on both ML and Bayesian inference, *Izatha* is strongly supported as monophyletic (99% bootstrap/1 posterior probability, Figure 2).

Overall, the monophyly of Hoare's (2010) *apodoxa* group (Table 1) is well supported (100/1, Figure 2) as well as the *epiphanes* complex (100/1, Figure 2). However, none of the other groups defined by Hoare (2010) are monophyletic.

The monophyly of most species is highly supported with a bootstrap percentage of 90 or higher and/or posterior probabilities of 1. The exceptions are the sister species *I. haumu* and *I. mesoschista*, neither of which are monophyletic (Figure 2) and *I. convulsella*, which is paraphyletic with respect to *I. gekkonella* (Figure 2). Although there is high support for *I. copiosella* as a monophyletic species, there appears to be a large separation in the branch length of the specimen *I. copiosella* (026BR) from the other two specimens of this species (Figure 2).

Based on the molecular phylogeny, we here divide *Izatha* species into 4 major monophyletic clades and groups of related species that share similar character states are also highlighted for ease of discussion (Figure 2). Relationships within *Izatha* can be best explained as A+(B+(C+D)). At the root of the tree, *I. copiosella*, *I. minimira*, *I. gibbsi*, and

I. metadelta form a clade (clade A) with moderate support – 59/0.89, however, the position of *I. metadelta* is not as well supported - <50/0.48 (Figure 2). Clade A was recovered as sister group to all other species of *Izatha*, however, a soft polytomy occurs at this node – 59/0.89 (Figure 2).

Izatha dasydisca and *I. prasophyta* form a clade (clade B) with moderate support – 65/0.99, but the position of clade B is not as well supported (<50/0.44, Figure 2). This clade is a sister group to the two other major clades of *Izatha* – clade C and D.

Izatha oleariae, *I. phaeoptila*, and the sister species of *I. psychra* and *I. heroica* form a paraphyletic assemblage (Figure 2). The species *I. attactella*, *I. blepharidota*, and *I. voluptuosa* (*attactella* group) have good support as being monophyletic – 85/0.99, in addition to *I. hudsoni*, *I. huttonii*, *I. taingo* and *I. peroneanella* (*hudsoni* group) which have strong support for monophyly – 100/1. All the above species comprise a third larger clade – clade C, which are a sister group to species included in clade D.

The fourth major clade – clade D - includes *I. convulsella* and *I. gekkonella* 100/1, (Figure 2); *I. picarella*, *I. acmonias*, *I. katadiktya*, *I. apodoxa* and *I. notodoxa* (*picarella* group) – 100/1; and *I. churtoni*, *I. balanophora*, *I. epiphanes*, *I. mesoschista*, and *I. haumu* (*churtoni* group) – 100/1 (Figure 2) which all form well-supported monophyletic clades. However, the position of two species within clade D – *I. manubriata* and *I. austera* is not well supported - 55/0.48 (Figure 2). They are tentatively placed as sister to the remaining species in the clade, excluding *I. convulsella* and *I. gekkonella* (Figure 2).

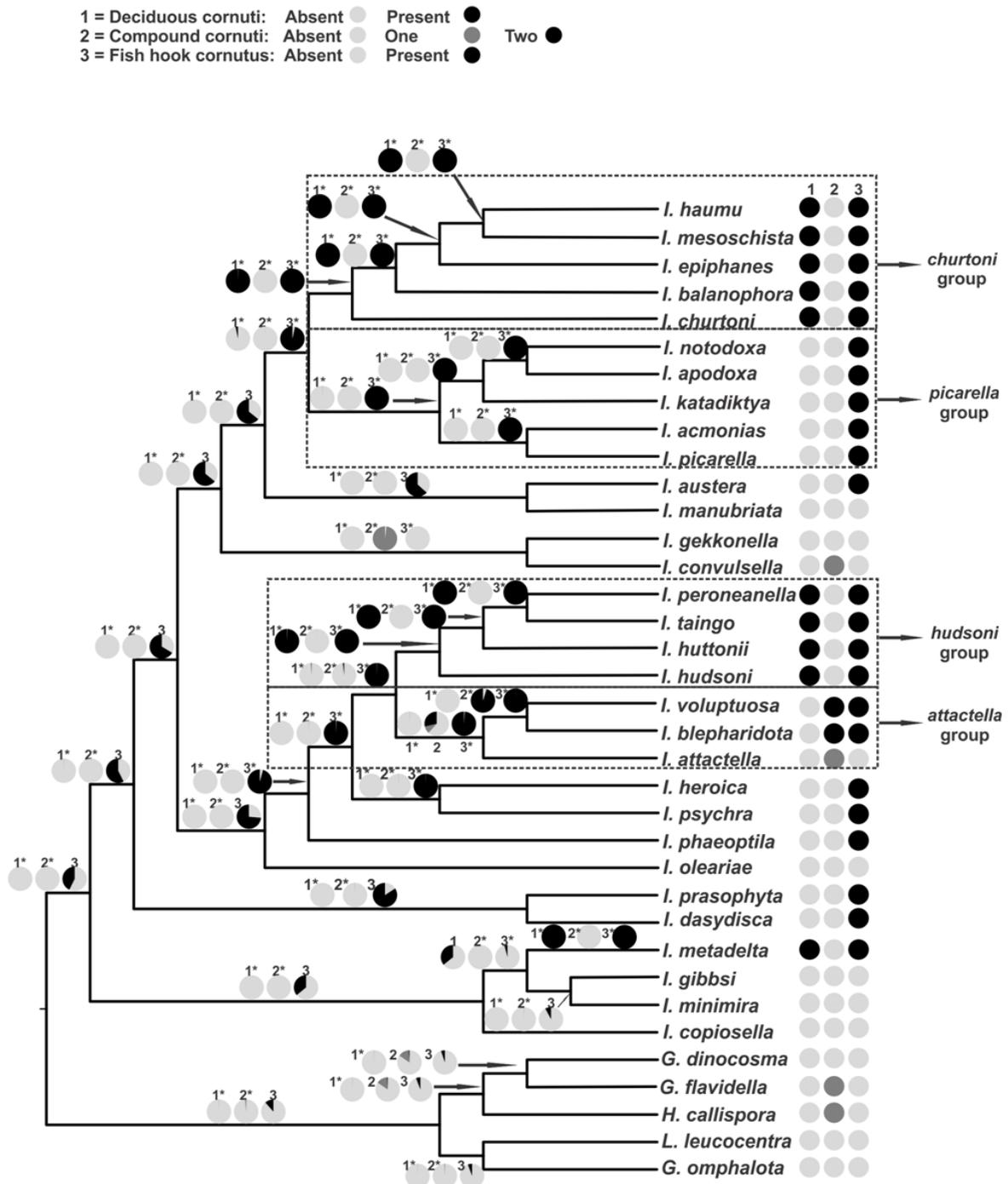


Figure 3. Ancestral state probabilities for cornuti traits mapped over the optimal ML topology. Pie charts at nodes represent 1 - the presence or absence of deciduous cornuti; 2 - the presence or absence and number of compound cornuti; 3 - presence or absence of a fish hook cornutus. Circles next to species name denote character states of that species. * = significant result. Distinct morphological groups are represented by dashed lines.

3.4.2 Ancestral states and direction of evolution of male genitalic characters

Deciduous cornuti

Deciduous cornuti (DC) are spines derived from the vesica that detach and are ejected into the female reproductive tract during copulation. There is a complete absence of DC in closely related out-group species (absence = 0.999*) and no other xyloryctids are known to have them (Hoare, R.J.B. pers. comm.). The absence of DC is also an ancestral state for *Izatha* (absence = 0.999*). The acquisition of these structures, however, has arisen on three separate occasions in the genus; once in *I. metadelta* and once in the *hudsoni* group in clade C (presence = 0.996*) and *churtoni* group within clade D (presence = 0.996*) (Figure 3).

Compound cornuti

Compound cornuti are sets of small spiny or leaf-like fixed cornuti attached to the inner vesica of the phallus. A possibly homologous compound cornutus is present in two species of closely related out group genera, *Gymnobathra flavidella* and *Hierodorus callispora*, but the absence of this structure is most prevalent (absence = 0.985*). The ancestral state of the absence of compound cornuti is significant for the *Izatha* genus (absence = 0.999*). However, the presence of one compound cornutus has been secondarily inherited twice in *Izatha*; in *I. attactella* (one = 0.998*) and *I. convulsella* (one = 0.980*). The development of two compound cornuti has also occurred in two

sister species of the *attactella* group – *I. blepharidota* and *I. voluptuosa* (two = 0.948) (Figure 3).

Fish hook cornutus

Like deciduous and compound cornuti, the fish hook cornutus is a sclerotised structure attached to internal vesica of the phallus. However, it is attached more closely to the base of the everted vesica during copulation. Fish hook cornuti are absent in out-group genera (absence = 0.918) and there is uncertainty about the ancestral state of this structure at the internal node to the *Izatha* genus (absence = 0.572; presence = 0.428). However, support for the development of this structure from an ancestor of the *picarella* (presence = 0.999*), *churtoni* (presence = 0.999*), *hudsoni* (presence = 0.999*), *attactella* (presence = 0.989*) groups and *I. psychra*/*I. heroica* (absence = 0.003; presence = 0.997*) is significant. This structure first appears in *I. metadelta* and is also present in clade B (presence = 0.810) and *I. phaeoptila* (Figure 3).

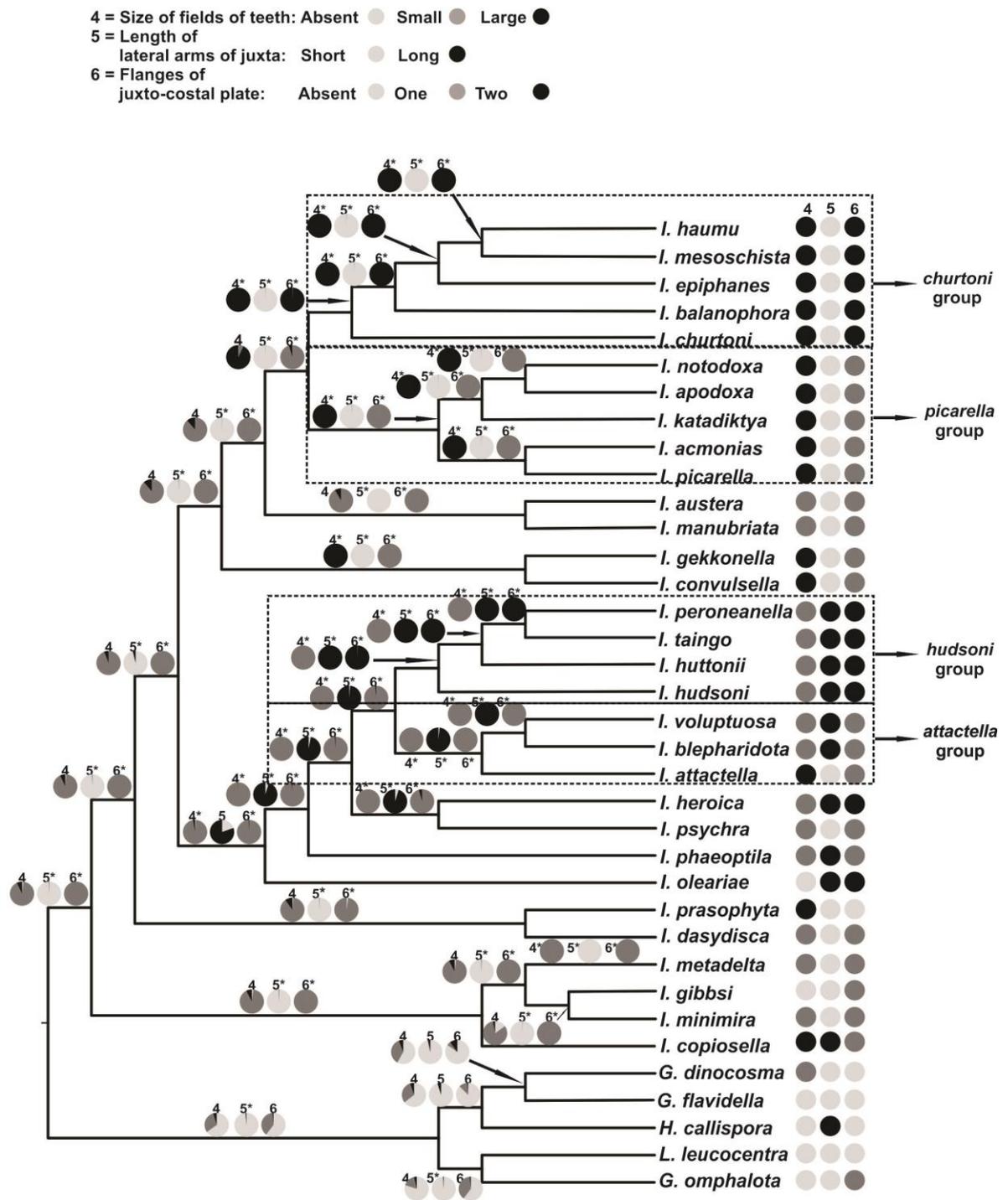


Figure 4. Ancestral state probabilities for non-cornutus traits mapped over the cladogram of phylogenetic relationships of *Izatha* and closely related outgroups based on the optimal ML tree. 4 = the presence or absence and size of teeth - small = $< 2.0E-4$, large = $> 2.0E-4$; 5 = the length of the arms of juxta – short = < 0.5 length of juxtal arm to valva ratio, long = > 0.5 length of juxtal arm to valva ratio; 6 = presence or absence and number of flanges on the juxto-costal plate of the genital capsule. Circles next to species name denote character states of that species. * = significant result. Distinct morphological groups are represented by dashed lines.

Size of teeth of phallus

With the exception of *Gymnobathra dinocosma*, which has a ridge of small teeth, all other out-group species lack teeth on the exterior of the phallus (absence = 0.650; small = 0.303; large = 0.047). There is strong support for small phallic teeth in the most recent common ancestor of *Izatha* (small = 0.910) and in clades A (small = 0.904), B (small = 0.886) and *manubriata/convulsella* group (small = 0.913). The ancestry of small teeth is significant for *I. psychra/I. heroica* (small = 0.998*), and the *attactella* (small = 0.994*) and *hudsoni* groups (small = 0.999*). The appearance of larger teeth occurs sporadically in the basal species *I. copiosella*, *I. prasophyta*, *I. attactella*, as well as the *convulsella* (large = 1.0*), *picarella* (large = 0.999*) and *churtoni* groups (large = 0.999*). The development of larger teeth appears to be a more derived character (Figure 4).

Length of arms of juxta

Most of the out-group species, with the exception of *Hierodorus callispora*, have short juxtal arms in the genital capsule (short = 0.989*). The ancestral condition for the genus *Izatha* is also the presence of short juxtal arms (short = 0.997*). Longer juxtal arms appear in *I. copiosella* (long = 0.843) and are also prevalent in species within clade C – *I. oleariae*, *I. phaeoptila*, *I. heroica*, and the *attactella* (excluding *I. attactella*) and *hudsoni* groups (long = 0.952*). The juxta is thought to provide guidance and support to the phallus during mating (Ever 1924; Klots 1970) (Figure 4).

Flanges of the juxto-costal plate

All but *Gymnobathra omphalota* of the outgroup genera, which has one flange present, lack flanges on the juxto-costal plate (absence = 0.603; one = 0.383; two = 0.014). The ancestry of at least one flange is significant for the node that defines *Izatha* monophyly (one = 0.999*), with all but *I. prasophyta* having at least one flange present. The presence of two flanges emerges three times - in the *churtoni* (two = 0.994*) and *hudsoni* groups (two = 0.995*), as well as in *I. heroica* and *I. oleariae* (Figure 4).

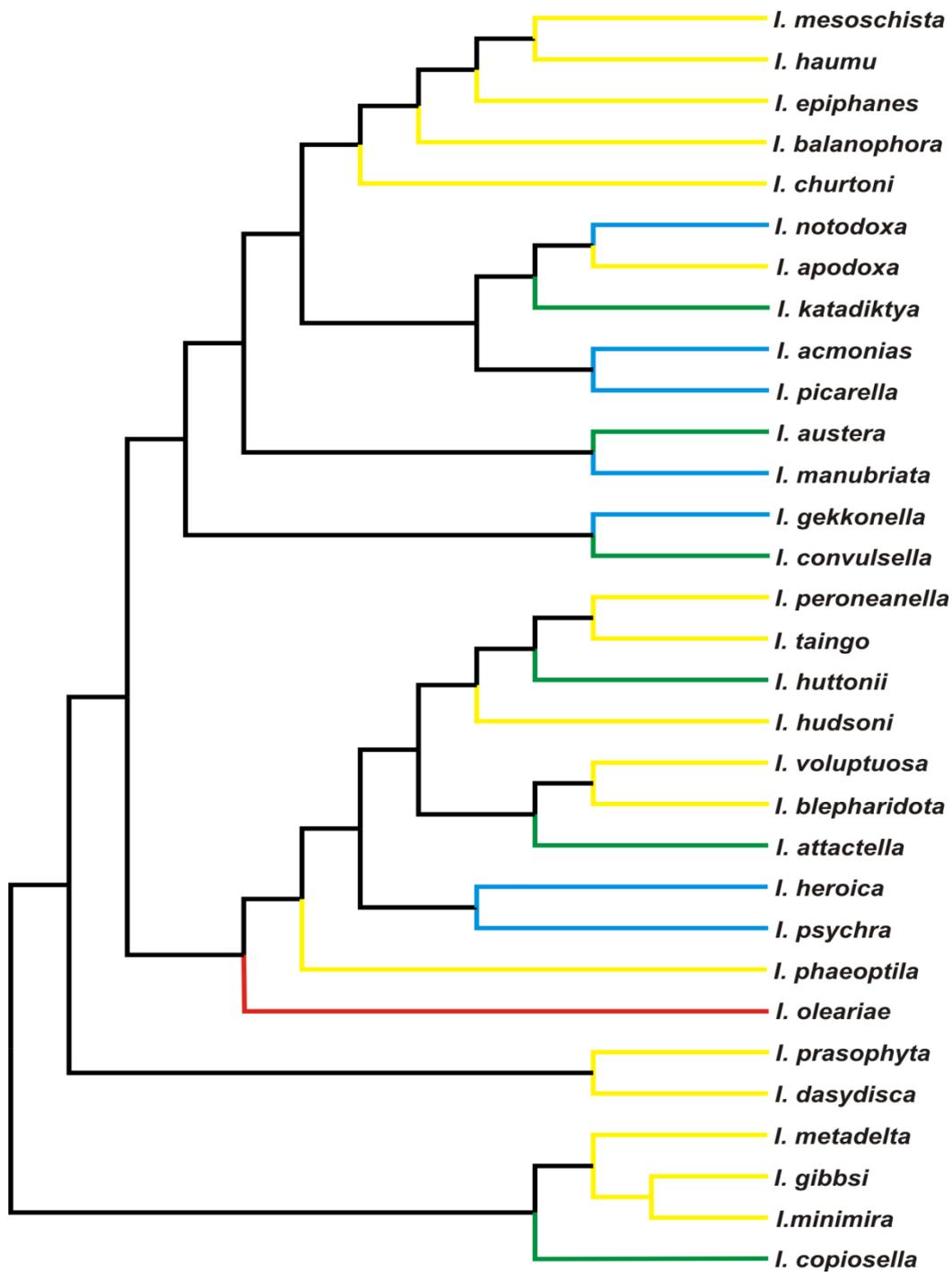


Figure 5. Cladogram of *Izatha* with terminal branches are colour coded to represent the distribution of species. Yellow = North Island; Blue = South Island; Green = both North and South Islands; Red = Subantarctic Snares Islands.

3.4.3 Biogeographical distribution within *Izatha*

Colour codes of the geographical location of species are depicted in Figure 5. There is no distinct progression from the North to South Island, mainland to offshore islands or vice versa. For species included in the phylogenetic analyses, seventeen are exclusive to the North Island, seven to the South Island, and six inhabit both islands. The species *I. oleariae* in red is restricted to the subantarctic Snares Island. There does, however, appear to be a recent radiation of species in the North Island that comprise species of the *epiphanes* complex (Table 1).

3.5 DISCUSSION

3.5.1 Do molecular phylogenetic relationships match proposed morphological species groups?

The molecular phylogeny is in agreement with the monophyly of Hoare's (2010) *apodoxa* group, but does not support the monophyly of his *balanophora*, *attactella*, *oleariae*, *caustopa* and *convulsella* groups. Hoare's (2010) *apodoxa* group is congruent with species of the molecular phylogeny's *picarella* clade. Although his *balanophora* group is not monophyletic, the *epiphanes* and *peroneanella* complexes (with the exclusion of *I. heroica*) he described within this group, which correspond to the *churtoni* and *hudsoni* clades of the molecular phylogeny are monophyletic.

Clade A at the root of the tree, which includes *I. copiosella*, *I. minimira*, *I. gibbsi*, and *I. metadelta*, appear to be the sister group of all other species within the genus. However,

the presence of a soft polytomy at the node separating this clade and clade B, which incorporates *I. dasydisca* and *I. prasophyta*, make it difficult to resolve which group should be closer to the root of the tree (Figure 2).

In terms of genital morphology, *I. copiosella* has some distinctive traits from the other three species within clade A. It has larger teeth present on the external surface of the phallus, and longer juxtal arms of the genital capsule. In contrast, *I. metadelta*, *I. gibbsi* and *I. minimira* have small teeth – or in the case of *I. gibbsi*, no teeth – and short juxtal arms. Three species of *Izatha* that were excluded from analyses were suggested to form a monophyletic group with *I. copiosella* due to similar external and genital morphology by Hoare (2010).

The two species included as clade B, *I. dasydisca* and *I. prasophyta*, are quite distinctive from other species of *Izatha* in terms of general and genital morphology. Genital characteristics of *I. dasydisca* most closely resemble those of *I. caustopa*, which was not included in the analyses. *Izatha prasophyta* was described as anomalous in terms of both general and genital morphology compared to other *Izatha* species (Hoare, 2010). It was tentatively placed with the Snares Island species, *I. oleariae* and *I. speniscella*, based on genital morphology; however, it lacks flanges on the juxto-costal plate, which is a relatively characteristic genital trait in *Izatha*.

Izatha oleariae and *I. phaeoptila* are included as part of a larger clade C, along with the *psychra*, *attactella* and *hudsoni* monophyletic clades. It is not surprising that the *attactella* and *hudsoni* clades are closely related as they share a distinctive male genitalic characteristic – the juxta base plate is split in two medially by a membrane or consists of ill-defined lateral sclerites that are joined together by a fine sclerotised strip (Hoare, 2010). The *hudsoni* clade was originally considered closer to the *churtoni* clade within clade D due to the presence of deciduous cornuti (Hoare, 2010), but it appears that a relationship based on that one trait alone does not adequately reflect molecular genetic relationships.

Support for the positioning and resolution of species within the larger clade D is strong. The positioning of *I. austera* and *I. manubriata*, however, is problematic. There is low nodal support for the positioning of these two species and the long branch length for *I. austera* suggests a large amount of change in the three genes included in the analyses for this species.

There is also incomplete phylogenetic resolution between *I. haumu* and *I. mesoschista*, and *I. convulsella* and *I. gekkonella*. This suggests recent speciation, which is not unexpected, as each species pair is very similar in external and genital morphology. *Izatha haumu* and *I. mesoschista* are difficult to identify in the field. There are subtle differences in external morphology, but identification is largely based on collection location and dissection of genitalia. Although *I. convulsella* and *I. gekkonella* can be identified on external characters, a single genital character, presence of the compound

cornutus in *I. convulsella*, is again the most reliable means of identification. Recent studies in which the incomplete lineage sorting of closely related species has occurred have often provided recent speciation as a reason for a lack of monophyly of species clades (Pestano et al., 2003; Hundsdoerfer et al. 2009; Sanders et al. 2013). Indeed, Nice & Shapiro (1999) found that morphological characteristics of a lycaenid butterfly genus had progressed more rapidly than the evolution of mitochondrial DNA and allozymes. Therefore, caution must be taken when interpreting recent divergence, as morphological characters, such as rapidly evolving genitalia, may precede genetic variation in slower evolving genes and may explain the lack of monophyletic resolution between the above mentioned two groups of closely related species. As the phylogenetic analyses are based on only one mitochondrial and two nuclear genes, additional gene regions may help to further resolve the monophyly of the species.

3.5.2 Ancestral states and direction of genital evolution within Izatha.

Deciduous cornuti

The ancestral state for *Izatha* is a lack of deciduous cornuti. These structures appear in more derived species and have arisen on at least three separate occasions; once in *I. metadelta*, and separately in all species of the *hudsoni* group within clade C and *churtoni* group within clade D. This is suggestive of the convergent evolution of these structures within the genus. Male *Izatha* eject the complete set of these structures in the first mating in which they are deposited with the spermatophore in the corpus bursae of the

female reproductive tract. Cordero (2010) proposed that DC may be under natural selection and work in conjunction with the contraction of a sclerotized structure in the corpus bursae of females – the signum – to assist with the breaking of the spermatophore. Other theories proposed suggest they are under sexual selection via sperm competition, sexual conflict or to influence female choice. Males may use these structures to displace or break up previous males' spermatophores before their spermatozoa migrate to the spermatheca. Alternatively, they may cause damage to the female to delay remating, or function to stimulate a female into utilising that male's spermatophore (Cordero, 2010). However, recent studies propose that they primarily function to assist in breaking up the spermatophore (Galicia et al. 2008; Lincango et al. 2013).

In describing the male genitalia of olethreutine moths (Family Tortricidae), Horak (2006) noted the presence of deciduous cornuti and proposed that this was an ancestral condition, with a progressive reduction in number and modification of these structures, with fixed cornuti the derived state. This is apparently not the case in *Izatha*, as the earliest appearance of deciduous cornuti is in *I. metadelta*, which has one of the lowest numbers (3-5) of these structures and is a sister species to the other two groups, the *hudsoni* and *churtoni* clades in which they have evolved separately. The highest number of these structures is found within the *hudsoni* clade; *I. peroneanella* possessing 24 – 43 and *I. taingo* between 29 – 48 deciduous cornuti. Within the *churtoni* clade, there is no direct progression from fewer to a higher number of DC. *Izatha mesoschista* has a range of 9 – 17 and *I. haumu* has between 11 – 16 deciduous cornuti. Therefore, although the

possession of deciduous cornuti appears to be a derived state within *Izatha*, the number of DC appears to follow alternative paths in different clades. There is also a potential coevolution between the presence of DC in males and the elaboration of a female sclerotised structure – the signum - within *Izatha*. Further examination of these two structures may help reveal the evolutionary origins and selective pressures behind the development and maintenance of these genital characters.

Compound cornuti

A lack of compound cornuti appears to be the ancestral state within *Izatha*. The presence of compound cornuti has arisen twice; once in *I. convulsella* and in the three species of the *attactella* clade: *I. attactella*, *I. blepharidota*, and *I. voluptuosa*. *Izatha convulsella* and *I. attactella* possess a single compound cornutus, whereas *I. blepharidota* and *I. voluptuosa* both have two compound cornuti (Hoare, 2010). The compound cornuti in these species resemble the over-lapping cornuti of closely related species *Hierodoris electrica*, *H. s-fractum* and *H. pachystegiae* (Hoare, 2005). However, the compound cornutus in *I. convulsella* does differ structurally to those found in the *attactella*-group, so the homology of this structure between these two groups is tentative (Hoare, 2010). As previously mentioned, Cordero (2010) proposed a number of hypotheses as to the function of fixed or non-deciduous cornuti such as the compound cornuti exhibited in *Izatha*. Interestingly, the four species that possess compound cornuti do not have deciduous cornuti. The position of the compound cornuti in the everted vesica suggests they are most likely to make contact with the ductus bursae slightly anteriorly to the external phallic teeth. As this region of the female reproductive

tract is fairly uniform in species in which compound cornuti are present, the lock and key hypothesis is not likely to account for them. Species that possess these structures – with the exception of *I. convulsella* - tend to have smaller external phallus teeth, hence they may provide additional stabilization of the phallus through sexual selective pressures to avoid potential displacement by rival males (sperm competition) or the female (sexual conflict) during copulation, or stimulate the female during or post-copulation (cryptic female choice). They could also have a natural selective origin for guidance of the vesica or spermatophore into the female's corpus bursae or assist in the breaking up of the spermatophore. Extensive scarring of the ductus bursae is evident in one species of *Izatha* – *I. convulsella* – but not in the other species that possess compound cornuti, however, spines on the compound cornuti in *I. convulsella* are larger in comparison to the other species may indicate that this is causing at least part of the damage.

Fish hook cornuti

The evolution of a fish hook cornutus appears to be a derived character within *Izatha*, or possibly a defining synapomorphy of the genus (though absent in some species). This structure is attached to the base of the everted vesica and probably reaches only just beyond the tip of the phallus into the ductus bursae during copulation. This cornutus is absent among closely related out-groups, whereas the ancestral probability for *Izatha* was evenly split. The majority of species possess one and it has arisen on a number of occasions and among certain groups within the genus. It is present in all members of the *churtoni*, *picarella*, *hudsoni*, *psychra* and *dasydisca* clades, as well as *I. austera*, *I. phaeoptila*, *I. metadelta* and *I. voluptuosa* and *I. blepharidota* from the *attactella* clade.

The function of this structure could potentially be similar to the hypotheses proposed by Cordero (2010) for general fixed cornuti. Alternatively, as suggested by Hoare (2010) it may help guide the collum of the spermatophore into position close to the ductus seminalis inception.

Development of teeth of the phallus

The presence of small teeth is recovered, with significant support, as an ancestral character of the outgroup taxa and *Izatha*. All but *I. gibbsi* and *I. oleariae* have teeth present on the exterior of the phallus. The development of larger teeth has occurred in *I. copiosella*, *I. convulsella*, and *picarella* and *churtoni* group within clade D (Figure 4).

There is no distinct direction of evolution of the enlargement of phallic teeth, as they occur in both basal and more derived species and have arisen on a number of occasions. Interestingly, the presence of larger teeth tends to coincide with shorter lateral juxta arms. If the function of the juxta is to provide support and guide the phallus (Ever 1924; Klots 1970), and/or aid in remaining attached to the female during copulation, then larger teeth may compensate for the presence of shortened juxtal arms by performing a similar function. Damage to the female ductus bursae of species with larger teeth in the region where they would most likely make contact is commonly evident during dissection. Therefore, these structures could also be used to delay or deter females from remating. If this is the case, females may respond with counter-adaptations to reduce the amount of damage incurred by these structures. Many common species of *Izatha* display polyandrous mating systems, as suggested by the presence of more than one spermatophore or sets of deciduous cornuti in the female reproductive tract. However,

little is known about the general ecology and mating systems of most species within the genus and the evolution of phallic teeth may coincide with a potential shift from non-monogamous mating systems to lengthen the female's post-copulatory refractory period as mentioned and/or to provide a tighter grip to avoid interruption from other males during copulation.

Length of juxtal arms

Short juxtal arms of the genital capsule are characteristic of all outgroup species with the exception of *Hierodoris callispora* and are prevalent among many species of *Izatha*. Longer juxtal arms have arisen at least twice; once in *I. copiosella*, and in all but *I. psychra* and *I. attactella* within clade C (Figure 3). In the hesperiid butterfly *Carcharodus baeticus*, two structures of the genital capsule – the uncus and gnathos – have been shown to obtain a dorsal grip by claspings on to the 8th tergite of the females abdomen during copulation (Common 1975; de Jong 1978; Sihvonen 2007). In a *Hofmannophila pseudospretella* (Oecophoridae), even though the uncus appeared to have no function during copulation, the gnathos was used to grip onto the female (Dugdale, J.S., pers. comm.). However, both these structures are highly reduced in *Izatha*. As previously mentioned, the juxta is proposed to function to help anchor and guide the phallus during copulation (Ever 1924; Klots 1970), and as the musculature of the juxta in closely related Gelechiid moths is associated with the phallus (Ponomarenko 2008, 2009) this further supports its role in the positioning and movement of the phallus during mating. Therefore, the juxtal arms, in conjunction with the elaboration of the valvae (in particular the sacculus) of the genital capsule in *Izatha* may compensate for the

reduction of the uncus and gnathos. The juxta and valval processes may ventrally stabilize the aedeagus during copulation and the fact that some species have extra sclerotised processes, further suggests that they have a gripping function (Hoare, 2010). This implies they may be under natural selection to guide/support the phallus or under sexual selection to help avoid displacement during copulation from rival males (sperm competition), females (sexual conflict) or may stimulate the female into utilizing the current males spermatophore (cryptic female choice).

Flanges of the juxto-costal plate

One of the closely related groups included in the character analyses, *Gymnobathra omphalota*, has a flange at the junction of the juxto-costal plate. At least one flange is present in all *Izatha* except *I. prasophyta*, which as previously mentioned shows fairly unique general and genital morphology. Many species have one flange, except for the *churtoni* and *hudsoni* clades, and *I. heroica* and *I. oleariae*, in which there are two. In addition, species closer to the root of the phylogeny tend to have less prominent juxto-costal flanges, in comparison to more derived species. A few species also possess scobination of the flanges. In *I. picarella*, the flanges are covered with teeth and along with a broadening at the tip of the phallus and large teeth, are suggestive of sexually antagonistic coevolution (Hoare, 2010). In correspondence with the juxtal arms, the flanges of the juxto-costal plate may also provide a further supplementary gripping function due to the reduction of other key lepidopteran gripping structures, the uncus and the gnathos. As mentioned, many species of field caught *Izatha* females have multiple spermatophores and/or sets of deciduous cornuti present in the corpus bursae, indicating a polyandrous mating system. If males are under direct competition from

other males, obtaining and maintaining a strong connection with a female during copulation becomes paramount, not only in order to prevent rival males from usurping mating attempts, but potentially in mate guarding to prevent immediate re-mating efforts. Therefore, with the reduction of the uncus and gnathos, the flanges of the juxto-costal plate and juxtal arms may compensate for this.

3.5.3 *Potential processes behind the current distribution and radiation of species within Izatha.*

Within *Izatha*, there appears to have been multiple exchanges between the North and South Island. Dynamic changes to the New Zealand landscape from about 5 million years ago – formation of mountains such as the Southern Alps, volcanism, land extension of the southern north Island, and climactic and land shifts associated with Pleistocene glaciations (Campbell & Hutching, 2007; Graham, 2008) would have produced ecological barriers and influenced the direction of dispersal of ancestral species. As a possible consequence, areas of high levels of endemism of New Zealand insects are often recorded in the northern North Island and northern and southern South Island (Craw, 1989; Wallis & Trewick, 2009; Trewick et al. 2011; Trewick & Bland, 2012). This is consistent with the distribution patterns of some species of *Izatha*, especially those at the root of the phylogeny, i.e. *I. copiosella*, *I. minimira*, and *I. gibbsi*. *Izatha minimira* and *I. gibbsi* are limited to the northern North Island and *I. copiosella* is prevalent over much of the South Island, but has populations that are present in the south-east part of the modern day North Island. These long branched species therefore show distribution patterns expected of the proposed area of New Zealand landmass not

submerged during the Pliocene. During the Pliocene, much of the lower North Island was submerged, with a thin strip of the Manawatu strait more closely connecting the South Island to the central North Island (Bunce et al. 2009; Trewick & Bland, 2012), which may explain the presence of populations of primarily South Island species such as *I. huttonii*, *I. copiosella* and *I. katadiktya* in the lower and south-eastern regions of the North Island.

Izatha oleariae inhabits the offshore subantarctic Snares Islands. The Snares Islands are one of several subantarctic island archipelagoes that make up the Campbell Plateau south-east of the New Zealand mainland (Michaux & Leschen 2005; Leschen et al. 2011). Previous descriptions and more recent phylogenetic research on insects (Gressitt & Wise 1971; Sublette & Wirth, 1980; Craw, 1999; Michaux & Leschen 2005), and plants (Mitchell et al. 1997; Swensen & Bremer, 1997; Wagstaff & Garnock-Jones, 1998; Fraser et al. 2009) inhabiting the subantarctic islands showed that taxa either share a distribution with or have a proposed mainland New Zealand origin of their sister groups. This is consistent with the placement of *I. oleariae* in the phylogeny of the genus, as this species has sister groups that are found only on the mainland.

As mentioned earlier, potentially recent speciation events are observed between *I. haumu*/*I. mesoschista* (Figure 2). *Izatha haumu* is limited to the top of the North Island, whereas, *I. mesoschista* is found all over the rest of the North Island, south of this area. A similar geographical distribution is seen between the closely related, derived sister species *I. taingo* and *I. peroneanella* - *I. taingo* is also restricted to northern Northland

and *I. peroneanella* prevalent all over the North Island south of the former's range. The northern most tip of the North Island encompasses the Te Pahi ecological district which spans about 30, 917 hectares to the neighbouring Aupouri ecological district further south within the region of Northland (Lux et al. 2009). This area is a known biodiversity hotspot with many endemic animal and taxa (de Lange 2003; Chapple et al. 2008; de Lange & Rolfe 2008; Lux et al. 2009; Winterbourn 2009; Buckley & Bradler 2010; Ball et al. 2013). Approximately 3mya, Pliocene inundations separated the northern tip of New Zealand which encompasses the Te Pahi ecological district from the rest of the mainland (Ballance & Williams 1992; Buckley & Leschen 2013) allowing for the potential range division and speciation of taxa from closely related North Island species. This may explain the range division and genital morphological deviations in between these species, as the submergence of the Northern tip of the North Island from the rest of the North Island may have impeded genetic flow, promoting speciation in isolation. Another apparent recent speciation is seen between *I. convulsella* and *I. gekkonella* (Figure 2).

Most members of the *churtoni* group within clade D and the *hudsoni* group, with the exception of *I. huttonii* within clade C, are restricted to the North Island, and appear to have undergone a more recent rapid radiation. Even though *Izatha* larvae are probably not restricted to dead wood of specific plant taxa, preferred plant species and wood in an appropriate state of decay, or with suitable fungal content, may be more abundant within larger areas of forest. During glaciations, there would have been a more diverse range of remnant native plant species in the North Island (Hall & McGlone, 2006; Hoare, 2010). Therefore, the higher radiation of *Izatha* species in the North Island may be partly

attributed to more extensive forest refugia during the last glaciation (McGlone 1985). Similar trends may be evident in other sapro-xylophagous invertebrate taxa within New Zealand. For example, other insect groups such as species of flat bugs (Hemiptera: Aradidae) that are associated with decaying wood and fungi show comparable patterns of distribution (Lariviere & Laroche, 2004). A recent study of three New Zealand log-dwelling beetles showed dispersal trends consistent with areas of forest refugia and geological barriers produced from the LGM (Marske et al. 2012), which may be consistent with dispersal patterns within *Izatha*.

3.5.4 Summary

The molecular phylogeny of *Izatha* agrees with some of the previous morphological taxonomic groupings within the genus, but also shows some interesting deviations. Among this is the independent evolution of the deciduous cornuti within the genus. Previous assumptions were that these structures had evolved once and contributed to the original monophyletic grouping of species who possess these structures (Hoare 2010).

Nevertheless, many species that were described as highly homogeneous in terms of external and genitalic morphology (eg. *apodoxa* group; *epiphanes* and *peroneanella* complexes (Hoare, 2010), have correspondingly come out as strongly supported monophyletic groups eg. *picarella*, *churtoni* and *hudsoni* clades. Morphological species groupings that were considered tentative and displayed autapomorphies frequently

showed divergences in the molecular phylogeny. As a consequence, the morphology of male genitalic structures have been useful characters for identifying species groups, but caution must be taken for cases in which convergent evolution may mislead inferences of monophyly and the addition of a molecular phylogenetic assessment has highlighted important discrepancies of the inter-generic relationships within *Izatha*. The further addition of species excluded from the phylogenetic analyses, especially other species classified in Hoare's (2010) mira group would help get a more complete picture of the evolutionary relationships and adaptation of this genus within New Zealand. Species such as the Three Kings Island's *I. quinquejacula* and Poor Knights *I. dulcior* are similar in external and genital morphology to *I. mesoschista*/*I. haumu* and *I. epiphanes* respectively, suggesting recent speciation. Therefore, it is highly likely that these two species would be included in the *churtoni* clade. The inclusion of additional nuclear gene sequences may also provide further resolution to the apparent recent speciation events within the two sets of closely related species groups of *Izatha*.

Izatha have some distinct and diverse modifications in the genitalia, especially of males. There is a reduction of the gnathos and uncus within *Izatha*, which are often prominent (Alma Solis & Metz, 2011) or show varying degrees of development in other Lepidoptera taxa (Hodges 1998; Horak, 2003; Horak, 2006; Wang, 2009). However, there has been development and enlargement of the lateral juxtal arms and flanges of the juxto-costal plate within *Izatha*. The evolution of these structures could potentially compensate for the significant reduction of the uncus and gnathos and provide an equivalent function, i.e. grasping and maintaining grip of the female during copulation. Interestingly, species

which have longer juxtal arms and flanges often possess smaller teeth on the phallus and vice versa, with species displaying large teeth, possessing shorter juxtal arms and flanges. If teeth on the phallus do largely function to stabilize and grip on to the female during copulation, then those species with larger teeth may offset the necessity for the elaboration of the juxtal arms and juxto-costal flanges.

Indeed, genital characters within *Izatha*, such as those mentioned in the previous paragraph tend to show a high level of homogeneity within monophyletic clades. The possession of fish hook cornuti also appears to be a synapomorphy for the genus. Although there are a lot of hypotheses to explain function, the exact way these structures operate during copulation is still not certain, as we have no observations of the coupling of male and female *Izatha* genitalia during copulation and a paucity of knowledge of the mating systems of many species. Furthermore, there is also the question of whether similar structures that have evolved in different clades are functionally homologous. Ponomarenko (2008) highlights the importance of investigating the underlying skeletal and muscular connections of genital structures in order to assess homology and gain an insight into functionality.

In terms of biogeography, there are some possible explanations for the current distribution of species within *Izatha*. *Izatha oleariae* and *I. spheniscella* of the subantarctic Snares Islands most likely had an ancestor on mainland New Zealand early in the evolution of the genus. Dynamic land changes during the Pleistocene would have had an effect on dispersal and distributions, although other events such as Pliocene

inundation may have played a role in current ranges or speciation events. Evolutionary time scale dating would help further explain what may have influenced species or clade dispersal and allow us to measure the rapidity of the radiation of North Island species. However, different areas of New Zealand have markedly variable landscapes and climates which provide distinctive environmental niches that may be conducive to speciation.

4.

Comparative morphology and sexually antagonistic coevolution in the lichen tuft moths (Lepidoptera: *Izatha*)

4. Comparative morphology and sexually antagonistic coevolution in the lichen tuft moths (Lepidoptera: *Izatha*)

4.1 ABSTRACT

Under sexual selection, an individual will try and maximize its overall fitness by ensuring its own reproductive success. Generally interactions between males and females reflect the major differences in investment in gametes such that females invest in high well-provisioned eggs and show strong mate preferences, whereas males produce much larger numbers of sperm and aim for increased number of mates. This fundamental difference between males and females may often lead to conflict between the sexes. Sexual conflict may influence the evolution of genital traits: in females, adaptations that permit favoured males to mate and resist unfavourable males or superfluous mating attempts; in males, traits to manipulate or overcome female resistance to mating. As a result, male persistence may result in tactics or structures that negatively influence female fitness. This in turn may lead to counter-adaptations in females to counteract the negative effects caused by males, leading to an arms race between the sexes or sexual antagonistic coevolution (SAC). Moths of the genus *Izatha* are excellent candidates for examining the potential occurrence of SAC, as they show large variation in male and female genital structures. Males of some species possess spines termed deciduous cornuti attached to the endophallus (i.e. vesica) that are ejected into the female reproductive tract with the spermatophore and some possess large external phallic teeth. In contrast, females show a varying degree of the presence and area of the

signum – a sclerotised structure on the inner wall of the corpus bursae which receives the spermatophore during copulation - and of the reinforcement of the ductus bursae with which the phallus makes contact. Physical injury to this region of the reproductive tract from the males' phallic teeth is often evident during dissection. Comparative phylogenetic analyses were applied to test the prediction that SAC has driven the evolution of these male and female traits. There was a significant correlation between the number, area and length of the deciduous cornuti and the area of the signum in females; between the area and length of the phallic teeth and the thickness of the ductus bursae (female reproductive tract); and between the shape of the phallus and the area of scarring of the reproductive tract. Due to the injurious nature of the male phallic teeth and apparent counter-adaptive thickening of the female reproductive tract, sexual conflict which has escalated to SAC is the most likely scenario behind the evolution of these two genital traits within *Izatha*. Additionally, the evolution of the signum appears to precede the evolution of deciduous cornuti in *Izatha*, suggesting that these male traits are in fact a counter-adaptation driven by the female. The potential evolutionary origins of the relationship between the female signum and male DC are also discussed.

4.2 INTRODUCTION

The evolution of male genitalia has often been referred to as a rapid and divergent process. Many taxa, especially arthropod species, require morphological examination of the male genitalia for species identification and delimitation, with many groups showing a large degree of diversity and elaboration of the male genitalia (Eberhard 1985, 1996).

Early theories proposed species isolation (the lock and key hypothesis) or pleiotropy as an explanation for such divergent genitalia (Shapiro & Porter, 1989; Mayr, 1963).

However, recent comparative research has provided compelling evidence for postcopulatory sexual selection being a key driver in genital evolution (Arnqvist 1998, Arnqvist & Danielsson 1999, Arnqvist et al. 2000, Eberhard 2004b).

As females often invest more energy into the production of offspring, they tend to be much more selective in their choice of mates (Thornhill & Alcock 1983). In polyandrous mating systems, females may derive some benefits from mating multiply, such as indirect benefits due to an increase in genetic variation that may increase offspring fitness (Eberhard & Cordero 1995; Arnqvist & Nilsson 2000). However, there may also be some potential costs such as increased time, energy, risk of predation, parasite infection or physical injury (Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005). Therefore, multiple prolonged matings have the potential to reduce overall female fitness.

It is expected that females will try to resist mating attempts under certain conditions, whereas males are expected to try and force copulation (Parker 1979; Arnqvist & Rowe, 2005). Male competition for access to the fertilization of females' eggs favours the evolution of male genitalic structures that provide a competitive advantage over other males. In addition, conflict between the sexes may lead to the evolution of physiological, morphological and/or behavioural adaptations that lend males a selective advantage in overcoming female resistance. As a consequence, in some cases, the evolution of

complex male genitalia may cause harm to the female during copulation (Crudgington & Siva-Jothy 2000; Siva-Jothy 2006). Females are predicted to counter-adapt to reduce the amount of harm incurred by male advantage, initiating an evolutionary arms race between the sexes or Sexually Antagonistic Coevolution (SAC) (Arnqvist & Rowe 2002a; Arnqvist & Rowe 2005).

One example of SAC is exhibited in plant bugs within the genus *Coridromius*. Males and females within *Coridromius* show a diversity of modifications in both male and female genital structures. Male species of *Coridromius* show a divergent range of modifications in the length, curvature, angle and thickness of the tip, and the amount of twisting that occurs along the axis of the intromittent organ (Tatarnic & Cassis 2010). Similarly, females show variation in the modification of the region in which the male's intromittent organ makes contact. In some species, males inseminate by piercing the intersegmental membrane that connects abdominal segments, whereas in other species, females have developed hardened cuticular indentations that function as a guide for the male's intromittent organ and inhibit the amount of potential damage from these structures (Tatarnic & Cassis 2010). Tatarnic & Cassis (2010) took a macroevolutionary approach to examine the occurrence of SAC in this genus. By developing a phylogenetic reconstruction of these primary structures involved in reproduction within *Coridromius*, they showed that the male intromittent organ exhibits variation between species and strongly correlates with the complexity of the female spermatheca, providing further evidence of the SAC of traumatic insemination.

Another example of SAC is observed in bruchid seed beetles. Different species exhibit varying development of spines on the external surface of the phallus, which have shown to produce scarring of the female reproductive tract during copulation (Crudgington & Siva-Jothy 2000; Ronn et al. 2007). In a recent study, comparative and experimental manipulation of several species was used to investigate evidence of sexual conflict and SAC in the evolution of the genital spines that these beetles possess (Ronn et al. 2007). Results showed that the evolution of more elaborate and harmful male genitalia, i.e. genital spines, were highly correlated with a thickening of the connective tissue of the female reproductive tract, indicative of a female counter-adaptation to minimize the amount of harm inflicted by these structures (Ronn et al. 2007). Neither the degree of harmfulness of the genital spines nor the degree of reinforcement of the female connective tissue of the reproductive tract was significantly associated with reproductive or lifetime fitness in females, suggesting SAC as the most likely driver of the coevolution of these structures in these beetles (Ronn et al. 2007). However, the relative degree of male harmfulness when compared to reinforcement of the female reproductive tract did correlate with female fitness. This suggests that female counteradaptations to male traits have on the most part successfully mitigated harm, but those species in which male spines are more harmful than average and female reinforcement is lower showed a high degree of reduced fitness in females (Ronn et al. 2007)

Copulatory wounding as a result of phallic spines has been recorded in a number of insect taxa (Blankenhorn et al. 2002; Baer & Boomsma 2006; Flowers & Eberhard 2006;

Ronn et al. 2007; Kamimura 2007; Kamimura & Mitsumoto 2012). Lange et al. (2013) defines trauma arising from such structures, in the relative absence of associated secretions as “traumatic penetration”. There are two proposed hypotheses behind the evolution of harmful male structures. The first is that it is a pleiotropic side-effect of structures that originated for another function (Parker 1979). The second is the adaptive harm hypothesis, in which males may benefit from harming their mates (Johnstone & Keller 2000). Traumatic penetration may provide benefits to the male in terms of providing anchorage of the phallus to avoid unwanted detachment from the female during copulation (Baer & Boomsma 2006; Lange et al. 2013) and/or prolonging copulation (Edvardsson & Tregenza 2005; Eady et al. 2007; Hotzy & Arnqvist 2009). In polygynous mating systems, repairing trauma incurred during copulation may delay female time to remating (Lange et al. 2013) or manipulate the female into putting all of her investment into the current reproductive event (Hosken & Price 2009), which would be of advantage to the female’s most recent mating partner. A recent study showed that males of the seed beetle species *Callosobruchus maculatus* with longer genital spines had higher fertilization success; however, this was achieved by perforating the female reproductive tract to allow the passage of seminal proteins and males with longer spines transferred a larger amount, which had an influence on male fertilization success (Hotzy et al. 2012).

Further research has revealed the occurrence of SAC in many other insect taxa such as water striders (Arnqvist & Rowe 2002a; Perry & Rowe 2012), bed bugs (Morrow & Arnqvist 2003), plant bugs (Tatarnic & Cassis 2010) and diving beetles (Bergsten & Miller 2007). However, evidence of sexually antagonistic interactions within the lepidoptera

has been lacking until recent publications have shown that the evolution of thicker spermatophore envelopes appear to be under SAC with the evolution of a female structure – the signum (Ambriz Jimenez et al. 2011; Sanchez et al. 2011; Sanchez & Cordero 2014). The signum is located on the inner wall of the corpus bursae – the sac-like structure that receives the spermatophore during copulation and function in the break-up the spermatophore (Galicia et al. 2008; Lincango et al. 2013).

Deciduous cornuti (sclerotized spines) attached to the inner phallus wall (vesica) within the moth genus *Izatha* are detached during mating and are transferred with the spermatophore to the female corpus bursae in which the signum is located (Hoare 2010). Cordero (2010) proposed hypotheses for the potential function of deciduous cornuti, which could be influenced by natural or sexual selection. These include 1) assisting females in breaking up the spermatophore (natural selection); 2) reducing other male's mating success by hindering the adequate positioning of spermatophores or prematurely breaking apart subsequent spermatophores before their spermatozoa have an opportunity to travel to the female spermatheca (intrasexual selection); 3) gaining favour by stimulating the female post-copulation; or 4) to try and decrease female remating propensity (intersexual selection). However, as the signum is proposed to have evolved in response to the evolution of tougher spermatophores, it is possible that deciduous cornuti have evolved to prevent the contraction of the corpus bursae and hence the utilization of the signum to easily break up the spermatophore. As males developed hard spermatophores to potentially delay the mating process and increase the female refractory period (Ambriz Jimenez et al. 2011; Sanchez et al. 2011), the

evolution of the signa may have counteracted this and favoured the evolution of structures that may gain back male control of sperm utilization.

Therefore, moths of the endemic NZ genus *Izatha* (Family: Xyloryctidae), are excellent candidates for assessing the potential role of sexual conflict in driving the evolution of genital morphology and the occurrence of SAC. There are 40 species of *Izatha*, with a recent taxonomic review revealing 15 new species (Hoare 2010). During the morphological identification of species groups among this genus, it became apparent that species very similar in general morphology have rather distinct male genitalia, with a large amount of variation in shape and elaboration of genitalic structures between species (Hoare 2010). Some species have relatively simplistic male genitalia, others have a varying degree of sclerotised teeth on the external surface of the phallus, and one group possesses a number of deciduous cornuti (detachable spines) (Hoare 2010).

Although female *Izatha* reproductive tracts appear more uniform across the genus than the male structures, there is variation in the reinforcement of the ductus bursae, the part of the reproductive tract with which the phallus makes contact. Among species there is also variation in the presence, size, shape and level of sclerotisation of the signum (plural = signa). Some species of *Izatha* display polyandrous mating systems, as multiple spermatophores and sets of deciduous cornuti are evident within the female reproductive tract of field caught specimens (Figure 1). Dissected males also have either a complete set of cornuti, or all of their cornuti detached, suggesting they eject all of their deciduous cornuti in the first mating. Damage to the reproductive tract of mated

females from teeth on the external surface of the phallus is also evident during dissection (Figure 1).

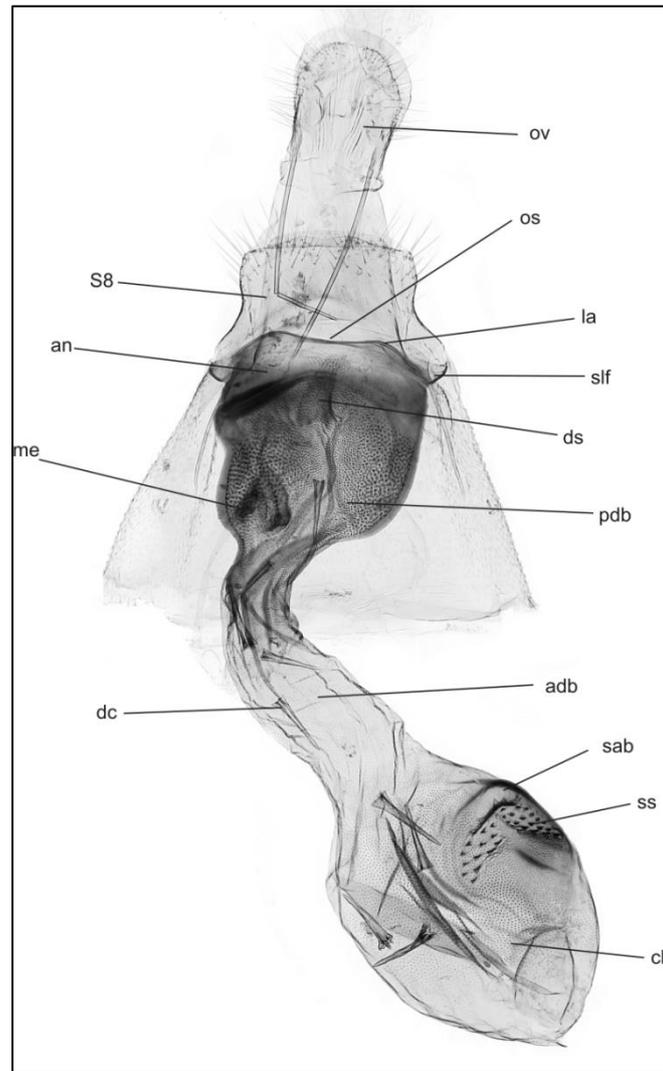


Figure 1. Mated female *Izatha mesoschista* reproductive tract. Note: me = melanised area of ductus bursae, probably resulting from damage by *I. mesoschista* male phallus (there are 3 areas that correspond to the 3 phallic teeth); (adb = anterior portion of ductus bursae; an = antrum; cb = corpus bursae; dc = deciduous cornutus from male vesica; ds, ductus seminalis inception; la = lamella antevaginalis; os = ostium; ov = ovipositor; pdb = posterior (scobinate) portion of ductus bursae; S8 = sternite 8; sab = apical boss of signum; slf = scoop-like lateral flange of S8; ss = spinose transverse strip of signum.) (Automontage photograph by R. J. B. Hoare and B. E. Rhode.) (Hoare 2010).

In the present study, comparative morphological techniques were used to evaluate the level of variation in shape and complexity of key male and female genitalic structures. For male traits, the shape variation and measurements of sclerotised external teeth of the phallus; and measurements and average number of deciduous cornuti were analysed. For female genitalic traits, the thickness of the connective tissue of the ductus bursae, in which the phallus makes contact, area of scarring and area of the signa were determined. Phylogenetically controlled comparative methods (PGLS) were then used to test the following predictions 1) that external teeth on the male phallus should correlate with a female counteradaptation to harm, such as the reinforcement of the female ductus bursae with which they make contact and 2) deciduous cornuti have coevolved with signum under sexually antagonistic coevolution.

4.3 METHODS

4.3.1 Study specimens

Thirty one moth species from the genus *Izatha* were chosen as a starting point for comparison of the female signum and male deciduous cornuti based on existing molecular phylogenetic data from chapter 3. Chosen specimens were representative of various clades and of a diverse degree of elaboration of genitalic structures within the genus. Nine species were excluded due to sampling limitations and the lack of rare species in collections. Therefore, 3 specimens of each of the thirty one species – many female signum measurements taken from previously dissected and microscope prepared specimens in the NZAC collection – were used for the relationship between the signum and deciduous cornuti. Comparisons of other male and female structures were further complicated by the paucity of females in collections; this is explained by

the lesser attraction of females to light (Altermatt et al. 2009) (which also resulted in low numbers of field caught female specimens) and the fact that females are unknown in four species. Therefore, the number of female specimens available limited how many species could be compared. As fresh specimens were required for histology, a minimum of three specimens of thirteen species was used for the relationship between the external teeth of the phallus and the thickness of the female ductus bursae wall. For the relationship between external phallic teeth, 10 specimens of 10 species were included for analysis; and for the interaction between mating status, size of phallic teeth, and the area of scarring, a minimum of 20 specimens of 6 species were included.

4.3.2 Dissection of male and female genitalia

Moths collected were pinned and dried for later dissection. Dissections were performed using techniques for microlepidoptera modified from Robinson (1976) and Hoare (2005). Once the genitalia were extracted from the abdomen, the phallus was separated from the genital capsule and both were prepared onto microscope slides. The female genitalia were prepared as for the males, but with the abdomen separated from the entire reproductive tract at the seventh segment.

4.3.3 Measurement of deciduous cornuti and external phallic teeth

Measurements of the area, length and number of deciduous cornuti and external phallic teeth were carried out in cell[^]D software from images taken using an Olympus SZX16 microscope with camera attached. Measurements of the deciduous cornuti were taken for species that were included in the *Izatha* phylogeny from Chapter 3 (n=10).

Measurements for the external phallic teeth included the thirteen species in which

female specimens were available for comparison with the thickness of the ductus bursae wall. A minimum of three deciduous cornuti and three phallic teeth were measured within an individual (as only 3 DC are common in *I. mesoschista* and 3 phallic teeth in *I. mesoschista*), and then averaged to get an overall value for each specimen. All values were then averaged for each species.

4.3.4 Male phallus shape

Based on the number of available female specimens, 13 species of male *Izatha* were chosen for the analysis of male phallus shape variation for comparison with the thickness of the female ductus bursae wall and 10 species for the relationship between male phallus shape variation and area of female scarring. Digital images of the male phallus of three specimens of each species were taken using a Leica microscope and compiled with NIS- Elements and Helicon Focus Pro imaging software. These images were then imported into tpsDig2 (Rohlf 2010). An equal number of landmarks were then applied to each image to produce quantitative coordinates of phallic shape variation in order to employ geometric morphometric analyses (Adams et al. 2004). Elliptic Fourier Analysis (EFA) (Lestrel 1997) was conducted in PAST (Hammer et al. 2001) to quantitatively describe phallic shape variation by converting the digitized outlines produced in tpsDig2 into a series of harmonics, each of which is comprised of four Fourier coefficients, which collectively define the shape of a structure – in this case, shape of the phallus. Analyses were made invariant to rotation, size and starting position. Size was removed from the resulting dataset in order for the Fourier coefficients to be calculated solely on shape variation.

EFA has proven a useful and accurate tool in analyzing shape variation in previous studies of insect genitalia (Arnqvist 1998; Holwell 2008; Holwell et al. 2010; Rowe & Arnqvist 2012). This resultant dataset was then reduced in PAST using a Principal Components Analysis (PCA) to produce a more compact series of variables that described shape variation among the species of interest.

The factor loadings for the first four PCA scores are presented in the results for both sets of relationships as this explained 85% of the cumulative percentage variance for the 13 species included for the relationship between phallic shape variation and thickness of the ductus bursae wall; and 84% for the 10 species included for the relationship between phallic shape and the amount of scarring.

4.3.5 *Measurement of signa*

The signum is a sclerotised structure located on the inner wall of the corpus bursae – the part of the female lepidopteran reproductive tract that receives the spermatophore during copulation (Drummond 1984; Holloway et al. 2001; Cordero 2005). At least three female specimens of the 22 species of *Izatha* in which a signum is present were prepared onto slides using the same techniques as for the male genitalia. From these preparations, photographs of the female reproductive tract were taken and measurements of the area of the female signum were taken for each specimen in cell[^]D imaging software. The mean area of the signa for each species was then used as a quantitative indicator of the elaboration of this structure. Nine species in which the

signum is absent were included in the comparative analyses, bringing the total number of species to 31.

4.3.6 *Measurement of the thickness of the female ductus bursae wall*

The ductus bursa is part of the reproductive tract in female Lepidoptera with which the male phallus often makes primary contact during copulation (Fanger & Naumann 1998; Sihvonen 2007; Mikkola 2008) and scarring that corresponds to the size, shape and number of teeth of the male phallus has been observed during dissection in female *Izatha*. To measure the thickness of the ductus bursae wall, fresh female abdomens were fixed in 10% formaldehyde and prepared for histological sectioning and staining. A minimum of three specimens of 13 species of *Izatha* – *I. attactella*, *I. austera*, *I. churtoni*, *I. convulsella*, *I. epiphanes*, *I. huttonii*, *I. katadiktya*, *I. mesoschista*, *I. metadelta*, *I. peroneanella*, *I. phaeoptila* and *I. picarella* were sectioned and measurements of the width of the connective tissue of the ductus bursae of each specimen was taken at several different points and averaged to get a mean measurement of the thickness in millimetres. The mean thickness of each specimen within a species was then averaged to get an overall measurement of the thickness of the ductus bursae for each species.

4.3.7 *Measurement of scarring of the ductus bursae*

Scarring is recognized as melanised areas on the ductus bursae wall (Figure 1). The amount of scarring was quantified by the area of overall scarring present in the ductus bursae of dissected field-caught specimens (n=10) for 10 species of *Izatha* – *I. acmonias*, *I. austera*, *I. churtoni*, *I. convulsella*, *I. epiphanes*, *I. huttonii*, *I. mesoschista*, *I. metadelta*,

I. peroneanella and *I. picarella*. Although the virginity of field-caught specimens cannot be guaranteed, instances in which the corpus bursae was obviously deflated (after copulation the corpus bursae inflates in the presence of the spermatophore, and despite the rupture and movement of the spermatophore for fertilisation, does not deflate again) and in which no scarring, spermatophores or deciduous cornuti were evident were counted as virgin specimens. A number of lab-reared virgin female specimens were also included. No virgin female specimens showed any evidence of scarring of the ductus bursae.

4.3.8 *Izatha* phylogeny

In order to display the phylogenetic relationships between *Izatha* genitalia, male and female genital traits were mapped onto a molecular phylogeny of *Izatha* based on maximum likelihood (ML) trees constructed in PAUP*4.0b10 (Swofford 2002) from chapter 3. For the comparison of the area of the signum and the presence of deciduous cornuti, 31 species of *Izatha* were included. For the association between size of phallic teeth and thickness of the ductus bursae wall, a ML tree was reconstructed using methods described in chapter 3 for the 13 species listed in section 4.3.4; and for scarring, size of phallic teeth and thickness of the ductus bursae wall, the 10 species listed in section 4.3.5.

4.3.9 Comparative analyses

All phylogenetic comparative analyses were executed in Rstudio (R 3.0.2) using Caper (Orme et al. 2012), APE (Paradis et al. 2004) and GEIGER (Harmon et al. 2008) packages.

Firstly, Pagel's λ was calculated (Pagel 1997; 1999) for all continuous variables for 31 species: Average area of signum (where present), number of DC, average area of DC and average length of DC. For the 13 species where sufficient material was available Pagel's λ for the following continuous variables were calculated: Average thickness of the ductus bursae wall, average area of phallic teeth, average length of phallic teeth, and the first four PCA scores; and for the 10 species where sufficient females were available Pagel's λ for the following continuous variables were calculated: Average area of female scarring, average area of phallic teeth, average length of phallic teeth, average thickness of the ductus bursae wall, and the first four PCA scores. Essentially, Pagel's λ provides a value between 0 and 1. A value of 0 signifies a lack of association between a trait or set of traits and phylogeny (i.e. closely related species tend not to share similar trait values). A value of 1 suggests that species' traits covary precisely as would be predicted by a Brownian motion model of evolution (i.e. closely-related species would share highly similar trait values) (Symonds & Elgar 2013). The significance of the measured phylogenetic signal for each trait was then assessed by testing whether the calculated λ value for each trait differed significantly from a λ value of 0. Phylogenetic generalized least squares (PGLS) regression was then applied to test the relationship between 1) the area of the female signa and the number, area, and length of male deciduous cornuti and phallus PCA scores; 2) the thickness of the female ductus bursae wall and the area and length of external teeth on the male phallus, and phallus PCA scores; 3) the area of scarring in the female ductus bursae and the area and length of external teeth on the male phallus, thickness of the female ductus bursae wall, and phallus PCA scores.

4.3.10 Scarring as a function of species, mating status and size of teeth on the phallus

The male and female reproductive tracts of a minimum of 20 specimens of six species of *Izatha* – three with smaller teeth, three with larger teeth – were dissected and the average area of scarring of the ductus bursae for each species calculated. The size of the teeth for each of the six species was defined by the average area scores calculated for the comparative analyses. The influence of species, mating status and their interaction was then assessed for the average area of scarring (mm^2) in the ductus bursae using two-way factorial ANOVA and Tukey post hoc tests conducted in Rstudio (R 3.0.2).

4.4 RESULTS

4.4.1 Phylogenetic signal for all traits

There was a significant amount of phylogenetic signal in all but three traits examined (Table 1). Therefore overall, both male and female genitalic structures (with the exception of the number of phallic teeth for the 13 species included for analysis against thickness of the ductus bursae wall and area of phallic teeth for the 10 species included for analysis against area of female scarring) and shape variation of the phallus (excluding PC 3 for the 13 species included for analysis with the thickness of the ductus bursae wall and PC 4 for the 10 species included for analysis with the area of scarring) showed high levels of similarity between closely related species. Although the λ value for the number of teeth for 13 species and area of teeth for the 10 species was not significant, there is still a moderate level of phylogenetic signal of these two traits.

Table 1. Measure of phylogenetic signal in a number of genitalic traits of *Izatha* using the ML phylogenies developed for analysis (figs. 2a & b; 4a & b; 6a & b). Significant Pagel's λ values are denoted by an asterix.

Trait	λ value
Ave area of signa	0.98*
Ave number of deciduous cornuti	1.0*
Ave area of deciduous cornuti	1.0*
Ave length of deciduous cornuti	1.0*
Ave thickness of the ductus bursae	0.92*
Ave area of phallic teeth	0.53*
Ave length of phallic teeth	0.86*
Ave number of phallic teeth	0.49
PCA 1 scores for 13 species	1.0*
PCA 2 scores for 13 species	0.99*
PCA 3 scores for 13 species	0
PCA 4 scores for 13 species	0.90*
Ave area of scarring	0.69*
Ave number of phallic teeth	0.58*
Ave area of phallic teeth	0.47
Ave length of phallic teeth	0.84*
Ave thickness of ductus bursae	0.91*
PCA 1 scores for 10 species	1.0*
PCA 2 scores for 10 species	1.0*
PCA 3 scores for 10 species	1.0*
PCA 4 scores for 10 species	0

4.4.2 Relationship of signum area to deciduous cornuti traits

Deciduous cornuti have arisen on three separate occasions within *Izatha* (Figure 2a) and correspond to the presence of a larger signum in conspecific females (Figure 2b).

However, for species in which deciduous cornuti are not present, females sometimes still have a signum present, but it is much smaller (Figure 2a & 2b).

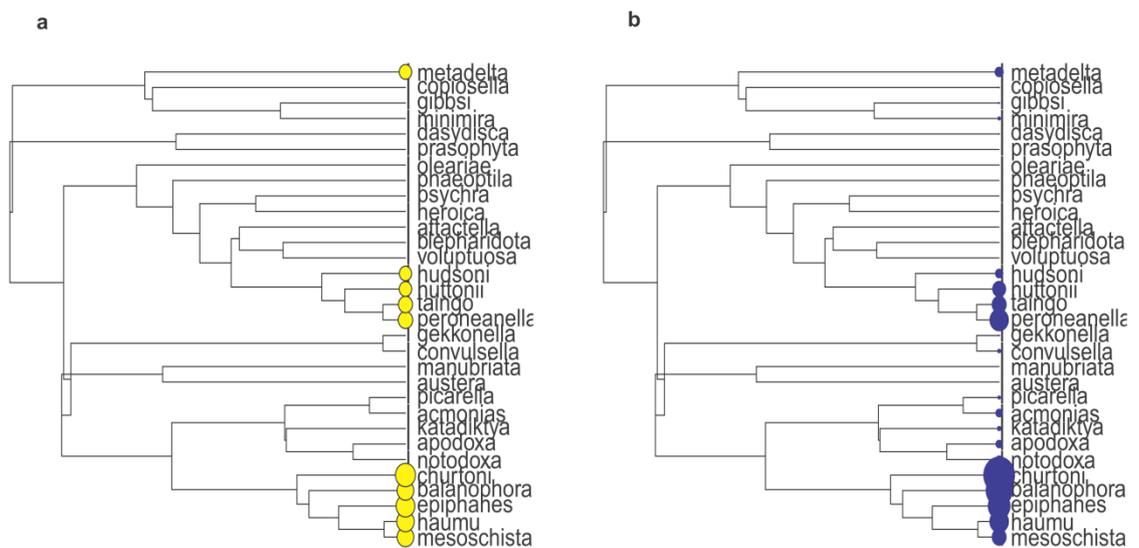


Fig 2. Phylogeny developed for PGLS analyses of 31 species of *Izatha*, with size of the coloured circles proportional to a) Average area of deciduous cornuti b) Average area of the female signum mapped onto the ML tree.

In PGLS analyses of the 31 species of *Izatha* examined, the average area of the female signum showed a significant correlation with the average number, area and length of deciduous cornuti in conspecific males (Figure 3; Table 2). This indicates a relationship between the presence and area of the female signa and the presence and development of DC in males. In species whose females have a larger signum, males tend to have a larger number of deciduous cornuti, and a larger area and length of deciduous cornuti (Figure 3; Table 2).

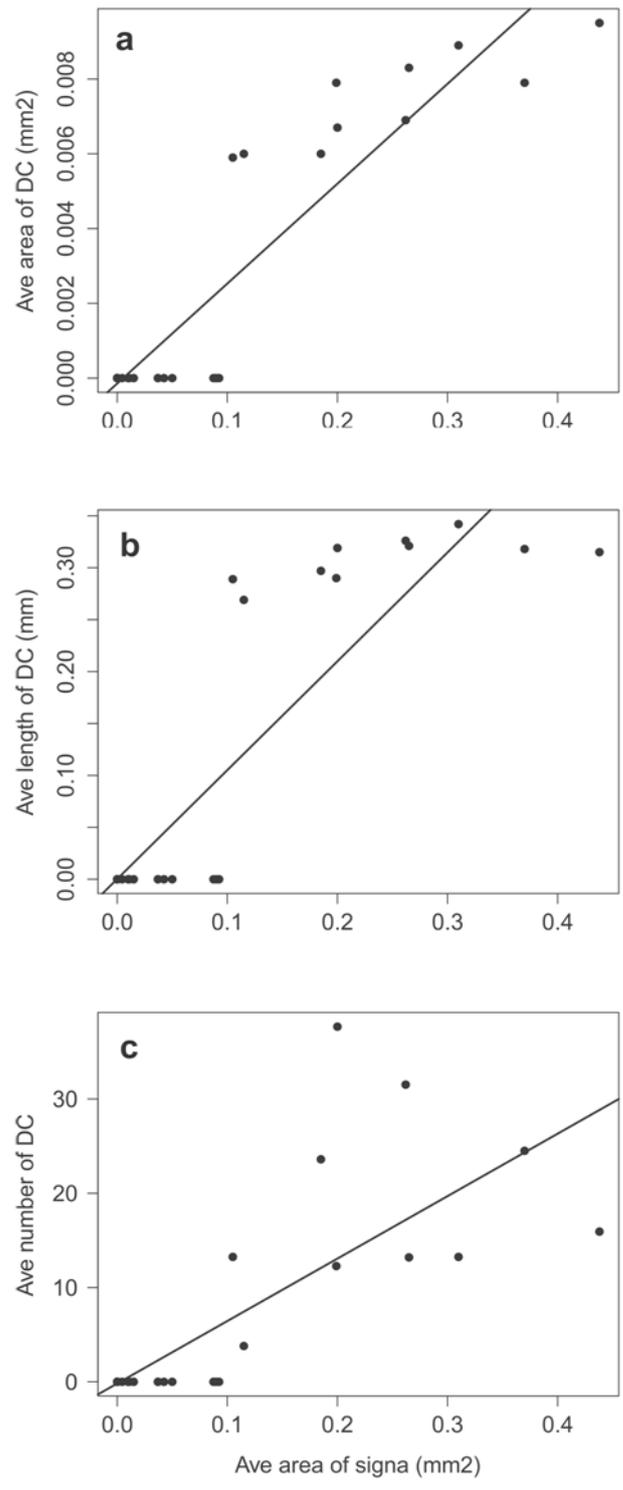


Figure 3. Linear regressions of average area of the female signum and a) Average area of deciduous cornuti (mm^2), b) Average length of deciduous cornuti (mm) and c) Average number of deciduous cornuti for 31 species of *Izatha*.

Table 2. PGLS regression of the relationship between average area of the signum and the average number, area and length of deciduous cornuti in 31 species of *Izatha*. Significant relationships are indicated in bold.

PGLS Regressions	Ave area of signa (mm ²)		
	β (\pm s.e.)	R^2	<i>p</i> -value
Intercept	-0.557 (\pm 3.241)	0.326	0.860
No. of DC	53.158 (\pm 13.505)		<0.001
Intercept	0.001 (\pm 0.001)	0.586	0.442
Ave area of DC (mm ²)	0.019 (\pm 0.003)		3.15E⁻⁰⁷
Intercept	0.026 (\pm 0.031)	0.519	0.406
Ave length of DC (mm)	0.752 (\pm 0.130)		2.94E⁻⁰⁶

4.4.3 Relationship of measurements of teeth on phallus and shape of the phallus to the thickness of the female reproductive tract

Figure 4a shows the relative length of teeth on the phallus and figure 4b shows the thickness of the female ductus bursae. The comparison suggests that the length of the teeth and the thickness of the ductus bursae wall have coevolved (Figure 4a & 4b).

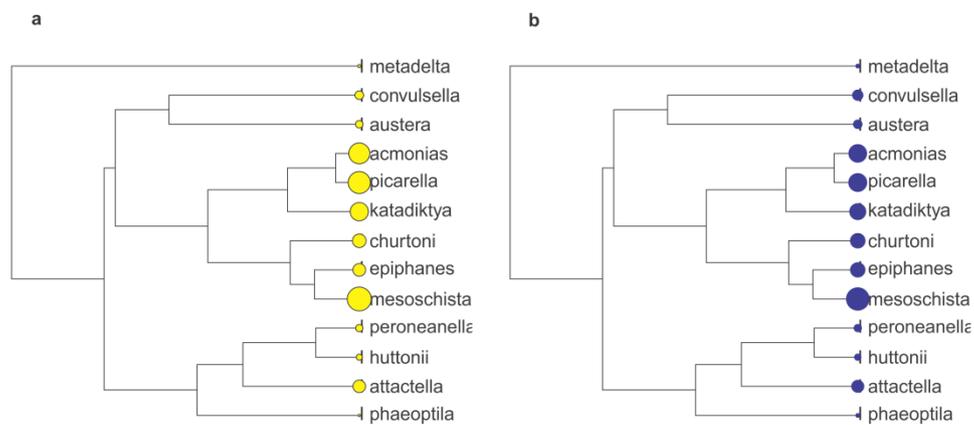


Figure 4. Phylogeny developed for PGLS analyses of 13 species of *Izatha*, with size of the coloured circles proportional to a) Average length of teeth on the phallus and b) Average thickness of the female ductus bursae mapped onto the ML tree.

Applying PGLS analyses of these 13 *Izatha* species, the thickness of the ductus bursae wall showed a significant relationship with the area and length of teeth on the male phallus (Figure 5 a & b; Table 3). In species with larger teeth, females have an increased thickness of the connective tissue of the ductus bursae, the region with which these teeth make contact during copulation. Shape variation represented by PCA scores on the other hand, did not show a significant correlation with thickness of the ductus bursae; indicating that overall shape of the phallus alone does not show a distinct association with the thickness of the female reproductive tract (Figure 5c; Table 3). Consequently only one graph of the first PCA scores against thickness of the ductus bursae wall is presented.

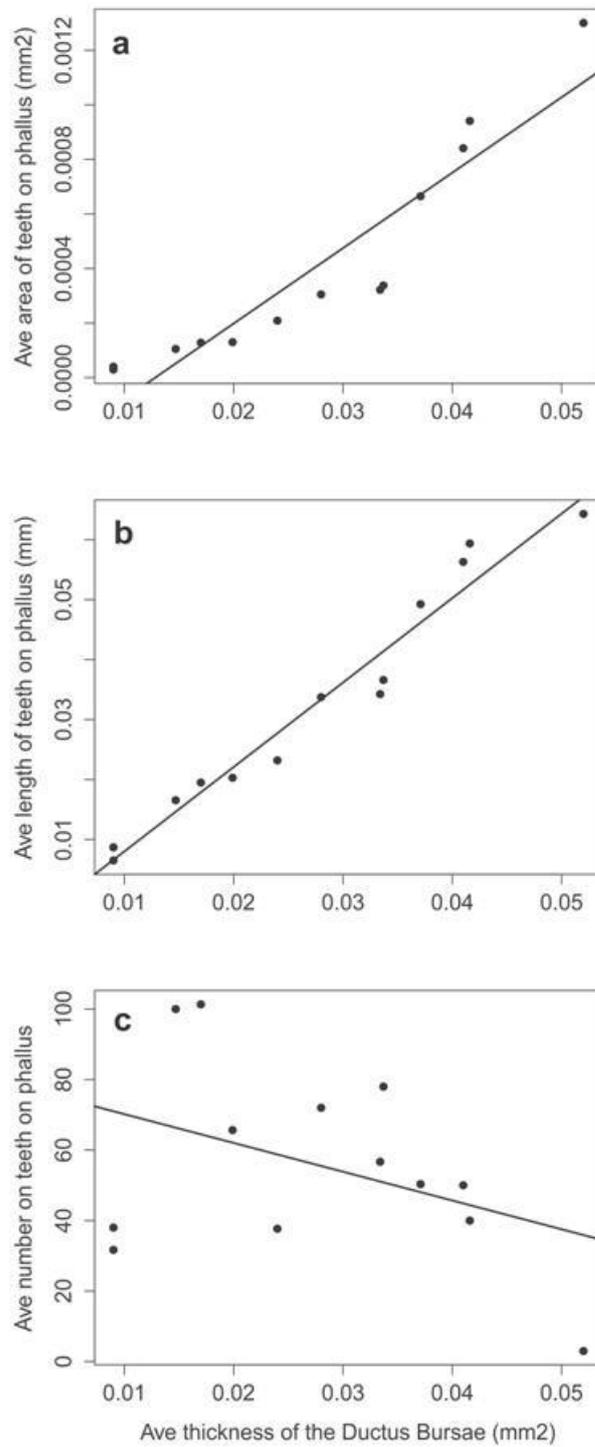


Figure 5. Linear regressions of average thickness of the female ductus bursae wall and a) Average area of teeth on the phallus (mm²), b) Average length of teeth on the phallus (mm) and c) Ave number of teeth for 13 species of *Izatha*.

Table 3. PGLS regression of the relationship between average thickness of the female ductus bursae (mm) and the average area and length of teeth on the phallus and PCA 1, 2, 3, 4 scores for male phallus shape variation in 13 species of *Izatha*. Significant relationships are indicated in bold.

PGLS Regressions	Ave thickness of the ductus bursae (mm)		
	β (\pm s.e.)	R^2	<i>p</i> -value
Intercept	-0.001 (\pm 0.001)		0.015
Ave area of phallic teeth (mm²)	0.029 (\pm 0.004)	0.799	3.847E⁻⁰⁵
Intercept	-0.007 (\pm 0.003)		0.038
Ave length of phallic teeth (mm)	1.483 (\pm 0.103)	0.950	1.716E⁻⁰⁸
Intercept	65.384 (\pm 19.255)		0.006
Ave number of phallic teeth	-701.386 (\pm 710.222)	0.081	0.345
Intercept	-0.021 (\pm 0.142)		0.885
PC 1	1.915 (\pm 4.530)	0.016	0.681
Intercept	-0.027 (\pm 0.073)		0.717
PC 2	0.426 (\pm 2.359)	0.003	0.860
Intercept	0.040 (\pm 0.032)		0.237
PC 3	-1.466 (\pm 1.096)	0.140	0.208
Intercept	0.011 (\pm 0.048)		0.820
PC 4	-0.535 (\pm 1.651)	0.009	0.752

4.4.4 Relationship of scarring to measurements of teeth on phallus, shape of the phallus and thickness of the female ductus bursae

Figure 6a shows the relative length of teeth on the phallus and figure 6b the average area of scarring of the female ductus bursae. Although there appears to be a phylogenetic trend that longer teeth are associated with an increased level of scarring, the area of scarring does not appear to be consistent relative to the length of external phallic teeth.

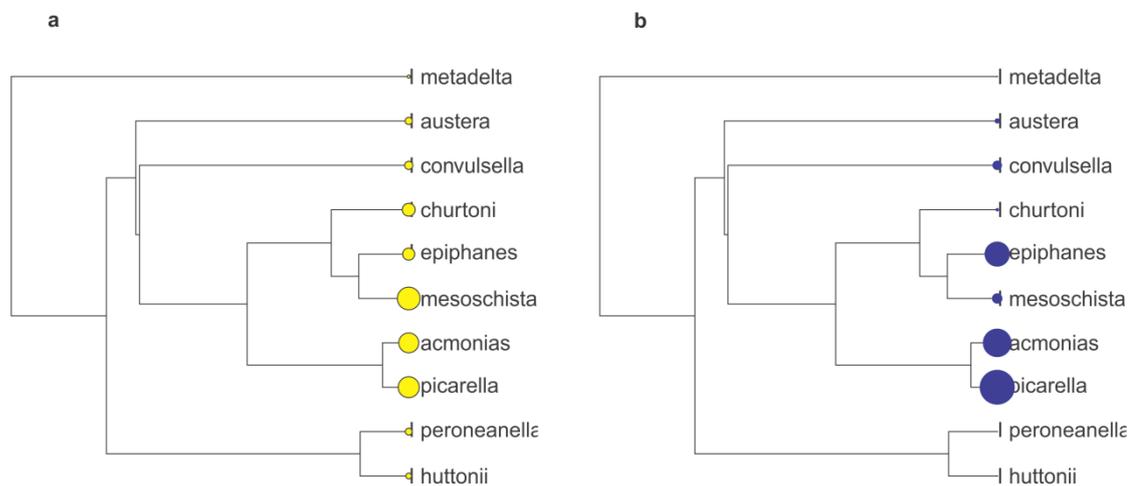


Figure 6. Phylogeny developed for PGLS analyses of 10 species of *Izatha*, with size of the coloured circles proportional to a) Average length of teeth on the phallus and b) Average area of scarring of the female ductus bursae mapped onto the ML tree.

PGLS analyses of these 10 species found a significant correlation between the area of scarring and PCA1 scores (Figure 7; Table 4). Even though other traits did not show a significant relationship with the area of scarring, there is still a trend evident that a

larger area and length of the phallic teeth and thicker ductus bursae is associated with a larger area of scarring (Figure 7; Table 4).

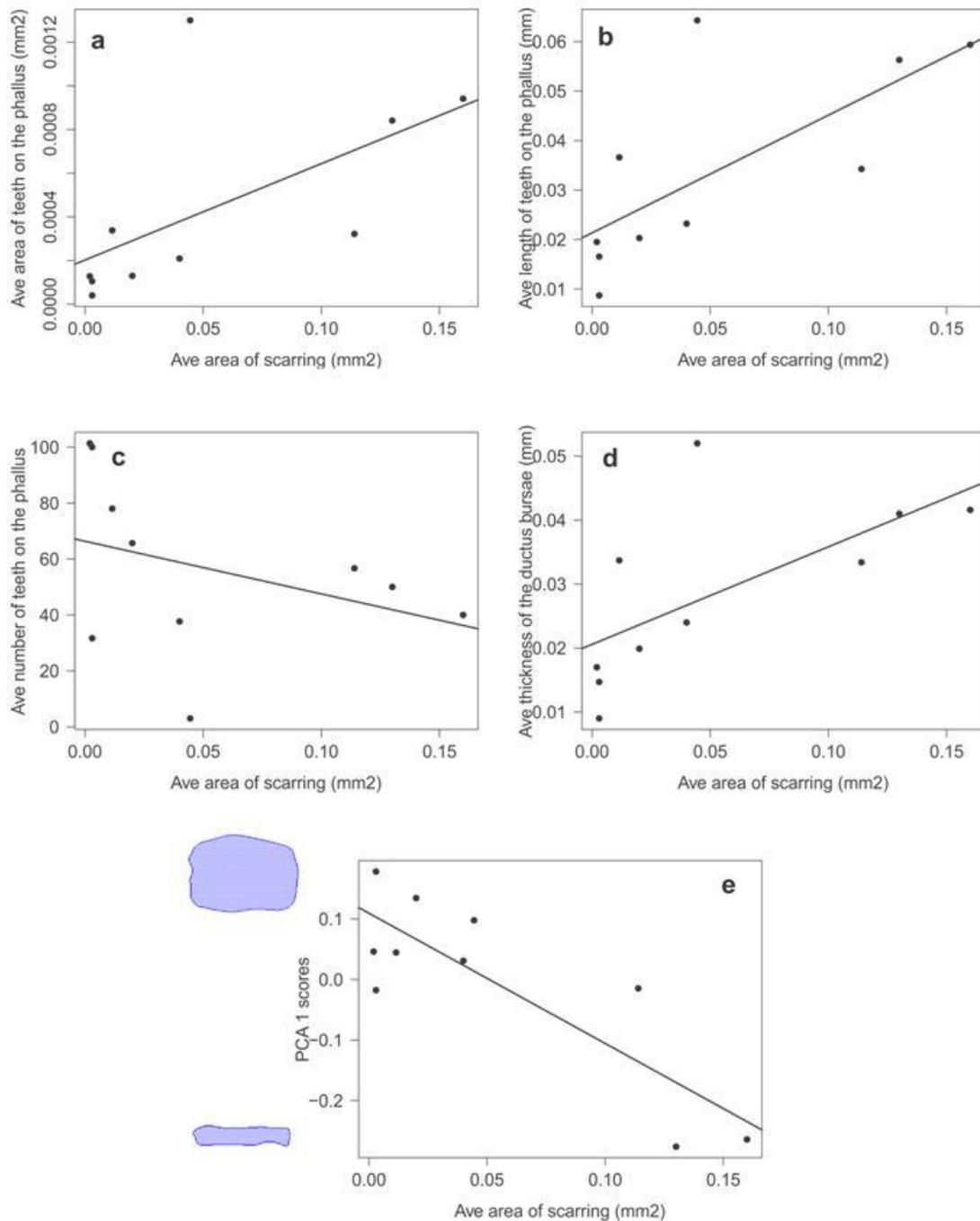


Figure 7. Linear regressions of average area of scarring of the female ductus bursae and a) Average area of teeth on the phallus (mm²), b) Average length of teeth on the phallus (mm) and c) Ave number of teeth on the phallus d) Average thickness of the ductus bursae for 10 species of *Izatha* and e) PC 1 with EFA phallic shape that corresponds with minimum and maximum scores displayed.

Table 4. PGLS regression of the relationship between average area of scarring (mm²) and the average area and length of teeth on the phallus; thickness of the female ductus bursae (mm); and PCA 1, 2, 3, 4 scores for male phallus shape variation in 10 species of *Izatha*. Significant results are indicated in bold.

PGLS Regressions	Ave area of scarring (mm ²)		
	β (\pm s.e.)	<i>R</i> ²	<i>p</i> -value
Intercept	0.014 (\pm 0.027)		0.624
Ave area of phallic teeth (mm ²)	66.531 (\pm 43.016)		0.160
		0.230	
Intercept	0.017 (\pm 0.01)		0.134
Ave length of phallic teeth (mm)	0.104 (\pm 0.097)		0.315
		0.126	
Intercept	57.708 (\pm 16.942)		0.009
Ave number of phallic teeth	-136.680 (\pm 190.079)		0.493
		0.061	
Intercept	-0.015 (\pm 0.042)		0.727
Thickness of DB wall (mm)	2.170 (\pm 1.617)		0.217
		0.184	
Intercept	0.044 (\pm 0.081)		0.604
PC 1	-1.405 (\pm 0.572)		0.0396
		0.430	
Intercept	-0.034 (\pm 0.073)		0.658
PC 2	0.333 (\pm 0.512)		0.534
		0.050	
Intercept	0.004 (\pm 0.04)		0.922
PC 3	0.334 (0.281)		0.268
		0.150	
Intercept	0.012 (\pm 0.026)		0.657
PC 4	-0.211 (\pm 0.363)		0.577
		0.041	

4.4.5 Scarring as a function of the size of teeth of the phallus

Two-way factorial ANOVA analyses revealed a significant interaction between the average area of scarring and size of phallic teeth among species ($p = <0.001$), the average area of scarring and mating status within species ($p = <0.001$) and average area of scarring and the interaction between size of phallic teeth and mating status ($p = 0.013$) (Table 5). Unmated specimens did not show any signs of scarring. Mated specimens of species with small phallic teeth did not always show evidence of scarring. In species with larger phallic teeth, all mated specimens exhibited scarring and a larger area of scarring to the female ductus bursae than species with small phallic teeth (Figure 8; Table 5). A Tukey post hoc test revealed where the most significant differences occurred. Significant differences were between species with small phallic teeth that were unmated and those with large phallic teeth that were mated (0.006); species with small phallic teeth that were mated and those with large phallic teeth that were mated (<0.001); and species with large teeth that were mated or unmated (<0.001).

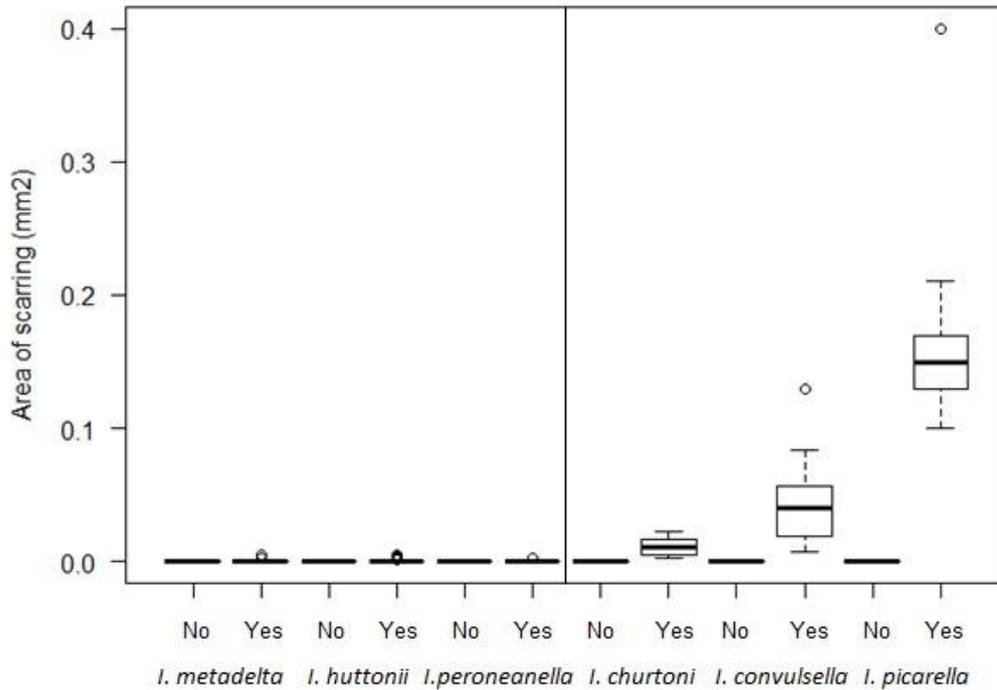


Figure 8. Boxplot of the range of scarring with standard error for each of the 6 species of *Izatha* examined, showing the division between 3 species with small phallic teeth – *I. metadelta*, *I. huttonii*, and *I. peroneanella* on the left of the graph and 3 species with large phallic teeth – *I. churtoni*, *I. convulsella*, and *I. picarella* on the right.

Table 5. Two way ANOVA values of the dependent variable of the average area of scarring against factors of size of teeth (Large, small) and mating status (Mated, not mated). Significant results are indicated in bold.

	df	F value	P
Size of teeth	1	17.304	<0.001
Mating Status	1	14.377	<0.001
Size of teeth*MatingStatus	1	6.31	0.013

4.5 DISCUSSION

4.5.1 *Coevolution of signa and deciduous cornuti*

There was a strong correlation between the number, area and length of deciduous cornuti and the elaboration of the female signum within *Izatha* consistent with the coevolution of these male and female genitalic structures. This conclusion supports the hypothesis that deciduous cornuti have coevolved with the female signum and the fact that some females of some species that lack DC still have a signum, although small in comparison to those in which the DC are present, suggests that the female structure (the signum) may be driving the evolution of a male trait (the DC). In *Coridromius* species of plant bugs, modification/complexity of the male intromittent organ has not been met with a corresponding development of an external spermatheca in females that is a common counter-adaptation in bed bugs (Morrow & Arnqvist 2003). This would suggest that the exaggeration in the male paramere of these species precedes female adaptation and may therefore be the driving factor of coevolution of this mating strategy (Tatarnic & Cassis 2010). In *Izatha*, it appears that the evolution of the presence of a signum may precede the development of deciduous cornuti. Therefore, it may be this structure and hence females that may be instigating the coevolution of these structures. This is perhaps one of the first examples using phylogenetic comparative methods that demonstrate the likely driver of male genital evolution by a female genital trait in Lepidoptera as no other documented cases have been found.

In polyandrous mating systems, lepidopteran females have been shown to become temporarily sexually unreceptive post-copulation and the refractory period before

regaining sexual receptivity is related to the amount of spermatophore that remains in the corpus bursae (Sugawara 1979; Drummond 1984; Kaitala & Wiklund 1995; Wedell 2005). Under these circumstances, sperm competition would be predicted to promote the evolution of spermatophore envelopes with a thicker wall that take longer for the female to digest, increasing the post-copulation refractory period (Drummond 1984; Cordero 2005). Recent research has found a strong correlation between thickness of the male spermatophore and the presence of signa in several species of Lepidoptera, with further support for the influence of sexually antagonistic coevolution between these two traits (Sanchez et al. 2011; Ambriz Jimenez et al. 2011; Sanchez & Cordero 2014).

In this scenario of an evolutionary arms race between the sexes, the female counter-adaptation of the development of the signum may have put the female ahead of the race; counteracting the effect of the development of tougher spermatophore envelopes by reducing the female latency period to remating. Theories on the directional selection of SAC driven by male persistence and female resistance propose that coevolutionary counteradaptations may be selected for until there is a stabilizing effect (neutralization), or until the extreme elaboration of a trait selects for male mutations that females preferentially select in mates, thus perpetuating this alternative trait within the population (i.e. de-escalation or reversal) (Gavrilets et al. 2001; Rowe et al. 2005).

However, before this point may be reached in evolutionary time sexual conflict may put males under pressure to try and regain control of the fertilization process by adapting further physiological, morphological or behavioural traits that provided them again with the advantage (Holland & Rice 1998; Kazancioglu & Alonzo 2012). Deciduous cornuti

may have evolved to prevent the contraction of the corpus bursae and delay the breakup of the spermatophore. Males would then benefit from again increasing the female refractory period to remating and increasing the chances of the utilization of their spermatophore.

Alternatively, DC could be used to stimulate the female post-copulation as a form of cryptic female choice. Research into the presence of stimulatory receptors in the wall of the female corpus bursae would help provide support for this theory. However, in *Izatha*, the wall of the corpus bursae – excluding the signum – appears relatively thin and during dissection pressure on the corpus bursae in which deciduous cornuti are present results in these sharply pointed structures tearing through its wall. In light of this, it is unlikely that these structures would be used as a female stimulatory device, as there is potential for damage to the delicate lining of the corpus bursae. In turn, it is also improbable that DC would operate to assist the signum in the breaking of the spermatophore, as similarly, contraction of the corpus bursae could also result in physical injury to the female. Furthermore, it is unclear why males would evolve a harder spermatophore envelope only to assist the female in breaking it up. This provides further support for sexual conflict and SAC being the potential driver behind the evolution of DC in *Izatha*.

4.5.2 Scarring and evolution of external phallic teeth and thickness of the female reproductive tract

Within *Izatha* phallic teeth appear to be structures that have evolved in the context of sexual conflict. The evolution of larger external phallic teeth has resulted in an increased level of damage to the female ductus bursae and there is strong evidence of the coevolution of the elaboration of these structures and the increased thickness of the female reproductive tract. The infliction of harm infers a cost to females and infers sexual conflict between the sexes. One way to judge whether the harm incurred is a result of sexually antagonistic coevolution, there should firstly be evidence of a female counteradaptation. The results of this study confirm this. However, the evolutionary origins of the genital structures that cause harm may or may not have arisen as a form of direct sexual antagonism from the male, but as another trait that provides an advantage to the male during mating. For example, in the fruit fly *Drosophila ananassae*, genital spines have been found to be imperative for securing adequate copulatory positioning. Experimental manipulation of spine length had an adverse effect: males with shortened spines were less successful at achieving copulation under male-male competition (Grieshop & Polak, 2012). In this case there is evidence that copulatory wounding from male genital spines is a pleiotropic side effect to the primary functionality of anchoring the genitalia during copulation. In contrast, the anchoring function of phallic spines in the seed beetle *Callosobrunchus maculatus* was not supported, despite previous hypotheses of this functionality and therefore the evolutionary origin of these structures in these beetles may be driven by the male under the adaptive harm hypothesis (Morrow et al. 2003; Ronn & Hotzy, 2012) as a premeditated form of sexual antagonism. Puncturing of the female reproductive tract is also advantageous to males of this species

of seed beetle as it allows the transfer of seminal proteins that have a beneficial effect on male fertilization success (Hotzy et al. 2012).

Males benefit from securing multiple and successful matings, but it is hard to understand why males may evolve harmful traits such as genital spines that inflict injury on the female when there is the potential that this could also be harmful to the male (Parker 1979). However, if female responses to the harm imposed directly benefits the male, then harmful structures may be selected for in spite of the potential costs – the adaptive harm hypothesis (Johnstone & Keller 2000; Morrow et al. 2003; Hotzy & Arnqvist 2009). Beneficial female responses include delaying remating, and increased investment in current reproduction or in utilization of the current partner's sperm (Johnstone & Keller 2000; Lessells 2005; Hotzy & Arnqvist 2009).

Nevertheless, females may prefer to mate with males that are more effective at overcoming resistance, resulting in cryptic female choice of males with a superior ability to maintain phallic positioning/and or stimulation of their reproductive tract via phallic spines. This may also subsequently result in increased scarring and highlights the complexity of disentangling traits driven by sexual conflict and cryptic female choice; perhaps neither is mutually exclusive (Cordero & Eberhard 2003, 2005; Hosken & Stockley 2004; Arnqvist & Rowe 2005). These two types of selection may be acting simultaneously on a female trait: in some instances reinforcing and in other circumstances opposing each other (Cordero & Eberhard 2005). Consequently, models explaining the evolution of female preference for a male trait may often appear like

female resistance (see Rice & Holland 1999). Regardless of whether a male trait is favoured and hence chosen by the female, or whether the male trait is successful at manipulating the female to overcome resistance, the proliferation of the exaggerated trait may be evident in subsequent generations. The type of selective process that drives the evolution of male traits may help to distinguish between a sexual conflict and cryptic female choice origin (Rice & Holland 1999), with a more definitive way of determining the selective driver of a specific trait resolved by establishing the quantitative balance between female gains and losses that include both direct and indirect costs and benefits (Cordero & Eberhard 2003). However, the perpetuation of the type of selection driving the coevolution of, for example, the exaggeration of phallic spines and counteradaptation of female reproductive fitness may be dependent on the distribution of variation of the male trait in a population (eg. small to larger spines) and the female response to this at any given time (eg. stronger resistance pressure to avoid direct costs and indirect gains from increased resistance of daughters vs. indirect gains from sons better at manipulation) – resistance vs. cooperation (Cordero & Eberhard 2005; Eberhard 2010a).

Successful mating experiments, investigating both female mate preference, and direct and indirect cost to benefit ratios that a female may be subjected to, may help further establish what kind of selection is driving the coevolution of these genital traits.

Furthermore, a male trait may be deemed sexually antagonistic if it has a direct negative impact on female reproductive success (Cordero & Eberhard 2005). Therefore, the intrinsic consequence of harm to certain female *Izatha* species in the form of physical

injury from interspecific males with exaggerated phallic spines (female scarring) provides convincing evidence for initial female resistance and SAC to such a male trait.

Ronn et al. (2007) made two key predictions about establishing the occurrence of SAC in genital spines. One is that the elaboration of genital spines should coevolve with a resistance trait in the female. Another is that if the coevolution between the sexes is stabilized, the adaptation of a trait in one sex should be balanced by a counteradaptation in the other. In other words, the evolution of increasingly harmful male genitalia may not necessarily result in increased injury to the female if the female has adapted by building resistance to the development of more harmful structures. With these assumptions in mind, *Izatha* have shown a strong correlation between the enlargement of the phallic teeth and the thickness of the female ductus bursae. However, larger phallic teeth and a thicker reproductive tract did not significantly correlate with the level of scarring when taking phylogeny into account. A reason for this could be that different species are at various stages in the coevolutionary arms race. Nevertheless, this supports Ronn et al's. (2007) second prediction about the occurrence of SAC, as larger size or number of phallic teeth did not always result in a greater amount of scarring to the female reproductive tract, showing that a male persistence trait may have been balanced by the evolution of a female resistance trait.

Out of all the genital traits, only PCA 1 scores of phallic shape variation showed a significant relationship with the level of scarring. This is interesting, as the most distinct feature of shape variation that corresponds to the first PC score is the breadth of the

phallus. A broader phallus is associated with a smaller area of scarring and a narrower phallus with a larger area of scarring. Species of *Izatha* that have a broader phallus tend to have smaller teeth and vice versa. Males with a broader phallus may have been able to better anchor themselves and maintain a tighter connection with the female during copulation, whereas males with a narrower phallus may have compensated for this by developing larger teeth rather than increasing the breadth of the phallus. This provides support for a pleiotropic side-effect origin of phallic teeth size. However, a potential “two birds, one stone” scenario may have occurred, where larger teeth not only allow a tighter grip during copulation, but have the potential to cause damage to the female and provide males with reproductive benefits such as an increase in the female remating refractory period.

Other factors that should be taken into account are the mating systems and mating propensity of different species. Unfortunately the mating system of many species of *Izatha* is still unknown; however, as mentioned in the introduction, some species do display evidence of polyandry. The larger area of scarring could additionally be attributed to higher levels of remating in specific species. Even though the results for other genital traits were not significant, there are still trends between a larger area and length of phallic teeth and thicker ductus bursae wall and an increased area of scarring. The small number of species sampled may have concealed the apparent relationships between these traits and the scarring. Therefore, there may be a complex interaction of a number of traits or behaviours that could be contributing to the amount of damage and subsequent scarring a female is subjected to. Alternatively, another explanation

may be due to the discrepancy between the relative size of phallic teeth in one species in particular, *I. convulsella*, and the elevated amount of scarring observed in this species. *Izatha convulsella* is one of only a few species (n=4) that also possess a compound cornutus that supposedly makes contact with the ductus bursae in the female in the same region as the phallic teeth. The greater area of scarring exhibited in *I. convulsella* may be due to the combined effects of the phallic teeth and the compound cornutus.

Nevertheless, further analyses showed that mating does cause damage as there is a lot of variation in scarring between mated and unmated females for three species in which males have larger phallic teeth, whereas species in which males had smaller phallic teeth the same did not occur (Figure 8). Therefore, there is evidence that it is the phallic teeth, and potentially in the case of *I. convulsella*, the compound cornutus and not another factor causing the scarring.

4.5.3 Summary

Even though sexually antagonistic coevolution produces adaptations that may be costly to both sexes, it is quite difficult to identify empirically, as we expect an adaptation in one sex to be balanced by a counter-adaptation in the other, the costs and conflict occurring may be easily concealed (Chapman & Partridge 1996). Furthermore, discriminating which mechanism of sexual selection is driving the evolution or coevolution of specific structures is difficult as all infer fertilization success, correlations in genital form and to a certain extent the premise of a “good fit” between male and female genital structures (Hosken & Stockley 2004). Nevertheless, the coevolutionary

relationships between the male and female genital structures within *Izatha* examined in the current study reveal a high level of correlation and the potential functional relationship between these sets of structures suggestive of SAC.

Additional analyses, especially in regards to variation in reproductive success among and within species are needed to provide further support for SAC being a key driver in *Izatha* genital evolution. The current study provides compelling evidence of a counter-adaptive female trait –reinforcement of the reproductive tract – which has co-evolved with a harmful male trait – exaggeration of male phallic teeth. The evolution of deciduous cornuti appears to be a male counter-adaptation to the evolution of the signum, which in turn is proposed to have evolved in response to thicker spermatophore envelopes. This provides evidence of a male adaptation driven by sexual antagonism from the female. A comparison of the thickness of the spermatophore envelope and observations of the mating systems that occur in different species of *Izatha* would provide further support to this theory. Clearly, there are some very interesting coevolutionary relationships and novel genitalic structures within this genus that warrant further investigation.

5.

Seasonality and determinants of mating success in *Izatha* *peronenealla*, *I. churtoni* and *I.* *epiphanes*

5. Seasonality and determinants of mating success in *Izatha peroneanella*, *I. churtoni* and *I. epiphanes*

5.1 ABSTRACT

Biased sex ratios, protandry, and sexual size dimorphism can have an impact on mating systems. These three phenomena are common within Lepidoptera and life history traits such as these may provide essential information in understanding the processes influencing mating systems within specific taxa. In this study, adult seasonal variation patterns and mating success of *Izatha peroneanella*, *I. churtoni* and *I. epiphanes* were determined from three locations over a single seasonal flight period. *Izatha* are an endemic genus of moths that show an interesting diversity of genitalic traits, but little is known about the general biology of species within this genus. All three species began appearing in October, trailing off in February for *I. churtoni* and *I. epiphanes*, and April in *I. peroneanella*. Numbers were greatest during December and January for all three species, but also February and March for *I. peroneanella*. Protandry and seasonal consistency in male-biased sex ratios were evident in *I. peroneanella*, *I. churtoni* and *I. epiphanes*, and tests revealed female-biased sexual size dimorphism in *I. peroneanella* and *I. churtoni*. There was a statistically significant decrease in male and female *I. peroneanella*, and male *I. churtoni* and *I. epiphanes* body size over the flight period, with larger individuals emerging first and smaller ones appearing progressively through the summer. Logistic regression showed a strong negative correlation between mating success and body size for *I. peroneanella* and *I. churtoni* males; which may explain why

there was a strong positive correlation between male *I. peroneanella* mating success over time, as smaller males were more likely to have mated, but also emerged later over the breeding season. Data on the ecology and mating success of *Izatha* are essential to gain a better understanding of the mating systems within this genus and insight into the factors that may have driven sexual selection within these three species, as mate choice, male or scramble competition may be selecting for smaller body size in males.

5.2 INTRODUCTION

In insects, ontogenetic processes that play a role in determining longevity and reproductive success often represent a complex interaction between physiological constraints and adaptive plasticity to environmental dynamics (Berger et al. 2012). Therefore, the effect of life history traits on individual fitness is often a complicated one and involves many combinations of interactions between traits that influence individual survival and reproductive success (Stearns 1992). In organisms such as insects that are generally short-lived, time constraints are often imposed due to limitations in the timeframe of the adult life cycle and subsequent mating period (Johansson & Rowe 1999; Yang et al. 2005). Therefore, in seasonal environments individuals are expected to optimize developmental and eclosion (emergence) times in accordance with dynamic conditions such as temperature, availability of food resources, predation risk and mating opportunities (Atterholt & Solensky 2010; Morbey 2013). Furthermore, the conceptualization and spread of research on adaptive growth in the 1990's (Morbey 2013) proposed that growth should not solely be attributed to environmental conditions, but that strategic "decisions" may influence the growth rate of individuals,

and trade-offs may occur to maximize either direct or reproductive fitness (Abrams et al. 1996; Ludwig & Rowe 1990; Morbey 2013; Rowe & Ludwig 1991).

Common life history traits in Lepidoptera that may exert an influence on developmental pathways include selection for protandry, sexual size dimorphism and male biased sex ratios (Fairbairn 1997; Underwood & Shapiro 1999; Adamski 2004; Allen et al. 2011; Boughton & Pemberton 2012; Gerard 2013). Protandry is defined as the earlier eclosion of males during the mating season (Neve & Singer 2007; Muralimohan & Srinivasa 2008). Several hypotheses have been proposed to explain the general evolution of protandry, which include indirect selection on: 1) the territorial sex, often the male, arriving earlier than competitors 2) females avoiding adverse environmental conditions earlier in the season 3) traits correlated with the earlier arrival of males; or direct selection: 4) males arriving earlier to optimize the opportunity for mating 5) females delaying arrival to minimize the chance of waiting for males and risking mating failure 6) a female mate assessment strategy by the removal of less fit males earlier in the season and 7) as a strategy to avoid inbreeding (Morbey & Ydenberg 2001). However, different mechanisms may be more applicable to smaller seasonal animals such as insects (Singer 1982), with three hypotheses suggested as the most relevant explanation of earlier male eclosion in Lepidoptera – which include the ‘constraint’ (hypothesis 3), ‘mate opportunity’ (hypothesis 4), and ‘wait cost’ (hypothesis 5) hypotheses (Wiklund 1995; in Morbey 2013). The constraint hypothesis proposes that protandry is a side-effect of selection for larger body size in females (Wiklund & Solbreck 1982). The mate opportunity hypothesis suggests that protandry is viewed as the result of intrasexual competition between males for mating opportunities, promoting earlier eclosion to try to maximize individual mating success and gain access to virgin females when they

emerge (Wiklund & Fagerstrom 1977; Rutowski 1997; Morbey & Ydenberg 2001; Muralimohan & Srinivasa 2010). The wait cost hypothesis proposes that females would benefit from waiting until sexually receptive males have arrived, rather than risk the possibility of pre-reproductive mortality (Fagerstrom & Wiklund 1982). The mate opportunity hypothesis has received the most attention in the literature and is often suggested as the primary driver of protandry in Lepidoptera (Muralimohan & Srinivasa 2010). However, conflicting evidence for the specific driver behind the evolution of protandry (Muralimohan & Srinivasa 2010) and the potentially multiple selective origins for its occurrence has led to a lack of consensus between hypotheses (Morbey et al. 2012).

Many external factors may contribute to biased sex ratios within mating populations. These include predation (Tabadkani et al. 2013), seasonal changes (Charnov et al. 1981), disease (Jiggins et al. 1998; Hassan et al. 2013), protandry (Wiklund et al. 1992), and nutritional stress (Charnov et al. 1981; Quezada-Garcia et al. 2014). Biased sex ratios have the potential to alter mating dynamics and thus reproductive strategies, in addition to influencing the operational sex ratio (OSR) of a population, which is defined as the ratio of sexually receptive males and females present at a given point in time (Emlen & Oring 1977; Kvarnemo & Ahnesjö 1996). Therefore, the sex ratio within a population may affect individual reproductive success, as temporal variation in sex ratios may result in alternative or fluctuating mating strategies and stronger competition for the limiting sex (Clutton-Brock & Vincent 1991). For example, a strong male-biased sex ratio in the almond moth (*Ephestia cautella*) has been shown to significantly reduce female

longevity (Soffan et al. 2012); a potential consequence of this being more intense competition for females and higher levels of polyandry.

Sexual size dimorphism – relative difference in body size between the sexes - is another frequent life history characteristic in Lepidoptera and a recent analysis has shown it to be largely female-biased in this order (73% of 48 species) (Stillwell et al. 2010). Variation in sexual size dimorphism, including disparity among populations of a species could be due to the different parameters of body size plasticity between the sexes as a reaction to ecological or other environmental variables (Stillwell et al. 2010). However, many physiological and/or developmental mechanisms may be influencing variation in sex dependent body size plasticity and sexual size dimorphism, and how they differ between the sexes is largely unknown (Stillwell et al. 2010). Rensch's rule describes the common pattern in which there is a larger variation in male body size among closely related species (Fairburn 1997). Therefore, male body size may evolve faster than body size in females. Selection on sexual size dimorphism in Lepidoptera, as for all forms of sexual dimorphism has often been debated, with explanations invoking sexual selection often receiving the most attention (Fairburn 1997; Allen et al. 2011).

Body size may influence reproductive success in numerous ways, such as influencing mate search ability and mate choice, both prior to and post-copulation and may also be under different selection between the sexes (Morbey 2013). Natural selection may influence large body size in females due to fecundity benefits (Berger et al. 2008; Fairburn 1997; Xu & Wang 2009), whereas in males, smaller size may be under sexual

selection as less energy requirements can afford more time spent mate searching or investing energy in sperm competition (Thornhill & Alcock 1983; Blanckenhorn 2005). Therefore, body size and sexual size dimorphism may be under sexual selection if one sex preferentially chooses to mate with a specific phenotype to gain direct or indirect benefits, determining the mating or fertilization success of an individual. From a physiological point of view, larger body size is often associated with a longer development period (Berven & Gill 1983), so body condition may also influence adult emergence time and subsequent body size (Morbey 2013). Larval body condition towards the end of development may determine the decision whether to emerge early under continuous development, but with a smaller body size; or continue feeding, but run the risk of pre-reproductive mortality in order to emerge with a larger body size (Poykko & Hyvarinen 2012).

However, a larger body size, emergence time or age may not always infer greater mating success. As females are often the limited and choosier sex, there is often greater selection pressure on males to entice female mate choice or counteract female resistance (Arnqvist & Rowe 2005), and many previous studies have found conflicting evidence of the effect of body size and age or experience on female mate choice and male mating success.

In some species of Lepidoptera larger males have higher mating success, as observed in mating trials of the moth *Rothschildia lebeau*, where larger males were more likely to be observed mating and were more likely to have mated (Agosta 2010). Similarly, in the

butterfly *Colias eurytheme* there was a significant tendency for mating males to be larger (Kemp 2006). However, in the pyraloid moths, *Parapediasia teterrella* and *Agriphila plumbifimbriella* females did not show a specific preference for male body size in both species, and accepted males of *Agriphila plumbifimbriella* tended to be lighter in weight (Marshall 1988). In contrast other studies have found no evidence of mating success being related to body size in Lepidoptera (Suzuki & Matsumoto 1992; Van Dongen et al. 1999).

As well the potential effect of body size on mating success, individual age or mating experience may influence female mate choice and male mating success. The effect of a number of factors, including age, body size, nutritional status and wing damage were tested for their influence on mating success on male *Bicyclus anynana* (Lepidoptera: Nymphalidae). The results showed no evidence for female preference based on body size or overall condition, but older males had much higher mating success compared to their younger counterparts, irrespective of prior mating status (virgin vs. non-virgin) (Fischer et al. 2008). However, studies on other species have also found that higher mating success was attributed to mating experience rather than adult age (Suzuki & Matsumoto 1992; Schlaepfer & McNeil 2000; Iyengar 2009).

These studies highlight the complexity of determining the effect of body size and time – in respect to eclosion timing, maturity or mating experience – on male mating success in lepidoptera as this may be specific to the species under examination; with many other factors such as mating system dynamics and size and age correlating with other traits

such as male courtship sex pheromones (Ivengar et al. 2001; Nieberding et al. 2008) having an influence on female mate choice and male mating success. Therefore, knowing the physiological constraints and mating system dynamics helps to understand what is driving mate choice and mating success within a species.

Moth species within the genus *Izatha* (Lepidoptera: Xyloryctidae) are primarily dead wood feeders, or are associated with lichens and fungi (Hoare 2010). There are forty species of *Izatha* and they display divergent male genitalia with evidence that sexually antagonistic coevolution has driven the adaptation of male and female genital structures (Chapter 4). In order to put evolutionary patterns into context it is important to understand other life history traits, such as mating system dynamics. Males of some species possess a number of spines attached to the inner wall of the phallus termed deciduous cornuti, in which they eject the entire set into the female reproductive tract during their first mating (Hoare 2010). During dissections, female *Izatha* frequently have more than one spermatophore or sets of deciduous cornuti within the corpus bursae, suggesting polyandrous mating systems in at least several species within the genus. This also provides a means of assessing the number of mates a female has had, and the absence of cornuti in males demonstrates that they have at least mated once.

Additionally, many species of male *Izatha* have external teeth on the phallus, which makes contact with part of the female reproductive tract known as the ductus bursae. The thickness of the ductus bursae wall is positively correlated with the size of the phallic teeth and scarring in this region of the female reproductive tract is often evident during dissection, providing further evidence of female mating history. Polyandry

increases male competition for females, which often leads to sexual selection for behavioural, physiological or genital traits that provide individual males an advantage over rivals (Arnqvist 1998). In order to test the following hypotheses the present study aims to examine seasonal variation in mating patterns in three common species of *Izatha*

- 1) Is protandry evident in *Izatha*, and if so what is potentially driving this?
- 2) Is there a bias in sex ratios and if so what consequences this may have in a mating population?
- 3) Does body size fluctuate during the flight season and between sexes (sexual size dimorphism), and if so what might be driving these patterns?
- 4) Is mating success related to body size or temporal variation in emergence time?

5.3 METHODS

5.3.1 Field sites and specimen collection

Adults of the *Izatha* species *I. peroneanella*, *I. churtoni*, and *I. epiphanes* were collected from three field sites in Auckland, New Zealand throughout the 2012 and 2013 summer season. The flight period for *I. peroneanella* has previously been recorded from September to early April; and between October to February for both *I. churtoni* and *I. epiphanes* (Hoare 2010). The first site was located in Albany, North of Auckland city (36°41.70S, 174°41.80E). The second site was in Waitakere, West Auckland (36°56.09S, 174°31.19E); and the third site at Kakamatua inlet, south-west of Auckland (37°0.04S, 174°35.59E). Each site was sampled fortnightly and all specimens collected at a 125watt

MV light trap. The light was set up at sunset, and run for a minimum of 3 hours, maximum of 4 hours. All adult specimens of the common *Izatha* species that came to light were collected.

5.3.2 *Sex ratios and rearing records*

The sex ratio for each species was calculated as the number of males divided by the total number of adult specimens caught. Additionally, the sex ratio for individuals collected during each month was calculated. Larvae were also collected from dead wood in the field and reared on three different artificial diets, as well as in the wood they were found. From these records, the sex ratio of *I. churtoni* – the species that yielded the highest number of individuals – was determined for comparison with field-caught specimens.

5.3.3 *Body size and mating status*

All specimens collected were pinned and dried for dissection. The sex of each specimen was recorded and three body traits – forewing length, thorax length, and foreleg length were measured as an indicator of body size. All three body measurements were highly correlated (presented in Table 1). Therefore, forewing length was used in subsequent analyses as a measure of body size, as it has been documented as a good indicator of lepidopteran adult body size (Miller, 1977; Noriyuki et al. 2010; Stillwell et al. 2010) and is a common character used in research that incorporates scaling relationships in other Lepidoptera (Sullivan & Miller, 2007). Specimen abdomens were then detached to dissect genitalia for quantifying mating status. Dissections were performed following techniques modified from Hoare (2005). Once the genitalia were extracted from the abdomen, the phallus was separated from the genital capsule and both prepared on to

microscope slides. The female genitalia were prepared as for the males, but with the abdomen separated from the entire reproductive tract at the seventh segment. Each specimen slide was examined and the mating status, number of deciduous cornuti in the male vesica, presence of scarring in the female ductus bursae, number of spermatophores and deciduous cornuti in the female corpus bursae of all specimens were recorded. Male mating status was defined by the presence or absence of deciduous cornuti in males, as males eject all of these in the first mating. Female mating status was defined by the presence of a spermatophore or spermatophore remnants and the presence of deciduous cornuti. Dissections of lab-reared female *Izatha* also reveal that in virgin females, the corpus bursae is obviously deflated, therefore females with a lack of any of the latter signs and a deflated corpus bursae were categorized as unmated.

Table 1: Pearson's correlation coefficient matrix comparing three body characters of both male and female *Izatha peroneanella*, *I. churtoni* and *I. epiphanes*. Data is the combined specimens collected from three different Auckland sites during the 2012/2013 season.

<i>Izatha</i> (N = 253)	Thorax length (TL)	Foreleg length (FL)
Forewing length (WL)	0.96	0.94
Thorax length (TL)		0.94

5.3.4 Statistical analysis

Sex ratios, Mann-Whitney and t-tests of sexual size dimorphism, and linear regressions for seasonal variation in body size and mating success were conducted in SigmaPlot11.0. For sexual size dimorphism analyses, *I. peroneanella* passed the Shapiro-Wilks normality test, but failed the equal variance test, so was re-run with a Mann-Whitney rank sum test. Logistic regression of the relationship between mating success and body size were run in Rstudio (R 3.0.2). Table 2 displays the total number of specimens of the three species of *Izatha* collected over the flight season, as well as the total number of males and females and overall sex ratio.

Table 2. Total abundance of male and females of three common *Izatha* species – *I. peroneanella*, *I. churtoni*, and *I. epiphanes*. All specimens collected over the 2012/2013 field season at three different field sites in the Auckland region. Sex ratio calculated as the number of males divided by the total number of adult specimens caught.

	Total no. specimens	No. males	No. females	Sex ratio
<i>I. peroneanella</i>	157	125	32	0.80
<i>I. churtoni</i>	59	45	14	0.76
<i>I. epiphanes</i>	38	33	5	0.87

5.4 RESULTS

5.4.1 Sex ratio of lab reared Izatha churtoni

The total number of specimens was 22, including 14 males and 8 females. A binomial exact test was applied to assess the deviation of the reared specimens from a 1:1 sex ratio and results are presented in table 3, showing a significant male-biased deviation from a 1:1 ratio.

Table 3: Binomial exact test results for probability that the sex ratio in reared specimens follow a 1:1 male:female ratio.

Binomial exact test	P: 8 or fewer out of 22 individuals	
	One-tailed test	Two-tailed test
Exact binomial calculation	0.143	0.286
Approximation via normal distribution	0.142	0.285

5.4.2 Flight period and sex ratios

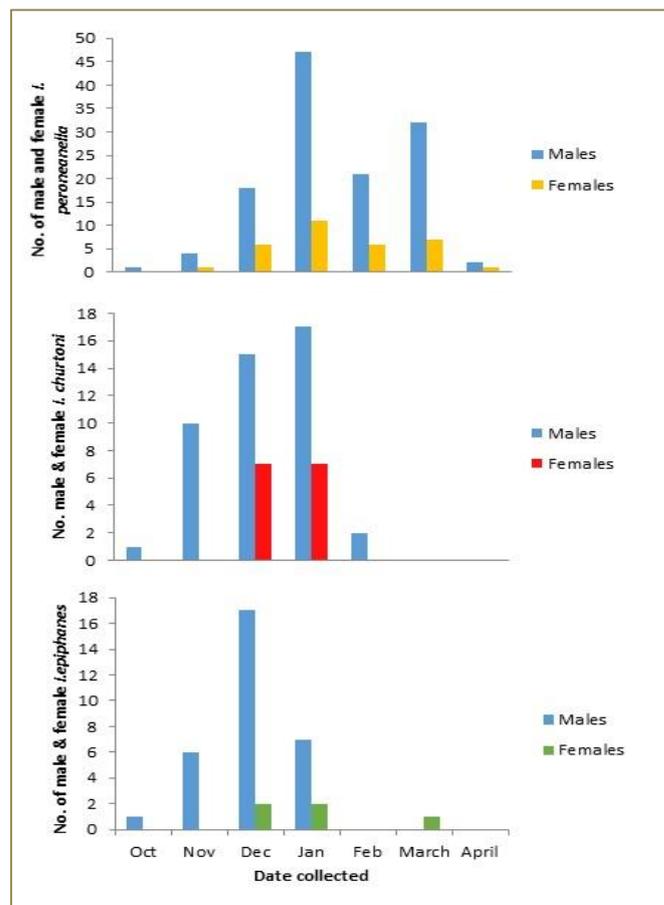


Figure 1. Number of male and female adult *Izatha peroneanella* (a), *Izatha churtoni* (b), *Izatha epiphanes* (c) collected at three sites in the Auckland region over the 2012/2013 field season.

Flight periods for all three species of *Izatha* matched those described in Hoare (2010) (Figure 1). The sex ratio appears to be male biased during the entire flight period (Figure 1; Table 4). The presence of males also seems to precede female emergence indicating protandry in these three species of *Izatha* (Figure 1; Table 4). Peak flight times for *I. peroneanella* occur between December and March, and between December and January for *I. churtoni* and *I. epiphanes* (Figure 1).

Table 4. Seasonal variation in sex ratio of *I. peroneanella*, *I. churtoni* and *I. epiphanes* over three sites in the Auckland region during the 2012/2013 field season.

<i>I. peroneanella</i>	Oct	Nov	Dec	Jan	Feb	March	April
Male	1	0.8	0.75	0.81	0.78	0.82	0.67
Female	0	0.2	0.25	0.19	0.22	0.18	0.33
Total	1	5	24	58	27	39	3
<i>I. churtoni</i>	Oct	Nov	Dec	Jan	Feb	March	April
Male	1	1	0.68	0.71	1	NA	NA
Female	0	0	0.32	0.29	0	NA	NA
Total	1	10	22	24	2	NA	NA
<i>I. epiphanes</i>	Oct	Nov	Dec	Jan	Feb	March	April
Male	1	1	0.89	0.78	0	0	NA
Female	0	0	0.11	0.22	0	1	NA
Total	1	6	19	9	0	1	NA

5.4.3 Difference in body size between the sexes

There was a significant difference between the body size of males and females in *I. peroneanella* ($p = <0.001$) and in *I. churtoni* ($p = 0.002$) with females possessing a larger average body size in both species. The results for *I. epiphanes* were not significant ($p = 0.156$), however the results for all three species combined were ($p = <0.001$) (Table 5).

Table 5. Results of the Mann-Whitney and t-tests for difference in body size between male and female *I. peroneanella*; male and female *I. churtoni*; male and female *I. epiphanes*; and males and females of all three *Izatha* species.

<i>I. peroneanella</i>	N	Median	25%	75%
Male Body Size (mm)	125	8.703	7.957	9.383
Female Body Size (mm)	32	9.687	8.634	10.802
<i>I. churtoni</i>	N	Mean	Std Dev	SEM
Male Body Size (mm)	45	10.044	1.231	0.184
Female Body Size (mm)	14	11.241	1.123	0.300
<i>I. epiphanes</i>	N	Mean	Std Dev	SEM
Male Body Size (mm)	33	9.235	0.918	0.160
Female Body Size (mm)	4	9.874	0.921	0.412
All 3 <i>Izatha</i> species	N	Mean	Std Dev	SEM
Male Body Size (mm)	203	9.088	1.251	0.0878
Female Body Size (mm)	51	10.224	1.519	0.213

5.4.4 Seasonal variation in body size

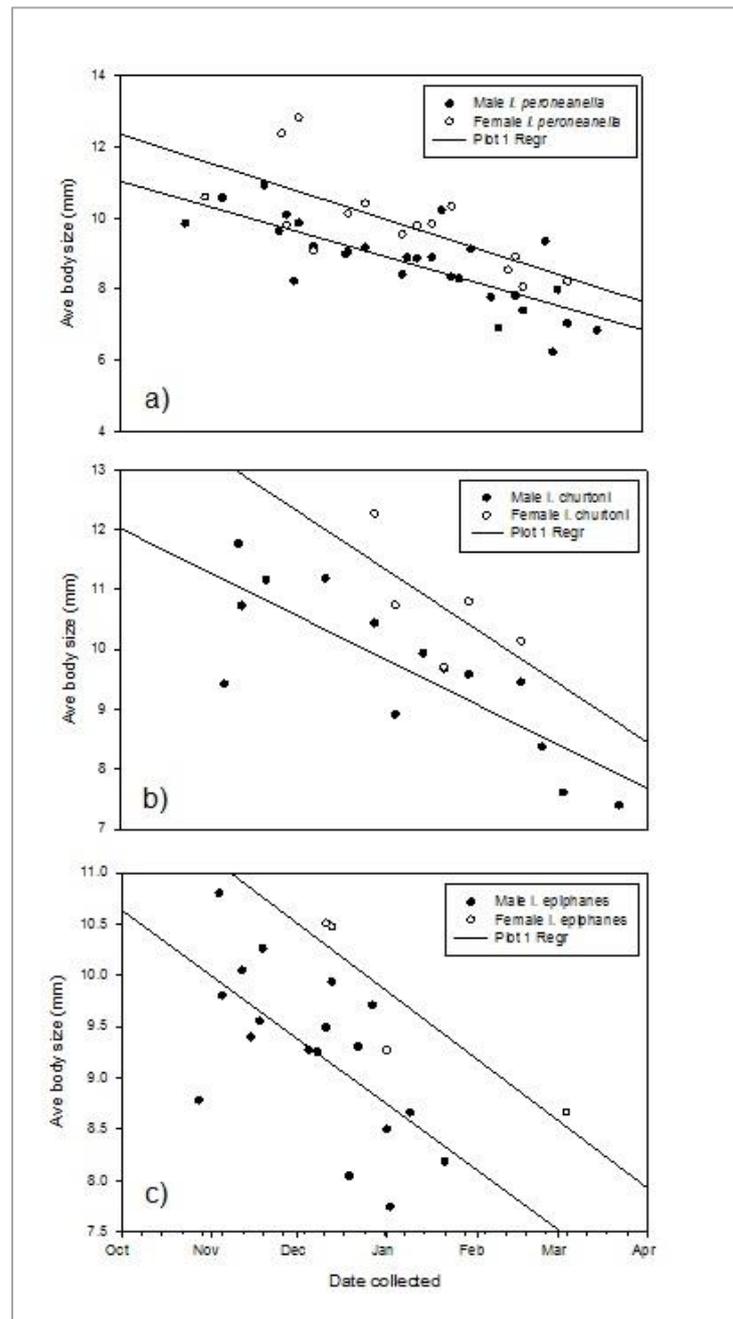


Figure 2. Seasonal variation in average body size of both male and female *I. peroneanella* (a), *I. churtoni* (b), and *I. ephanes* collected over the 2012/2013 flight season.

In *I. peroneanella*, body size showed a significant decrease over the flight period in both males and females (Males: $R^2 = 0.598$, p value = >0.001 ; females: $R^2 = 0.502$; p value = 0.003) (Figure 2a). There was also a significant decrease in body size over the season for *I. churtoni* males ($R^2 = 0.674$, p value = >0.001), but not for *I. churtoni* females ($R^2 = 0.444$, p value = 0.220) (Figure 2b). Similarly, *I. epiphanes* males showed a significant decrease in body size over time ($R^2 = 0.409$, p value = 0.004), whereas females of that species showed no significant change in body size over the flight period ($R^2 = 0.821$, p value = 0.094) (Figure 2c).

5.4.5 Mating success and body size

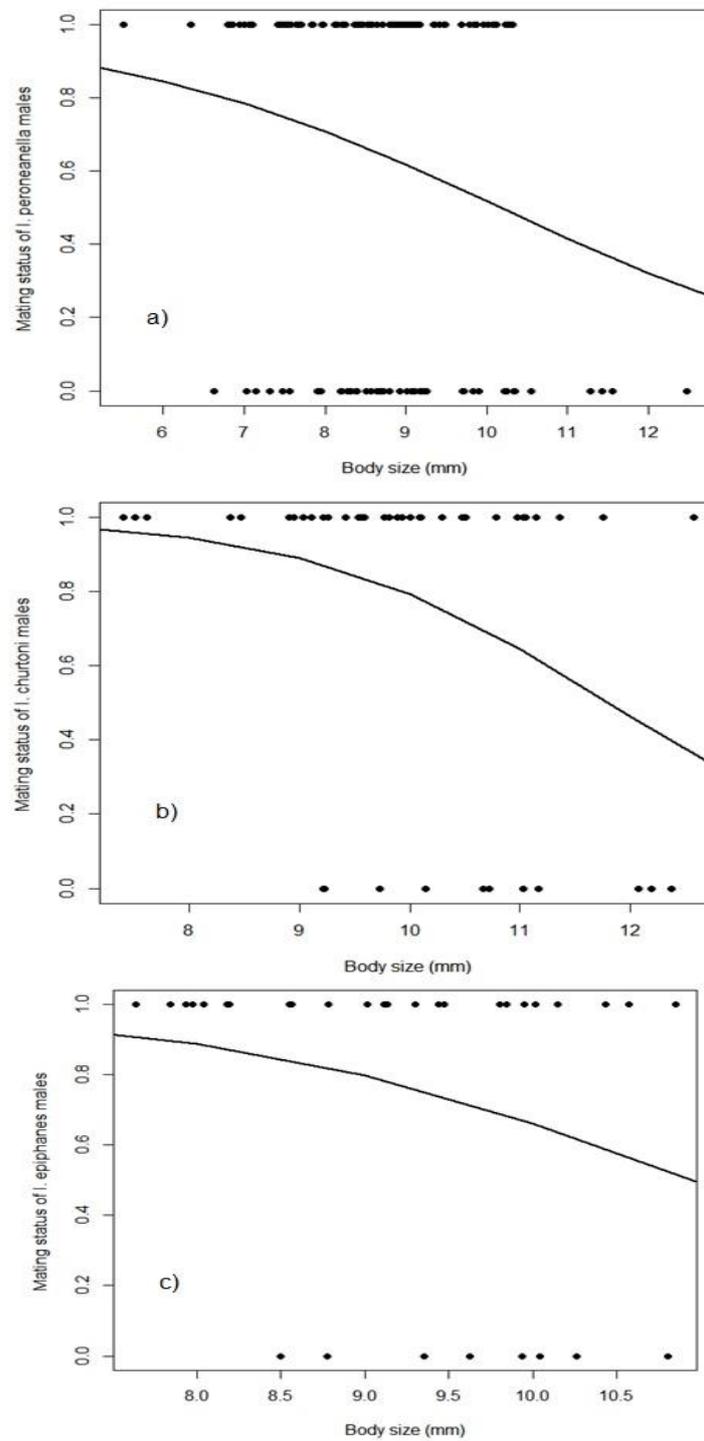


Figure 3. Relationship between mating status (0 = Not mated, 1 = Mated) and body size for *I. peroneanella* (a), *I. churtoni* (b), and *I. epiphanes* (c) males.

Logistic regressions showed a significant relationship between male body size and mating status ($p = 0.01950$) for *I. peroneanella* males (Figure 3a; Table 6) but not for the females ($p = 0.654$). Similarly, there was a significant relationship for male *I. churtoni* ($p = 0.0298$) (Figure 3b; Table 6), but not the females ($p = 0.159$). Overall, smaller males were more likely to have mated than larger males in both *I. peroneanella* and *I. churtoni*. The relationship between body size and mating status was not significant for *I. epiphanes* males ($p = 0.157$) (Figure 3c; Table 6), and could not be analysed for *I. epiphanes* females as only 4 specimens were collected and all were mated. Although there were no significant results for the relationship between body size and mating status for *I. epiphanes* they do show a similar trend to *I. peroneanella* and *I. churtoni* in the respect that smaller males are more likely to have mated.

Table 6. General linear model coefficient values for the relationship between mating status - mated, unmated - and body size for *Izatha peroneanella*, *I. churtoni* and *I. epiphanes* males.

Logistic regression coefficients: <i>I. peroneanella</i> males				
	Estimate	std Error	Z value	Pr(> z)
Intercept	4.1642	1.5582	2.672	0.00753**
Body size	-0.4091	0.1751	-2.336	0.01950*
Logistic regression coefficients: <i>I. churtoni</i> males				
	Estimate	std Error	Z value	Pr(> z)
Intercept	8.8315	3.6354	2.429	0.0151*
Body size	-0.7481	0.3444	-2.172	0.0298*
Logistic regression coefficients: <i>I. epiphanes</i> males				
	Estimate	std Error	Z value	Pr(> z)
Intercept	7.7094	4.7604	1.619	0.150
Body size	-0.7037	0.4976	-1.414	0.157

5.4.6 Mating success over time

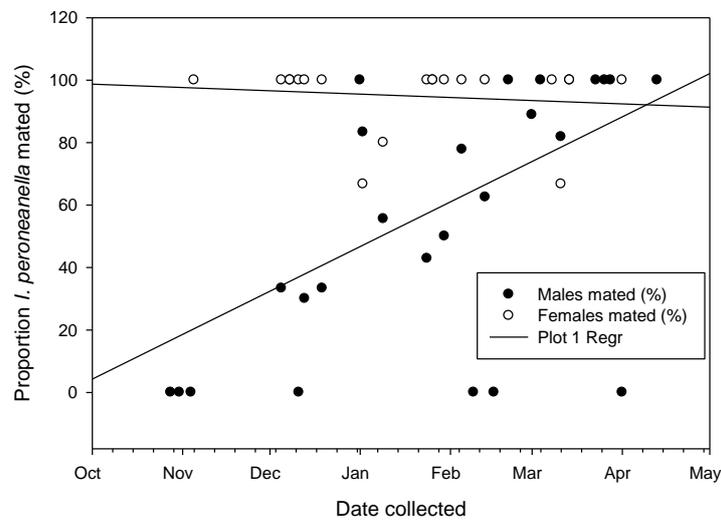


Figure 4. Relationship between the proportion of individuals mated and collection date for *I. peroneanella* males and females.

The only significant relationship between the proportion of males mated and date of collection was in *I. peroneanella* males ($R^2 = 0.294$; p value = 0.003) (Figure 4; Table 7).

Table 7. Linear regression of the proportion of *I. peroneanella* mated and collection date.

	df	SS	MS	F	<i>P</i>
Regression	1	13541.707	13541.707	10.829	0.003
Residual	26	32511.975	1250.461		
Total	27	46053.682	1705.692		

5.5 DISCUSSION

5.5.1 Flight period and sex ratios

The flight periods of *I. peroneanella*, *I. churtoni*, and *I. epiphanes* resemble those recorded in Hoare's (2010) revision of the genus. *Izatha peroneanella* has the longest flight period, with individuals collected from the end of October through to the middle of April. *Izatha churtoni* and *I. epiphanes* have a shorter flight period, appearing from October until February, with one specimen of *I. epiphanes* collected in early March. Emergence of all three species began in October, with *I. churtoni* and *I. epiphanes* numbers peaking in December and January. *Izatha peroneanella* numbers also rose during this time, but remained steady during February and March, before dropping off in April. The relatively normal distribution of seasonal abundance is also suggestive of a univoltine life cycle in *I. churtoni* and *I. epiphanes*, and potentially *I. peroneanella*.

Sex ratios in all three species also appear to be consistently biased towards males over the flight season, however, specimens collected at light may not be a true representation of sex ratios in the natural environment (Altermatt et al. 2009), as males flying in search of receptive females may be more likely to arrive to light. Most *Izatha* females collected at light were already mated, and may be flying in search of an oviposition site post-copulation. Virgin females are predicted to remain stationary, releasing sex pheromones to attract males (Roelefs et al. 2002; Lienard et al. 2008). Previously mated *Izatha* females may also remain stationary, either during a post-copulatory refractory period, or concealing themselves from predators during recurring sexual receptivity. Sex ratios of lab reared *I. churtoni* showed a more balanced sex ratio, but numbers were still slightly male biased. However, this was based on a sample size of

22 lab reared specimens, so further data are needed to make a strong conclusion about the naturally occurring sex ratio. Therefore, the male-biased sex ratios of field caught *I. peroneanella*, *I. churtoni*, and *I. epiphanes* are most likely an artifact of the female propensity to cryptic behavior in contrast to males (Adamski 2004). However, to gain insight into how sex ratios may influence the mating systems within *Izatha* it is more appropriate to calculate the operational sex ratio; which may be difficult to obtain in field-collected specimens given the potentially cryptic nature of sexually receptive females. However, if the observed male-biased sex ratio in the three *Izatha* species is in fact a true representation of the naturally occurring sex ratios, the amount of competition among males for mates will be intense, which in turn may alter reproductive strategies and potentially impose stronger sexual selection on traits (Allen et al. 2011), including genital structures. As mentioned above, contributing factors to biased sex ratios in Lepidoptera may include predation (Miler et al. 2008), nutritional stress (Quezada-Garcia et al. 2014), protandry (Wiklund et al. 1992), and disease (Jiggins et al. 1998). As the three *Izatha* species examined are nocturnal and it is the males (which are generally of smaller size) that are flying in search of mates, it is less likely that predation would contribute to the male-biased sex ratio as males are the ones that are at most risk. Nutritional stress could be a factor, but without an in-depth knowledge of the nutritional requirements of each of these species of *Izatha* this cannot be tested. The larvae of *I. peroneanella*, *I. churtoni*, and *I. epiphanes* are sapro-xylophagous and have been reared from several plant species. This would require ascertaining the specific compounds in the wood that are paramount to their diet, which may include certain types of fungi or state of decomposition. To date, there are no known diseases among populations of *Izatha* that may contribute to an unbalanced sex ratio; however, red

mites (unidentified species) have been noted on field caught specimens. The effect these red mites have on the moths is unknown, but observations suggest a bias of presence on females.

5.5.2 *Protandry*

During the flight period males of all three *Izatha* species also appear to emerge first, with female numbers increasing later in the season, supporting the occurrence of protandry in these three species. Protandry – the earlier emergence of males to breeding sites – is common within Lepidoptera (Morbey & Ydenberg 2001; Allen et al. 2011) and is generally attained by males increasing their growth rate (Wiklund et al. 1991) or decreasing developmental time (Zijlstra et al. 2002). The mate opportunity hypothesis proposes that male competition for access to females may select for protandry (Morbey & Ydenberg 2001), however, this is often more characteristic of species that exhibit monandrous mating systems that tend to mate shortly after eclosion (Wiklund & Fagerstrom 1977; Singer 1982; Muralimohan & Srinivasa 2010). In monandrous mating systems there would be stronger pressure on males to secure a mate early in the season to avoid missing out on mating than in these polyandrous *Izatha* species. If females are sexually receptive repeatedly during the mating season, it is expected there should be less urgency for males to emerge first. Nevertheless, adaptive strategies during the season due to the extent sexual size dimorphism and protandry could result in less competition at the beginning of the season, with progressive intensity for polyandry as the flight period progresses. Males may also gain benefits from mating with virgin females, as virgin females may be more likely to lay a higher proportion of their lifetime supply of eggs (Thornhill & Alcock 1983). This places

selection on males to time emergence during the period that more newly emerged females, as opposed to older, previously females are more likely to be present (Thornhill & Alcock 1983). The wait cost hypothesis proposes that females would benefit from ensuring male presence at breeding sites to avoid the probability of mortality before the opportunity to mate (Fagerstrom & Wiklund 1982; Morbey & Ydenberg 2001). This is an unlikely mechanism influencing protandry in *I. peroneanella*, *I. churtoni* and *I. epiphanes*, as the flight period lasts longer than many other lepidopteran species, ranging from 5 to 7 months. Males and females of these species have also survived up to four weeks in captivity (pers. obs.), so they are not as time constrained as other shorter-lived taxa. Besides the possibility of high levels of predation in their natural environment or extended latency periods to sexual receptivity in newly emerged adults, females potentially have a high probability of successfully mating. The constraint hypothesis suggests that protandry may be interrelated with female biased sexual size dimorphism (SSD) (Wiklund & Solbreck 1982; Morbey & Ydenberg 2001). For example a shorter developmental period in males would result in a disparity in body size between the sexes. However, the mere co-occurrence of these traits does not necessarily infer that one causes the other.

5.5.3 Female-biased sexual size dimorphism and seasonal variation in body size

Izatha peroneanella and *I. churtoni* females do have a significantly larger body size relative to conspecific males, i.e. they show female-biased sexual size dimorphism. Results for the difference between male and female body size in *I. epiphanes* were not significant, however, the sample size of this species was relatively low with few female specimens, so any significant results may have been obscured. Previous research on the

relationship between sexual size dimorphism, polyandry and protandry in 23 species of butterfly from two families revealed a positive correlation between the degree of male-biased sexual size dimorphism and the extent of polyandry (Wiklund & Forsberg 1991). In mating systems where females mate more than once, there may be stronger selection on body size differences between the sexes with males larger than females (Blanckenhorn 2005). This is likely due to the increased fitness of larger males owing to superior competitive ability, or through female preference for larger males (Hedrick & Temeles 1989). However, species of Lepidoptera in which direct precopulatory female mate choice and male-male contests are not observed, such a relationship may not be expected. In fact, perhaps a common form of competition between male moths occurs as scramble competition (see Wickman 1992, Stevenson & Harris 2009), which may select for smaller body size, such as that found in *Izatha*. As mentioned in the previous section, protandry has been proposed to be interrelated with sexual size dimorphism (Wiklund & Solbreck 1982; Morbey & Ydenberg 2001). Protandry may result from the shortened development time of males, which in turn may have an effect on emerging adult body size and thus contribute to sexual size dimorphism. However, a shortened development period does not explain gradual decreases in body size over the season. Both male and female *I. peroneanella*, and *I. churtoni* and *I. epiphanes* males showed a significant decrease in body size over the flight period (Figure 2). Although, the results for *I. churtoni* and *I. epiphanes* females were not significant, the lack of female specimens, especially in *I. epiphanes* (n = 4) may have obscured true patterns, as there does appear to also be a drop in female body size for these species over the season (Figure 2). A larger sample size of females is required to confirm this. Alternatively, selection for maintaining a larger body size may be greater in females, presenting as

lower variation in body size over the season relative to males. According to Rensch's rule male body size has the potential to vary more among and within a single species than female body size (Allen et al. 2011) and this could be what is occurring in this case. Nevertheless, the results presented here show a gradual decrease in body size for males of all three species and if adult body size was directly related to the length of the developmental period, the opposite pattern should be expected, i.e. smaller males at the beginning of the flight period. *Izatha*, much like other deadwood feeders appear to have a univoltine lifecycle. The difference in body size over the flight season could be attributed to when the eggs were oviposited in the previous breeding period. Eggs that are laid at the beginning of the breeding season, which starts in spring may benefit from having an extended larval period feeding on wood during autumn and winter, during which nutritional resources would be in a appropriate condition (e.g. moist and fungal laden). However, eggs laid toward the end of the breeding season could be resource-poor compared to their earlier developing counterparts during the larval stage and may be trying to make up body size in late spring/summer when the wood will be drier and less nutrient rich, resulting in a smaller adult body size. Other explanations are that body size in males may be under sexual selection either by cryptic female choice for some trait that accompanies smaller male size or for sperm competition, or small male size selected for in scramble competition for females, as smaller, lighter males may fly more easily. If body size was under stronger environmental influence such as nutritional stress, then we would expect to see a decrease in body size in both sexes over the flight period. Data suggests that environmental factors may be playing a primary role in the decrease in body size over the flight period, but this does not mean that the influence of

sexual selection can be ruled out, as mating success could be an indicator of sexually selected preferences for smaller body size.

5.5.4 Relationship between mating success, body size and time

For the relationship between body size and mating success, only male results were displayed as females were usually mated and there were smaller sample sizes of females. As mentioned previously, most females that come to light are potentially in search of oviposition sites post-copulation. Rather than flying in search of males, the females in most moth taxa emit sex pheromones in order to attract males (Roelefs et al. 2002; Lienard et al. 2008), and a recent discovery of a compound that is involved as a male sex attractant for *Izatha peroneanella* confirms this scenario in this species (Twidle et al. 2013). Therefore, the number and mating status of field-collected female *Izatha* may be biased. Nevertheless, the data provides important information about male mating success. Larger males appear first and are less likely to be mated, whereas smaller males that tend to hatch later in the season appear more likely to have mated (Figure 3). Newly emerged females may have a specific latency period before releasing sex pheromones, with earlier emerging collected males not having had the opportunity to mate before being collected, especially in the presence of protandry, where females arrive later than males. However, mating success was not correlated with the time of emergence during the breeding season, except in *I. peroneanella*, in which males appear to be more likely to have mated as the season progresses (Figure 4). The smaller sample size of *I. churtoni* and *I. epiphanes* may have obscured any similar trends in these two species. Nevertheless, the higher probability of mating in *I. peroneanella* and *I. churtoni* males may actually be attributed to body size, as smaller males that tend to emerge later in the flight season were more likely to have mated. Furthermore, the higher mating success observed over time may actually be the result of a strong link between body size and time in the probability of being mated. Males were more

likely to be mated as the season progressed, however, smaller males were also more prevalent later in the season.

It is possible that females prefer to mate with smaller males; as smaller body size may correlate with smaller genitalia and less chance of a female being injured by harmful structures. As discussed in Chapter 4, some females of field-caught *I. peroneanella* that had mated showed a small amount of scarring to the ductus bursae wall in which teeth of the male phallus make contact, whereas others did not. In contrast, all mated females of *I. churtoni* and *I. epiphanes* exhibited a varying level of scarring. One of the contributing factors to the area of scarring of the female ductus bursae could be the relative size of the male. The size of the male(s) with which field-caught female specimens had mated is unknown, but it would be interesting to examine what effect body size and relative size of the male genitalia within a species may have on the amount of injury the female may receive during copulation.

In *Izatha*, although smaller males were more likely to have mated this does not imply lifetime mating success, as the overall number of matings smaller males secured compared to larger males is unknown. Lab-based preference tests would help decipher whether field-based results match this and whether smaller males gain increased numbers of copulations. Although the spermatophore primarily functions in the transfer of spermatozoa, other accessory substances may be transferred with it and provide direct benefits to female and increase male reproductive success (South & Lewis 2011). In the Indian meal moth (*Plodia interpunctella*), Lewis et al. (2011) found that smaller males - as a product of larval resource limitation - had lower mating success in terms of

number of lifetime matings than higher provisioned larger males. However, smaller males still transferred a similar amount of fertile sperm as larger males, but also a higher amount of nonfertile sperm in terms of energy rich resources for the female (Lewis et al. 2011). In the crambid moth *Neoleucinodes elegantalis* spermatophore size was not related to male body size (Pontes et al. 2013), therefore male body size may not be a good indicator of the relative gamete and accessory substances an individual may transfer to their mate. Furthermore, a seasonal trade-off due to physiological limitations and fluctuating levels of competition (operational sex ratios) may result in a shift of investment and mating strategies, as under increased levels of sperm competition, male indian meal moths showed increased levels of sperm investment and sperm allocation (Ingleby et al. 2010). For example, smaller *Izatha* males may transfer a higher amount of energy rich resources with the spermatophore that may influence female utilization of the current male's spermatozoa or put a larger investment into sperm production, giving smaller males an advantage under more intense sperm competition later in the season. Therefore, smaller body size in males may be under sexual selection as a form of sperm competition, but also postcopulatory mate choice in females. In the comma butterfly (*Polygonia c-album*, Nymphalidae), females were shown to be employing postcopulatory mate choice, as females adjusted their reproductive effort in accordance with the quality of the male's sperm in terms of a higher amount of associated proteins for egg provisioning (Wedell 1996). Therefore, male size could also correlate with other traits selected for in females, such as courtship pheromones (Kelly et al. 2012). Male size may be an honest signal of male quality and/or smaller males may compensate for the lack of size by putting their energy into traits that promote female choice pre or post copulation. Ultimately, a bigger body size in *Izatha* may not necessarily be better and

trade-offs occurring over the mating season may reflect adaptive strategies under physiological and social limitations.

Another explanation behind the higher probability of smaller males that progressively emerge during the season could be scramble competition for females. In scramble competition, competitors, i.e. males may not prevent access to resources, i.e. females, but rather focus on getting to or having the ability to gain greater access to females (Gomendio et al.1998). Scramble competition may select for smaller *Izatha* males, which are likely to be lighter in weight and therefore have increased flight ability that is advantageous in quickly locating females.

5.5.5 Summary

The three species of *Izatha* examined exhibit protandry, female-biased sexual size dimorphism and male-biased sex ratios. The mate opportunity hypothesis seems to be the best explanation for protandry within *Izatha*. The evolution of protandry and other traits, in addition to the potential selection for smaller body size in males may have a large influence on sexual selection in these species. Smaller males seem to a higher probability of mating, however, more controlled mating trials also accounting for number of matings and fertilization success are warranted to confirm this. Furthermore, smaller male size may provide an advantage under more intense male competition. Most research to date reveals contrasting factors influencing mating success in Lepidoptera. This highlights the importance of biological and ecological constraints, and mating system dynamics within species or closely related taxa in establishing the type of natural and sexual selection mechanisms that may be operating in determining mating and/or fertilization success in both sexes.

6.

General discussion

6. General discussion

6.1 SUMMARY

In this thesis I have highlighted the patterns of genital evolution and sexual selection in two genera of moths from different families – the New Zealand *Glaucocharis* (family Crambidae) and *Izatha* (family Xyloryctidae), as well as defining mating system characteristics and proponents of mating success in a few representative *Izatha* species. Comparing closely related species allows the determination of coevolutionary relationships between male and female traits and hence the role of sexual selection in the evolution of genitalia (Arnqvist 1998; Arnqvist & Rowe 2002b). Males often show the greatest amount of divergence in genital traits (Eberhard 1985; Simmons et al. 2009; Holwell et al. 2010) and this was very much evident in both genera of moths examined. It is because of this obvious variance in genitalic traits that the effects of sexual selection are often more pronounced in males. In **Chapter 2** the construction of a molecular phylogeny for New Zealand *Glaucocharis* enabled the interpretation of evolutionary relationships and patterns of male genital evolution, as well as a phylogenetically controlled comparison of a male and female trait that have coevolved, most likely as a result of sexual conflict between the sexes. In **Chapter 3** a molecular phylogeny for *Izatha* was also constructed to clarify the evolutionary relationships and patterns of the evolution of male genital traits within this genus. Processes that may be responsible for the current distribution of *Izatha* species were also discussed as the high species diversity in the North Island is an unusual pattern among New Zealand Lepidoptera and may be in part a product of a rapid radiation of North Island species. In **Chapter 4** comparative phylogenetic analyses revealed the coevolution of the unusual

lepidopteran male and female genital traits within *Izatha* – male deciduous cornuti and female signum; and of the male external phallic teeth and the extent of reinforcement of the female reproductive tract. In both cases results strongly suggest that the driver of this coevolution is sexual conflict resulting in sexually antagonistic coevolution. Of greatest interest is that it appears that the female may be driving the evolution of a male structure – deciduous cornuti.

In addition to comparing the evolution of genital traits and the subsequent selective mechanisms behind their development, it is important to consider the type of mating system characteristic of a species, as this may contribute to variation in mating strategies that define the parameters of sexual selection. For example, in monandrous mating systems it is expected that there is less competition for mates, which may result in the relaxation of sexual selection on genitalia and often manifests as less divergent and less complex genitalia (Arnqvist 1998). **Chapter 5** defines seasonal variation patterns and potential determinants of mating success among three species of *Izatha* – *I. peroneanella*, *I. churtoni* and *I. epiphanes*. This study has revealed that these three *Izatha* species display protandry, female-biased sexual size dimorphism and male-biased sex ratios. In addition, male body size showed strong relationship with mating status in *I. peroneanella* and *I. churtoni*, with smaller males more likely to have mated. Mating status was also related to collection date in *I. peroneanella*, as males collected later in the season were more likely to have mated.

6.2 GENITAL EVOLUTION IN NEW ZEALAND *GLAUCHARIS* AND *IZATHA*, AND CURRENT DISTRIBUTION PATTERNS IN *IZATHA*.

The construction of molecular phylogenies for New Zealand *Glauccharis* and *Izatha* allowed for a more in-depth examination of the evolutionary relationships of species within these genera. The resulting relationships were partly in agreement with morphological species groupings inferred by Gaskin (1985) for *Glauccharis* and Hoare (2010) for *Izatha*; however, there were also discrepancies in the monophyly of some groups in both genera. This was largely a reflection of male genital characters, with more closely related species sharing more similar traits. Therefore, male genitalia appear to be a good indicator of species relationships. However, this has proven not always to be the case and monophyly cannot be assumed based on specific male genital characters. For example, there was an original assumption that the evolution of deciduous cornuti evolved once and belonged to one closely related group within the phylogeny, leading to the classification of a group based on the presence of these structures (Hoare 2010). However, the molecular results show that these structures have evolved on more than one occasion and in three separate clades within *Izatha*. Compound cornuti have also arisen twice, suggesting strong selection of these structures in particular species to serve a particular function, which could potentially relate to differences in mating system between species.

Ancestral state reconstruction for the male genitalia in New Zealand *Glauccharis* revealed traits that are characteristic of this genus and clades within it. In the New Zealand *Glauccharis* there appears to be differential development of gripping

structures, the uncus and gnathos, the separation of the valva to possess an elongated process, the loss of the external spur of the phallus, and the development of large and smaller rows of fixed cornuti. Ancestral state reconstruction for the male genitalia of *Izatha* revealed that several unique structures that were originally absent have evolved within *Izatha*, including deciduous cornuti, compound and fishhook cornuti (which were terms coined by Hoare, 2010). Within *Izatha*, there has also been a reduction of the uncus and gnathos, but an elaboration of the juxta, which is suggested to play a supplementary grasping role.

The inability to induce mating in *Glaucocharis* and *Izatha* in captivity and consequent lack of observations on the functional morphology of specific genital structures during copula has hindered the conclusive interpretation of functionality of structures.

Perfecting lab rearing and captive mating in both genera would help support proposed roles by demonstrating which male and female structures are interacting during copula.

Nevertheless, the shape and location of genital structures allows inference on functions.

For example, the function of pouches arising from the ostium in female New Zealand *Glaucocharis* is unknown; however, the shape and length of valval processes – structures on the male genital capsule – suggests they have coevolved and may play an anchoring role during copulation. The shape and location of attachment of different forms of fixed cornuti in *Glaucocharis* and *Izatha* have allowed some inference of the function for each type. Furthermore, the fixed cornuti in *Glaucocharis* are unlikely to be functionally homologous to the deciduous cornuti of *Izatha*, which is corroborated by the lack of signa in all but one species of New Zealand *Glaucocharis*.

Unlike *Izatha*, which is endemic to New Zealand, other species of *Glaucocharis* are found worldwide. Adding other species of *Glaucocharis* from other countries into the molecular phylogeny would allow testing of one or multiple origins of species within New Zealand. Furthermore, this may add to information about the genital evolution and potential coevolution of male and female structures in this genus.

In addition to examination of the evolution of male genitalia, the potential processes behind the current distribution and radiation of species within *Izatha* were also discussed. Current distributions of *Izatha* reflect previous landscape and environmental changes in New Zealand's history and probable ecological adaptations. The incomplete genetic resolution of two sets of sister species – *I. haumu* + *I. mesoschista*, and *I. convulsella* + *I. gekkonella* - is suggestive of recent speciation, which corroborates the proposed rapid radiation exhibited in North Island species of *Izatha*. The application of evolutionary time scaling would help to validate these patterns.

6.3 SEXUAL COEVOLUTION OF MALE AND FEMALE GENITAL TRAITS

Comparative analyses of several species of New Zealand *Glaucocharis*, accounting for phylogeny, revealed that the size of the apical spur on the phallus has coevolved with a reinforcement of the connective tissue in the region of the female reproductive tract with which it most likely makes contact. The comparison of male and female traits of several species of *Izatha* also revealed a similar pattern of a significant positive correlation between the size of harmful external teeth on the male phallus and the reinforcement of the female ductus bursae – the part of the reproductive tract with

which these teeth make contact. Scarring of this region in female *Izatha* is also evident and correlated with the breadth of the phallus. A broader phallus generally has smaller teeth and a narrower phallus larger teeth, with a larger area of scarring of species in which the males have a narrower phallus and larger teeth. The relationship of a trade-off between the girth of the phallus and size of external teeth in *Izatha* may be occurring. This is worth further investigation, along with the type of selection driving the evolution of the apical spur in *Glaucocharis*, as the potential for female physical harm from these structures is suggestive of sexual conflict playing a role. For example, Ronn & Hotzy (2012) used several populations of the *Callosobruchus* seed beetle to test the prediction that genital spines function under natural selection to anchor the male during copulation. They did this by assessing whether males with longer spines had enhanced attachment during mating. However, males from populations with longer spines did not engage in longer copulations than those with smaller spines, and females mated with males that possessed longer spines experienced the fitness cost of lower overall offspring production (Ronn & Hotzy 2012). Such experimental applications would help resolve the roles of natural or sexual selection and any costs to female reproductive success indicative of sexual conflict. However, the fact that females of both genera had similar responses to different forms of harmful traits in males and that in *Izatha* sexual antagonism appears to be driving this coevolution, suggests that sexual antagonism may be the best explanation of the driver of such harmful male phallic spines.

The presence of and differential measurements of deciduous cornuti were also highly positively correlated with the area of the female signum in *Izatha*. In species in which

males possess deciduous cornuti, the area of the conspecific female signum is greater. Previous research has shown that the signum appears to have evolved as a counter-adaptation to the hard spermatophores developed by males in order to reduce sperm competition by prolonging the female refractory period to remating (Galicia et al. 2008; Sanchez & Cordero 2014). This evidence and the fact that females of some species in which males do not have deciduous cornuti do possess a signum – although reduced in size compared to those who do – suggest that signa may have been a precursor to and driver of the evolution of deciduous cornuti in males. Therefore, deciduous cornuti in *Izatha* appear to have arisen to counteractively delay the break-up of the spermatophore and recover female control of sperm utilisation. In other words, deciduous cornuti appear to be a male response to the adaptation of the signa which favours minimizing the female refractory period back to her optimum. Therefore, deciduous cornuti appear to be a sexually antagonistic trait, which have in turn evolved in response to the development of the signum. No other cases of a female sexually antagonistic trait driving the evolution of a male genital trait appear to have been documented.

6.4 MATING TRAITS AND MATING SUCCESS

Protandry, female-biased sexual size dimorphism and male-biased sex ratios are mechanisms characteristic of many Lepidoptera (Rutowski 1997; Underwood & Shapiro 1999; Adamski 2004; Allen et al. 2011; Boughton & Pemberton 2012) and were evident in three species of *Izatha* – *I. peroneanella*, *I. churtoni* and *I. epiphanes*. These three species have also shown evidence of polyandry, with multiple spermatophores and sets of deciduous cornuti observed in the female reproductive tract during dissection. The

mate opportunity hypothesis (see Morbey & Ydenberg 2001) appears the most relevant explanation of the evolution of protandry in *I. peroneanella*, *I. churtoni*, and *I. epiphanes*. Males may gain benefits of emerging early and mating with virgin vs. older, previously mated females, as virgin females may contribute a higher proportion of eggs or energy into the first oviposition (Thornhill & Alcock 1983). Female-biased sexual size dimorphism is also apparent in *I. peroneanella* and *I. churtoni* and body size was also shown to decrease significantly in males of all three species over the flight period. This may be in part due to environmental dynamics such as nutritional stress during the larval developmental period. However, sexual selection may be favouring a smaller body size as the season progresses, since mating dynamics may alter over the course of the flight period. Sex ratios over the season were consistently male-biased. Preliminary data from lab reared specimens suggest that this bias may be naturally occurring. However, the substantial male bias of sex ratios in field collected specimens over the flight period may be largely due to the cryptic behaviour of females. Females are more likely to be quiescent unless in search of food or oviposition sites in contrast to males that will be flying in search of mates. Therefore, the greater numbers of males to light may be an artefact of this.

The mating status of *I. peroneanella* and *I. churtoni* showed a high negative correlation with body size, with smaller males more likely to have mated. In *I. peroneanella*, males appearing progressively later in the season were also more likely to have mated. The results for *I. epiphanes* did not show a significant correlation, however, low sample numbers may have obscured this, as there is still a pattern of smaller males being more

likely to have mated. The results of female mating status were not presented as they were not significant either for the relationship between body size and mating status or for the relationship between emergence time and mating status. This may be due to the reasons suggested above, i.e. females coming to light may largely be those in search of oviposition sites post-copulation. Therefore, the vast majority of females collected were mated, but also because the sample size for each species was perhaps too small to gain any reliable results. However, it is likely in natural mating systems of these species, most females, regardless of emergence time or body size have a higher likelihood of mating, being the limiting sex. In regards to male mating status, although smaller males were more likely to have mated, this does not imply lifetime mating success or female preference for smaller males. Smaller body size in males may correlate with some other trait that holds an advantage in sperm competition, or may be due to a shift in mating strategies during the course of the season. For example, the increasing numbers of receptive females as the flight period progresses may select for smaller, lighter males with an advantage due to less energetic costs in flight-searching behaviour. The presence of scramble competition for females later in the season may also select for smaller male body size. The progressive increase of mated *I. peroneanella* males over the flight period may be related to body size rather than time *per se*. However, the presence of a higher number of females that emerge later in the season may also place a bearing on this. Nevertheless, these two factors of smaller body size and later collection date that are related to mating status in *I. peroneanella* are likely to be strongly linked. If smaller males that are more likely to have mated are those which are also flying later, then both male body size and collection date may be linked to the probability of being mated. Replications of field collected data over a number of seasons may help

corroborate these findings. Mate choice trials and mating and fertilisation success in lab reared specimens would also further substantiate the determinants of reproductive success in male and female *Izatha*.

6.5 LIMITATIONS

Earlier on during the thesis research process, many *Izatha* larvae were collected in the field and lab-reared on the deadwood they were found in or on three artificial diets. Rearing of the larvae was reasonably successful, with an approximately 40% survival rate to adulthood (see Appendix Three). Numerous attempts were then made to mate several species of *Izatha* in the lab to investigate the effect on female reproductive success and longevity of number of matings, and mating with virgin versus mated males, using lab reared specimens. Unfortunately, all attempts at inducing captive mating with reared specimens were unsuccessful, and several attempts with field caught specimens only yielded one record of copulation in *Izatha peroneanella*, which occurred at 21.14pm on the 24th February 2013 and lasted for approximately 5 minutes 20 seconds. Efforts were also made to mate *Glaucocharis* in captivity, however, this too proved fruitless. Although clearly difficult, perfecting conditions that stimulate captive mating or successful mating in the field would certainly help to experimentally quantify the costs involved in mating in *Izatha* and *Glaucocharis*, and to document the functional morphology of the genitalia in copula. The examination of life history traits and determinants of mating success was incorporated later in the project, so was restricted to one field season. Replication and larger sample sizes would help support these findings.

Another limitation has been the rarity, or potentially cryptic behaviour of female *Izatha* and it is for this reason that not all *Izatha* species could be included in the molecular phylogeny. Older specimens from the New Zealand Arthropod Collection (NZAC) were useful in examining the female reproductive tract of a representative number of *Izatha* species, however, the requirement of fresh abdomens of multiple specimens for histological sectioning of the female ductus bursae and examination of scarring restricted the number of species that could be included in the analyses. Nevertheless, a good representation of different clades was achieved, covering a broad spectrum of genital development within this genus.

6.6 FURTHER DIRECTIONS

There are numerous areas for additional research; however, many are dependent on the successful mating of a sufficient number of individuals of *Glaucocharis* and *Izatha*. In addition to previous suggestions of investigating the reproductive success and individual fitness of females mated once or with multiple males, there are many other hypotheses that may be addressed via successful experimental trials. For example, it is interesting that males eject all of their cornuti in the first mating. They put all these potentially costly structures into that initial copulation, even though they have the opportunity to remate. Does the ejection of deciduous cornuti associate with increased fertilisation success in males? It has been suggested that females may evolve indifference to male traits or perhaps an ability to discriminate against them (Rowe et al. 2005). In this case, can females detect the virginity status of males? If so, are they more likely to choose to mate with non-virgins in order to avoid receiving antagonistic structures such as the

deciduous cornuti? This could further support my findings of the correlation of higher mating success later in the season, as females may prefer to mate with non-virgin males that are more likely to be common in the population then.

6.7 CONCLUDING REMARKS

This thesis has presented original molecular phylogenies for New Zealand moths of the genera *Glaucocharis* and *Izatha* allowing further interpretation of evolutionary relationships within both genera. I have estimated the ancestral states and described patterns of evolution and the potential function of male genitalia, laying the groundwork for further investigation of these diverse structures. I have shown that in three species of *Izatha* the mating system is characterised by protandry – the earlier emergence of adult males, female-biased sexual size dimorphism and male-biased sex ratios, and that male mating success is correlated with smaller body size in two species (*I. peroneanella* and *I. churtoni*) and later emergence for *I. peroneanella*. By comparing male and female traits among several *Glaucocharis* I was able to reveal the coevolution of the apical phallic spur with the thickening of the ductus bursae wall. In comparing male and female traits in several *Izatha* species, I also found evidence of sexually antagonistic coevolution between the external teeth on the male phallus and the thickening of the female ductus bursae wall, and deciduous cornuti in males and the female signum. Furthermore, females appear to be driving the evolution of deciduous cornuti in *Izatha*, which is possibly the first case of female driven sexually antagonistic coevolution documented. It is my hope that this thesis inspires further research into the mating system and evolution of unusual and divergent male genitalia; as well as additional examination of

the interesting sexual coevolutionary patterns occurring within *Glaucocharis*, *Izatha* and other Lepidoptera.

Appendix One

APPENDIX ONE: *Glaucocharis* collection records

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Cutty Grass Track	Waitakeres	AK	12-Nov-12	<i>G_leucoxantha</i>	11	9	2	R.M.B. + R.J.B.Hoare	MV Light
Fletchers Creek	Inangahua	BR	27-Nov-12	<i>G_leucoxantha</i>	6	1		R.M.B. + S.L.Bennik	MV Light
Craigieburn forest park	Craigieburn	MC	4-Dec-12	<i>G_leucoxantha</i>	1	1	0	R.M.B. + D. Newman	MV Light
Two mile creek	Queenstown	OL	13-Jan-13	<i>G_leucoxantha</i>	1	1	0	R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	5-Mar-13	<i>G_leucoxantha</i>	1	0	1	R.M.B. + K.A.Broadbent	MV Light
Fletchers Creek	Inangahua	BR	27-Nov-12	<i>G_pyrsophanes</i>	4	3	1	R.M.B. + S.L.Bennik	MV Light
Bullock Creek Rd	Punakaiki	BR	9-Dec-12	<i>G_pyrsophanes</i>	1	1	0	R.M.B. + D. Newman	MV Light
The Brook	Nelson	NN	13-Dec-12	<i>G_pyrsophanes</i>	2	0	2	R.M.B. + D. Newman	MV Light
Two mile creek	Queenstown	OL	13-Jan-13	<i>G_pyrsophanes</i>	1	1	0	R.M.B. + K.A.Broadbent	MV Light
Waihinahina park	Newlands	WN	28-Dec-12	<i>G_pyrsophanes</i>	1	1	0	R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	<i>G_parorma</i>	2	2	0	R.M.B. + N.Hudson	MV Light
Cutty Grass Track	Waitakeres	AK	26-Feb-13	<i>G_parorma</i>	1	1	0	R.M.B. + K.A.Broadbent	MV Light
Kerr Bay	Lake Rotoiti	BR	10-Dec-12	<i>G_microdora</i>	1	1	0	R.M.B. + D. Newman	MV Light
Totara flat carpark	Pelorus Bridge	MB	24-Nov-12	<i>G_interrupta</i>	3	2	1	R.M.B. + S.L.Bennik	MV Light
Kerr Bay	Lake Rotoiti	BR	26-Nov-12	<i>G_interrupta</i>	1	0	1	R.M.B. + S.L.Bennik	MV Light
Kerr Bay	Lake Rotoiti	BR	10-Dec-12	<i>G_interrupta</i>	2	1	1	R.M.B. + D. Newman	MV Light
Pelorus Bridge	Rai Valley	MB	12-Dec-12	<i>G_interrupta</i>	5	3	2	R.M.B. + D. Newman	MV Light
The Brook	Nelson	NN	13-Dec-12	<i>G_interrupta</i>	1	0	1	R.M.B. + D. Newman	MV Light
Two mile creek	Queenstown	OL	13-Jan-13	<i>G_interrupta</i>	1			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	<i>G_bipunctella</i>	1	1	0	R.M.B. + N.Hudson	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Faulkners Bush	Wakefield	NN	22-Nov-12	G_harmonica	1	1	0	R.M.B. + S.L.Bennik	MV Light
Pelorus Bridge	Rai Valley	MB	12-Dec-12	G_harmonica	1			R.M.B. + D. Newman	MV Light
Bullock Creek Rd	Punakaiki	BR	9-Dec-12	G_holanthes	1			R.M.B. + D. Newman	MV Light
Pelorus Bridge	Rai Valley	MB	12-Dec-12	G_holanthes	2			R.M.B. + D. Newman	MV Light
Spraggs Bush	Waitakeres	AK	18-Feb-13	G_holanthes	1			R.M.B. + K.A.Broadbent	MV Light
Trotters Gorge	Moeraki	DN	1-Dec-12	G_chrysochyta	2			R.M.B. + D. Newman	MV Light
Fletchers Creek	Inangahua	BR	27-Nov-12	G_chrysochyta	1			R.M.B. + S.L.Bennik	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	G_chrysochyta	1	1	0	R.M.B. + N.Hudson	MV Light
Cutty Grass Track	Waitakeres	AK	5-Feb-13	G_chrysochyta	3		1	R.M.B. + K.A.Broadbent	MV Light
Spraggs Bush	Waitakeres	AK	18-Feb-13	G_chrysochyta	3			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	26-Feb-13	G_chrysochyta	2			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	5-Mar-13	G_chrysochyta	4			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	8-Mar-13	G_chrysochyta	1			R.M.B. + K.A.Broadbent	MV Light
Faulkners Bush	Wakefield	NN	22-Nov-12	G_elaina	3	2		R.M.B. + S.L.Bennik	MV Light
Totara flat carpark	Pelorus Bridge	MB	24-Nov-12	G_elaina	3	1		R.M.B. + S.L.Bennik	MV Light
Kerr Bay	Lake Rotoiti	BR	26-Nov-12	G_elaina	1			R.M.B. + S.L.Bennik	MV Light
The Brook	Nelson	NN	13-Dec-12	G_elaina	2			R.M.B. + D. Newman	MV Light
Two mile creek	Queenstown	OL	13-Jan-13	G_elaina	2			R.M.B. + K.A.Broadbent	MV Light
Pelorus Bridge	Rai Valley	MB	12-Dec-12	G_auriscriptella	1			R.M.B. + D. Newman	MV Light
Sycamore Farm	Levin	WN	23-Dec-12	G_auriscriptella	2			R.M.B. + V.W.Linskill-Bennik	MV Light
Cutty Grass Track	Waitakeres	AK	5-Feb-13	G_auriscriptella	12		5	R.M.B. + K.A.Broadbent	MV Light
Kakamatua Inlet	Huia	AK	13-Feb-13	G_auriscriptella	2			R.M.B. + K.A.Broadbent	MV Light
Spraggs Bush	Waitakeres	AK	18-Feb-13	G_auriscriptella	4			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	21-Feb-13	G_auriscriptella	3			R.M.B. + R.J.B.Hoare	MV Light
Cutty Grass Track	Waitakeres	AK	24-Feb-13	G_auriscriptella	1			R.M.B. + K.A.Broadbent	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Cutty Grass Track	Waitakeres	AK	26-Feb-13	G_auriscriptella	4			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	5-Mar-13	G_auriscriptella	2		1	R.M.B. + K.A.Broadbent	MV Light
Harvey Bay campground	Tennyson Inlet	SD	23-Nov-12	G_lepidella	3	3	0	R.M.B. + S.L.Bennik	MV Light
Totara flat carpark	Pelorus Bridge	MB	24-Nov-12	G_lepidella	3	1	3	R.M.B. + S.L.Bennik	MV Light
Fletchers Creek	Inangahua	BR	27-Nov-12	G_lepidella	4	2		R.M.B. + S.L.Bennik	MV Light
Pelorus Bridge	Rai Valley	MB	12-Dec-12	G_lepidella	13		3	R.M.B. + D. Newman	MV Light
Trotters Gorge	Moeraki	DN	1-Dec-12	G_lepidella	1			R.M.B. + D. Newman	MV Light
Mt Fyfe Rd Track	Kaikoura	KA	11-Dec-12	G_lepidella	2			R.M.B. + D. Newman	MV Light
Bullock Creek Rd	Punakaiki	BR	9-Dec-12	G_lepidella	6		1	R.M.B. + D. Newman	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	G_lepidella	1			R.M.B. + N.Hudson	MV Light
Twelve mile creek	Queenstown	OL	13-Jan-13	G_lepidella	9			R.M.B. + K.A.Broadbent	MV Light
One mile creek	Queenstown	OL	19-Jan-13	G_lepidella	10			R.M.B. + K.A.Broadbent	MV Light
Kakamatua Inlet	Huia	AK	30-Jan-13	G_lepidella	1			R.M.B. + N.Hudson	MV Light
Spraggs Bush	Waitakeres	AK	18-Feb-13	G_lepidella	4			R.M.B. + K.A.Broadbent	MV Light
Kakamatua Inlet	Huia	AK	11-Mar-13	G_lepidella	1			R.M.B. + K.A.Broadbent	MV Light
Bullock Creek Rd	Punakaiki	BR	9-Dec-12	G_selenaea	2			R.M.B. + D. Newman	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	G_selenaea	3	2		R.M.B. + N.Hudson	MV Light
Twelve mile creek	Queenstown	OL	13-Jan-13	G_selenaea	1			R.M.B. + K.A.Broadbent	MV Light
Kakamatua Inlet	Huia	AK	30-Jan-13	G_selenaea	1			R.M.B. + N.Hudson	MV Light
Spraggs Bush	Waitakeres	AK	18-Feb-13	G_selenaea	2			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	21-Feb-13	G_selenaea	4			R.M.B. + R.J.B.Hoare	MV Light
Cutty Grass Track	Waitakeres	AK	24-Feb-13	G_selenaea	1			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	26-Feb-13	G_selenaea	1			R.M.B. + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	5-Mar-13	G_selenaea	4			R.M.B. + K.A.Broadbent	MV Light

Appendix Two

APPENDIX TWO: *Izatha* collection records

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Lyell DoC campground	Lyell	BR	30-Nov-10	<i>I_acmonias</i>	4	2	2	R.M.Bennik + A.A.Hitchcock	MV Light
Fletchers Creek	Inangahua	BR	1-Dec-10	<i>I_acmonias</i>	26	15	11	R.M.Bennik + A.A.Hitchcock	MV Light
Greyneys Shelter	Arthur's Pass	NC	2-Dec-10	<i>I_acmonias</i>	31	29	2	R.M.Bennik + A.A.Hitchcock	MV Light
Smithy Creek	Fiordland NP	FD	4-Feb-11	<i>I_acmonias</i>	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Fletchers Creek	Inangahua	BR	27-Nov-12	<i>I_acmonias</i>	1	1	0	R.M.Bennik + D. Newman	MV Light
North Mavora Lake	Mavora Lakes	OL	15-Jan-13	<i>I_acmonias</i>	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
Sycamore Farm	Buller Rd, Levin	WN	20-Dec-11	<i>I_austera</i>	1	0	1	R.M.Bennik	
Cutty Grass Track	Waitakeres	AK	11-Jan-12	<i>I_austera</i>	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
Wiltons Bush	Karori	WN	17-Jan-12	<i>I_austera</i>	4	3	1	R.M.Bennik + S.L.Bennik	MV Light
Cutty Grass Track	Waitakeres	AK	16-Feb-12	<i>I_austera</i>	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
Kakamatua Inlet	Huia, Waitakeres	AK	19-Dec-12	<i>I_austera</i>	1	1	0	R.M.Bennik + N.Hudson	MV Light
Sycamore Farm	Buller Rd, Levin	WN	23-Dec-12	<i>I_austera</i>	1	0	1	R.M.Bennik + E.Thompson	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-12	<i>I_austera</i>	1	0	1	R.M.Bennik + K.A.Broadbent	MV Light
Kakamatua Inlet	Huia, Waitakeres	AK	2-Jan-13	<i>I_austera</i>	2	1	1	R.M.Bennik + N.Hudson	MV Light
Kakamatua Inlet									
Matuku Reserve	Bethells	AK	24-Oct-10	<i>I_attactella</i>	2	2	0	R.J.B.Hoare	Tree trunk by day
The Brook	Nelson	NN	23-Nov-10	<i>I_attactella</i>	1	1	0	R.M.Bennik + J.S.Dugdale	MV Light
Fletchers Creek	Inangahua	BR	1-Dec-10	<i>I_attactella</i>	1	0	1	R.M.Bennik + A.A.Hitchcock	MV Light
Mt. Robert carpark	Lake Rotoiti	BP	14-Dec-10	<i>I_attactella</i>	1	0	1	R.M.Bennik + A.A.Hitchcock	MV Light
Mt. Robert carpark	Lake Rotoiti	BR	25-Nov-12	<i>I_attactella</i>	1	1	0	R.M.Bennik + S.L.Bennik	Tree trunk at dusk
Honeydew walk	Lake Rotoiti	BR	25-Nov-12	<i>I_attactella</i>	1	1	0	R.M.Bennik + S.L.Bennik	Tree trunk by day
Honeydew walk	Lake Rotoiti	BR	26-Nov-12	<i>I_attactella</i>	1	0	1	R.M.Bennik + S.L.Bennik	Tree trunk by day
Rangataua Scenic Reserve	Rangataua	TO	14-Jan-11	<i>I_balanophora</i>	2	2	0	R.M.Bennik + A.A.Hitchcock	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Sycamore Farm	Buller Rd, Levin	WN	27-Dec-11	I_balanophora	3	3	0	R.M.Bennik + S.L.Bennik	MV Light
Konini Road	Titirangi	AK	21-Feb-12	I_balanophora	1	1	0	R.J.B.Hoare	
Sycamore Farm	Buller Rd, Levin	WN	23-Dec-12	I_balanophora	3	3	0	R.M.Bennik + E.Thompson	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	I_balanophora	1	1	0	R.M.Bennik + N.Hudson	MV Light
Cutty Grass Track	Waitakeres	AK	1-Apr-13	I_balanophora	1	1	0	N. Hudson	MV Light
Sycamore Farm	Buller Rd, Levin	WN	27-Dec-13	I_balanophora	27	27	0	R.M.Bennik + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	I_blepharidota	1	1	0	R.M.Bennik + N.Hudson	MV Light
Konini Road	Titirangi	AK	17-Jan-13	I_blepharidota	1	1	0	R.J.B.Hoare	Window light
Cutty Grass Track	Waitakeres	AK	24-Jan-13	I_blepharidota	1	1	0	R.J.B.Hoare + N. Hudson	MV Light
Wright Road	Albany	AK	26-Jan-13	I_blepharidota	1			A.W.Emmerson	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-10	I_churtoni	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Rangataua Scenic Reserve	Rangataua	TO	14-Jan-11	I_churtoni	19	13	6	R.M.Bennik + A.A.Hitchcock	MV Light
Cutty Grass Track	Waitakeres	AK	20-Dec-11	I_churtoni	15	13	2	R.M.Bennik + K.A.Broadbent	MV Light
Sycamore Farm	Buller Rd, Levin	WN	27-Dec-11	I_churtoni	2	1	1	R.M.Bennik + S.L.Bennik	MV Light
Sycamore Farm	Buller Rd, Levin	WN	23-Feb-12	I_churtoni	6	5	1	R.M.Bennik + E.Thompson	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-12	I_churtoni	2	2	0	R.M.Bennik + K.A.Broadbent	MV Light
Kakamatua Inlet	Cornwallis	AK	12-Nov-13	I_churtoni	1	1	0	R.M.Bennik + R. Simpson	MV Light
Ewing Rd	Laingholm	AK	16-Nov-13	I_churtoni	2	1	1	R.M.Bennik	MV Light
Spraggs Bush	Waitakeres	AK	2-Dec-13	I_churtoni	9	8	1	R.M.Bennik + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	12-Dec-13	I_churtoni	4			R.M.Bennik + K.A.Broadbent	MV Light
Pelorus Bridge	Rai Valley	MB	18-Nov-10	I_convulsella	1	0	1	R.M.Bennik + C.J.Bennik	MV Light
Harvey Bay Campsite	Tennyson Inlet	SD	19-Nov-10	I_convulsella	5	3	2	R.M.Bennik + C.J.Bennik	MV Light
The Brook	Nelson	NN	23-Nov-10	I_convulsella	4	1	3	R.M.Bennik + J.S.Dugdale	MV Light
Mount Robert Carpark	Lake Rotoiti	BR	14-Dec-10	I_convulsella	13	9	4	R.M.Bennik + A.A.Hitchcock	MV Light
Prices Valley	Little River	MC	1-Dec-12	I_convulsella	1	0	1	B.Patrick + T.Patrick	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Prices Valley	Little River	MC	8-Dec-12	<i>I_convulsella</i>	3	1	2	R.M.Bennik + B.Patrick + T.Patrick	MV Light
Kerr Bay	Lake Rotoiti	BR	10-Dec-12	<i>I_convulsella</i>	1	1	0	R.M.Bennik + D.Newman	MV Light
Pelorus Bridge	Rai Valley	MB	12-Dec-12	<i>I_convulsella</i>	3	1	2	R.M.Bennik + D.Newman	MV Light
Craigieburn Forest Park	Craigieburn	MC	3-Dec-10	<i>I_copiosella</i>	5	5	0	R.M.Bennik + A.A.Hitchcock	MV Light
Mount Robert Carpark	Lake Rotoiti	BR	14-Dec-10	<i>I_copiosella</i>	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
One Mile Creek	Queenstown	OL	11-Jan-11	<i>I_copiosella</i>	1	1	0	R.M.Bennik + R. Bright	MV Light
Craigieburn Forest Park	Craigieburn	MC	4-Dec-12	<i>I_copiosella</i>	1	1	0	R.M.Bennik + D.Newman	MV Light
Kerr Bay	Lake Rotoiti	BR	10-Dec-12	<i>I_copiosella</i>	1	1	0	R.M.Bennik + D.Newman	MV Light
One Mile Creek	Queenstown	OL	19-Jan-13	<i>I_copiosella</i>	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
North Mavora Lake	Mavora Lakes	OL	15-Jan-13	<i>I_copiosella</i>	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
Kakamatua Inlet	Huia, Waitakeres	AK	11-Nov-10	<i>I_epihanes</i>	1	1	0	N.Hudson	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-10	<i>I_epihanes</i>	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Cutty Grass Track	Waitakeres	AK	20-Dec-11	<i>I_epihanes</i>	2	1	1	R.M.Bennik + K.A.Broadbent	MV Light
Sycamore Farm	Buller Rd, Levin	WN	27-Dec-11	<i>I_epihanes</i>	4	3	1	R.M.Bennik + S.L.Bennik	MV Light
Wiltons Bush	Karori	WN	17-Jan-12	<i>I_epihanes</i>	4	3	1	R.M.Bennik + S.L.Bennik	MV Light
Cutty Grass Track	Waitakeres	AK	16-Feb-12	<i>I_epihanes</i>	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
Sycamore Farm	Buller Rd, Levin	WN	23-Feb-12	<i>I_epihanes</i>	11	9	2	R.M.Bennik + S.L.Bennik	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-12	<i>I_epihanes</i>	3	2	1	R.M.Bennik + K.A.Broadbent	MV Light
Ewing Rd	Laingholm	AK	16-Nov-13	<i>I_epihanes</i>	5	5	0	R.M.Bennik	
Henderson Bay Road	Pukenui	ND	2-Nov-12	<i>I_haumu</i>	13	12	1	R.M.Bennik + K.A.Broadbent	MV Light
One Mile Creek	Queenstown	OL	27-Jan-11	<i>I_heroica</i>	1	1	0	R.M.Bennik + R.Bright	MV Light
Routeburn Track Carpark	Glenorchy	OL	29-Jan-11	<i>I_heroica</i>	1	1	0	R.M.Bennik + R.Bright	MV Light
Smithy Creek	Fiordland NP	FD	4-Feb-11	<i>I_heroica</i>	7	6	1	R.M.Bennik + A.A.Hitchcock	MV Light
Paparoa DoC visitor centre toilet	Punakaiki	BR	16-Feb-11	<i>I_heroica</i>	1	1	0	R.M.Bennik + A.A.Hitchcock	Toilet, By day

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Mt. Robert Carpark	Lake Rotoiti	BR	17-Feb-11	I_heroica	1	0	1	R.M.Bennik + A.A.Hitchcock	MV Light
North Mavora Lake	Mavora Lakes	OL	15-Jan-12	I_heroica	2	2	0	R.M.Bennik + K.A.Broadbent	MV Light
Pelorus Bridge	Rai Valley	MB	18-Nov-10	I_huttonii	11	8	3	R.M.Bennik + C.J.Bennik	MV Light
Harvey Bay campsite	Tennyson Inlet	SD	19-Nov-10	I_huttonii	19	18	1	R.M.Bennik + C.J.Bennik	MV Light
Duncan Bay	Tennyson Inlet	SD	20-Nov-10	I_huttonii	5	5	0	R.M.Bennik + C.J.Bennik	MV Light
Faulkners Bush	Wakefield	NN	21-Nov-10	I_huttonii	7	7	0	R.M.Bennik + J.S.Dugdale	MV Light
Montrose Drive	Atawhai	NN	22-Nov-10	I_huttonii	1	1	0	R.M.Bennik	MV Light
The Brook	Nelson	NN	23-Nov-10	I_huttonii	4	4	0	R.M.Bennik + J.S.Dugdale	MV Light
Lyell campsite	Buller Gorge	BR	30-Nov-10	I_huttonii	5	4	1	R.M.Bennik + A.A.Hitchcock	MV Light
Fletchers Creek	Inangahua	BR	1-Dec-10	I_huttonii	7	7	0	R.M.Bennik + A.A.Hitchcock	MV Light
Greyneys Shelter	Arthur's Pass	MC	2-Dec-10	I_huttonii	7	7	0	R.M.Bennik + A.A.Hitchcock	MV Light
Craigieburn forest Park		MC	3-Dec-10	I_huttonii	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Peel forest	Geraldine	SC	6-Dec-10	I_huttonii	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Trotters Gorge Scenic Reserve	Moeraki	DN	7-Dec-10	I_huttonii	2	2	0	R.M.Bennik + A.A.Hitchcock	MV Light
Ohinetahi Gardens	Governors Bay	MC	10-Dec-10	I_huttonii	3	3	0	R.M.Bennik + A.A.Hitchcock	MV Light
Lakehead Hut	Lake Rotoiti	BR	13-Dec-10	I_huttonii	2	1	1	R.M.Bennik + A.A.Hitchcock	MV Light
Mount Robert Carpark	Lake Rotoiti	BR	14-Dec-10	I_huttonii	3	3	0	R.M.Bennik + A.A.Hitchcock	MV Light
Onamalutu Scenic Reserve	Wairau Valley	MB	15-Dec-10	I_huttonii	3	3	0	R.M.Bennik + A.A.Hitchcock	MV Light
Puhi Puhi Loop Track	Hapukui	MB	19-Jan-11	I_huttonii	3	2	1	R.M.Bennik + A.A.Hitchcock	MV Light
One Mile Creek	Queenstown	OL	27-Jan-11	I_huttonii	5	5	0	R.M.Bennik + R.Bright	MV Light
Routeburn Track Picnic Area	Glenorchy	OL	29-Jan-11	I_huttonii	1	0	1	R.M.Bennik + R.Bright	MV Light
North Mavora Lake	Mavora Lakes	OL	2-Feb-11	I_huttonii	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Smithy Creek	Fiordland NP	FD	4-Feb-11	I_huttonii	7	4	3	R.M.Bennik + A.A.Hitchcock	MV Light
Twelve Mile Creek	Queenstown	OL	9-Feb-11	I_huttonii	2	1	1	R.M.Bennik + A.A.Hitchcock	MV Light
Bobs Cove Reserve	Queenstown	OL	10-Feb-11	I_huttonii	2	2	0	R.M.Bennik + A.A.Hitchcock	MV Light
Moke Lake	Queenstown	OL	11-Feb-11	I_huttonii	2	1	1	R.M.Bennik + A.A.Hitchcock	MV Light
Mt. Robert Carpark	Lake Rotoiti	BR	17-Feb-11	I_huttonii	4	2	2	R.M.Bennik + A.A.Hitchcock	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Paparoa DoC visitor centre toilet	Punakaiki	BR	16-Feb-11	I_huttonii	2	1	1	R.M.Bennik + A.A.Hitchcock	Toilet, By day
Harvey Bay campsite	Tennyson Inlet	SD	23-Nov-12	I_huttonii	1	1	0	R.M.Bennik + S.L.Bennik	MV Light
Faulkners Bush	Nelson	NN	22-Nov-12	I_huttonii	2	2	0	R.M.Bennik + S.L.Bennik	MV Light
Totara Flat Carpark, Pelorus Bridge	Rai Valley	MB	24-Nov-12	I_huttonii	3	3	0	R.M.Bennik + S.L.Bennik	MV Light
Fletchers Creek	Inangahua	BR	27-Nov-12	I_huttonii	2	2	0	R.M.Bennik + S.L.Bennik	MV Light
Trotters Gorge Scenic Reserve	Moeraki	DN	1-Dec-12	I_huttonii	1	1	0	R.M.Bennik + D.Newman	MV Light
Prices Valley	Little River	MC	8-Dec-12	I_huttonii	15	10	5	R.M.Bennik + B. Patrick + T. Patrick	MV Light
Bullock Creek Road	Punakaiki	BR	9-Dec-12	I_huttonii	5	3	2	R.M.Bennik + D.Newman	MV Light
Paparoa DoC visitor centre toilet	Punakaiki	BR	10-Dec-12	I_huttonii	1	0	1	R.M.Bennik	Toilet, By day
Twelve Mile Creek	Queenstown	OL	13-Jan-12	I_huttonii	4	3	1	R.M.Bennik + K.A.Broadbent	MV Light
North Mavora Lake	Mavora Lakes	OL	15-Jan-12	I_huttonii	4	3	1	R.M.Bennik + K.A.Broadbent	MV Light
Craigieburn Forest Park	Craigieburn	MC	3-Dec-10	I_katadiktya	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Ohinetahi Gardens	Christchurch	MC	10-Dec-10	I_katadiktya	3	3	0	R.M.Bennik + A.A.Hitchcock	MV Light
One Mile Creek	Queenstown	OL	27-Jan-11	I_katadiktya	5	5	0	R.M.Bennik + R.Bright	MV Light
Twelve Mile Creek	Queenstown	OL	9-Feb-11	I_katadiktya	6	6	0	R.M.Bennik + A.A.Hitchcock	MV Light
Prices Valley	Little River	MC	8-Dec-12	I_katadiktya	4	3	1	R.M.Bennik + B.Patrick + T.Patrick	MV Light
Twelve Mile Creek	Queenstown	OL	13-Jan-13	I_katadiktya	9	9	0	R.M.Bennik + K.A.Broadbent	MV Light
North Mavora Lake	Mavora Lakes	OL	14-Jan-13	I_katadiktya	3	3	0	R.M.Bennik + K.A.Broadbent	MV Light
North Mavora Lake	Mavora Lakes	OL	15-Jan-13	I_katadiktya	4	4	0	R.M.Bennik + K.A.Broadbent	MV Light
One Mile Creek	Queenstown	OL	19-Jan-13	I_katadiktya	2	2	0	R.M.Bennik + K.A.Broadbent	MV Light
North Mavora Lake	Mavora Lakes	OL	2-Feb-11	I_manubriata	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
North Mavora Lake	Mavora Lakes	OL	15-Jan-13	I_manubriata	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Kakamatua Inlet	Huia, Waitakeres	AK	11-Nov-10	I_mesoschista	2	2	0	R.M.Bennik + R.J.B.Hoare + A.A.Hitchcock	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-10	I_mesoschista	4	3	1	R.M.Bennik + A.A.Hitchcock	MV Light
Sycamore Farm	Buller Rd, Levin	WN	27-Dec-11	I_mesoschista	13	13	0	R.M.Bennik + S.L.Bennik	MV Light
Wiltons Bush	Karori	WN	17-Jan-12	I_mesoschista	1	1	0	R.M.Bennik + S.L.Bennik	MV Light
Sycamore Farm	Buller Rd, Levin	WN	23-Feb-12	I_mesoschista	9	7	2	R.M.Bennik + E.Thompson	MV Light
Ewing Rd	Laingholm	AK	16-Nov-13	I_mesoschista	1	0	1	R.M.Bennik	MV Light
Ohakune Mountain Road	Ohakune	TO	13-Jan-11	I_metadelta	1	0	1	R.M.Bennik + A.A.Hitchcock	MV Light
Rangataua Scenic Reserve	Rangataua	TO	14-Jan-11	I_metadelta	1	0	1	R.M.Bennik + A.A.Hitchcock	MV Light
Wiltons Bush	Karori	WN	17-Jan-12	I_metadelta	8	4	4	R.M.Bennik + S.L.Bennik	MV Light
Lakehead Hut	Lake Rotoiti	BR	13-Dec-10	I_notodoxa	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Mount Robert Carpark	Lake Rotoiti	BR	14-Dec-10	I_notodoxa	9	9	0	R.M.Bennik + A.A.Hitchcock	MV Light
Kakamatua Inlet	Huia, Waitakeres	AK	11-Nov-10	I_peroneanella	4	3	1	R.M.Bennik + R.J.B.Hoare + A.A.Hitchcock	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-10	I_peroneanella	18	16	2	R.M.Bennik + A.A.Hitchcock	MV Light
Whakapapanui Track	Tongariro NP	TO	12-Jan-11	I_peroneanella	2	1	1	R.M.Bennik + A.A.Hitchcock	MV Light
Ohakune Mountain Rd	Ohakune	TO	13-Jan-11	I_peroneanella	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Rangataua Scenic Reserve	Rangataua	TO	14-Jan-11	I_peroneanella	14	12	2	R.M.Bennik + A.A.Hitchcock	MV Light
Sycamore Farm	Buller Rd, Levin	WN	23-Feb-11	I_peroneanella	27	25	2	R.M.Bennik	MV Light
Te Hape Bay	Lake Taupo	TO	24-Feb-11	I_peroneanella	2	1	1	R.M.Bennik	MV Light
Cutty Grass Track	Waitakeres	AK	20-Dec-11	I_peroneanella	9	9	0	R.M.Bennik + K.A.Broadbent	MV Light
Sycamore Farm	Buller Rd, Levin	WN	27-Dec-11	I_peroneanella	6	3	3	R.M.Bennik	MV Light
Wiltons Bush	Karori	WN	17-Jan-12	I_peroneanella	1	0	1	R.M.Bennik + S.L.Bennik	MV Light
Raurimu Road	Raurimu	TO	26-Feb-12	I_peroneanella	2	1	1	R.M.Bennik + K.A.Broadbent	MV Light
Mill Bay	Huia, Waitakeres	AK	25-Oct-12	I_peroneanella	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
Sycamore Farm	Buller Rd, Levin	WN	23-Dec-12	I_peroneanella	13	12	1	R.M.Bennik + E.Thompson + V.W.Linskill- Bennik	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-12	I_peroneanella	4	4	0	R.M.Bennik + K.A.Broadbent	MV Light
Kakamatua Inlet	Cornwallis	AK	12-Nov-13	I_peroneanella	1	1	0	R.M.Bennik + R. Simpson	MV Light
Ewing Rd	Laingholm	AK	16-Nov-13	I_peroneanella	2	1	1	R.M.Bennik	MV Light
Spraggs Bush	Huia, Waitakeres	AK	2-Dec-13	I_peroneanella	13	13	0	R.M.Bennik + K.A.Broadbent	MV Light
Cutty Grass Track	Waitakeres	AK	12-Dec-13	I_peroneanella	3	2	1	R.M.Bennik + K.A.Broadbent	MV Light
Spraggs Bush	Huia, Waitakeres	AK	6-Jan-14	I_peroneanella	3	3	0	R.M.Bennik + N. Hudson	MV Light
Konini Road	Titirangi	AK	14-Feb-12	I_phaeoptila	1	1	0	R.J.B.Hoare	MV Light
Konini Road	Titirangi	AK	21-Feb-12	I_phaeoptila	2	0	2	R.J.B.Hoare	MV Light
Cutty Grass Track	Waitakeres	AK	9-Jan-13	I_phaeoptila	3	3	0	R.M.Bennik + N.Hudson	MV Light
Kakamatua Inlet	Huia, Waitakeres	AK	30-Jan-13	I_phaeoptila	1	1	0	R.M.Bennik + N.Hudson	MV Light
Cutty Grass Track	Waitakeres	AK	5-Feb-13	I_phaeoptila	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light
Pelorus Bridge	Rai Valley	MB	18-Nov-10	I_picarella	6	3	3	R.M.Bennik + C.J.Bennik	MV Light
Duncan Bay	Tennyson Inlet	SD	20-Nov-10	I_picarella	1	1	0	R.M.Bennik + C.J.Bennik	MV Light
The Brook	Nelson	NN	23-Nov-10	I_picarella	2	1	1	R.M.Bennik + J.S.Dugdale	MV Light
Lakehead Hut	Lake Rotoiti	BR	13-Dec-10	I_picarella	4	0	3	R.M.Bennik + A.A.Hitchcock	MV Light
Mount Robert Carpark	Lake Rotoiti	BR	14-Dec-10	I_picarella	22	10	12	R.M.Bennik + A.A.Hitchcock	MV Light
Harvey Bay campsite	Tennyson Inlet	SD	23-Nov-12	I_picarella	1	1	0	R.M.Bennik + S.L.Bennik	MV Light
Totara Flat carpark	Pelorus Bridge	MB	24-Nov-12	I_picarella	1	0	1	R.M.Bennik + S.L.Bennik	MV Light
Kerr Bay	Lake Rotoiti	BR	10-Dec-12	I_picarella	5	3	2	R.M.Bennik + D.Newman	MV Light
Pelorus Bridge	Rai Valley	MB	12-Dec-12	I_picarella	6	1	5	R.M.Bennik + D.Newman	MV Light
Sycamore Farm	Buller Rd, Levin	WN	29-Dec-10	I_prasophyta	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Kakmatua Inlet	Huia, Waitakeres	AK	18-Jan-13	I_prasophyta	1	1	0	N.Hudson	MV Light
Kakmatua Inlet	Huia, Waitakeres	AK	1-Mar-13	I_prasophyta	1	1	0	N.Hudson	MV Light
Pukaki Scientific Reserve	Mount Cook	MK	15-Feb-11	I_psychra	3	3	0	R.M.Bennik + A.A.Hitchcock	MV Light

Site	Location	Region	Date	Species	Total	No. males	No. females	Collectors	Ecology
Henderson Bay Road	Pukenui	ND	2-Nov-12	<i>I_taino</i>	6	2	4	R.M.Bennik + K.A.Broadbent	MV Light
Rangataua Scenic Reserve	Ohakune	RI	14-Jan-11	<i>I_voluptuosa</i>	1	1	0	R.M.Bennik + A.A.Hitchcock	MV Light
Cutty Grass Track	Waitakeres	AK	20-Dec-11	<i>I_voluptuosa</i>	1	1	0	R.M.Bennik + K.A.Broadbent	MV Light

Appendix Three

APPENDIX THREE: Larval rearing records of species of *Izatha* on three different diets: GPD, Brintons, and Huhu or the dead wood they were collected in.

Extraction Date	Location	Region	Date collected	Plant	Diet	Pupated	Hatched	Date Hatched	Parasitised	Notes
11-Oct-10	Smiths Rd	AK	11-Jul-10	Unknown	GPD (100%)	Y	N		N	Died as pupa (Dried out)
	Mill Bay	AK	24-Jul-10	<i>Coriaria arborea</i>	Brintons	N	N		N	Died as a larva
	Mill Bay	AK	24-Jul-10	<i>Coriaria arborea</i>	Brintons	Y	N		N	Died as pupa (Dried out)
	Little Muddy Creek	AK	25-Jul-10	<i>Coriaria arborea</i>	GPD (100%)	Y	Y	19-Oct-10		<i>I. churtoni</i> ♂
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Brintons	Y	N		N	Died as pupa (Dried out)
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	N	N		Y	Parasitised by Hymenoptera
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	Y	Y	15-Feb-11	N	Unidentified moth
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	Y	N		N	Died as pupa (Dried out)
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	Y	N	27-Oct-10	N	<i>I. churtoni</i> ♂
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	Y	N		N	Died as pupa (Dried out)
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Huhu	Y		8-Oct-10		<i>I. churtoni</i> ♀
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	Brintons	Y	Y	8-Oct-10	N	<i>I. churtoni</i> ♂
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>	GPD (100%)	N	N		N	Died as a larva
	Whareroa Village	TO	22-Aug-10	<i>Sophora microphylla</i>	Huhu	N	N		N	Died as a larva
	Whareroa Village	TO	22-Aug-10	<i>Sophora microphylla</i>	Huhu	N	N		N	Died as a larva
	Whareroa Village	TO	22-Aug-10	<i>Sophora microphylla</i>	Huhu	Y	Partially		N	Died as a pupa (<i>I. churtoni</i>)
	Whareroa Village	TO	22-Aug-10	<i>Sophora microphylla</i>	Huhu	N	N		N	Died as a larva

Extraction Date	Location	Region	Date collected	Plant	Diet	Pupated	Hatched	Date Hatched	Parasitised	Notes
	Whareroa Village	TO	22-Aug-10	<i>Sophora microphylla</i>	Huhu	Y	N		N	Died as pupa (Dried out)
	Whareroa Village	TO	22-Aug-10	<i>Sophora microphylla</i>	Huhu	13-Jan-11	N		N	Died as pupa (Dried out)
	Whareroa Village	TO	22-Aug-10	<i>Sophora microphylla</i>	Huhu	Y	N		N	Died as pupa (Dried out)
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	Y	Y	Unknown	N	Died as an adult
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	N	N		Y	Parasitised by nematode
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	Y	Partially	Unknown	N	Died as a pupa
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	Y	N		N	Died as pupa (Dried out)
	Blue Springs	WO	9-Oct-10	Unknown	Huhu	11-Oct-10	Y	9-Nov-10		I. mesoschista ♂
22-Oct-10	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	Unknown	Y	Unknown		I. mesoschista ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva (mould)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva (mould)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	Y	N		N	Died as a pupa
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva (mould)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	Y	Partially		N	Died as a pupa
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	Y	Y	15-Feb-11	N	Died as an adult (malformed)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	13-Jan-11	N		N	Died as pupa (Dried out)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva (mould)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	16-Feb-11	N		N	Died as pupa (Dried out)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Huhu	Y	N		N	Died as pupa (Dried out)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Brintons	Y	N		N	Died as pupa (Dried out)
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	Brintons	N	N			
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	GPD (100%)	N	N		N	Died as a larva

Extraction Date	Location	Region	Date collected	Plant	Diet	Pupated	Hatched	Date Hatched	Parasitised	Notes
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>	GPD (100%)	Y	Y	19-Oct-10	N	I. austera ♂
25-Oct-10	Motutapa Gardens	WI	12-Sep-10	<i>Sophora microphylla</i>	Huhu	N	N		N	Died as a larva
	Motutapa Gardens	WI	12-Sep-10	<i>Sophora microphylla</i>	Huhu	N	N		N	Died as a larva
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	Huhu	N	N		N	Died as a larva
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	Huhu	N	N		N	Died as a larva
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	Huhu	N	N		N	Died as a larva
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	Brintons	N	N		N	Died as a larva
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	Brintons	N	N		N	Died as a larva
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	GPD (100%)	N	N		N	Died as a larva (mould)
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	GPD (100%)	N	N		N	Died as a larva (mould)
	Kowhai Park	WI	12-Sep-10	<i>Sophora microphylla</i>	Huhu	N	N		N	Died as a larva
26-Oct-10	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva (mould)
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Huhu	Y	N		N	Diptera larvae
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Brintons	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	Brintons	N	N		N	Died as a larva
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>	GPD (100%)	N	N		N	Died as a larva
	Karamatura Loop Tk	AK	12-Aug-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva (mould)
	Karamatura Loop Tk	AK	12-Aug-10	<i>Coriaria arborea</i>	Huhu	N	N		N	Died as a larva (mould)
	Rangemore Tk	AK	25-Jul-10	<i>Coprosma sp.</i>	Huhu	N	N		N	Died as a larva
1-Nov-10	Kauri Grove Tk	AK	31-Oct-10	<i>Coprosma sp.</i>	Huhu		Y	15-Feb-11	N	I. austera ♂
	Blue Springs	WO	15-Aug-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	15-Aug-10	Unknown	Huhu	N	N		N	Died as a larva

Extraction Date	Location	Region	Date collected	Plant	Diet	Pupated	Hatched	Date Hatched	Parasitised	Notes
	Blue Springs	WO	15-Aug-10	Unknown	Huhu	N	N		N	Died as a larva
	Blue Springs	WO	15-Aug-10	Unknown	Huhu		N		Y	Parasitised by fly
	Blue Springs	WO	15-Aug-10	Unknown	Huhu	N	N		N	Died as a larva (mould)
	Blue Springs	WO	15-Aug-10	Unknown	Huhu	Y	Y	Unknown		I. mesoschista ♂
	Blue Springs	WO	15-Aug-10	Unknown	Huhu	Y	Y			
	Blue Springs	WO	15-Aug-10	Unknown	Brintons	Y	Y			
	Blue Springs	WO	15-Aug-10	Unknown	GPD (100%)	Y	Y			
	Karamatura Loop Tk	AK	12-Aug-10	<i>Coproasma sp.</i>	Huhu	N	N		N	Died as a larva
2-Nov-10	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	N	N		N	Died as a larva
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	N	N		N	Died as a larva
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	Y	N		N	Died as a pupa (mould)
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	Y	Y	Unknown	N	Died as an adult (I. peroneanella?)
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	N	N		Y	
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	N	N		N	Died as a larva
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	Y	Y	Unknown	N	Died as an adult (malformed)
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	Y	N		N	Died as pupa (Dried out)
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	13-Jan-11	N		N	Died as pupa (Dried out)
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	16-Feb-11	N		N	Died as pupa (Dried out)
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu	16-Feb-11	Y	Mar-11	N	Not Izatha
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Huhu		Y	Unknown	N	
	Kauri Grove Tk	AK	31-Oct-10	<i>Coproasma sp.</i>	Brintons	Y	N		N	Died as pupa (Dried out)
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	Huhu	N	N		N	Died as a larva
	Esplanade (Pnorth)	WI	11-Sep-10	Unknown	Huhu	N	N		N	Died as a larva
11-Oct-10	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>		11-Oct-10	Y	18-Oct-10		I. churtoni ♀
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>		11-Oct-10	Y	21-Oct-10		I. churtoni ♂
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>		11-Oct-10	Y	22-Oct-10		I. churtoni ♂
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>		11-Oct-10	Y	22-Oct-10		I. churtoni ♀

Extraction Date	Location	Region	Date collected	Plant	Diet	Pupated	Hatched	Date Hatched	Parasitised	Notes
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>		11-Oct-10	Y	22-Oct-10		I. churtoni ♂
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>		11-Oct-10	Y	24-Oct-10	N	I. churtoni ♀
	Little Muddy Creek	AK	7-Oct-10	<i>Coriaria arborea</i>		11-Oct-10	N		N	Died as pupa (Dried out) ♂
	Blue Springs	WO	9-Oct-10	Unknown		11-Oct-10	Y	27-Oct-10		I. peroneanella ♂
22-Oct-10	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	Unknown		I. epiphanes ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	N		N	Died as pupa (Dried out) ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	N		N	Died as pupa (Dried out) ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	N		N	Died as pupa (Dried out) ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	N		N	Died as pupa (Dried out) ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	N		N	Died as pupa (Dried out) ♀
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	2-Nov-10	N	I. churtoni ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	28-Oct-10	N	I. churtoni ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	1-Nov-10	N	I. churtoni ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	26-Oct-10	N	I. churtoni ♀
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	5-Nov-10	N	I. churtoni ♀
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	5-Nov-10	N	I. churtoni ♀
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	27-Oct-10	N	I. churtoni ♂
	Whareroa Village	TO	22-Aug-10	<i>Coriaria arborea</i>		22-Oct-10	Y	1-Nov-10		I. peroneanella ♂
25-Oct-10	Kowhai Park	WI	12-Sep-10	<i>Sophora microphylla</i>		25-Oct-10				
	Kowhai Park	WI	12-Sep-10	<i>Sophora microphylla</i>		25-Oct-10				
26-Oct-10	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>		26-Oct-10	Y	29-Oct-10		I. peroneanella ♀
	Ridge Rd Track	AK	5-Sep-10	<i>Coprosma sp.</i>		26-Oct-10	Y	Unknown		I. austera ♂
	Rangemore Tk	AK	25-Jul-10	<i>Coprosma sp.</i>		26-Oct-10		Unknown	N	I. peroneanella ♀
1-Nov-10	Blue Springs	WO	15-Aug-10	Unknown		1-Nov-10	N		N	Died as pupa (Dried out)
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	27-Oct-11		I. austera ♂
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	12-Nov-11		I. austera ♀
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	15-Nov-11		I. austera ♂
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	15-Nov-11		I. austera ♂

Extraction Date	Location	Region	Date collected	Plant	Diet	Pupated	Hatched	Date Hatched	Parasitised	Notes
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	15-Nov-11		I. austera♂
	Sycamore Farm	WN	13-Sep-11	Unknown			Y	23-Oct-11		I. austera♀
	Sycamore Farm	WN	13-Sep-11	Unknown			Y	4-Nov-11		I. austera♂
	Sycamore Farm	WN	13-Sep-11	Unknown			Y	4-Nov-11		I. austera♂
	Cascade Track	AK	8-Nov-11	<i>Coriaria arborea</i>			Y	30-Dec-11		I. austera♂
	Mill Bay	AK	25-Oct-11	<i>Coriaria arborea</i>			Y	20-Nov-11		I. epiphanes♂
	Slip Track	AK	7-Dec-11	<i>Coriaria arborea</i>			Y	21-Dec-11		I. epiphanes♂
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	8-Sep-11		I. attactella♀
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	16-Sep-11		I. attactella♂
	Sycamore Farm	WN	2-Sep-11	Unknown			Y	20-Oct-11		I. attactella♂
	Mill Bay	AK	11-Nov-11	<i>Coriaria arborea</i>			Y	2-Dec-11		I. mesoschista♂
	Mill Bay	AK	6-Dec-11	<i>Coriaria arborea</i>			Y	9-Dec-11		I. peroneanella♂
	Mill Bay	AK	6-Dec-11	<i>Coriaria arborea</i>			Y	12-Dec-11		I. peroneanella♂
	Slip Track	AK	7-Dec-11	Unknown			Y	10-Mar-11		I. peroneanella♂
	Mill Bay	AK	11-Nov-11	<i>Coriaria arborea</i>			Y	14-Nov-11		I. peroneanella♂
	Mill Bay	AK	11-Nov-11	<i>Coriaria arborea</i>			Y	26-Nov-11		I. peroneanella♀
	Mill Bay	AK	25-Oct-11	<i>Coriaria arborea</i>			Y	28-Oct-11		I. churtoni♂
	Mill Bay	AK	25-Oct-11	<i>Coriaria arborea</i>			Y	1-Nov-11		I. churtoni♂
	Mill Bay	AK	25-Oct-11	<i>Coriaria arborea</i>			Y	1-Nov-11		I. churtoni♂
	Mill Bay	AK	25-Oct-11	<i>Coriaria arborea</i>			Y	17-Nov-11		I. churtoni♀
	Mill Bay	AK	25-Oct-11	<i>Coriaria arborea</i>			Y	9-Dec-11		I. churtoni♂

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