ABSTRACT

TURNER, STEVEN PAUL. The Evolution of Sexually Selected Traits in Dance Flies. (Under the direction of Dr. Brian Wiegmann.)

The Diptera family Empididae is a successful and species rich radiation of the superfamily Empidoidea. Flies in this family are well documented to engage in a myriad of elaborate courtship displays, which has made them model organisms of choice for behavioral and evolutionary biologists studying the evolution of sexual selection. Chapter 1 of this thesis aims to summarize the variation of courtship strategies of flies in the empidid subfamily, Empidinae. I focus on nuptial gift presentation and variation in types of nuptial gifts. I also briefly discuss the occurrences of sex role reversed mating systems and the reasons why these may have evolved. The evolution of potential exploitation of female sensory biases by males in systems where males "cheat" concludes the first chapter.

Chapter 2 focuses on how sexual selection drives rapid radiations in nuptial gift giving flies. The focus for this study is Enoplempis a sub genus of Empis. Representatives of this clade have been observed to engage in almost all nuptial gift giving interactions seen across the entire subfamily Empidinae. These range from males presenting unwrapped nuptial prey to females, males constructing "balloons" in which to wrap the prey before presenting it to the female, the production of empty balloons by the males for courtship interactions and sex role reversed systems where males choose swarming females, the converse is normally the case. The study involved behavioral observations, the coding of morphological traits and the use of molecular phylogenies to reconstruct the evolution of courtship traits across Enoplempis. I also used methods

in macroevolution to recover the ancestral conditions of characters mapped onto the phylogenetic tree and used divergence time estimation to explore the origins of the Enoplempis radiation and the temporal appears of key traits.

Chapter 3 focuses on the classification and temporal diversification of the subfamilies of the Empididae. This study involved Maximum Likelihood and Bayesian Inference based phylogenetic analyses with the aim of recovering the sister group to Empididae and to resolve the poorly understood subfamily relationships within Empididae. A key focus of this study was also to establish the closest related subfamily to the highly diverse Empidinae. Divergence time estimations were also done using fossil calibrated molecular clocks in order to identify important events in the evolutionary history of the Empidoidea families and to better understand temporal diversification of empidid subfamilies.

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The Evolution of Sexually Selected Traits in Dance Flies

by
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DEDICATION

For my family, and their support over the years, my parents in particular for supporting me in my academic persuits. I also dedicate this to the Wiegmann lab and colleagues at North Carolina State University for their support and valuable input into my research.

BIOGRAPHY

Steven Paul Turner was born in the industrial town of Bradford, West Yorkshire, England. From a young age he took an interest in many subject areas and was torn between pursuing an academic career in Ancient History and Biological Sciences. His passion for biology ultimately won over and in fall 2004 Steven embarked on his undergraduate degree in Ecology which he completed with honors at the University of Derby. It was during this time that he found his passion for invertebrate biology, behavioral ecology and phylogenetics. In 2007, he embarked on his Masters degree in Advanced Methods in Taxonomy and Biodiversity at the Imperial College of Science, Technology and Medicine. During this period, Steven became focused on molecular phylogenetics and the use of phylogenetic comparative methods as an essential tool of evolutionary biology in general. Steven completed his degree with a research project on Molecular Phylogeny and Evolution of Tarantulas. Following his Masters, Steven applied to work on his PhD with Professor Brian Wiegmann at NCSU. His research has focused on the phylogeny and macroevolution of flies in the family Empididae.

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CHAPTER 1

Mating Systems in Empidinae: A Review

Mating systems and sexual selection are gaining more attention from researchers in many areas of biology as important mechanisms of diversification (Ritchie 2007). Many groups of vertebrates and invertebrates have been studied to understand how mating preferences might result in advantageous traits. Insects in Mecoptera and Orthoptera and spiders in Salticidae are becoming important model systems for studying the evolution of sexual selection (Thornhill 1978, Gwynne 2008, Vahed 2007, Maddison and Masta 2002, Bonduriansky 2001). Hyperdiverse lineages offer an excellent opportunity to assess the importance of sexual selection in contrast with natural selection as a primer for mass speciation events in closely related organisms with diverse mating systems. The insect order Diptera is one such lineage and has been shown to have had several large episodic radiations due to an ability to evolve and radiate rapidly when novel niches become available (Wiegmann et al. 2011). Diptera exhibit a large diversity of body plans and many groups exhibit marked sexual dimorphism. The dance flies (Empididae) represent a species rich lineage of Empidoidea with over 3,000 known species and a large undescribed diversity. They are notable for their varied mating systems and exhibit many sexually selected morphologies and behaviors. Many of these systems often include the presentation of a nuptial gift which was once hypothesized to be a male defense against sexual cannibalism (Kessel 1947, Aldrich 1899) but have since been demonstrated to be primarily used in courtship. These attributes are thought to be used in several ways including honest indication of genetic fitness by males (Hamilton and Zuk 1982, McLean et al. 2012), resource and investment shifts that result in sex role reversed mating systems and the evolution of the exploitation of female sensory biases by males which effectively cheat the females (Vahed 1998, 2007, Gwynne 2008, LeBas 2005). The purpose of this review is to briefly summarize what is known about the mating behaviors of the diverse dance fly subfamily Empidinae.

I. Systems where males display fitness to females

The Empididae and in particular the empidid subfamily Empidinae exhibit a range of nuptial feeding behaviors. The majority of these observed behaviors involve males hunting a prey item suitable for presentation to the female as a nuptial gift (Cumming 1994, Gwynne and and Bussie`re 2002). In many empidoids the larvae are often predatory with both males and females feeding primarily on the larvae of other semi-aquatic diptera. The adults however fill differing ecological niches. The females are primarily nectar feeding and may be important pollinators of many types of meadow wildflowers. However in order to induce the maturation of their oocytes female Empidinae and many other types of Diptera, including hematophagous groups such as Tabanidae and Culicidae rely on an extra source of protein. In tabanids and culicids this comes in the form of the blood of a host organism and is parasitic in its function (Yuval 2006). The requirement of nutrition for developing eggs in Empididae evolved an alternate strategy which involves predatory males presenting the females with nuptial prey (Daugeron et al. 2011). The extra proteins and lipids extracted from this prey item are directly beneficial to the developing offspring and therefore enhance the reproductive fitness of both the males and females. This system usually revolves around female choice of the male displaying the "best" attributes, which indicates that he has strong genetics. It is thought to have evolved from a mating system where both partners were predatory as adults as seen in the closely related empidoid family Hybotidae (Downes 1968). Basal empidid subfamilies, such as the Clinocerinae also exhibit this groundplan. Both sexes are predaceous in the clinocerine *Wiedemannia* which uses swarming behavior for both mating and hunting; some species of this fly rely on complex aggregation patterns on rocks whilst grounded to trigger mating behavior (Rogers 1981). Over time selection pressures drove the females to lose prey foraging abilities as adults. This is thought to be an evolved response to the risk faced by foraging females to their own predators. Being the egg producing sex, predation upon females may have became detrimental to the fitness of the species. This resulted in males providing the nutrition required by the females to successfully develop eggs in the form of a nuptial gift.

Across Empidinae there are many examples of nuptial feeding behaviors where males capture prey, typically swarm and then are chosen by a female. Downes (1968) composed a summary based on primary observations on the nuptial feeding habits of Empididae. This study found that many species of empidids swarm and mate in flight. Males and females of several species of *Empis* and *Rhamphomyia* landed to mate, either alighting on trees, bushes or tall grasses and directly on the ground. Downes also suggests that the nuptial gift in empidids is directly associated with female requirements during oocyte maturation, as the males and females have both been observed to visit flowers. The typical sequence of nuptial feeding interactions observed by Downes is that males seek out and hunt nuptial prey and take it to a swarming site, usually a landmark of a low lying tree or shrub, or a large rock. The swarming sites are often used continuously by successive generations, but the mechanism which allows newly emerged adults to recognize swarming sites is currently unknown (Sadowski 1999). Males then begin to fly in a specific courtship dance whilst holding on to the prey item. Male competition within these swarms can be very fierce with dominant males of some species chasing sub-ordinate males far out of the swarm. Female choice then comes into play as females fly into the swarm and choose the males with the optimal courtship display. This does not necessarily correlate with nuptial gift size and it is thought that empidid females generally choose larger males with intermediate sized gifts (Sadowski et al. 1999). It has been hypothesized that larger nuptial gifts result in inability to perform optimal courtship flight so is selected against by female choice (Sadowski et al. 1999). Pairs of males and females then leave the swarm to mate on foliage or on the ground. In species which mate in the air, pairs of males and females leave the swarm and either fly away from the singles or form mating swarms. This behavior is thought to be a defense against sperm competition by leading females away from potential rival males (Rogers 1981, Evans 1988).

The mating behaviors of Empidinae can be highly variable even between closely related species and very similar in distantly related species (Kessel 1955, Turner, in Prep). This makes species recognition in Empidinae important. Alcock (1970) observed the courtship behaviors of two relatively large species of *Empis E. barbatoides* and *E.*

poplitea. Both species occur in overlapping habitats in the Western USA and look similar in appearance. Both species typically prey on many families of Diptera including smaller empidid species and males of both species are usually found around trees with abundant sources of swarming prey. In *E. barbatoides* small groups of 5-10 males swarm over tree tops and females choose the most attractive males based on the size of the nuptial gift and their flight ability. The pairs of males and females then engage in an erratic mating flight which culminates in a rapid and steep dropping phase. Nuptial prey is transferred from the male to the female during landing. These two phases often result in the splitting of pairs and result in failed courtship. If the landing is successful the pairs will mate until the female has finished feeding on the nuptial gift. *In E.poplitea* the courtship is similar up to the point following female choice taking place high above the ground within small male swarms. When female choice occurs, instead of erratically dropping and attempting prey transfer on landing, *E.poplitea* pairs attempt to ascend. Again this often results in pairs breaking up and falling if it is not properly synchronized. It is hypothesized that these differing copulation strategies are species recognition systems which prevent courtship interactions and saves incompatible males and females of both species resources (Alcock 1970). Slight variations in color between the two species may also be important for conspecific recognition.

Recent molecular phylogenetic results and macro-evolutionary studies have revealed that male swarming and prey transfer in Empidinae dance flies is likely the ground plan for courtship behavior in this group (chapter 2). This usually involves the presentation of a captured insect prey item (or less commonly small spiders). Evans

(1988) studied the hunting and swarming habits of *Rhamphomyia sociablis*. The vast diversity of prey items appear to be other types of Diptera, up to 70%, with other Empididae and Empidoidea being relatively common nuptial prey. Conspecifics are never found to be given as nuptial prey, providing more evidence that sexual cannibalism is not a trait of female empidids. Six other orders of insects comprised the vast majority of the rest of the chosen nuptial prey. Reproductive males of Formicidae were the most common prey item (per family), possibly due to their vulnerability and inability to sting and their highly synchronous phenology.

In many species of hilarine flies and a smaller group of empidines, courtship involves a nuptial gift wrapped in silk or enclosed in frothy bubbles being presented to the female. Courtship interaction observed in these species is similar in sequence to others already discussed, consisting of male swarms of varying size over a specific landmark and culminating with female choice. Mating takes place with the transfer of the balloon in the same way as nuptial prey is transferred. The prey found in these "balloons" is highly variable. In *Empis aerobatica*, it is usually a complete prey item, usually a dipteran, fully incorporated into the balloon (Aldrich 1899). In an unidentified *Hilara* species from Eastern Australia, males skim the surface of streams and use a sticky silken pad to make a nuptial gift entirely composed of hundreds of diatoms (Young et al. 2003, Sutherland et al. 2007). Some ballooning empidines such as *Empis snoddyi* and *E.geneatis* produce empty balloons. The function of these balloons is unclear and the impact of the lack of a nuptial gift and any female alternate source of protein has yet to be studied. It is thought that the primary function of empty balloons is as a sign stimulus which triggers mating (Kessel 1955, Sadowski et al. 1999, Vahed 2007).

II. Examples of sex role reversed systems in Empidinae

In many groups of organisms, particularly insects which engage in costly courtship interactions, role reversed behavior evolve. This is hypothesized to be a result of external ecological pressures on the fitness of foraging males (Ritchie 2007, Vahed 2007, Gwynne 2008). This can be surprisingly common and has been observed in many species of Orthoptera (Vahed 1998) and is also common in the Empidinae, with up to one third of all species engaging in role reversed courtship. There are many examples of swarming females and females with secondary sexual ornamentations in the form of pinnate scales and eversible sacs and decorated wings. (Svennson 1987, Funk and Tallamy 2000, Gwynne and and Bussie`re 2007). Females in these systems still do not hunt and therefore require a nutritional gift as they do in the more common Empidinae mating systems; however, the increased investment from the male shifts the choosy sex from female to male. This is what is thought to have led to the role reversed courtship. In most cases females swarm as males in non-reversed systems do in order to attract members of the opposite sex. Males choose the most attractive females and present their nuptial prey to them; the pair then alights near the swarming site and copulates. A newly discovered (undescribed) Californian species of *Empis* in the subgenus *Enoplempis* has been observed to have females which swarm with secondary sexual ornaments (Turner, *in prep*). The ornamentation of females in this species is difficult to notice until the females are observed swarming and push out orange eversible sacs. Groups of 4-5 females have been observed swarming above a landmark, typically a low lying rock or shrub and evert their ornaments. Males were observed to fly in to the swarms whilst carrying nuptial prey and interact with the females but copulation was not observed (Turner, *in prep*). The ornamentations seen in *Rhamphomyia longicauda* and *R. pectinata* are much more elaborate than the previous example. In *R. pectinata*, the entire body of the female is covered in pinnate scales, which are vividly coloured on the abdoman and thorax. In *R. longicauda*, the females have robust eversible sacs which inflate during swarming. They also have heavily scaled legs which are often dangled when swarming. Typically 15-20 females swarm over a landmark in both species. Statistical analyses suggest that males usually choose larger females with more robust ornaments (Gwynne and and Bussie`re 2002, Funk and Tallamy 2004). It has been hypothesized that these individuals are more fecund and represent a good choice for the male to maximize his own reproductive fitness.

Female signalling in Empidinae is not restricted to specialized secondary sexual organs on the legs, thorax and abdoman. In *Empis borealis*, females have broad wings which are shaded with patterns. In this species female swarm size is very flexible with as few as a single individual circling over a landmark to as many as 40 individuals making up a swarm. Svensson et al. (1995) found that males preferred larger females with more visible traits.

Sex role reversal may have a detrimental impact on female fitness in some cases. For example, Gwynne and and Bussie`re (2002, 2007) found that ornamented females of *Rhamphomyia longicauda* suffered higher levels of mortality due to being trapped more easily in spider webs than males.

III. Examples of possible exploitation of female sensory biases

Most mating systems seen across the tree of life appear to have evolved into complex interactions comprise varying levels of trade offs which directly impact the fitness of each of both sexes. Recent studies focussing on the mating systems of varied organisms have found many examples, obvious or not, where males take advantage of female sensory biases (Thornhill 1978, Ritchie 2007). For example, it has recently been discovered that the large protein capsules produced by some male Orthoptera, the spermatophylax, in some species is largely filled with fluid and offers the female little nutritional benefit but allows the male a prolonged copulation period (Vahed 2007). It is hypothesised that in some mating systems males are evolutionarily taking advantage of female sensory biases and cheating the system by investing less resources in their offspring than they initially appear to be (Gwynne 2008,). Other examples of cheating strategies include in Mecoptera where males will reuse and feed on their own degraded nuptial gifts (Thornhill 1978).

Deceptive behavior has also been observed in several groups of empidids and some systems which were once thought to have been derived signs of fitness such as the empty balloons of some empidine flies have also been hypothesized to be possible examples of exploitation of female sensory biases (Ritchie 2007, Sadowski 1999). One hypothesis for the evolution of empty balloons in *E. snoddyi* and *E. geneatis* is that it has evolved to take advantage of the nuptial feeding system (including the presentation of silk wrapped prey) which was the ancestral condition in *Enoplempis,* the sub genus to which these species belong. The balloon has evolved to the point where it is simply a sign stimulus to initiate courtship and balloon transfer towards the end of courtship is the stimulus that triggers mating (Ritchie and Vahed 2007). It is not clear whether females evolved an alternate way of acquiring the necessary nutrients to produce eggs and that the relic balloon is now fundamentally an indication of male fitness. In *E. snoddyi* balloon size is important for female choice so may indicate that male fitness is being assessed through the balloon (Sadowski 1999). The cost or benefit of producing empty balloons has yet to be studied in Empididae.

Another potential courtship system which may involve exploitation of female sensory biases is *Empis jaschhoforum* which displays extreme polymorphism, including asymmetry of the basitarsi of the forelegs (Daugeron et al. 2011). The three variations of this character are I. Absent, the basitarsi are not enlarged; II. Asymmetrical, one basitarsus is swollen and one is unmodified; III: Both legs have modified basitarsi. The modified basitarsi in this species appear to mimic a nuptial prey item. This species was newly described in 2011 and its courtship behavior has not yet been observed; however, females have secondary sexual characters on their hind legs, suggesting that this species also has female swarms. It has been hypothesized that several courtship strategies are used by males. Males with unmodified legs display honestly and have to capture nuptial prey in order to successfully choose a mate. Males with unmodified legs have evolved a cheating strategy where the female is deceived by the ornamented basitarsi and it is possible that selection for this trait is currently shaping the evolution of this species (Daugeron et al. 2011, Ritchie and Vahed 2011).

A final example of cheating evolving in a system where nuptial feeding is known to be taking place is *Rhamphomyia sulcata* (LeBas 2005). Males in this species typically collect nuptial prey and swarm to attract a female. However, some males in this species have been recorded to collect seeds instead of hunting nuptial prey and present this to the female as a nuptial gift. The females are kept occupied attempting to feed on the seeds, as they would a prey item whilst copulation takes place, allowing the male to transfer most of his sperm at no cost at all.

It is unclear why cheating behaviors evolve alongside genuine gift giving. One reason may be that the risks faced by males from their own predators whilst hunting nuptial prey have begun to outweigh the benefit of amplified male reproductive fitness (measured by investment in and number of offspring). The strategies used by males including empty balloons, mimicry ornaments and replacing prey with a worthless object may all be responses to outside selection pressures. Conversely, males may be deceiving females to their benefit as they enjoy reduced exposure to predators and prolonged copulation with females, resulting in reduction in female fitness.

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CHAPTER 2

A molecular phylogeny of dance flies in the Empis subgenus Enoplempis and the evolutionary origins of mating system associated traits.

Introduction

Much of the biological diversity seen today has been the result of a number of large species radiations. These radiations are often attributed to specific adaptations or 'key innovations' that are associated with specialization in behavioral, ecological and/or physiological traits—and this specialization, in turn, produces differential mating among diverging populations —the primary requirement for speciation (Simpson 1944, Mitter et al. 1988; Winkler and Mitter 2010). Natural selection is often the main driving force behind the evolution and diversification of traits. In many systems, however, Darwin's theory of sexual selection plays an important a role in evolution within populations and ultimately across species (Bateman 1948, Trivers 1972, Ritchie 2007). In modern studies, this theory has been further developed with hypotheses such as Zahavi's (1975) handicap hypothesis and the male brightness hypothesis of Hamilton and Zuk (1982, McLean et al. 2012), where male morphological and colour ornamentation is perceived as signaling "good genes" to females. The impact of sexual selection appears to have had a profound effect on the evolution of many lineages, being studied in many present-day radiations that exhibit a wide variety of secondary sexual ornaments, behaviors and other traits primarily driven by female mate choice. Examples include observations in stickleback fish (Boughman 2001), reptiles (PerryandGarland 2002), amphibians (Emerson 1998), land snails (Sauer and Hausdorf

2009), birds (Moller 1988) and many insect groups (Vahed 1998, Emlen et al. 2005, Gwynne 2008).

When the preference for a variant of the same trait becomes polarized within or between populations, such that individuals with the new trait have differential mating success, this trait can rapidly become fixed within a given population (Ritchie et al. 2007). As mating success remains tied to these trait differences, the result is reproductive isolation and the evolution of novel species. In this way, sexual selection can lead to the rapid evolution of many species over surprisingly short periods of time (Seehausen 2000, Sauer and Hausdorf 2009).

The use of phylogenetic methods for testing evolutionary hypotheses has become a standard tool for biologists interested in the origin and diversification of traits (Felsenstein 1985, Lajeunesse 2009, Garamszegi and Møller 2010). These methods track the sequence of trait modifications and place the independent origins of features through time. Phylogenetic methods can reveal macroevolutionary patterns following acquisition of a given trait and provide insight into ancestral conditions that might have shaped present day distributions, biotic interaction, and patterns of biodiversity. Recently, there has been a surge of studies investigating the impact of sexual selection on the evolution of lineages in a phylogenetic framework; these studies have given more weight to the idea of sexual selection as a common mechanism in species diversification (Basolo 1995, Baker and Wilkinson 2001, Burns 1998, Emlen et al. 2005, Masta and Maddison McLean et al. 2012).

Hyperdiverse groups of insects provide ideal systems to study the evolution of sexually selected traits due to the large amount of variation seen across species and for the larger sample of potential independent origins. For example, the coleopteran superfamily Lamellicornia (the lucanids, scarabs and dynastines) has many representatives which exhibit wide variation in size dimorphism, secondary sexual ornamentation and courtship interactions. Males of some sublineages display varying strategies for attracting mates, including the evolution of weapons used in fighting for dominance and the evolution of less ornamented sneaker males (Emlen et al. 2005). Sexual dimorphism is also notable in many representatives of Orthoptera (Gwynne 2008, Vahed 2007). These ornamentations serve to demonstrate male fitness and survivorship as many of these traits can make an individual more conspicuous to predators or susceptible to disease and have even been hypothesised to be "honesty mechanisms" (Hamilton and Zuk 1982). Many of these secondary sexual morphologies are driven by female choice (Svennson 1987, Eberhard 1996, LeBas 2004, Vahed 1998, Gwynne 2008).

The necessity for males to advertise their genetic fitness to females has also led to the evolution of nuptial feeding systems. There are many examples of this type of behavior across arthropods, including silk wrapped prey items in the spider *Pisaura mirabilis* (Stålhandske 2001), the spermatophylax of tettigoniid crickets, the capture of nuptial prey in Mecoptera and the silken pads of hilarine dance flies (Thornhill 1982, Vahed 2007, Young 2003, Sutherland et al. 2007). There have also been many documented cases of cheating by male insects in systems where males take advantage of ancestral sensory biases in females and present apparently worthless gifts that stimulate female acceptance (LeBas et al. 2005, Vahed 2007). The opposite also occurs in some insect groups where males risk survival to increase their own reproductive fitness to a greater extent than females. The result is sex role reversed systems, where males choose their mates and females evolve elaborate ornamentation to impress the males (Bonduriansky 2001, Gwynne 2008, Vahed 1998).

Sexually selected traits appear to have played a role in at least one of the large species radiations in the insect order Diptera — within the superfamily Empidoidea, a lineage which dates back to the Late Jurassic (155 MYA). The earliest fossils of the monophyletic family Empididae (Moulton and Wiegmann 2007) date back to the Early Cretaceous some 94 million years ago and appear to have a long and rich evolutionary history (Grimaldi and Cumming 1999). The Empididae are well known for their elaborate mating systems and variation in courtship strategies that have been observed across the group (Alcock 1973, Hamm 1928, Kessel 1947, Kessel 1955, LeBas 2005, Svensson and Petersen 1987, Sadowski 1999, Gwynne 2008, Vahed 2007). The subfamily Empidinae in particular is notable for variation in nuptial gifts provided by the male, usually in the form of a captured prey item. In some species of *Empis* and *Hilara,* balloon like structures are produced by silk glands and possibly anal secretions and used in nuptial gift presentation (Becker 1888, Aldrich and Turley 1899, Melander 1940, Kessel 1955, Cumming 1994, Sutherland et al. 2007).

Kessel (1955) hypothesized an eight-stage evolutionary sequence in nuptial feeding interactions beginning with both sexes being predaceous and no nuptial gift, as seen in basal Empidid lineages. Progressive transitions follow with prey presentation from males to females and then to prey wrapped in silk and culminating in empty balloons (Fig i). There have also been studies on male exploitation of female sensory biases, where the males present worthless nuptial gifts, even within species where males usually present a valuable gift to the females (LeBas et al. 2005). Sex role reversed mating systems occur in a high percentage of empidine species with up to 1/3 of known species having heavily ornamented swarming females such as those of *Rhamphomyia longicauda* and *Empis borealis* (Funk and Tallamy 2000, Svennson 1987). The *Empis* subgenus *Enoplempis* includes species with multiple combinations of nuptial feeding traits and thus provides an opportunity to test Kessel's hypothesis in a phylogenetic framework. This subgenus has been observed to exhibit many of the secondary sexual characters seen across Empidinae. Males of most *Enoplempis* species also have femoral-trochantal leg modifications which have been observed to be used in courtship interactions in other dipterans (Ingram et al. 2008); however, the use of these ornamentations has not been observed.

In this study, I use nucleotide data to estimate phylogenetic relationships of *Enoplempis* and to test the monophyly and constituency of the group. Once clades are established, the evolution of nuptial gift types, swarming behaviors and leg modifications can be mapped onto the phylogeny to evaluate their possible role as key traits in the diversification of *Enoplempis*. I also use the new phylogenetic estimate to infer ancestral character states and delimit the origins and effects of newly acquired features during the diversification of *Enoplempis*. In addition, I estimate divergence times to better place the age of the *Enoplempis* split from the main *Empis* lineage*.* A more fully resolved and quantitative analysis of *Enoplempis* provides new understanding of the role of mating systems and sexually selected morphologies in species diversification.

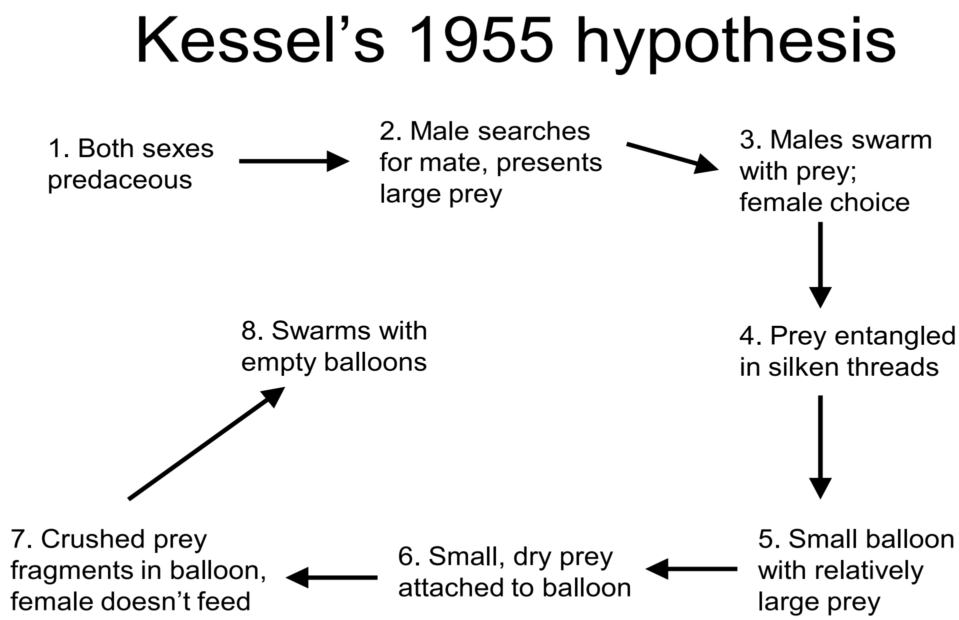

Fig i. Evolutionary sequence of nuptial feeding behaviors hypothesized by Kessel (1955).

Material and Methods

Specimen collection. *Enoplemis* species were sampled in the summer of 2009, spring of 2010 and early summer of 2011 on the Pacific Coast from Southern California to the Canadian border. On the East Coast, specimens were sampled from North Carolina to

Pennsylvania across the Appalachian ranges, Michigan and Ontario were sampled for eastern *Enoplempis* species in summer 2010. During this sampling event, pinned museum specimens, consisting of both western and eastern *Enoplempis* species, were borrowed from the Canadian National Collection of Arthropods and Nematodes for DNA extraction. Where possible, swarm composition and nuptial feeding interactions were recorded in the field at collection localities. Additional behavioral information was obtained from previous studies. All field collected specimens were placed directly into 100% ETOH and stored in the -20°C freezer. All specimens used in this study, collecting and storage information are summarized in supplementary material I.

Taxon sampling. Twenty empidine outgroup taxa were added to the data matrix in order to root the tree; 45 ingroup *Enoplempis* species were included in the final datasets giving a total taxon sample of 65 taxa. An additional 21 empidid outgroups were added for divergence time estimation giving a total dataset of 86 taxa in these analyses (supplementary material I).

DNA extraction. *Enoplempis* species were extracted using the Qiagen DNEasy kit. Abdomens were cut from the specimens and used in the digestion master mix composed of 180µl ATL buffer and 20 µl proteinase K per specimen. Specimens were left to digest at 55 °C for 36 hours. The extractions were then purified and eluted into the final collection tubes. Two elutions of 30 µl were done per specimen giving a total of 60 µl of template.

Gene sampling. For phylogenetic reconstruction, six molecular markers were sampled. 16S rRNA, ND1 and the bar-coding region of COI were amplified from the mitochondrial genome, and regions I and III of the nuclear protein coding gene CAD were amplified. Weak PCR products from CAD PCR reactions were reamplified using alternate internal primer pairs. Amplified products were quantified on agarose gel and bands of the correct length were cut. Genes were purified using QIAquick PCR purification kit (Qiagen) and sequenced by the Genome Sciences Laboratory at North Carolina State University. Primers are summarized in supplementary material II.

Data processing. Contigs were assembled and base ambiguities edited manually in the program Sequencher Version 5.0 (Gene Codes Corporation). Consensus sequences for all genetic fragments were then exported in FASTA concatenated format and initially aligned using the program MUSCLE (Edgar 2004). The aligned data were then imported into the program Mesquite vers. 2.75 (Maddison and Maddison 2011) and manually adjusted to preserve translated amino acid reading frame for protein coding genes, and positional homology or secondary structure for 16S rDNA. Individual gene matrices were concatenated in Mesquite to assemble the final alignment of 3893 base pairs for the phylogenetic analysis.

Phylogenetic analysis. Maximum Likelihood (ML) analyses were carried out using the program RAxML vers. 7.0.4 (Stamatakis 2006). Each data set was analyzed twice with third codon positions of protein coding regions excluded in the second run. In all ML analyses, 1000 iterations were performed and the tree with the best likelihood score was selected as the optimal topology. Support for evolutionary lineages within *Enoplempis* was estimated using 1000 bootstrap replicates. Bayesian inference was also estimated using the program MrBayes vers 3.1.2 (Huelsenbeck and Ronquist 2001).

Again, all matrices were analyzed twice, including and excluding third codon positions in protein coding regions. Four simultaneous MCMC chains were set to 20 million generations for all analyses with trees being sampled every 2000 generations. The burn-in fraction was set to 0.25, discarding the first 25% of all trees. The remaining trees were summarized into a consensus topology with posterior probabilities being used as a measure of clade support. J model test 0.1.1 (Posada 2008) was used to determine the optimal model of sequence evolution for all analyses, which was GTR+r+I for all ML and BI analyses.

Character mapping. Three traits associated with nuptial gift behavior were mapped onto the final tree topology to infer the number of origins and phylogenetic position of specific traits. Nuptial feeding states were binary coded, (0) for nuptial prey presentation and (1) for use of balloons as gifts. Male leg clasper modifications were also mapped on the tree. Femoral-trochantal spines were coded as presence or absence character states, with (0) present and (1) absent. Known swarming sizes were also mapped onto the phylogeny but not used for ancestral state reconstruction due to the low number of observations. Table I summarizes character states. Supplementary table III shows full coding with respect to taxa.

Table 1. Summary of character states.

Nuptial feeding	Prey Presented (0)	Balloon Presented (1)
Leg Modification	Absent (0)	Present (1)
Balloon Type	Contains prey (0)	Empty (1)

Ancestral state reconstructions. Mesquite vers. 2.75 (Maddison and Maddison 2011) was used to reconstruct ancestral states on the phylogenetic tree estimate using ML and a Brownian motion model of trait evolution where transitions between states are equally likely. This was done to further study the evolutionary sequence of nuptial gift giving strategies. To investigate the ancestral ballooning condition, an analysis was conducted on a smaller subset of species known to balloon. States are summarized in Table I.

Divergence time estimation. BEAST vers 1.7.1 (Drummond and Rambaut 2007) was used with the molecular data to estimate the age divergence of *Enoplempis* from its nearest *Empis* sister lineage and to estimate the time of ancestral origin of ballooning behavior in the subgenus. The divergence times analysis was calibrated using two Cretaceous fossil Empididae: *Protoreogeton admirabilis (*165 mya) [minimum origin of Empidoidea], and *Turonempis styx* (93 mya) [minimum age of the subfamily Empidinae] (Grimaldi and Cumming 1999). All genes were individually partitioned and the codon positions for each gene were partitioned to allow positions 1+2 and 3 to be analyzed under differing parameters. A relaxed lognormal molecular clock model was chosen allowing for rate variation for different gene partitions over time. BEAST requires that a bifurcating tree is used for the divergence time estimations, so the best maximum likelihood topology was chosen as the starting tree. The Yule speciation model was chosen for the model of species evolution. In order to further test the hypothesis that ballooning *Enoplempis* species share a common ancestry, the clade containing all ballooners was constrained to be monophyletic in this analysis. Representatives of the subfamily Empidinae were also constrained as monophyletic in BEAST analyses.

Results

Phylogenetic relationships of Enoplempis (Fig II). Both maximum likelihood and Bayesian analysis yielded trees that agree on the major groupings within *Enoplempis*. *Enoplempis* was strongly supported as monophyletic (pp=1, BS=97). The type species, *Empis mira,* was placed as sister to all other members of the subgenus (node A). This group was divided into two well supported monophyletic groups (BandC) (pp=1,BS=96 and pp=1, BS=99, respectively). Four (Nodes D-G) subgroups delimited major clades within these groups (pp=0.98, BS=65). Groupings within these lineages were less certain, with low to moderate support for relationships among terminals. Subgroup four (Node G), which contained all known ballooners and had relatively strong support from the Bayesian result, was moderately supported in the maximum likelihood analysis (pp=0.94, BS=55). Subgroup three was placed as closest relative to the ballooner clade (Figure ii).

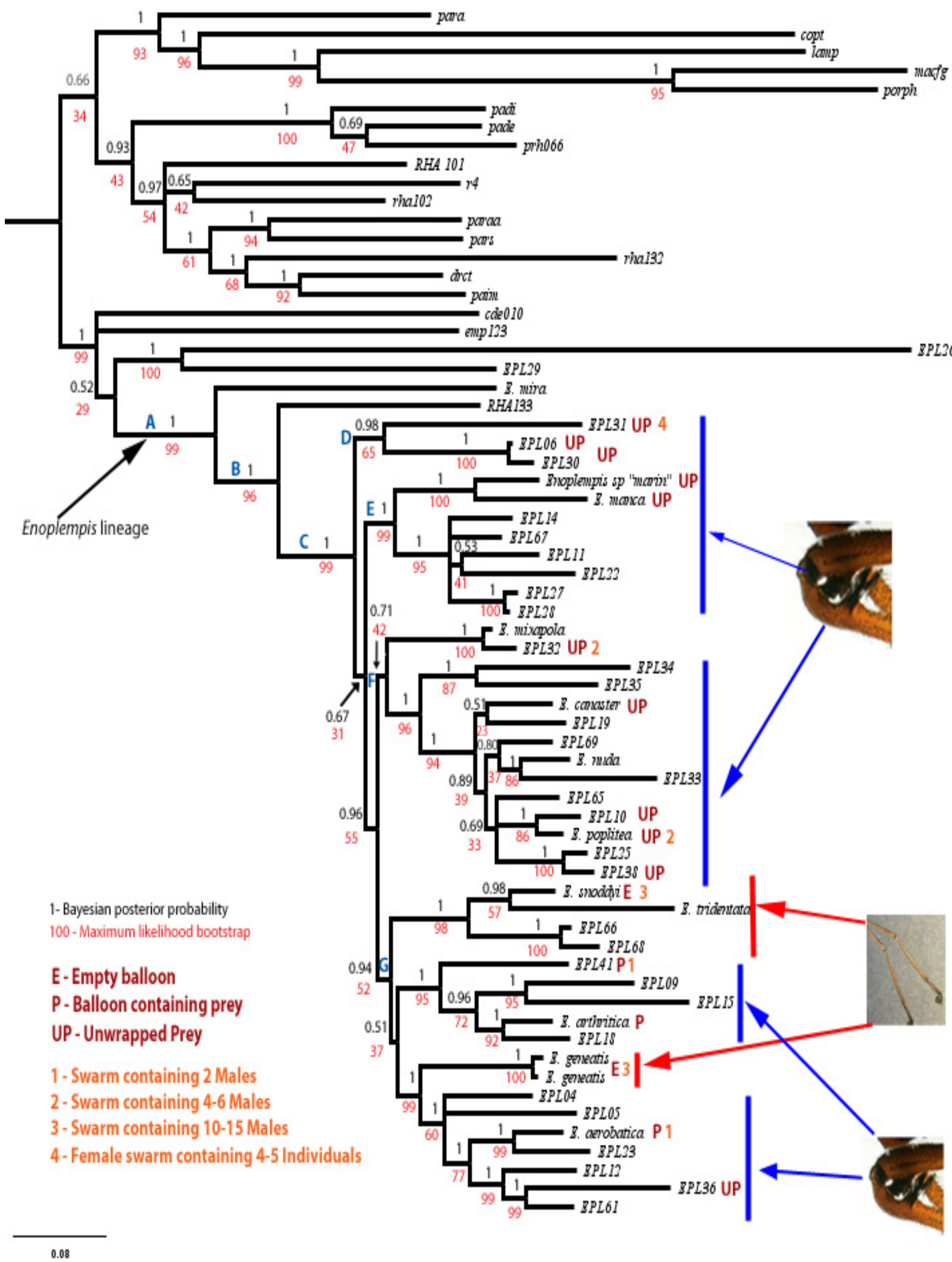

Figure ii. Molecular phylogeny and character mapping of known *Enoplempis* traits. Blue bars cover species with modified legs. Red bars cover species with unmodified legs.

Character mapping. Mapping nuptial feeding states across the *Enoplempis* phylogeny revealed that groups one, two and three contain representatives that exhibit full, unwrapped prey presentation behaviors. All five known ballooning species occured within the same strongly supported subgroup despite the variations seen in ballooning behavior. Leg modifications were found in representatives of all groups. Only group four contained representatives that had no leg modifications, and these were nuptial balloon making species (*E. snoddyi* and *E. geneatis,* and related taxa with unknown nuptial behavior, EPL66 and EPL68). Swarming behaviors were variable across the phylogeny with no distinct pattern for any given subgroup. The number of individuals comprising swarms was highly variable across the tree, varying widely even in closely related species. For example, EPL41 contained just two swarming males (Turner, *in prep*) whilst *E. snoddyi* had up to 15 (Sadowski et al. 1999). Sex role reversal was only found in subgroup one. The distribution of these characters is summarised in Figure ii.

Ancestral state reconstruction. Maximum likelihood based ancestral state reconstruction of nuptial feeding behaviors (Fig. iii) in the subgenus *Enoplempis* revealed that the ancestral condition is whole prey presentation. This trait appears to be distributed over all *Enoplempis* subgroups with the exception of the balloon fly-making subgroup four. The ballooning condition appears to be a derived nuptial gift presenting condition in *Enoplempis*. This result strongly supports the hypothesis that ballooning behaviors arose from a single common ancestor in this sub genus and that the balloon-making flies are monophyletic. The reconstruction of the evolution of the leg modification in *Enoplempis* males revealed that clasping modifications arose in the common ancestor of all *Enoplempis* and these were lost independently in both *E. snoddyi* and *E. geneatis.* (Fig. iv). Finally, identifying the exact origin of ballooning condition was ambiguous due to missing information on this characteristic for many of the taxa included here (Fig. v).

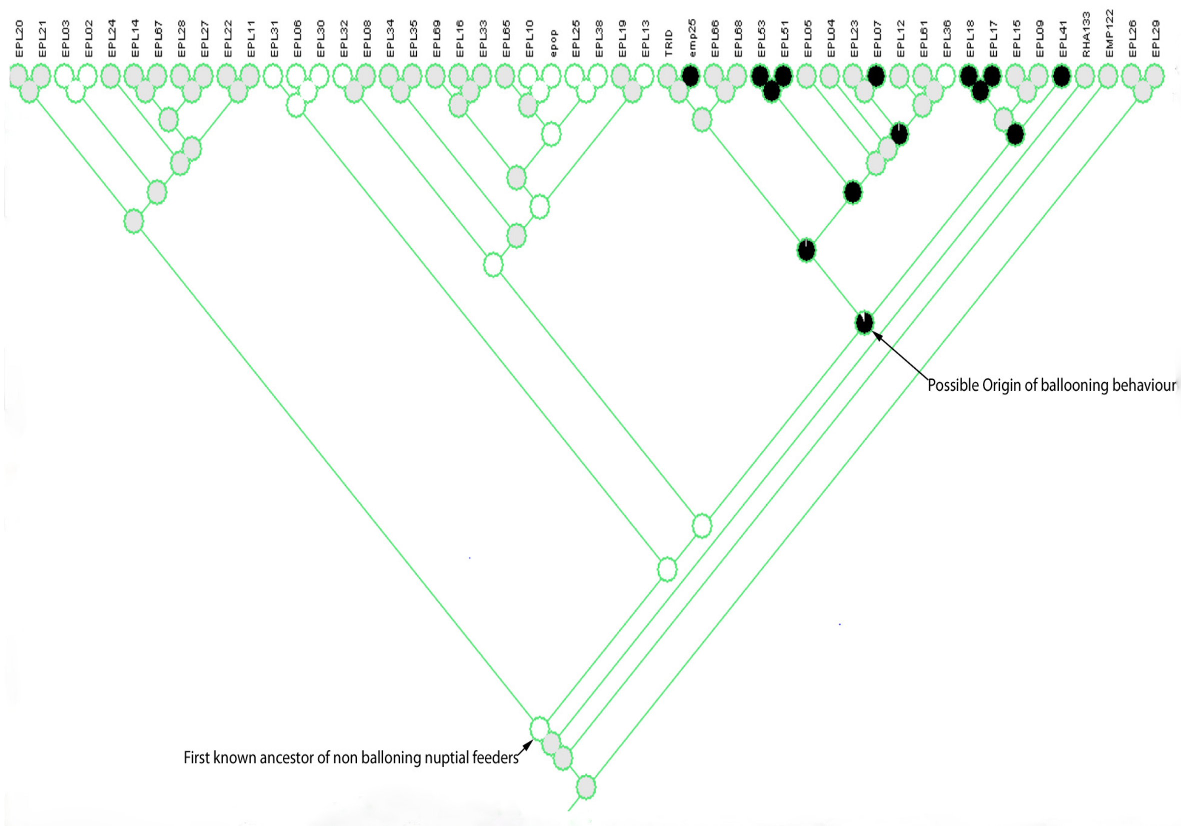

Fig. iii. Ancestral nuptial behaviors within *Enoplempis*. Black states represent ballooning behaviors; white states represent unwrapped prey. Grey represents unknown.

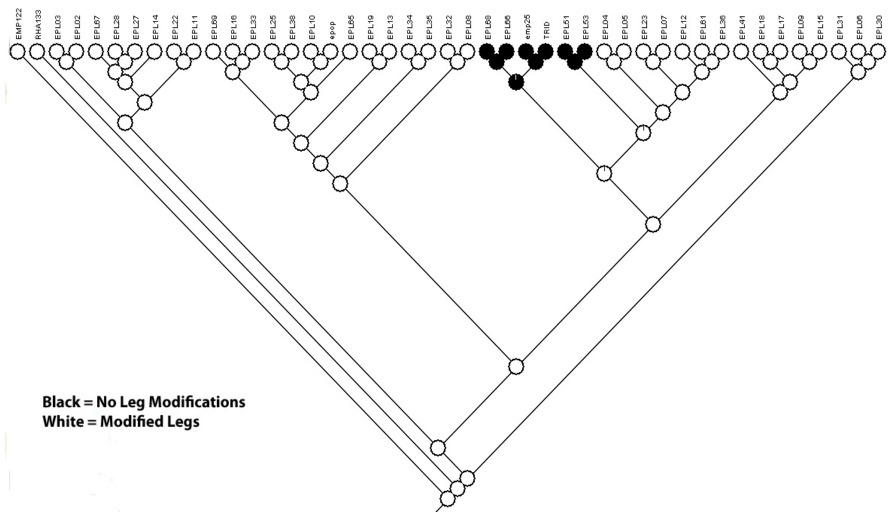

Fig iv. Maximum likelihood reconstruction of ancestral condition of leg modification.

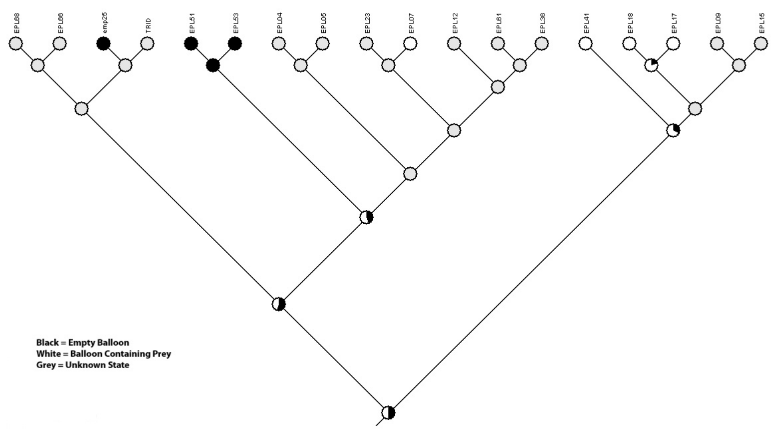

Fig. v. Maximum likelihood reconstruction of ancestral ballooning condition in *Enoplempis.*

Divergence time estimations (Fig vi). Calculation of divergence times in BEAST resulted in the same topology as the ML best tree and the MrBayes run for the *Enoplempis* lineage. Tree congruence from these three sources indicates general agreement in support for the current best estimate tree topology. The BEAST analysis showed that the empidid subfamilies began to split from their most recent common ancestor 151.33 million years ago. Within the subfamily Empidinae, the Hilarini and Empidini began to diverge from one another around 95 million years ago. The *Enoplempis* lineage split from its closest sister taxon 44.46 million years ago. The rapid diversification of *Enoplempis* subgroups began 19.72 million years ago. According to our data, the ballooning dance flies arose from their last common ancestor 17.81 million years ago. The *Enoplempis* sub lineages appear to have undergone successive rapid speciation events; this is reflected in the short periods between divergence times and is mirrored by the branch lengths.

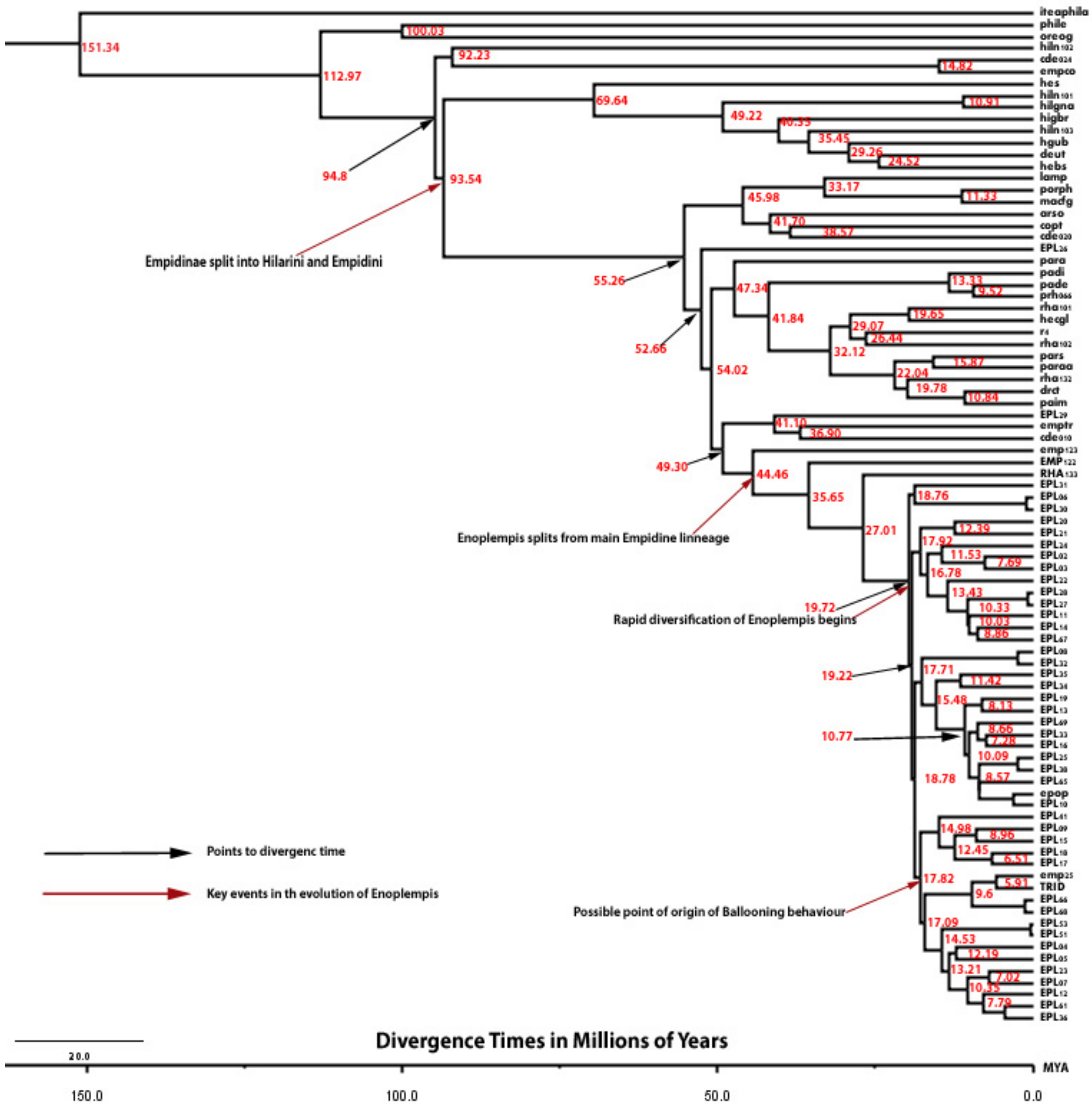

Figure vi. Divergence time tree showing important events in the evolutionary history of *Enoplempis*.

Discussion

Phylogenetic relationships of Enoplempis

Our phylogenetic analyses (Fig ii) show that *Enoplempis* is a monophyletic lineage (pp=1, BS=99). Based on the current data, the sister lineage to *Enoplempis* is not certain, but the placement of the group is near the *Empis (Xanthempis)* lineage or the *Rhamphomyia (Holoclera) flava* lineage. The placement of a *Rhamphomyia* sub lineage close to a strongly supported *Empis* group reveals the current classification system is comprised of a series of smaller subgroups and two basal lineages. The type species, *E. mira,* and unidentified taxon RHA133 are basal to the larger radiation of the subgroup. The Bayesian and ML analyses both agree on the common tree shape; however, the ML bootstrap is less well supported than the Bayesian tree, with several nodes being weakly supported in this framework. Both trees agree that the monophyly of subgroups I-IV is strongly supported (PP=1, BS=99). Subgroup 1 is also strongly supported in both analyses (pp=0.98 BS = 65). However, the support values for the leading to the other *Enoplempis* subgroups are in conflict between the two tree topologies. The monophyly of subgroups two, three and four is weakly supported in both trees (pp=0.67, BS =31). The monophyly of the balloon flies to their closest sister group and the monophyly of the ballooning clade itself (Node G) are both strongly supported in the Bayesian phylogeny (pp=0.96 and 0.94 respectively) but only moderately supported in the ML analysis (BS =55 and 52 respectively). This conflict may be due to the fact that Bayesian posterior probabilities tend to overestimate support for nodes and ML bootstraps underestimate support for nodes; the nature of this rapid radiation may amplify the disagreement between the two support scores (Zhang et al. 2012, Alfaro et al. 2003). The subgroups all stem from a series of short branches (recovered in both ML and BI analyses), which suggests that a rapid radiation occurred in *Enoplempis* as the sub lineages within the group became more derived. Sister group relationships between the major groupings within subgroups I-IV are mostly well resolved with some ambiguities occurring at the tips of the phylogeny, regarding sister species relationships. This is most noticeable in subgroup II. In contrast, subgroup IV has good resolution at the tips with the only ambiguous node being the split between the second and third sub sets of ballooning flies (pp=0.51, BS=37). These uncertainties could likely be resolved by the addition of extended taxon sampling and the inclusion of additional nuclear and mitochondrial genes to the current dataset. Overall, the resulting phylogeny has a robust structure against which evolutionary hypotheses on character evolution and splitting of lineages can now be tested.

Evolution and diversification of traits

Character mapping (Fig. ii) reveals that all subgroups have representatives which have known nuptial feeding and swarming behaviors. Unfortunately, limited behavioral observations and the difficulty of finding *Enoplempis* mating swarms have made it impossible to completely map out this characteristic for all taxa included in this study. However, information from the literature and several primary observations by the authors has allowed the mapping of these traits in order to better understand their variation among taxa. Empidids are known to have excellent vision and have been observed to recognize conspecific swarming patterns (Alcock 1973). This is important as it may function as a barrier to the hybridization of closely related species or divergent populations. The mapping of known swarming states show that there is considerable variation across *Enoplempis*. *Empis poplitea*, *E. snoddyi, E.geneatis, E. arthritica, E. aerobatica* and new species EPL 41 all have male swarms; however, the number of individuals in the swarm varies considerably from as few as two males in the case of EPL41, to up to 15 in *E. snoddyi* (Turner, *in prep*, Sadowski et al. 1999). Species EPL 31 shows another variation to swarming behavior and exhibits sex role reversal, containing swarming females with secondary sexual ornaments. This is the first time this has been observed in *Enoplempis* (Turner, *in prep*). The variation in swarming strategy across the phylogeny suggests that preference of this trait and its polarization is a potentially important isolation mechanism and has been observed in other dipterans, suggesting that swarming cues are important for species recognition in diverse groups of flies (Downes 1969, Diabaté et al. 2011).

Enoplempis group IV contains species where only ballooning behavior has been observed, supporting our hypothesis that balloon making in *Enoplempis* is a derived behavioral state. Within this subgroup it appears that the ballooning behaviors themselves have become derived with representatives exhibiting behaviors varying from balloons containing complete prey insects to completely empty balloons. The ancestral state reconstruction (Fig. v) of ballooning did not reveal the exact origins of the ballooning condition. This was due to the lack of behavioral data points for this trait; additional observations are required on the tips of the phylogeny in order to answer this question. The single point of origin for all ballooning species is also supported by the results of the divergence time estimations, which show that group IV (and all known ballooning species) split from the prey presenting *Enoplempis* lineages 17.81 million years ago, shortly after the initial rapid diversification of the four main subgroups that began 19.72 million years ago. These findings do have some concordance with Kessel's 1955 hypothesis in that the ballooning condition is the most derived; however, it is not clear which ballooning behaviors (e.g., with or without included prey) are the most derived based on the data presented here. There is also no observable sequential pattern on the evolution of swarm complexity; however, this may be due to lack of observations. The utilization of silk to produce nuptial gifts has been recorded in several species of hilarine dance flies (Young 2003, Eltringham 1928, Sutherland et al. 2007). This behavior has only been found in *Enoplempis* in the empidine lineage, suggesting two highly divergent, independent (at least 94 million years since the last common ancestor) origins of this nuptial gift strategy in Empidinae.

The femoral-trochantal spines seen in many species of *Enoplempis* are found across all subgroups. In groups I, II, and III all species sampled show a wide range of species specific modifications. In group IV, most species have these leg modifications, with the exception of two subgroups: 1) *Empis* snoddyi + *E. tridentata* + EPL 68 and 69 and 2) *Empis geneatis*. Both *E. snoddyi* and *E. geneatis* produce empty balloons and have no leg modifications. The ancestral state reconstruction of this trait shows that in *Enoplempis,* the ancestral species had leg modifications and that legs lacking these structures are the derived condition. The current taxon sampling suggests that this trait evolved twice independently in the groups containing individuals that are known to produce empty balloons. Although the use of this morphology has yet to be observed, a pattern is emerging that may associate leg modification in *Enoplempis* species with prey capture. Sepsid flies have similar structures which have been observed to be used by males for hooking onto the wings of the female during mating interactions (Ingram et al. 2008). However, in *Enoplempis,* these traits are only present in species which capture prey for nuptial feeding interactions, or in ballooning species which capture and incorporate prey items into their balloons. This trait is not observed in *E. snoddyi* or *E. geneatis,* which are known to produce empty balloons, or in closely related species such as *E. tridentata,* for which no observations have currently been made. Sepsid males use this trait in order to prolong mating with the female. It may be the case that males who have captured and invested nuptial prey in *Enoplempis* species which have modified legs use them to prolong mating and thereby ensure that they gain maximum benefit from this investment. Conversely, based on the current knowledge of *Enoplempis* mating behaviors and the results of the ancestral state reconstruction, it is that the leg modification is associated with prey capture, given its distribution among the prey capturing taxa. Alternatively, this morphology may be used for the manipulation of prey during swarming and transfer to the female during courtship in species that capture prey. In addition, these structures may also be used for manipulating prey during balloon making in ballooning species. The loss of such characteristics in empty balloon making species makes sense under these hypotheses as they swarm and interact with females in a similar way to species which incorporate prey into their balloons. This may be evidence that this structure may not be used for copulation in *Enoplempis* as it is in Sepsidae because the potential loss of a characteristic beneficial to a mating interaction is detrimental to the reproductive fitness of the males (Kirkpatrick 1982). In contrast, the loss of a secondary sexual structure (or any morphology) that has become redundant may be advantageous because no resources are invested into developing this structure, as may be the case in *E. snoddyi* and *E. geneatis.* It is possible that the loss of hunting behavior and the leg modifications may be due to external ecological pressures such as the increased risk of predation resulting in the male not investing in capturing nuptial prey. The loss of any morphology would have a profound effect on the behavior and ecology of most organisms (Kopp and True 2002, Ellers et al. 2012).

Sexually selected traits increase rates of species diversification

Both phylogenetic analyses and divergence time estimates suggest that a rapid radiation occurred within *Enoplempis*. The distribution of secondary sexual characters including nuptial feeding behavior, swarming behavior and secondary sexual morphologies indicates that these many variations of courtship interaction across *Enoplempis* may have driven this rapid evolution of taxa. The short branch lengths in both the phylogeny and time tree, and the short periods between branch times seen in the main lineage of *Enoplempis* support this hypothesis. The basal taxa including the type species, *E. mira*, split from the main lineage of *Empis* 45.5 million years ago (Fig. vi) over a time period of approximately 7.5 million years. Compared with this, all sub lineages appear to have split over a relatively short time period. All subgroups split over the course of 1.91 million years, diverging between 19.7 and 17.8 million years ago. The frequency of splits in subgroups two, three and four continued to be rapid with most of the present known diversity of *Enoplempis* appearing by 7 million years ago. Group four in particular shows a rapid diversification of species. The increase in diversification rate as indicated by short branch lengths and short branch times, along with the known courtship traits of *Enoplempis*, suggest that sexual selection may play a role in the rapid evolution of this subgenus. Behavioral observations and experiments to determine mating success and effects of nuptial gifts, sexual ornaments, and swarming on fitness and mating preferences are required to support this general phylogenetic pattern.

However, sexual selection may not solely be responsible for the diversification of *Enoplempis*. From the information obtained from the divergence time estimates, the ancestral species of the current representatives of *Enoplempis* appeared during the late Eocene/early Oligocene 34 million years ago. It was not until the early Miocene around 15 million years later that the rapid diversification of the wider radiation occurred. During the course of the Miocene, in several geographic regions, including North America, grasses replaced large areas of forest (Strömberg 2004). Thus, environmental change may have impacted the evolutionary direction of *Enoplempis* due to the fact that these flies are typically found in, or at the fringes of, wooded areas (Sadowski 1999). Habitat fragmentation caused by the regression of forests and the rise of grasses may have resulted in the geographic isolation of *Enoplempis* populations which then continued on separate evolutionary paths into different species.

Darwinian natural selection may also have contributed to the evolution of some lineages of *Enoplempis*. It has been recorded in other groups of dance flies that secondary sexual ornamentation correlates closely with an increased risk of predation. For example, in *Rhamphomyia longicauda*, heavily ornamented females appear to be at increased risk from entanglement in spider webs whilst swarming (Gwynne and Bussie`re 2002, 2007). This might be the case in males of *Enoplempis*, and unmodified legs may have evolved as a result. If this trait is associated with the evolutionary loss of prey capture, it is possible to hypothesize that the risk of foraging for prey had began to outweigh the benefits of a nutritional gift, resulting in its loss. Similarly, a shift in investment and risk to individual fitness by both parents, where male foraging becomes increasingly risky, probably resulted in sex-role reversal in EPL31. Male investment and risk to fitness has been observed in many groups of vertebrates and invertebrates (Vahed 1998, Thornhill 1976).

Finally, some of the variation seen in *Enoplempis* species may be due to male exploitation of female sensory biases. In the case of the balloon flies, it may be possible that males in species that produce empty balloons have taken advantage of the nuptial feeding system by evolving a nutritionally worthless gift, and therefore invest minimally in their offspring. It has been observed in *Rhamphomyia suclata* that males of this species utilise alternate courtship strategies; some males present a nutritional gift whilst others present the female with seeds (LeBas 2005). “Cheating” in various forms as alternative strategies has also been observed in other insect groups with the most notable examples being in Orthoptera and Mecoptera (Vahed 2007, Thornhill 1976).

Conclusion

This study represents the first attempt to examine the influence of sexually selected characters on the diversification of a group of dance flies from a phylogenetic perspective. The results of the phylogenetic and macroevolution analyses suggest that sexually selected traits can be important in the diversification of *Enoplempis* species. These traits also appear to affect the rate of diversification in the main radiation of *Enoplempis*. Sexually selected traits may have driven the diversification of the wider Empididae, which have groups which exhibit even broader variation in some of the traits discussed in this study. The results of this study also appear to agree with some aspects of Kessel's 1955 hypothesis, in that the ballooning flies appear to exhibit the most derived behaviors; however, there are not sufficient data points here to fully address how this system evolved. Additional behavioral observations are also needed in order to better understand the ancestral conditions for swarming and nuptial feeding traits for the four sub lineages of *Enoplempis*. Further observations of behavioral traits and marker sampling are needed in this group to fully resolve some of the ambiguous sister group relationships and to increase the support for ambiguous nodes, in particular the subdivision of the ballooning species. This study has provided a focused example of some of the characteristics that have driven the evolution of the wider Empididae over a much longer time period. Future work should involve the study of other selected focal groups to enable the meta-analysis of mating systems and traits across Empididae in a phylogenetic comparative context (Felsenstein 1985, Lajeunesse 2009, Garamszegi et al. 2010). This study may also be useful as a starting point for studying the effect of secondary sexual characters on the evolution of populations of wide ranging species of *Enoplempis* such as *E. snoddyi* and *E. poplitea* to investigate how they are influencing the possible divergence of these populations in the present day. Future work should involve a combination of continued behavioral observations and molecular genetic analyses to recover both the evolutionary history of the species and traits being studied and to identify the genes which are important in behavioral pathways and for the expression of key morphological traits. Next generation sequencing technologies will make these goals achievable and may prove to be valuable tools for studying the impact of the evolution of sexual selection on insect groups at the deeper phylogenetic level and in present day populations of species.

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CHAPTER 3

Phylogenetic relationships of flies in the family Empididae (Insecta: Diptera: Empidoidea)

Introduction

The empidoid flies represent a large and phylogenetically challenging radiation of dipterans consisting of over ten thousand known species and with many more expected to be discovered. Since an initial effort by Chvála (1983) to place empidoid groups in a phylogenetic classification, there have been seven attempts to resolve the phylogenetic relationships of Empidoidea using both molecular and morphological data. The initial study which drove this flourish in empidoid research resulted in a four family classification system Atelestidae, Hybotidae, Microphoridae + Dolichopodidae, and Empididae s.str based on morphological evidence (Chvála 1983). Further phylogenetic based classifications of Empidoidea have been published, four based on morphological data (Wiegmann et al. 1993; Cumming et al. 1995, Yang 2004, and Sinclair and Cumming 2006) and three using molecular sequence data (Moulton and Wiegmann 2007, Collins and Wiegmann 2002 and Moulton and Wiegmann 2004). Moulton and Wiegmann (2007) established a solid framework for the classification of Empidoidea based on molecular phylogenetic data. Their molecular results agreed in large part with the original classification of Chvála suggesting that his 1983 study laid a valid framework for a multi–family classification of Empidoidea, which previously had been broken into just two families, the Empididae and Dolichopodidae. Despite the new evidence, there is still a great deal of uncertainty and controversy regarding placement, taxonomic composition and subfamily-level relationships within the empidoid families. For example, the family Empididae has not been thoroughly sampled in studies involving the wider Empidoidea and there are a large number of genera that are still considered "Empididae *insertae sedis*"—meaning, we still do not know where or whether these groups are placed within the family. Morphology based analyses have proven to be ambiguous when resolving the subfamily level relationships of Empididae and have resulted in polyphyletic subfamilies and several genera being unplaced into any subfamily. Sinclair and Cumming (2006) raised a new empidoid family the Brachystomatidae, comprised of the brachystomatine, ceratomerine, and trichopezine empidids. This family has not been recovered in any other study and the most recent molecular phylogeny of Empidoidea (Moulton and Wiegmann, 2007) places the constituent taxa in various positions nested within the Empididae. The same study suggests that a family Empididae (*s.s*) can be delimited as a strongly supported monophyletic group with its placement within the Empidoidea well understood. However, analyses of molecular data have been unable to clearly resolve ingroup relationships between the subfamilies of Empididae (Moulton and Wiegmann 2007).

The objective of the current study is to focus on the subfamily level relationships of Empididae in an attempt to delimit monophyletic lineages by building on the work of Moulton and Wiegmann (2007) using a selective based taxon sampling strategy and extending nuclear gene sampling. The nuclear genes AATS, PGD and TPI have been used recently in projects aimed at resolving dipteran relationships at multiple taxonomic levels (Wiegmann et al. 2011, Bayless 2012). New sequences obtained for these genes are added to an existing dataset composed of previously sampled genes (CAD, 28S, COI) from Moulton and Wiegmann (2007) for selected taxa chosen to test hypotheses about the phylogenetic position and taxonomic membership of clades in the family Empididae.

Specifically we will address the following questions:

1. What are the limits of the Empididae sensu stricto?

2. Which empidoid family is most closely related to the Empididae?

3. What are the relationships of the genera of the empidid subfamilies and are traditional subfamilies monophyletic?

4. What is the sister group to the subfamily Empidinae?

5. What is the divergence time of Empididae from its closest empidoid sister taxon and when did the empidoid subfamilies began to in the context of a molecular clock analysis?

Material and Methods

Additional gene sampling. To investigate placement of Empididae within Empidoidea and the position of several genera of uncertain placement, we added 19 mitochondrial cytochrome oxidase sequences (COI) to a phylogenetic dataset constructed from data In Moulton and Wiegmann (2007) for 32 empidoid and outgroup taxa (Table 1). The Moulton and Wiegmann (2007) data included a 3918 bp fragment of the CPSase region of the nuclear protein coding gene CAD, and 1337 bp of the large subunit ribosomal gene (28S). Sequences for outgroup taxa were available from the FLYTREE AToL project (Wiegmann et al. 2011). All taxa sampled and genes amplified for the chosen taxa are summarized in supplementary table I. Three additional nuclear markers were targeted and amplified for 15 taxa considered in previous studies to be close relatives or early diverging representatives of the Empididae. Representatives of all Empididae subfamilies were included in the novel amplifications, with the exception of the Clinocerinae and Ceratomerinae due to problems in amplifying the template DNA. 557 bases of the protein coding gene AATS, 772 bases of the nuclear protein coding gene PGD and 565 bases of the nuclear protein coding gene TPI were amplified for the selected taxa giving the alignment 1894 novel molecular characters and a total of 3274 additional characters to add to the data set of Moulton and Wiegmann (2007). The total final alignment consisted of 8529 molecular characters. All additional markers were amplified from existing templates from stored DNA from the previous molecular study by Moulton and Wiegmann (2007). Amplified products were quantified on agarose gel and bands of the correct length were cut. DNA fragments were purified using QIAquick PCR purification kit (Qiagen) and sent to the Genome Sciences Laboratory at North Carolina State University for sequencing. Primer pairs used for additional data acquisition are summarized in Table II.

Table I. Taxa and additional genes sampled.

Taxon	AATS	PGD	TPI
Apalocnemis sp.	YES	YES	NO
Chelipoda sp.	YES	YES	NO
Empidadelpha sobrina	YES	YES	YES
Gloma fuscipennis	YES	YES	NO
Hesperempis sp.	YES	YES	NO
Hilara sp. (Hiln 103)	YES	YES	YES
Hilara sp. (Hiln 106)	YES	YES	NO
Hilarempis sp.	YES	NO	YES
Hormopeza sp.	NO	NO	NO
Oreogeton scopifer	YES	YES	YES
Iteaphila sp.	YES	YES	NO
Metachela coousor	YES	NO	NO
Neoplasta concava	YES	YES	NO
Rhamphomyia sp.(Rha 101)	YES	YES	YES

Table II. Summary of primer pairs used to amplify additional genes.

Gene	Forward Primer	Reverse Primer
AATS	AATS 39F (Wiegmann et al. 2011)	AATS 244R (Wiegmann et al. 2011)
PGD	PGD 2F (Regier et al. , 2008)	PGD 4R (Regier et al. , 2008)
TPI	TPI 111Fb (Bertone et al. 2008)	TPI 277R (Bertone et al. 2008)

Data processing. Contigs were assembled from forward and reverse primed sequences and base ambiguities edited manually in the program Sequencher Version 5.0 (Gene Codes Corporation). All new sequences were aligned manually in the program Mesquite vers. 2.75 (Maddison and Maddison 2011). Protein coding regions were translated into amino acids to ensure reading frame accuracy.

Phylogenetic analysis. Maximum Likelihood (ML) analysis was done using the program RAxML vers. 7.0.4 (Stamatakis 2006). Each data set was analyzed twice with third codon positions of protein coding regions excluded in the second run. In all ML analyses, 1000 iterations were performed and the tree with the best likelihood score was selected as the optimal topology. 1000 bootstrap replicates were performed to assess reliability of empidoid relationships recovered in the ML analysis. Bayesian inference was also estimated using the program MrBayes vers 3.1.2 (Huelsenbeck and

Ronquist 2001). As with the ML analyses, all matrices were analyzed twice, including and excluding third codon positions in protein coding regions. Four simultaneous MCMC chains were set to 50 million generations for all analyses with trees being sampled every 5000 generations. The burn-in fraction was set to 0.25, discarding the first 25% of all trees. The remaining trees were summarized into a consensus topology with posterior probabilities being used as a measure of clade support. J-model test 0.1.1 (Posada 2008) was used to determine the optimal model of sequence evolution for all analyses, with GTR+r+I chosen as the most appropriate model for all ML and BI analyses, and for all gene regions. Two asiloid taxa and two cyclorrhaphans were used to root all resulting trees.

Divergence times estimations. BEAST vers 1.7.1 (Drummond and Rambaut 2007) was used to estimate divergence time for each of the empidoid and empidid lineages (e.g., subfamilies) found in this study. The program BEAUTI vers 1.7.1 (Drummond and Rambaut 2007) was used to build the XML files for the divergence time analysis. The Bayesian topology was used as the starting tree for BEAST analysis. Ten, minimum-age calibration points were based on fossil specimens of empidoid flies, including specimens considered to be the earliest known representatives of Empidoidea and the oldest known representatives of the extant empidoid families (Grimaldi and Cumming 1999, Gimaldi and Engel 2005). Nine clades were constrained as monophyletic to enforce topological agreement between BEAST, ML and BI analyses. In the BEAST analysis each gene was treated as a separate partition and codon positions for each protein coding gene was further partitioned by site. A relaxed lognormal molecular clock model was chosen allowing for rate variation for different gene partitions over time. The Yule speciation model was chosen for the model of species evolution. Finally the MCMC chain was set to run for fifty million generations with trees being sampled every 1000 generations. The final tree was obtained by importing the output of the BEAST analysis into the program Tree Annotator vers 1.7.2 (Drummond and Rambaut 2007). Burnin was set to 20,000 and the remaining 30,000 trees were summarized into a consensus tree topology. Tree topologies from all analyses were opened and edited using the program Fig Tree vers 1.3.1 (Drummond and Rambaut 2007). Fossil calibration dates and groups (including suborder and superfamily, family and subfamily groupings) constrained to monophyly are summarized in Table III. All fossil calibration points are taken from Grimaldi and Cumming (1999), Grimaldi and Engel (2005) and Sinclair and Kirk-Spriggs (2010).

Table III: Fossil calibrations and monophyly constraints of included empidoid families and empidid subfamilies.

Phylogenetic Group	Oldest Known Fossil (Million Years)	Monophyly constraint
Atelestidae	112	NO
Dolichopodidae+Microphorninae	125	YES
Empididae	138	YES
Empidoidea	155	YES
Empidinae	N/A	YES
Hemerodromiinae	N/A	YES
Hybotidae	125	YES
"Group A"	N/A	NO
"Group B"	N/A	NO
"Group C"	N/A	NO

Results

Phylogenetic analysis

The full 6-gene, 32 taxon, data set included 8529 aligned sites. Empidoidea is recovered as monophyletic with high support in all analyses (PP=1, BS=93). Four main groups of empidoid families are recovered. Atelestidae, Empididae and Hybotidae all form strongly supported monophyletic lineages (PP=1, BS=98; PP=1, BS=99; PP=1, BS=93 respectively). Dolichopodidae+Microphorinae form a less well supported group (PP=0.77, BS=48), however all groupings are consistent in the Bayesian consensus topology and the ML best tree topology. The genera found in the Empididae *sensu stricto*, from the classifications of Chvála (1983) and Moulton and Wiegmann (2007) are recovered as monophyletic .The sister lineage to Empididae s.s. is recovered as Dolichopodidae + Microphorinae but was weakly supported. Five subgroups of Empididae are recovered, only Hemerodromiinae and Empidinae are recovered as monophyletic. The Hemerodomiinae is strongly recovered here as sister to the Empidinae (PP=1, BS=84). The remaining three subgroups (labelled A, B and C) are comprised of taxa which are polyphyletic according to current classifications (Chvála 1983, Sinclair and Cumming 2006).

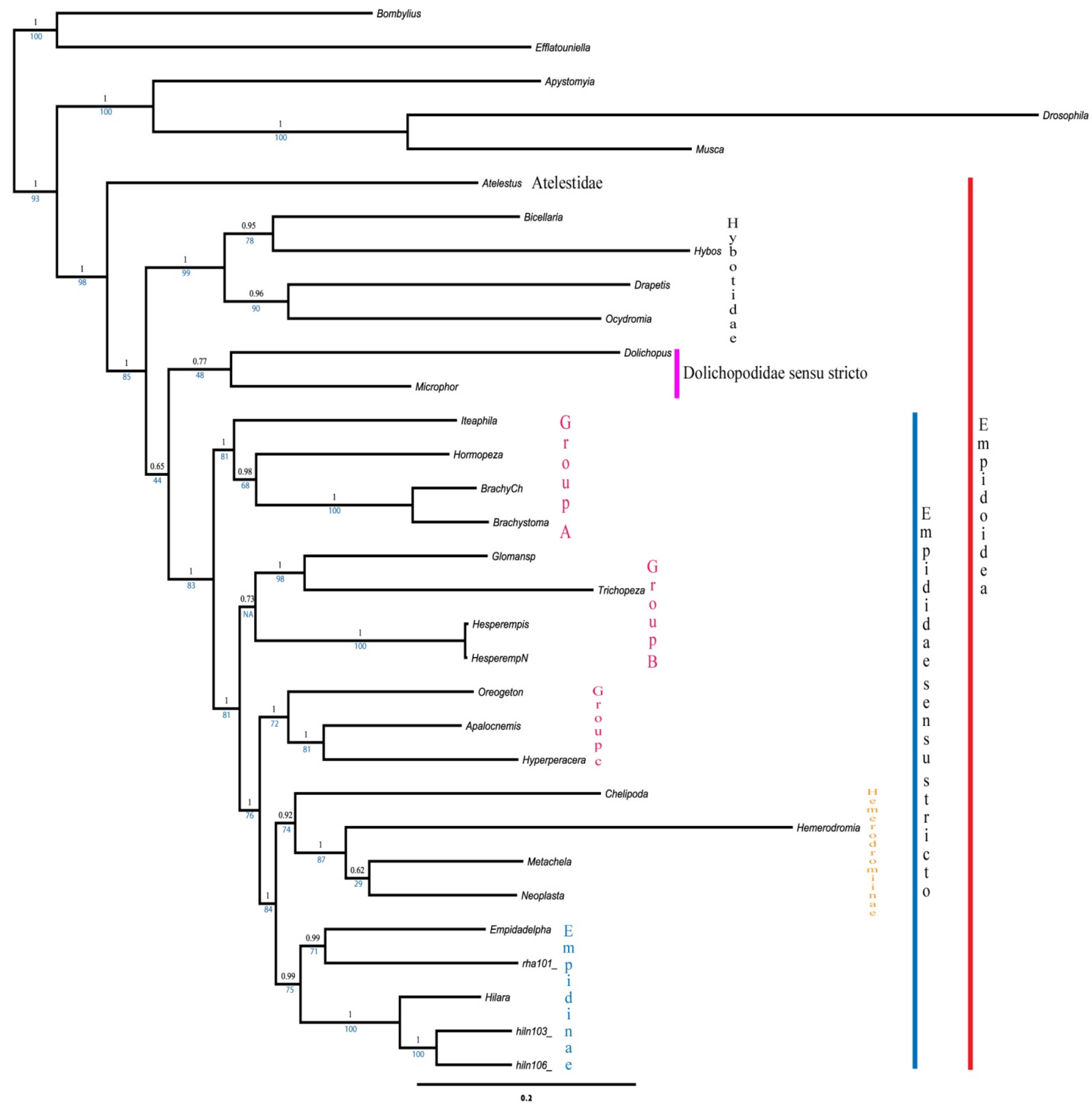

Fig I. Phylogenetic relationships of Empididoidea and Empididae subfamilies. Numbers below nodes represent ML bootstraps. Numbers above represent Bayesian posterior probabilities.

Divergence time estimates for empidoid families and empidid subfamilies

Divergence time analysis in BEAST places the split between empidoids and all other Eremoneura in the early Jurassic 184 MYA. Atelestidae is found to be the earliest diverging empidoid lineage, a result consistent with the Bayesian and ML topologies. The diversification of the empidoid families began 166 MYA. The time tree places the Dolichopodidae+Microphorinae as sister group to the Empididae sensu stricto with the two lineages sharing their last common ancestor approximately 152 MYA. The diversification of the represented subgroups of Empididae began 140.5 MYA. Fig II and Table IV summarize the divergence times of the Empidoidea and empidid subfamilies.

Table IV: Divergence times inferred from molecular data.

Taxon	Molecular Divergence Time (Million Years)
Apystomyia	166.81
Atelestidae	160.11
Bombylius+Effaltouniella	164.49
Brachycera	225.96
Dolichopodidae	145.28
Drosophila+Musca	76.90
Empididae	140.64
Empidinae	122.6
Hemerodromiinae	122.16
Hybotidae	152.10
“Group A”	145.28
“Group B”	140.63
“Group C”	136.48

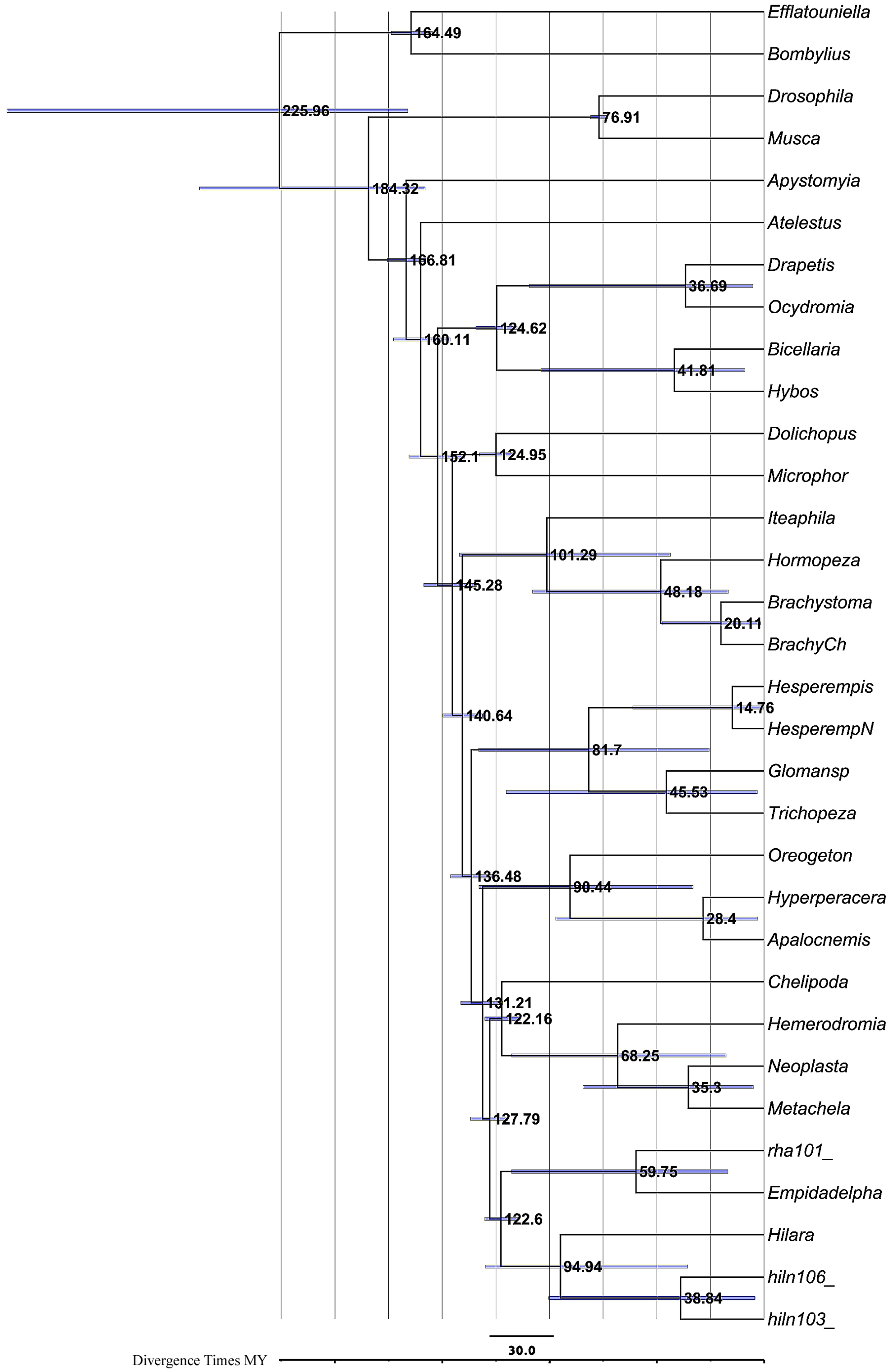

Fig II. Divergence times of Empidoidea and Empididae.

Discussion

Phylogenetic relationships (Fig I.)

The results of this study are concordant with previous studies which propose a four family classification system for the Empidoidea (Chvála 1983, Collins and Wiegmann 2002, Moulton and Wiegmann 2007). Including three additional nuclear markers (AATS, PGD and TPI) and a large mitochondrial fragment (COI) to the existing data greatly improved the support for the monophyly of the Empididae sensu stricto. The limits of Empididae are clearly defined by the inclusion of the additional character information and we see no reason to reduce or expand the family based on this information. The inclusion of these markers has re-affirmed the need for a revised taxonomic framework for the majority of empidid subgroups recovered here. The key problems found in this study are the polyphyly of the trichopezid and oreogetonine subfamilies of which representatives appear in several distantly related subgroups. A deeper taxonomic issue is the contentious family Brachystomatidae which is not only polyphyletic but clearly nested within the Empididae sensu stricto with its representative in this study forming a monophyletic group with *Iteaphila* and *Hormopeza* (Oreogetoninae). Our evidence suggests that this family is not valid and that its polyphyletic genera should be a focus of future research along with Oreogetoninae and Trichopezinae to develop an alternate classification for the supported unplaced subgroups (Fig I – groups A, B and C). Out of all of the subfamilies of Empididae included in this study only two, the Empidinae and Hemerodromiinae were recovered as monophyletic. These results are strongly supported by our data and are consistent

with previous molecular and morphological studies (Sinclair and Cumming 2006, Moulton and Wiegmann 2007). These two subfamilies are strongly supported as sister taxa for the first time. Previous studies have pointed to this conclusion but insufficient support from previous data sources prevented the formulation of this hypothesis. This finding has significance as it will allow a fuller understanding of the ancestral condition of mating behaviors in Empidinae, which contains many examples of courtship systems which are becoming important models for work focussing on the influence of sexual selection on evolution (Ritchie 2007). Understanding the evolutionary history of key traits such as differences in mating systems between these two subfamilies and the different trajectories in which they took in diversification will explain why the Empidinae, consisting of over 3,000 species is much more diverse than the Hemerodromiinae which consists of only 300 (Daugeron and Grootaert 2003).
Previous studies based on both molecular sequences and morphology have failed to establish the closest related group to Empididae within Empidoidea without uncertainty. Here our results are consistent with the findings based on sequence data of Moulton and Wiegmann (2007) and the morphology based work of Chvála (1983), that show Dolichopodidae + Microphorinae are the sister lineage to the Empididae s.s. This result is again weakly supported by the data analyzed here and will require further testing before this hypothesis can be confirmed.

Divergence times of Empidoid families and Empidid Subfamilies (Table IV, Fig II)

The importance of understanding the ages of evolutionary lineages cannot be understated. The estimated times of diversification directly provide information as to why present day species may be abundant and diversifying or in decline and relatively rare. Estimating divergence times accurately can be used to understand environmental conditions and selection pressures at the beginning of the diversification event. Here we used fossil calibrated molecular data to estimate the diversification of empidoid families. The results of our analysis here are remarkably consistent with what is known from existing fossils. Our results show that Empidoidea diverged from their closest relative around 167 million years ago, an age only slightly older than the earliest suspected fossil of the group, *Protoreogeton admirabilis*, dated at 165 million years (Grimaldi and Engel 2005). The results provide a different insight to the temporal origins of the respective families, however. The earliest dates of the Atelestidae, Hybotidae, Dolichopodidae + Microphorinae and Empididae are all pushed back from the earliest known fossil occurrences. In the case of Empididae it is only slightly older with the minimum occurrence being around 140 million years ago. Dolichopodidae is considerably older according to our data than the fossil calibration, appearing 20 million years earlier. The origins of Hybotidae and Atelestidae are also pushed back considerably by 17 and 50 million years respectively. We have also provided the first temporal framework for the subfamilies of Empididae using a calibrated molecular clock. The divergence times are largely consistent with the fossil calibration points and there is a high level of confidence that these are relatively accurate based on the error bars seen in Fig II. In our frame work, all major subgroups of Empididae had begun their diversification by 122 million years ago. The result also conforms to the findings of the phylogenetic analysis that the Hemerodromiinae is sister to the Empidinae as these two groups began to diversify into their respective genera at almost the same point in time, shortly after they diverged from each other. The time tree recovers polyphyletic groups A, B and C and gives estimated divergence times for them (140, 136 and 131 million years ago for each group, respectively) adding more weight to the polyphyly of the family Brachystomatidae and the subfamilies Oreogetoninae and Trichopezinae. The error bars for divergence events of Empididae subfamilies show a larger margin of error, so additional fossil calibration points may be required in future work to confirm the results found here. Empidoid flies are often seen visiting flowering plants and may be important pollinators (Forister et al. 2012); their diversification in the late Jurassic through to the mid Cretaceous may have been a result of a rapid radiation fueled by the exploitation of a new ecological niche.

Conclusions and future directions

This study has resulted in a more robust understanding of relationships within the family Empididae. It is in agreement with the previous findings of Moulton and Wiegmann (2007) and with the original four family classification of Chvála (1983).With the result of this study we have determined that several classification issues need to be resolved and have proposed our views on this. Several important questions still require to be answered however. This study did not include representatives of the Clinocerinae and Ceratomerinae and their placement within the Empididae is crucial for a full understanding to the subfamily level relationships of the family. This is of particular importance due to the polyphyletic nature of the included representatives of the disputed family Brachystomatidae to which Clinocerinae belongs. There are at least two clearly polyphyletic subfamilies revealed by our results here. The weight of support from molecular evidence suggests that these are true groupings and an alternate classification of Empididae subfamilies is necessary to resolve this issue. The results here could serve as a scaffold on which to build a morphology based classification. The divergence times estimated for the origin of the Empidoidea is slightly older than the oldest known empidoid fossil and we consider this to be a reasonable estimate. This study has also provided the first molecular based divergence time estimations for the represented subfamilies of Empididae. The sister group to the Empididae is a key question which is in need of clarification, evidence from the most recent molecular studies point to Dolichopodidae + Microphorinae being the most likely candidate and has again been recovered in this study, however additional work is required to confirm this result due to lack of statistical support.

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APPENDIX

Appendix 1

(Chapter 2)

Supplementary Table I: Taxon codes, geographic origins and genes targeted.

Supplementary Table I Enoplempis taxon codes and locality information							
Taxon code	Ingroup/OutGroup (IG/OG)	Geographic Region	CAD GenBank No.	AATS GenBank No.	16S GenBank No.	ND1 GenBank No.	COI GenBank No.
cde010	OG	France	XXX	XXX	XXX	XXX	XXX
copt	OG	Madagascar	XXX	XXX	XXX	XXX	XXX
drct	OG	USA	XXX	XXX	XXX	XXX	XXX
EMP122	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
emp123	OG	USA (WA)	XXX	XXX	XXX	XXX	XXX
emp25	IG	USA (NC)	XXX	XXX	XXX	XXX	XXX
EPL02	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL03	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL04	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL05	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL06	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL07	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL08	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL09	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL10	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL11	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL12	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL13	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL14	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL15	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL16	IG	USA (PA)	XXX	XXX	XXX	XXX	XXX
EPL17	IG	USA (WA)	XXX	XXX	XXX	XXX	XXX
EPL18	IG	USA (VA)	XXX	XXX	XXX	XXX	XXX
EPL19	IG	USA (NC)	XXX	XXX	XXX	XXX	XXX
EPL22	IG	USA (WA)	XXX	XXX	XXX	XXX	XXX
EPL23	IG	USA (WA)	XXX	XXX	XXX	XXX	XXX
EPL25	IG	USA (OR)	XXX	XXX	XXX	XXX	XXX
EPL26	IG	USA (AZ)	XXX	XXX	XXX	XXX	XXX
EPL27	IG	USA (OR)	XXX	XXX	XXX	XXX	XXX
EPL28	IG	USA (OR)	XXX	XXX	XXX	XXX	XXX
EPL29	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL30	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL31	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL32	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL33	IG	USA (NC)	XXX	XXX	XXX	XXX	XXX
EPL34	IG	USA (NC)	XXX	XXX	XXX	XXX	XXX
EPL35	IG	USA (NC)	XXX	XXX	XXX	XXX	XXX
EPL36	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL38	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
EPL41	IG	USA (OR)	XXX	XXX	XXX	XXX	XXX
EPL51	IG	USA (OR)	XXX	XXX	XXX	XXX	XXX
EPL53	IG	USA (OR)	XXX	XXX	XXX	XXX	XXX
EPL61	IG	USA (VA)	XXX	XXX	XXX	XXX	XXX
EPL65	IG	USA (TN)	XXX	XXX	XXX	XXX	XXX
EPL66	IG	USA (PA)	XXX	XXX	XXX	XXX	XXX
EPL67	IG	USA (NY)	XXX	XXX	XXX	XXX	XXX
EPL68	IG	USA (VA)	XXX	XXX	XXX	XXX	XXX
EPL69	IG	USA (PA)	XXX	XXX	XXX	XXX	XXX
epop	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
lamp	OG	Peru	XXX	XXX	XXX	XXX	XXX
macfg	OG	French Guiana	XXX	XXX	XXX	XXX	XXX
pade	OG	USA (WNEA)	XXX	XXX	XXX	XXX	XXX
padi	OG	USA (ENEA)	XXX	XXX	XXX	XXX	XXX
paim	OG	USA (WNEA)	XXX	XXX	XXX	XXX	XXX
para	OG	USA (WNEA)	XXX	XXX	XXX	XXX	XXX
paraa	OG	USA (ENEA)	XXX	XXX	XXX	XXX	XXX
pars	OG	USA (ENEA)	XXX	XXX	XXX	XXX	XXX
porph	OG	USA (WNEA)	XXX	XXX	XXX	XXX	XXX
prh066	OG	USA (ENEA)	XXX	XXX	XXX	XXX	XXX
r4	OG	USA (ENEA)	XXX	XXX	XXX	XXX	XXX
rha101	OG	USA (WNEA)	XXX	XXX	XXX	XXX	XXX
rha102	OG	USA (WNEA)	XXX	XXX	XXX	XXX	XXX
rha132	OG	USA (ENEA)	XXX	XXX	XXX	XXX	XXX
RHA133	IG	USA (CA)	XXX	XXX	XXX	XXX	XXX
TRID	IG	USA (NC)	XXX	XXX	XXX	XXX	XXX

Supplementary Table II: Primers.

Primer	Sequence
AATS1	
M13A1x92F	TGTAAAACGACGGCCAGTTAYCAYCAYACNTTYTTYGARATG
M13rA1x244R	CAGGAAACAGCTATGACCATNCCRCARTCNATRTGYTT
16s	
LR-J-12887	CCGGTTTGAACTCAGATCATGT
LR-N-13398	CGCCTGTTTAACAAAAACAT
CO1	
LC01490f	GGTCAACAAATCATAAAGATATTGG
HC02198r	TAAACTTCAGGGTGACCAAAAAATCA
ND1	
ND1-F	CGTAAAGTCCTAGGTTATATTCAGATTCG
ND1-R	ATCAAAAGGAGCTCGATTAGTTTC
CAD	
M13CAD581*	TGTAAAACGACGGCCAGTGGWGGWCAAACWGCWYTMAAYT
CAD581*	GGWGGWCAAACWGCWYTMAAYTGYGG
TM60F	TGTAAAACGACGGCCAGTGARGTNGTNTTYCARACNGGNAT
TM364R	CAGGAAACAGCTATGACCTCNACNGCRAANCCRTGRTT
CAD60F	GARGTNGTNTTYCARACNGGNAT
CAD54F	GTNGTNTTYCARACNGGNATGGT
M13CAD843R	CAGGAAACAGCTATGACGCYTTYTGRAANGCYTCYTCRAA
CAD 364R	TCNACNGCRAANCCRTGRTTYTG
CAD843R	GCYTTYTGRAANGCYTCYTCRAA

Supplementary Material III: Morphology coding.

	Character conditions		
Taxon	Nuptial feeding state 0=Prey 1=Balloon	Leg Modification 0=Absent 1=Present	Balloon Type 0=Contains Prey 1=Empty
EMP122	0	0	n/a
emp25	1	0	1
EPL02	0	1	n/a
EPL03	0	1	n/a
EPL04	Unknown	1	Unknown
EPL05	Unknown	2	Unknown
EPL06	0	1	n/a
EPL07	1	1	1
EPL08	0	1	n/a
EPL09	0	1	n/a
EPL10	0	1	n/a
EPL11	Unknown	1	Unknown
EPL12	0	1	n/a
EPL13			
EPL14	Unknown	1	Unknown
EPL15	Unknown	1	Unknown
EPL16	Unknown	1	Unknown
EPL17	1	1	0
EPL18	1	1	0
EPL19	Unknown	1	Unknown
EPL22	Unknown	1	Unknown
EPL23	Unknown	1	Unknown
EPL25	0	1	n/a
EPL27	Unknown	1	Unknown
EPL28	Unknown	1	Unknown
EPL30	0	1	n/a
EPL31	0	1	n/a
EPL32	0	1	n/a
EPL33	Unknown	1	Unknown
EPL34	Unknown	1	Unknown
EPL35	Unknown	1	Unknown
EPL36	0	1	n/a
EPL38	0	1	n/a
EPL41	1	1	0
EPL51	1	0	1
EPL53	1	0	1
EPL61	Unknown	1	Unknown
EPL65	Unknown	1	Unknown
EPL66	Unknown	0	Unknown
EPL67	Unknown	1	Unknown
EPL68	Unknown	0	Unknown
EPL69	Unknown	1	Unknown
epop	0	1	n/a
RHA133	Unknown	0	Unknown
TRID	Unknown	0	Unknown

Appendix 2

(Chapter 3)

Supplementary Table I.

Taxon	Locality Information	Current Subfamily	CAD	28S	COI	AATS	PGD	TPI
Apalocnemis	Australia	Empididae: Trichopezinae	YES	YES	NO	YES	YES	NO
Apystomyia	USA (CA)	Apystomyiidae	YES	YES	YES	NO	NO	NO
Atelestus	Europe (United Kingdom)	Atelestidae	YES	YES	YES	NO	NO	NO
Bicellaria	USA (NC)	Hybotidae: Ocydromiinae	YES	YES	YES	NO	NO	NO
Bombylius	USA (NC)	Bombyliidae	YES	YES	NO	NO	NO	NO
BrachyCh	USA (NC)	Empididae: Brachystomatinae	YES	YES	NO	NO	NO	NO
Brachystoma	USA (NC)	Empididae: Brachystomatinae	YES	YES	YES	NO	NO	NO
Chelipoda	Australia	Empididae: Hemerodromiinae	YES	YES	NO	YES	YES	NO
Dolichopus	USA (WA)	Dolichopodidae	YES	YES	NO	NO	NO	NO
Drapetis	USA (NC)	Hybotidae: Tachydromiinae	YES	YES	YES	NO	NO	NO
Drosophila	USA	Drosophilidae	YES	YES	YES	NO	NO	NO
Efflatouniella	Isreal	Therevidae	YES	YES	NO	NO	NO	NO
Empidadelpha	Chile (Chiloe Isld.)	Empididae: Empidinae	YES	YES	YES	YES	YES	YES
Glomansp	USA (CA)	Empididae: Trichopezinae	YES	YES	NO	YES	YES	NO
Hemerodromia	USA (NC)	Empididae: Hemerodromiinae	YES	YES	NO	NO	NO	NO
Hesperempis	USA (CO)	Empididae: Oreogetoninae	YES	YES	NO	YES	NO	YES
HesperempN	USA (CO)	Empididae: Oreogetoninae	YES	NO	YES	NO	NO	NO
Hilara	Europe (United Kingdom)	Empididae: Empidinae	YES	YES	NO	YES	YES	NO
hiln103	Chile	Empididae: Empidinae	YES	YES	YES	YES	YES	YES
hiln106	Argentina	Empididae: Empidinae	YES	YES	YES	YES	YES	NO
Hormopeza	USA (MN)	Empididae: Oreogetoninae	YES	YES	YES	NO	NO	NO
Hybos	Australia	Hybotidae:Hybotinae	YES	YES	YES	NO	NO	NO
Hyperperacera	Chile (Chiloe Isld.)	Empididae: Trichopezinae	YES	YES	YES	NO	NO	NO
Iteaphila	USA (UT)	Empididae: Oreogetoninae	YES	YES	YES	YES	YES	NO
Metachela	USA (UT)	Empididae: Hemerodromiinae	YES	YES	YES	YES	NO	NO
Microphor	Europe (United Kingdom)	Microphorinae	NO	YES	NO	NO	NO	NO
Musca	USA (NC, NCSU colony)	Muscidae	YES	YES	YES	NO	NO	NO
Neoplasta	USA (UT)	Empididae: Hemerodromiinae	YES	NO	NO	YES	YES	NO
Ocydromia	Europe (Germany)	Hybotidae: Ocydromiinae	YES	YES	YES	NO	NO	NO
Oreogeton	USA (NV)	Empididae: Oreogetoninae	YES	YES	NO	YES	YES	YES
rha101	USA (WNEA)	Empididae: Empidinae	YES	YES	YES	YES	YES	YES
Trichopeza	Europe (Germany)	Empididae: Trichopezinae	YES	YES	YES	NO	NO	NO