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Tracing the Evolution of Aquatic Life History in Diptera: from Ecology to Phylogeny

By

Nina Pak

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

In

Environmental Science, Policy, and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Neil Tsutsui, Chair
Professor Erica Bree Rosenblum
Professor Bruce Baldwin

Fall 2021

ABSTRACT

Tracing the Evolution of Aquatic Life History in Diptera: from Ecology to Phylogeny

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Doctor of Philosophy in Environmental Science, Policy, and Management

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Professor Neil Tsutsui, Chair

Diptera has more species with an aquatic stage than any other insect group, yet their evolutionary history and its association with other traits, like blood feeding and marine dwelling, remain unknown. A comprehensive phylogeny of Diptera (flies) has recently been proposed, allowing for studies of adaptations across this diverse group. Ancestral state reconstruction analyses suggest marine dwelling is not an ancestral trait in the most recent common ancestor to Diptera. In addition, several families (e.g. Culicidae and Canacidae) of flies have independently adapted to saltwater found in the marine environment. For example, Canacidae (beach flies) is a cosmopolitan family that includes species that are found in intertidal ecosystems where their larvae feed on algae. The genus *Procanace*, in particular, offers the opportunity to study biogeography and the adaptation to novel freshwater environments. In this dissertation, a phylogeny of *Procanace* and relatives is reconstructed, based on multiple mitochondrial and nuclear DNA sequences from 15 species representing 5 genera. The reconstructed phylogeny supports the monophyly of the *Procanace* genus, which confirms previous taxonomic studies. In addition, the reconstructed phylogeny suggests that canacids colonized Hawai'i twice, once by an ancestor of *Procanace* and again by an ancestor of *Canaceoides*. Speciation patterns within the genus *Procanace* follow the progression rule and demonstrate a single switch from saltwater to freshwater habitats in the Hawaiian Islands. Although understudied, these flies may yield insights into freshwater invasions and pathways behind osmoregulatory systems.

This dissertation also examines the evolution of aquatic life histories and their associations with blood-feeding across Diptera. The most recent common ancestor of Diptera is likely to have had an aquatic (but not marine) life history. When examining aquatic life history in relation to blood-feeding, we found that blood-feeding behaviors often occur in fly lineages that also have aquatic life histories, but not significantly more often. This pattern suggests that, in some cases, adaptations to larval aquatic life history were a precursor for flies expanding their diets to take blood meals. This work builds on our understanding of the ecological and evolutionary history of aquatic Diptera and its associations with blood-feeding.

To Monica,
Friends and Family,
And My Parents

Table of Contents

Dedication	i
List of Contents	ii
List of Figures	iii
List of Tables	iv
Acknowledgements	v
Introduction	vi
Chapter 1: Phylogenetic analyses of Hawaiian <i>Procanacae</i> : implications for biogeography and ecological adaptations	1
Chapter 2: The evolution of marine dwelling in Diptera	15
Chapter 3: Aquatic life history and blood-feeding: reconstructing their association in Diptera	27
Conclusion	41
Bibliography	42
Appendix A. Supplementary Information for Chapter 1	51
A.1 Supplementary Table	51
Appendix B. Supplementary Information for Chapter 3	53
B.1 Supplementary Table	54
B.2 Supplementary Table	59

List of Figures

1.1	Nuclear and mitochondrial tree of Hawaiian <i>Procanace</i>	13
1.2	Concatenated alignment phylogeny of Hawaiian <i>Procanace</i> with ML and BI analyses	14
2.1	Diptera phylogeny with marine dwelling, aquatic life history, and ambiguous states	24
2.2	Ancestral state reconstruction for marine dwelling flies	25
2.3	Stressors (abiotic and biotic) and physiological adaptations for marine flies	26
3.1	Diagram showing the rates of change from aquatic to blood-feeding	37
3.2	Ancestral state reconstruction for aquatic flies	38
3.3	Stochastic character mapping of aquatic life history	39

List of Tables

1.1 Loci used for phylogenetic analysis	9
1.2 Distribution and life history of canacid species	11
2.1 Trait Correlation Models and AIC scores	40
Supplemental Table A.1 Supplementary Table	50
Supplemental Table B.1 Supplementary Table	52
Supplemental Table B.2 Supplementary Table	58

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Introduction

This dissertation focuses primarily on understanding the transitions from marine to freshwater and vice versa. In this dissertation, I first explored a specific example of marine flies in **Chapter 1**, examining the evolutionary history of Hawaiian Canacidae (beach flies), particularly focusing on the genus *Procanace*. I tested for monophyly and the progression rule within the freshwater *Procanace* species. I constructed maximum likelihood trees based on mitochondrial and nuclear DNA sequences, and prepared the concatenated alignment for Bayesian and maximum likelihood analyses. From phylogenetic analyses and relatives of Hawaiian Canacidae lineages (freshwater and saltwater), I can compare freshwater flies to their saltwater relatives, and observe this ecological transition.

In **Chapter 2**, I took a broader view and examined the marine aspect of aquatic life across Diptera. This interdisciplinary study explores the evolutionary history of marine dwelling in Diptera using records from the World Register of Marine Species. For flies, I found that the shift to the marine environment did not always originate from other aquatic environments. I utilized ancestral state reconstruction tools to examine the evolution of marine dwelling across the fly tree of life. I found that some families show gradual transitions to the marine environment, while other marine families were likely to be one-off occurrences. In addition, I outlined the potential adaptations for marine flies, the barriers of colonizing the marine environment, and the implications to the mechanisms for salt tolerance.

Lastly, in **Chapter 3**, I investigated the evolution of aquatic life history in Diptera and its associations with blood-feeding behaviors. I used stochastic character mapping and ancestral state reconstruction tools to explore patterns of trait evolution. Looking across the fly tree of life, I examined whether the most recent common ancestor of Diptera likely had an aquatic life history. I conducted trait correlation analyses and found that most blood feeding flies have an aquatic life history. Finally, I discuss the rise of blood-feeding flies and specifically speculate on its relation to aquatic life history.

Chapter 1

Phylogenetic analyses of Hawaiian *Procanace* (Diptera: Canacidae: Canacinae): implications for biogeography and ecological adaptations

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Abstract

The genus *Procanace* offers the opportunity to study biogeography and the adaptation to novel freshwater environments. We reconstruct a phylogeny of *Procanace* and relatives, based on multiple mitochondrial and nuclear DNA sequences from 15 species representing 5 genera. The monophyly of the *Procanace* genus is strongly supported and confirmed previous taxonomic studies. The phylogeny suggests that Canacidae colonized Hawai'i twice, once by an ancestor of *Procanace* and again by an ancestor of *Canaceoides*. Within the genus *Procanace*, we observe speciation patterns following the progression rule and a single switch from saltwater to freshwater habitats in the Hawaiian Islands.

Introduction

The Hawaiian Archipelago is the most isolated island group in the world, situated almost 3000 km from the nearest continental land mass. This archipelago has formed over a more-or-less stationary “hot spot” in the Pacific Ocean where lava seeps through the ocean floor and builds up to form a subaerial volcano. The eight main Hawaiian Islands (Ni'ihau, Kaua'i, O'ahu, Moloka'i, Kaho'olawe, Lana'i, Maui, Hawai'i) range from ~1000 ft. to over 13,000 ft. above sea level. The motion of the Pacific Plate eventually moves the island to the northwest at the rate of roughly 2 cm per year (Carson, 1995; Carson et al., 1995). Once an island is no longer actively growing, it subsides and erodes over time, eventually sinking below the surface of the sea. Volcanic activity, coupled with plate tectonics, created a linear progression of islands, from the youngest near the hot spot in the southeast of the chain to the oldest in the northwest. The Hawaiian Islands have been active for over 60 million years and many of the older islands in the northwest of the chain are now low sea mounts, shoals, and reefs, rising barely above sea level (Price & Clague, 2002).

The Hawaiian Islands are also home to a number of evolutionary radiations across plant and animal groups (Price & Wagner, 2018; reviewed in Hembry *et al.*, *in press*). The diverse array of ecological niches, spanning habitats from coastal strand to subalpine tundra (Juvik et al., 1998) and the known geological ages of each Hawaiian island inferred from K-Ar dating (Price & Clague, 2002; Price & Elliott-Fisk, 2004) offer a powerful framework for understanding mechanisms of diversification. Studies on various plant and animal groups (Gillespie et al., 2012; Lim et al., 2019; O'Grady & DeSalle, 2008) have demonstrated multiple long-distance colonization events over the past 25 million years, repeated biogeographic patterns (*e.g.*, progression rule) within the islands, and the exploitation of novel ecological niches by many taxa. Previous studies have shown that patterns of species richness and diversification differ between lineages, with some groups restricted to one or a few taxa and others forming adaptive radiations with hundreds of species (Price & Wagner, 2018, reviewed in Hembry *et al.*, *in press*).

Perhaps the most common biogeographic pattern observed in the Hawaiian Islands is the progression rule (Funk & Wagner, 1995; Hembry *et al.*, *in press*; Hennig, 1999). This pattern, where species from progressively older islands are successively sister taxa to those endemic to younger islands, is common in hot spot archipelagoes (Bonacum et al., 2005; Goodman et al., 2014, 2016; Haines et al., 2014; Magnacca & Price, 2015; O'Grady et al., 2011). This biological pattern reflects the age of island formation and the history of colonization from older to younger

islands. While this is seen in many Hawai'i taxa, it is not ubiquitous, and many smaller lineages (e.g., Goodman and O'Grady 2013) do not display a clear progression from older to younger islands.

The family Canacidae, commonly known as beach flies, is a small cosmopolitan family of 308 species placed in 27 genera. Larvae and adults feed on kelp, green algae, and seagrass in intertidal zones (Ferrar, 1987). This family is widely distributed and abundant in cool-temperate and tropical beaches of the world. Over half of canacid species are found in the Australasian and Oceania regions (Evenhuis, 2014; Munari & Mathis, 2010). Despite being common locally in these saline environments, the natural history of this family of flies has remained poorly understood and no comprehensive studies have examined the relationships among genera or have elucidated classifications beyond the family (O'Grady & Pak, 2016).

Seventeen canacid species, placed in five genera, are present in the Hawaiian Islands (O'Grady & Pak, 2016). The genus *Procanace* includes nine Hawaiian taxa and can be found in a range of habitats, from coastal strand to high-elevation (> 4000 ft.) rainforests (Hardy & Delfinado, 1980; O'Grady & Pak, 2016). Interestingly, eight *Procanace* species are found in freshwater, rather than in marine habitats. Hawaiian Canacidae can serve as a model system for understanding marine-dwelling as well as the loss of salt tolerance, when some species adapt to freshwater environments.

Here, we present a phylogenetic hypothesis for the genus *Procanace* in Hawai'i based on multiple nuclear and mitochondrial loci. We use this phylogeny to examine the history of colonization of the Hawaiian Islands, as well as biogeographic patterns observed within the archipelago.

Materials and Methods

Taxonomic Sampling. Specimens were collected using sweep nets from sites across the Hawaiian Islands (Hawai'i, Maui, Moloka'i, O'ahu, and Kaua'i), French Polynesia, and along the coasts of California, Texas, and Mississippi. Hawaiian samples were collected along beaches and in freshwater streams. Flies were preserved in 95% ethanol. No endangered species were included as part of this work. Various keys were used to identify species based on where the material was collected (Mathis & Foster, 2007; McAlpine et al., 1981; O'Grady & Pak, 2016). Six of the nine known *Procanace* species were sampled. Outgroup taxa from nine other species, including representatives of *Canacea*, *Dasyrhicnoessa*, *Canaceoides*, *Noticanace*, and *Neopelomyia*, were included.

Full collection records are in Appendix A.1. *Canaceoides* included in this study are generally recognized across multiple islands and multiple regions in California. Representatives from each island and regions within a given species' known range were included when possible (Appendix A.1). Partial sequences from different populations within three species, *Canaceoides nudatus*, *C. angulatus*, and *C. hawaiiensis*, were combined to represent a single species for phylogenetic analyses.

DNA extraction, amplification and sequencing. Genomic DNA was extracted from individual flies using Qiagen DNeasy DNA extraction Kit (Qiagen, Inc). The only exception to the standard protocol was that individuals were soaked in Proteinase K overnight prior to extraction. Three mitochondrial genes (*cytochrome c oxidase subunit I (COI)* and *cytochrome c oxidase subunit II (COII)*, *16S*, *ND2*) were then amplified using Polymerase Chain Reaction (PCR). We performed PCR using standard master mixes in 26 mL final volumes. PCR conditions were as follows: 5 minutes denaturing at 95°C, followed by 30 cycles of the following amplification sequence of (1) denaturing at 95°C for 30 seconds, (2) annealing (between 52°C and 60°C, depending on gene) for 30 seconds, and (3) extension at 72°C for 30 seconds, then a final round of extension at 72°C for 5 minutes. PCR products were visualized on 1% agarose TAE gels and cleaned using the standard ExoSAP-IT protocol (USB/Amersham). Clean PCR products were sequenced in both directions via Sanger sequencing at the University of California, Berkeley Sequencing Facility using the same primers used for PCR amplification. Raw sequences were *de novo* assembled and edited into contigs in Geneious v. 9.0.5.

We used a multiplex approach based on Krehenwinkel et al. (2018) to amplify *12S*, *CytB*, *18S*, *28S*, *PGD*, and another region of *16S* under varying temperature conditions (Krehenwinkel et al., 2018). For each multiplex reaction, we used custom-made primers containing the 30-bp TruSeq tail on the 5' end of the forward primer and the 24-bp TruSeq tail on the 5' end of the reverse primer. For fragment size assessment, PCR products were visualized on 1.5 agarose gel with a 100-bp ladder (New England BioLabs), then cleaned of leftover primers with 1X AMPure beads XP (Beckman Coulter). Before we performed DNA sequencing on an Illumina MiSeq platform, PCR products were run through a second round of PCR for indexing. In this reaction, the PCR products were bound to forward and reverse Illumina TruSeq index primers containing a sequence complementary to the TruSeq tails, an 8-bp indexing barcode, and a sequencing tail designed to bind to the sequencing primers in the MiSeq flow cell. Every PCR sample included a unique combination of forward and reverse barcoding primers so the samples could later be identified. Afterwards, products were pooled together at roughly equal amounts (ng of DNA) based on gel band strengths. The pool was quantified with a Qubit fluorometer (Invitrogen) and diluted to a 4 nM concentration. The 4 nM sample was run on an Illumina MiSeq with V3 chemistry (600 cycles) and 2 X 300 bp reads at the California Academy of Sciences' Center for Comparative Genomics (San Francisco, CA, USA).

Sequencing Process. Using Paired-End read merger (PEAR), MiSeq sequences were demultiplexed by index barcode combinations and then assembled reads were converted to FASTA format by FastX-Toolkit (Gordon & Hannon, 2010; Zhang et al., 2014). Based on 97% similarity, we used USEARCH (Edgar, 2010) to cluster operational taxonomic units (OTUs) after FASTA files were simplified from their replicates. Lastly, sequences were trimmed from their primer sequences and renamed. Sequences were then checked against the NCBI nucleotide database using BLASTn (Altschul et al., 1990) to reduce sequencing error and to filter non-diptera hits.

The contigs of Sanger sequences and the processed MiSeq sequences were then assembled and edited using Geneious v.9.0.5. We used the MAFFT alignment plugin in Geneious to align each gene. Alignments were checked visually and adjusted manually when necessary before sequences were concatenated in MEGA11 (Tamura et al., 2021). Overall,

genetic data consist of a total of 9 genes (6,057 base pairs) from the mitochondrial genome (*COI*, *COII*, *12S*, *ND2*, *CytB*, and *16S*) and the nuclear genes (*28S*, *PGD*, and *18S*) (See Table 1.1). Table 1.1 shows the exact primers, references, and lengths used to amplify and align sequences. We show the distribution and life history of the sampled canacid in Table 1.2.

Phylogenetic analysis. Final concatenated alignments of 6,057 bp were used for phylogenetic reconstruction (See Table 1.1 for lengths of individual markers and Appendix A.1 for completeness of data matrix). Phylogenetic analyses were performed on mitochondrial and nuclear loci separately, as well as on a single concatenated DNA sequence data matrix.

For mitochondrial, nuclear, and combined phylogenetic analyses, we used Jmodeltest and the ModelFinder option from the IQ-TREE package to determine the optimal substitution model for each partition, using Bayesian Information Criterion (BIC) (Kalyaanamoorthy et al., 2017). In the maximum likelihood analyses, we inferred phylogenies with the program IQ-TREE (Nguyen et al., 2015) using a fully partitioned GTR + Gamma substitution model with 1,000 bootstrap replicates on the IQ-TREE server.

Phylogenetic analyses using Bayesian inference were then conducted in MrBayes (Ronquist et al., 2012), using the same partitioning schemes and model in the IQ-TREE analyses. MrBayes analyses were run two times with 20,000,000 generations, and sampled every 1000 generations with a burn-in of 10%. Convergence metrics were checked by examining the average standard deviations of split frequencies when they were below the proposed 0.01 threshold (Huelsenbeck & Ronquist, 2001). Trees were formatted into figures using FigTree v. 1.4.3 and Adobe Illustrator CC 25.4.1. (Adobe Systems, San Jose, CA, USA).

Results

Tree topology. We analyzed our matrix in several ways to thoroughly explore the data. All mitochondrial loci were combined into a single concatenated data matrix and analyzed using ML and Bayesian approaches. Likewise, all nuclear loci were also concatenated into a single matrix. Figure 1.1 shows the results of comparisons between nuclear and mitochondrial genes under a maximum likelihood analysis via IQ-TREE. Mitochondrial and nuclear trees differed in their topologies and support values (bootstrap percentage (BP) and posterior probabilities (PP)), but recovered many of the same monophyletic groups. The mitochondrial tree was generally consistent with the concatenated tree, but differed in topology for species like *Canaceoides angulatus* and *C. hawaiiensis*. These species, while recovered as sister species in the concatenated and the nuclear trees, appear paraphyletic in the mitochondrial tree. In addition, *Canace macateei* and *Dasyrhicnoessa ferruginea* constituted a clade with a relatively low support value (79%), but were resolved as distantly related in the nuclear tree (see Figure 1.1A and 1.1B).

Analyses of concatenated nuclear and mitochondrial data using both ML and Bayesian approaches yielded results that were largely congruent across methods and with the combined mitochondrial and nuclear datasets described above (Figure 1.1). Two major clades were evident, one containing the sampled Hawaiian *Procanace* (BP = 100; PP = 100) and the other clade containing the genera *Nocticanace*, *Canaceoides*, and *Neopelomyia* (BP = 100; PP = 100).

Procanace wirthi, a single island endemic species from Kauaʻi, is sister to all other *Procanace* sampled (BP = 100; PP = 100). A clade (BP = 100; PP = 100) containing two other single island endemics from Kauaʻi (*P. nigroviridis*, and *P. hardyi*) was recovered as the sister taxon to a clade (BP = 100; PP = 100) of species from the younger islands (*P. acuminata*, *P. constricta* and *P. confusa*) with strong support (BP = 99; PP = 100). Relationships within this latter clade are not well resolved, with *P. acuminata* and *P. confusa* being weakly supported (BP = 78; PP = 70) as sister taxa.

Relationships among the non-Hawaiian outgroup taxa provide the first insight into the relationships among some canacid genera. The genera *Nocticanacae* and *Canaceoides* sampled in this study were both paraphyletic, although this may be an artifact of the reduced sampling and correspondingly long branch lengths relative to *Procanace*. Only three of the nine described *Canaceoides* species and three of the thirty five known *Nocticanacae* species were sampled in the present study and, while these analyses suggest paraphyly of these taxa, additional sampling will be required to make a conclusive assessment concerning the status of these genera. Only a single member of the genera *Neopelomyia*, *Canacae*, and *Dasyrhicnoessa* were included in this study, so an assessment of the monophyly of these groups is not possible. Furthermore, statistical support for some of the nodes in this part of the phylogeny is not strong (BP < 80), so additional taxon sampling should be done in future work.

Discussion

Our phylogenetic analysis based on our concatenated alignment demonstrates that the analyzed Hawaiian *Procanace* represent a monophyletic group (Figure 1.2). Relationships among the species are reasonably well supported, with the exception of low support between *Procanace acuminata* and *P. confusa* (BP = 78, PP = 70). *Procanace* comprises species that are unique among canacids in that its endemic members are found in freshwater stream habitats across the Hawaiian Islands. In particular, five of the eight endemic species can be found in the Alakaʻi Swamp region of Kauaʻi (O'Grady, 2015). Future investigations should reveal the remaining placement of Hawaiian *Procanace* (*Procanace bifurcata*, *P. quadrisetosa*, and *P. williamsi*).

Monophyly of the Hawaiian *Procanace* suggests a single introduction to the Hawaiian Islands. Many lineages of Diptera have colonized the Hawaiian Islands once, while at least one family, the Hawaiian long-legged flies (Dolichopodidae) have undergone more than one colonization event (Goodman et al., 2016; Hembry et al., *in press*). Our phylogenetic analyses suggest that Canacidae, like Dolichopodidae, colonized Hawaiʻi twice, once in *Procanace* and again in the genus *Canaceoides*. The two colonists of canacids have undergone different diversification histories. *Canaceoides* consists of only two species in Hawaiʻi while its other seven counterparts are found in Mexico, Panama, and the United States (Evenhuis, 2012; Munari & Mathis, 2010). In comparison, *Procanace* includes thirty species, is found in the Palearctic, Nearctic, Afrotropical, and Australasian/Oceania regions, and contains a small radiation of at least six species in Hawaiʻi based on our study; a total of eight species have been described to be present in Hawaiʻi (O'Grady & Pak, 2016). Morphological and ecological adaptations may explain differences in diversity between the two genera, although estimations of divergence

times within the group would provide a better understanding of the timing and evolution of this group.

Hawaiian *Procanace* are found on all Hawaiian Islands. It was ambiguous whether these significant aquatic and semi-aquatic dipterans follow the progression rule, the biogeographical pattern in which lineage diversification occurred on progressively younger islands as they formed (Bonacum et al., 2005; Funk & Wagner, 1995). In comparison to other dipteran families, there is evidence that lineages in Hawaiian long-legged flies (Dolichopodidae) support the progression rule pattern, despite being generalist predators and strong fliers (Goodman et al. 2014). On the other hand, Hawaiian crane flies (Limoniidae) show little evidence of this biogeographic pattern, although they are broadly distributed on more than one island (Goodman & O'Grady, 2013). Interestingly, these three families have larvae that inhabit fresh and/or brackish water, which could be an advantageous dispersal capability to move from island to island. In our study, the diversification of Hawaiian *Procanace* adheres to the progression rule: we observe lineages within *Procanace* have evolved into new species on different islands (Figure 1.2). *Procanace wirthi*, *P. hardyi*, and *P. nigroviridis* emerged from Kaua'i with subsequent lineages, *P. acuminata*, *P. confusa*, and *P. constricta*, present on O'ahu, Moloka'i, Maui, and Hawai'i. The split between species on different islands is well supported by high support values (BS = 99, PP = 1). Without divergence time estimates, the timing of colonization within the Hawaiian Islands is unclear. Finer scale biogeographic patterns are not possible to resolve without more sampling and improvements on phylogenies.

Overall, members of the canacid family are almost exclusively of marine and intertidal habitats (McAlpine et al., 1981; Wirth, 1969; WoRMS, 2021). We resolved a single transition from saltwater to freshwater habitats in the Hawaiian Islands in *Procanace*. This pattern suggests that the initial colonizer adapted to a freshwater life history. This ecological shift is supported by molecular data, and is more parsimonious than the hypothesis of several independent colonizations of freshwater stream habitats. In contrast, the ancestral *Canaceoides* was most likely a generalist-type, almost oceanic species, invading marine nearshore habitats across all the Hawaiian Islands. Unlike in *Procanace*, the progression rule is not supported for the species of *Canaceoides*, and the species *C. angulatus* and *C. hawaiiensis* overlap in range and occurrences on multiple islands (O'Grady & Pak, 2016). O'Grady & Pak (2016) hypothesized that these two taxa arose from two distinct colonization events of the Hawaiian Islands as they have different biogeographic distributions: *Canaceoides angulatus* is widely distributed across the Pacific Islands and North American continent, while the endemic *C. hawaiiensis* has no known counterparts on the American coasts (O'Grady & Pak, 2016; Wirth, 1969). Our phylogenetic analysis shows evidence for a single colonization of the Hawaiian Islands for *Canaceoides*. It is likely that more transitions between saltwater and freshwater have occurred within the canacid family, but further taxon sampling and phylogenetic analyses are needed in future studies to resolve any additional habitat shifts.

How and when terrestrial insects moved between sea, saltwater, freshwater, and land remain as important questions in evolutionary biology and ecology. Ecological shifts to the freshwater environment are often hypothesized to have originated from the marine environment and terrestrial environments. The shift between freshwater and saltwater environments offers insight into the challenges and adaptations that have led to colonization of the marine

environment. Marine dwelling flies are generally understudied, although Diptera have independently colonized the marine environment several times (Pak et al., 2021). Canacids provide an example of marine flies that have made this ecological transition and reverted to freshwater at least once.

Conclusions

Here we present the first phylogenetic analysis of *Procanace* and some of its relatives within the Canacidae family. The results suggest that *Procanace* forms a monophyletic group, and that two colonization events have occurred across the Hawaiian Islands. We see evidence of the progression rule pattern of colonization from older (Kaua'i) to younger (Maui, Hawai'i) islands within *Procanace*. This study offers insight into the evolution of this small, yet fascinating, group of Hawaiian flies.

Table 1.1: Loci used for phylogenetic analysis

Locus and primer names	Length (bp)	Genome	Reference
<i>Cytochrome Oxidase I (COI)</i> (2183 and 3041)	823	mitochondrial	(Bonacum et al., 2001)
<i>Cytochrome Oxidase I (COI)</i> (LCO - 2198 and HCO - 1490)	715	mitochondrial	(Vrijenhoek, 1994)
<i>Cytochrome Oxidase II (COII)</i> (3037 and 3791)	759	mitochondrial	(Bonacum et al., 2001)
<i>NADH Dehydrogenase (ND2)</i> (192-732)	526	mitochondrial	(Nitta & O'Grady, 2008)
<i>16S</i> (LR-J-12887 and LR-J-013417)	525	mitochondrial	(DeSalle et al., 1992)
<i>16S</i> (F3 and R1)	347	mitochondrial	(Krehenwinkel et al., 2018)
<i>12S</i> (F1 and R1)	402	mitochondrial	(Krehenwinkel et al., 2018)
<i>Cytochrome B (CytB)</i>	356	mitochondrial	(Barraclough et al., 1999; Krehenwinkel et al., 2018)
<i>18S (SSU)</i>	368	nuclear	(Fonseca et al., 2010)
<i>18S (18SM)</i>	389	nuclear	(Machida & Knowlton, 2012)
<i>28S (rc28C and 28P)</i>	510	nuclear	(Gibson et al., 2014)
<i>PGD</i>	369	nuclear	(Gibson et al., 2014)

(PGD-Dipt3805F and 4R)			
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Table 1.2 : Distribution and life history of canacid species

Species	Distribution	Ecology	Collection ID
<i>Canaceoides</i>			
<i>C. angulatus</i> ¹	Kaua'i, O'ahu, Moloka'i, Lana'i, Maui, Hawai'i, Northwest Hawaiian Islands	marine	205640 205519 205520
<i>C. hawaiiensis</i> ²	Kaua'i, O'ahu, Moloka'i, Maui, Hawai'i, Northwest Hawaiian Islands	marine	205130 205641
<i>C. nudatus</i>	California, Mexico	marine	20600 205334 000001NP
<i>Procanace</i>			
<i>P. acuminata</i> ²	Moloka'i, Maui, Hawai'i	freshwater	205136
<i>P. confusa</i> ²	Maui, Hawai'i	freshwater	923.1
<i>P. constricta</i> ²	O'ahu, Moloka'i, Maui, Hawai'i	freshwater	205268
<i>P. hardyi</i> ²	Kaua'i	freshwater	205652
<i>P. nigroviridis</i> ²	Kaua'i	freshwater	205648

<i>P. wirthi</i> ²	Kaua'i	freshwater	205649
<i>Nocticanace</i>			
<i>N. arnaudi</i>	California	marine	205332
<i>N. peculiaris</i>	French Polynesia	marine	973.1
<i>N. texensis</i>	Texas	marine	1.01
<i>Neopelomyia</i>			
<i>N. rostrata</i>	French Polynesia	marine	826.4
<i>Dasyrhicnoessa</i>			
<i>D. ferruginea</i>	French Polynesia	marine	955.3
<i>Canacea</i>			
<i>C. macateei</i>	Mississippi	marine	8.4

1. This species is adventive in Hawaii. 2. This species is endemic to Hawaii.

Figure 1.1



Figure 1.1: Maximum Likelihood (ML) analyses of Hawaiian *Procanace* generated by IQ-TREE based on the nuclear genes (A) and mitochondrial genes (B). Node values show ML bootstrap support.

Figure 1.2

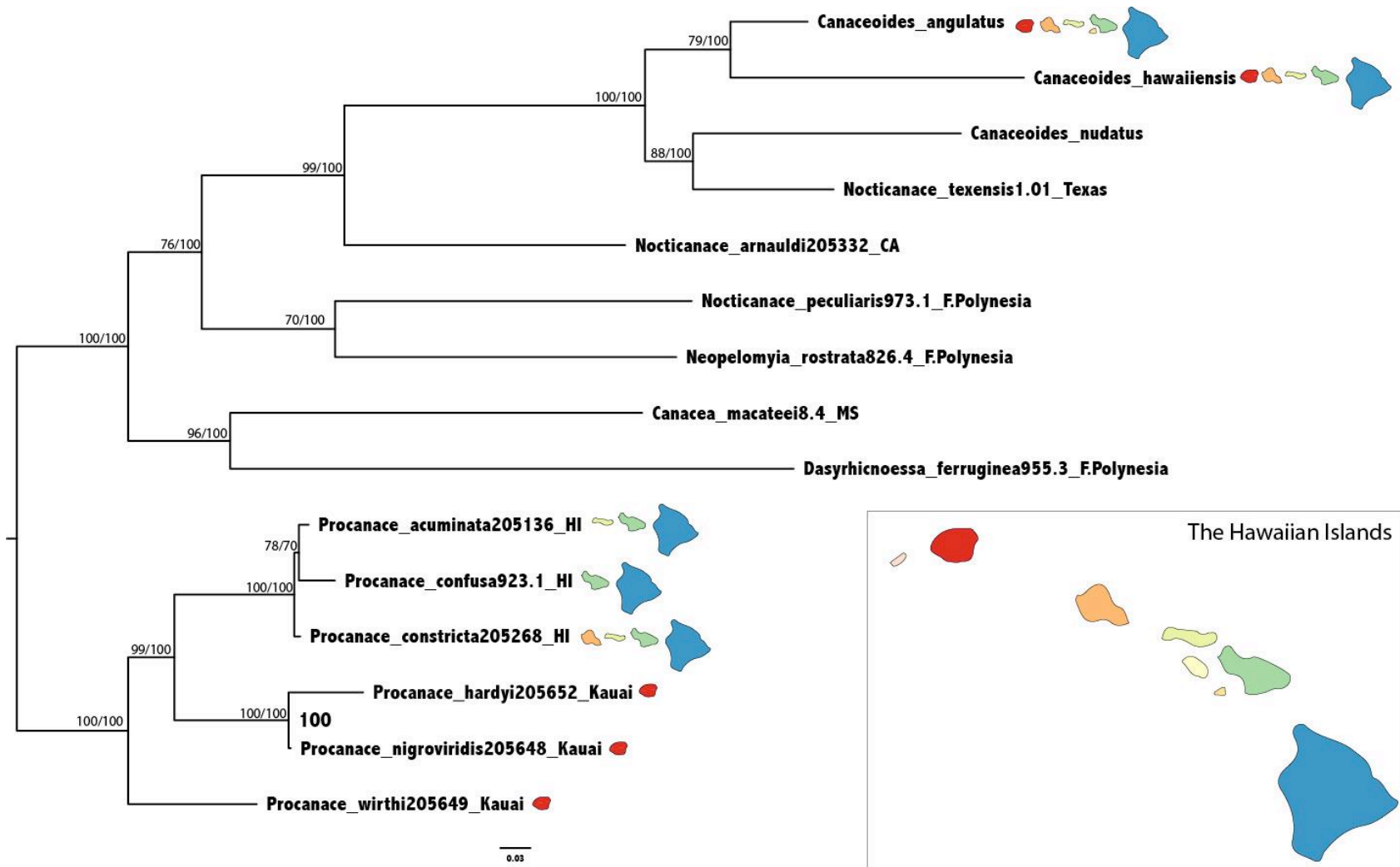


Figure 1.2: Maximum Likelihood (ML) phylogeny of Hawaiian *Procanace* generated by IQ-TREE. Symbols indicate the island(s) where the respective species occur. Node values show ML bootstrap support followed by Bayesian posterior probabilities.

Chapter 2

The Evolution of Marine Dwelling in Diptera

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Transition between chapter 1 and 2

Chapter 1 examined a subset of Canacidae, a fly family with a marine life history. Notably, one lineage of Hawaiian species lost the ability to tolerate saline habitats and now occupies high elevation freshwater streams. The discoveries described in Chapter 1 inspired the work done in Chapter 2. In Chapter 2, I examine multiple transitions between marine, freshwater, and terrestrial environments across the fly tree of life. This chapter further explores the occurrence of marine Diptera and the evolutionary history of marine dwelling.

Abstract

Marine dwelling in Diptera has been relatively unexplored and the frequency of transitions to the marine environment and the evolutionary history remain poorly understood. By reviewing records from the World Register of Marine Species and using ancestral state reconstruction methods, we build on the fly tree of life phylogeny and ecological descriptions of marine life history. Our ancestral state reconstruction analyses suggest marine dwelling is lacking as an ancestral trait for the most recent common ancestor to Diptera. While many transitions in Empidoidea, Sciomyzoidea, Tipulomorpha and Culicomorpha seem to have been gradual, other transitions in Tephritoidea and Tabanomorpha were found likely to have been stochastic occurrences. From the collection of 532 marine species, we reveal several independent transitions to the marine environment throughout the fly tree of life. Considering the results from our analysis, we outline potential adaptations for marine flies, discuss the barriers of colonizing the marine environment and the implications to the mechanisms for salt tolerance.

Introduction

Habitat transitions among marine, freshwater, and terrestrial environments give insights into the challenging barriers in colonizing new habitats. While major habitat transitions have been relatively rare, the ability to cope and interact with environmental changes across ecosystems provides an opportunity to examine the processes of colonization and evolutionary diversification. For example, transitioning from marine to freshwater habitats has allowed multiple radiation and speciation events for many taxa (Lee & Bell, 1999; Waters et al., 2020). On macroevolutionary time scales, this transition from marine to freshwater has occurred more frequently than the transition from freshwater to land (Grosholz, 2002). In addition, non-marine animal clades, when compared to marine clades, have shown higher rates of diversification (Wiens, 2015). Particularly, arthropods have been successful in exploiting habitats, radiating into the greatest species abundance of any extant phylum (Lee, 2016; Thomas et al., 2020). Insects were among the first animals to colonize and exploit terrestrial and freshwater ecosystems (Misof et al., 2014; Vermeij, 2020). The increasing number of insect phylogenies, both within and between orders, and the extensive work relating to major drivers of diversification brings opportunities to examine the broad-scale patterns of ecological transitions.

The species of Diptera are an ecologically diverse group and have colonized the aquatic environment (Adler & Courtney, 2019). Proposed phylogenies have illustrated the evolution of an aquatic life history in many families (Wiegmann et al., 2011). Within studies of aquatic

dipterans, those focusing on marine flies are sparse in comparison to the breadth of freshwater dipteran literature. The marine paradox highlights this gap in the literature: despite the estimated 1.6 million kilometers of coastline around the world, which can range from intertidal zones, estuaries, salt marshes, and dunes to rocky and sandy beaches, marine dwelling insects are rare – estimated to be about 2,037 species of insects in the sea and less than 1,000 fly species associated with the marine environment (Ayyam et al., 2019; R. W. Merritt et al., 2019; Vermeij, 2020). These numbers contrast with an estimated 5.5 million species of insects in total globally (Stork, 2018). As many of these coastal habitats are at risk and in decline across the world due to environmental changes and human activity, a greater understanding of the dipterans living in the marine environment, such as the coast and intertidal zones, would help to interpret responses to changes in sea levels and salinity as a result of global climate change (Beaumont et al., 2014; Kefford et al., 2016). An opportunity to examine the marine environment is also a chance to understand saline environments and the common means of coping with salt and stress.

Marine dwelling is a complex trait for many reasons. Salinity has been a source of stress for many animal phyla, shaping distributions and influencing community structures of ecosystems (Arribas et al., 2014). Evolving salt tolerance may mitigate the effects of osmotic and ionic stress of salinity while also allowing populations to escape predation, to reduce competition, and to avoid water loss (Arribas et al., 2014). However, Diptera are not well known as osmotically sensitive organisms. Previous literature documenting marine adaptation in Diptera is non-existent in contrast to other insect orders, with the exception of Cheng's (1976) compilation of articles on marine arthropods (Cheng, 1976). In contrast, relatives of Diptera have developed several pathways to combat salt. For example, species of Crustacea and Coleoptera have been used in several studies examining osmoregulatory systems in both the freshwater and marine environments (Lee, 2016; Pallarés et al., 2017).

In this paper, we focus on marine-dwelling Diptera. Almost all marine-dwelling insects live in the intertidal zone, spending a fraction of their lives underwater, most commonly in the egg, larval, and pupal stages. We can expect salt tolerant or halobiont (organisms that develop only in saline habitats) species to occur across the fly tree of life as rare events (Szadziwski, 1983). No species of Diptera is known to spend their entire life cycle fully submerged in the sea.

The purpose of this research is to outline an evolutionary model for understanding the few marine-dwelling fly species currently known. We do not make the assumption that salinity plays the only role in determining whether an organism can live in a marine environment, but the implications of inhabiting these environments can illuminate mechanisms and adaptations to saline environments. Macroevolutionary investigations and ancestral state reconstructions can reveal mechanisms that allow for evolutionary transitions (Edwards & Donoghue, 2013). By examining the phylogenetic distribution of marine-dwelling species of flies, we explore what the macroevolutionary perspective can reveal about the evolution of marine dwelling and to the extent of salt tolerance.

Here, we take advantage of the fly tree of life proposed by Wiegmann et al. (2011) to address how local-scale ecology (e.g., inhabiting a marine or terrestrial environment) relates to the diversity of Diptera, and to consider the phylogenetic distribution of marine dwelling Diptera (Wiegmann et al., 2011). A macroevolutionary approach takes a broader view, where the focus is

not on particular species or environments but on detecting any general patterns in the evolution of a trait by comparing a large number of lineages using phylogenetic analyses. We can estimate how often marine dwelling has evolved and its evolutionary history by examining the distribution of marine flies on a phylogeny.

Methods

WoRMS annotation. We compiled a list of 145 Diptera families based on the family-level fly tree of life with 206 species (Wiegmann et al., 2011). To find published reports of Diptera that have at least one life-history stage in a marine environment, we relied on the World Register of Marine Species (WoRMS, 2021; www.marinespecies.org). In WoRMS, species are attributed to the following environments: marine, brackish, freshwater, terrestrial, and combinations thereof (Costello et al., 2015; Horton et al., 2017). The mission of WoRMS, aside from being a global scale biodiversity inventory, is to integrate global marine species information and standardize the species names recorded worldwide since 2008 (Costello et al., 2015; Ng et al., 2017). Taxonomic information are checked by WoRMS's taxonomic experts as a quality assurance process (Ng et al., 2017).

We coded each fly family for ecological traits: marine dwelling and aquatic life history. Fly families containing at least one species from the WoRMS database were coded as “Marine” (Yes = 1) and those without as “Other” (No = 0). Families with an aquatic life history from the literature were recorded as well (Yes = 1/No = 0) (Adler & Courtney, 2019; Wiegmann, 2011). Marine families with 4 species or fewer were designated as ambiguous for potentially being misclassified or observed as rare events (See Supplementary Table 2). The dataset of all records is available as electronic supplementary material in file Supplementary X1. The database of WoRMS records for Diptera was collected in December 2020.

Ancestral state reconstruction. We investigated the evolution of marine dwelling in Diptera to assess how frequently the trait was gained or lost across the proposed phylogeny (Wiegmann et al. 2011). Using a rooted phylogeny as a scaffold and the presence or absence of at least one marine species within the fly family as a character, we employed PastML for ancestral state reconstruction (ASR) for discrete characters (Ishikawa et al., 2019).

Based on the marginal posterior probabilities approximation (MPPA) in PastML, we performed ASR by maximum likelihood. The analyses were performed based on the F-81 model, the Jukes Cantor model, and the estimate from tips (EFT) model. In an EFT model, equilibrium frequencies are calculated based on the tip state proportions. The Jukes Cantor model uses equilibrium frequencies where all frequencies between states are equal, instead of being estimated. Alternatively, the F-81 model allows marginal posteriors to be inferred with an optimized scaling factor. We then assessed the optimal model selected by the Akaike information Criterion (AIC), resulting in the EFT model (Akaike, 1974). Our assessment of the marine diversity, based on the number of species from WoRMS records, was then combined with ASR analyses.

Results & Discussion

Taxonomic distribution: Is marine dwelling confined to a few key specialist clades or does it occur throughout the dipteran phylogeny? Ecological classifications can be examined in Supplementary X1, which list the number of fly families that have been known to occupy the aquatic environment based on WoRMS. Supplementary Table 2 includes the number of Marine species in each family. From our full dataset, we present results of combined traits of marine dwelling, aquatic life history, and ambiguous ecologies in Figure 2.1. WoRMS and the tree from Wiegmann et al. 2011 do not include all flies in existence and all environments they inhabit, but both use the most complete set of fly genetic and ecological data ever collected thus far. By definition, aquatic Diptera includes species with at least one life history stage in either a marine, freshwater, or a combination of both in these environments. Specifically, it is a strong association with bodies of water that encompasses aquatic dipterans, not necessarily saline conditions (R. W. Merritt et al., 2019). In addition, for defining marine insects, Gibson and Choong (2021) state, “A marine insect species is a species that spends at least one of its developmental stages habitually in a marine habitat, that must feed, either as larvae or adults, on other marine organisms, or that has an ethology that is intimately linked to marine organisms, such as a reliance on other insects that depend on marine organisms as hosts”(Gibson & Choong, 2021).

We used the WoRMS database to quantify which families had the highest proportions of marine Diptera. We investigated if marine Diptera are distributed randomly across the fly tree of life with over 200 Dipteran taxa. Marine dwelling had multiple independent origins. Twenty-five families (17% of fly families, 25/145) have at least one species living in the marine environment based on the WoRMS database. The estimation could be far greater when including fly groups that live outside of the sea but in salty environments, such as saline lakes and other inland saline environments. While marine origins are more likely in some clades than in others (like Empidoidea and Sciomyzoidea), marine dwelling flies are dispersed widely on the phylogeny. The groups Bibionomorpha, Stratiomyomorpha, Phoroidea, and Nerioida all lacked any marine species, while other groups had one or a few lineages. Of the 532 WoRMS’ designated marine-dwelling Diptera, the family Therevidae had 180 records, which was the highest number of records. Overall, only three families, Therevidae, Ephydriidae, and Hybotidae, had more than 50 species. Nonetheless, fourteen of the twenty-five families were designated as ambiguous, suggesting that marine dwelling flies are the minority in many Dipteran families, generally constituting a small percentage of the described species (see Supplementary Table 2).

After examining the distribution of marine and aquatic designations across the fly tree of life, a few patterns emerge. Not all aquatic fly families include marine dwelling species and not all marine dwelling species are in families most notable for being aquatic (Figure 2.1). Only 36% (9/25) of marine flies were within otherwise non-aquatic groups (Figure 2.1). These families are Asilidae, Therevidae, Hybotidae, Sphaeroceridae, Anthomyiidae, Sepsidae, Ulidiidae, Australimyziidae, and Heleomyzidae (Figure 2.1).

Ancestral state reconstruction: When did marine dwelling evolve in Diptera? For habitat transitions between marine and other environments, we used maximum likelihood ancestral state reconstruction method in PASTML (Ishikawa et al., 2019). Under the three models of evolutions (JC, F-81, and EFT), the estimated ancestral state constructions were largely congruent, but EFT was concluded to have the best AIC score. We estimate that marine dwelling has evolved at least

20 times in the family-level phylogeny of Diptera (Figure 2.2). Our analysis is at the family level, although shifting to marine habitat has been gained within a superfamily several times (for example in the Empidoidea and Sciomyzoidea).

Our ancestral state reconstruction demonstrated that marine dwelling is likely to have evolved independently multiple times across the Diptera phylogeny. The most recent common ancestor of Diptera was inferred to have lived in an aquatic environment, but not necessarily a marine environment. In the EFT model, the earliest transition could have occurred in the Tipulomorpha, particularly within the Limoniidae lineage with a posterior probability (PP) value of 0.69. Families within Tipulomorpha are notable for their aquatic life history in freshwater ecosystems, and thus transitions to the marine environment could have taken place via the freshwater environment instead of terrestrial environment. Other lineages in Nematocera, specifically within Culicomorpha and Psychodomorpha, exhibited marine dwelling in families like Psychodidae (PP 0.58), Culicidae (PP 0.80), and shared between Ceratopogonidae and Chironomidae (PP 0.54). A direct transition to the marine environment could have taken place among many nematoceran lineages, but had several accelerated reversions to freshwater and terrestrial habitats. This could explain why the state of the most recent common ancestor of lineages in Culicomorpha and Psychodomorpha was ambiguous, but estimated with a posterior probability of 0.37 for marine dwelling.

Among the Empidoidea, fly families Dolichopodidae (PP 0.95) and Hybotidae (PP 0.96) shared a common ancestor, likely to have been capable of living in a marine environment (PP 0.80). Shifts to the marine environment could have originated from a freshwater environment for the notably aquatic family, Dolichopodidae, while in Hybotidae, the transition to the marine environment may have been derived from the terrestrial environment, as there are several terrestrial sister groups (McAlpine et al., 1981). This suggests differences in barriers to colonize the marine environment, and that multiple mechanisms for adapting to the marine conditions are likely at play.

In addition, in the more recent diverging groups in Sciomyzoidea, especially the families Coelopidae, Helcomyzidae and Heterocheilidae, all had marine species within the WoRMS database records. These families share notable aquatic ecologies, with the implication of a progression to the marine environment directly from the freshwater environment. With these lineages, losing the ability to live in the marine environment may not have occurred throughout evolutionary history. Given the absence of evidence for terrestrial species within these families, we earmark them and Canacidae as Diptera families where the most recent common ancestor specialized in the marine environment. These four families may represent the best case for “fully marine adapted” Diptera lineages.

The majority of transitions to the marine environment in Empidoidea, Tipulomorpha, Culicomorpha, and Sciomyzoidea seem to have been gradual. Other transitions in the Tephritoidea (fruit flies) and Tabanomorpha were found to have likely been stochastic occurrences within the lineage. The ancestral state reconstruction suggests that shifts to the marine environment has occurred within more recently diverging Dipteran clades; marine dwelling is lacking as an ancestral trait but arose several times.

The marine fly tree of life. Our distribution of marine dwelling Diptera has challenged the notion that marine flies are a rare phenomenon. The diversity of marine dwelling Diptera has not been demonstrated before in the context of the fly tree of life. Marine dipterans appear across the fly tree of life, lacking prominence across any one specific infraorder or superfamily. The lack of determination of an ancestral marine origin for any one clade could be explained by the limited number of species within the phylogeny or by the lack of literature exploring marine life histories and the ecophysiological adaptations to the aquatic environment. Future work will no doubt reveal the ecological mechanisms that have allowed transitions to the marine environment as well as address the literature gaps in the knowledge of their aquatic ecologies and life histories.

While the currently known marine Diptera species are taxonomically widespread, some groups of marine flies lack any overlap with known aquatic groups. Groups labeled as ambiguous with 4 or fewer species (Figure 2.1) are suspected to include transitions to the marine environment as a random occurrence. We highlight the families containing more than 4 species, suggesting these groups include species where the marine environment may play a significant role in its diversification (See Supplementary Table 2). In addition, we note that several of the designated marine fly families differ in their overall species-level diversity and proportion of marine species (See Supplementary Table 2). For instance, Canacidae (beach flies) is estimated to have 307 global species, where over 90% inhabit the coast and the intertidal zone (Munari & Mathis, 2010). In smaller families like Coelopidae (commonly known as kelp flies with nearly 30 species), and Helcomyzidae (12 species), all described species are exclusively marine, feeding on red and brown algae on the coast. In comparison, Therevidae have over 1,100 species within the family, but the marine species make up a minority of the known global diversity and its larval habitats more commonly range from arid desert environments to open woodlands. Despite what geographical or environmental factors may have influenced these differences in current diversity, future work must consider marine dwelling beyond the family level.

Insect species included in the WoRMS database range in their degree of ecological, physiological, and anecdotal data. WoRMS contains an ongoing list of marine dipterans, but relies entirely upon published literature and the scientific community to self-report ecological descriptions of species. Some groups with well-known marine life history such as *Thoracochaeta* (Sphaeroceridae: Limosiinae; (Hodge et al., 2017; S. A. Marshall, 1982)), *Oedoparena* (Dryomyzidae; (Gibson & Choong, 2021)), and *Telmatogeton* (Chironomidae: Telmatogetoninae; (Brodin & Andersson, 2009; Lorenz Simões et al., 2020; Nondula et al., 2004)), *Thalassomyia* (Chironomidae: Telmatogetoninae; (Qi et al., 2019)), and *Tanytarsus* (Qi et al., 2019) were missing from the overall list of marine species in WoRMS. Similarly, genera like *Fucellia* (Anthomyiidae: Anthomyiinae; (Kaczorowska, 2005) and *Pontomyia* (Chironomidae: Chironominae; (Huang & Cheng, 2011) were given marine designations in WoRMS as a family but were not among the taxa examined in the fly tree of life (Wiegmann et al., 2011). For example, Chironomidae is represented in the phylogeny by a single taxon although the family includes several thousand species, including many known marine dwellers (Morley & Ring, 1972). As more taxa become part of phylogenies, ecological descriptions and life history observations will become essential in further understanding the diversification of these marine lineages.

Our analysis suggests that the distribution of marine dwelling across fly families can be due to multiple evolutionary patterns and individual adaptations in different lineages. This cannot replace detailed understanding of marine adaptations at the physiological level or examination of the particular strategies employed by different species, but the hope is that the macroevolutionary patterns could reveal some of the underlying evolutionary forces that shape biodiversity and distribution of marine Diptera, which might then prompt more fine-scale studies that examine the links in more detail.

Potential macroevolutionary mechanisms. Examining the phylogenetic distribution of marine dwelling flies is a stepping-stone to understanding the evolution of salt tolerance in Diptera. Salt tolerance may be necessary before transitioning to the marine environment or other saline inland habitats, and may also be difficult to persist as a trait across macroevolutionary scales. One reason is that an investment in salt tolerance could be costly. The production of compatible solutes or transport of ions could be a costly evolutionary tradeoff – using up resources that could be put into other functions or behaviors like growth and reproduction. It is important to consider that transitions from one environment to another do not signify that organisms become independent of the environment they have partially left (Bromham, 2015; Vermeij, 2020).

Based on our ancestral state reconstruction, we observe marine dwelling can be a labile trait with an enhanced rate of loss - it is often gained, but then is typically lost several times. This scenario is likely given that marine dwelling may be a result of a combination of several ecophysiological traits and no one particular trait confers salt tolerance or stress tolerance within the marine environment. Within some families, particularly Canacidae and Coelopidae, adapting to the marine environment may have been gained early on in the evolutionary history and then occasionally lost as some of these lineages transitioned to freshwater environments (O’Grady & Pak, 2016). Absence of evidence of reversions to the terrestrial environment within these families suggests that their most recent common ancestor specialized in marine environments. Future work will investigate whether colonizations of the marine environment were via the freshwater or the terrestrial environment and if marine dwelling within a family is difficult to evolve or modify following Dollo’s law (Marshall et al., 1994; Price & Wagner, 2018).

Marine dwelling can vary across lineages based on the degree of stress – acute or chronic. Some putative associations with salt tolerance include cuticle formation, osmoregulation, fat storage, desiccation tolerance, drought tolerance, ion transportation, sodium and chloride pumps, detoxification, and speciation. Marine dwelling as a trait could be explained by several dependencies on other stress tolerant traits. Potential physiological adaptations to a wide range of marine environmental challenges include developing dark shades on the cuticle, short antennae, cremaster (hook structures), enhanced pulvilli (tarsal pads for clinging to surfaces), additional bristles, seasonal dormancy, and other changes in phenology (Brodin & Andersson, 2009; Vaz et al., 2021). Stress from the environment, as abiotic factors, could be in the form of UV radiation, wind, variable temperatures, fluctuating tides, humidity, and water surface tension from the nearby sea (Dionisio-Sese et al., 2001; Ikawa et al., 2012; Peace, 2020). Biotic factors that may be shaping marine fly populations include predation by birds, fish and other beach fauna, competition from other marine organisms, the degree of kelp, algae, and other forms of vegetation, as well as the pathogens and endosymbionts inhabiting the beach (Rechsteiner et al., 2018; Wickham et al., 2020). In addition, human activity has influenced the state of both

terrestrial and marine environments, possibly disrupting natural processes of nutrient cycling and vegetation growth (S. L. Nielsen et al., 2004). We summarize these environmental factors in Figure 2.3.

We speculate different physiological and environmental constraints for flies inhabiting the saline/hypersaline inland habitats (e.g., Ephydridae in Great Salt Lake). It seems likely that other factors may be equally or more critical in the saline inland habitats due to absence of competition from marine arthropods (i.e., crustaceans). Future work will investigate the potential differences between colonizations in the marine environments and the inland saline habitats, and extend to examining the relationships between diversification rates and degrees of salinity.

Conclusions

Understanding the abilities and constraints of dipteran populations to adapt to salt and the marine environment will become more critical as humans continue to impact the world's aquatic resources through climate change, landscape modification, and pollution, resulting in increasingly stressful habitats for aquatic Diptera. Through a macroevolutionary approach, we are making the case that marine Diptera may not be as rare as generally assumed, but that it also is very unlikely to be an adaptation that arose only once within Diptera. Through classifying habitats and studies, we conclude that the abilities of these species to locally adapt to coastal habitats require more work on less observed species and several evolutionary adaptations may be involved.

Our understanding of the evolutionary processes leading to this adaptation is also in its infancy. We summarize the existing knowledge on this subject and present a possible framework toward the development of an evolutionary model of dipteran adaptation to the marine environment and, by extension, salt. While no published list of marine flies will be complete, due to poor knowledge of salt tolerance in certain families and geographical regions, this will be the most extensive database of known marine flies.

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Data Accessibility Statement

Annotations, WoRMS records, and Fly Families (Supplementary X1 file) can be found on Dryad (<https://doi.org/10.6078/D1799Z>)

Figure 2.1: Diptera phylogeny from (Wiegmann et al. 2011) with marine dwelling, aquatic life history, and ambiguous (families with less than 4 marine species) ecologies labelled. Infraorders and superfamilies are colored.

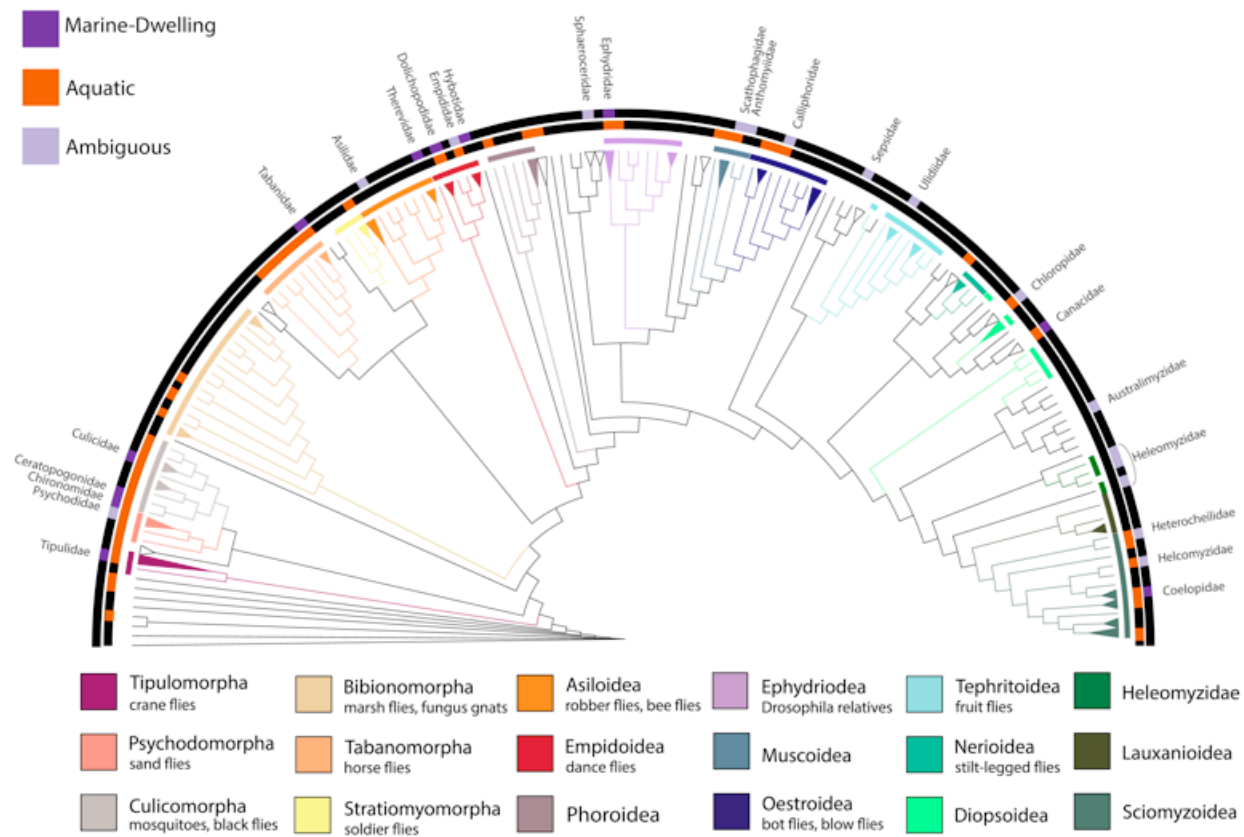


Figure 2.2: Ancestral state reconstruction for marine dwelling flies under EFT model using the Diptera phylogeny from Weigmann et al. 2011. Colored labels show infraorders and several superfamilies. Character states were made based on posterior probability estimates. The marine families based on WoRMS records were categorized into groups with 5 or more species (blue) and those with 4 or fewer species (yellow). Bracket numbers show number of species. Complete list of species, ecological descriptions, and families are found in Supplementary X1. Figures were made through PASTML (Ishikawa et al. 2019, Letunic and Bork 2019).

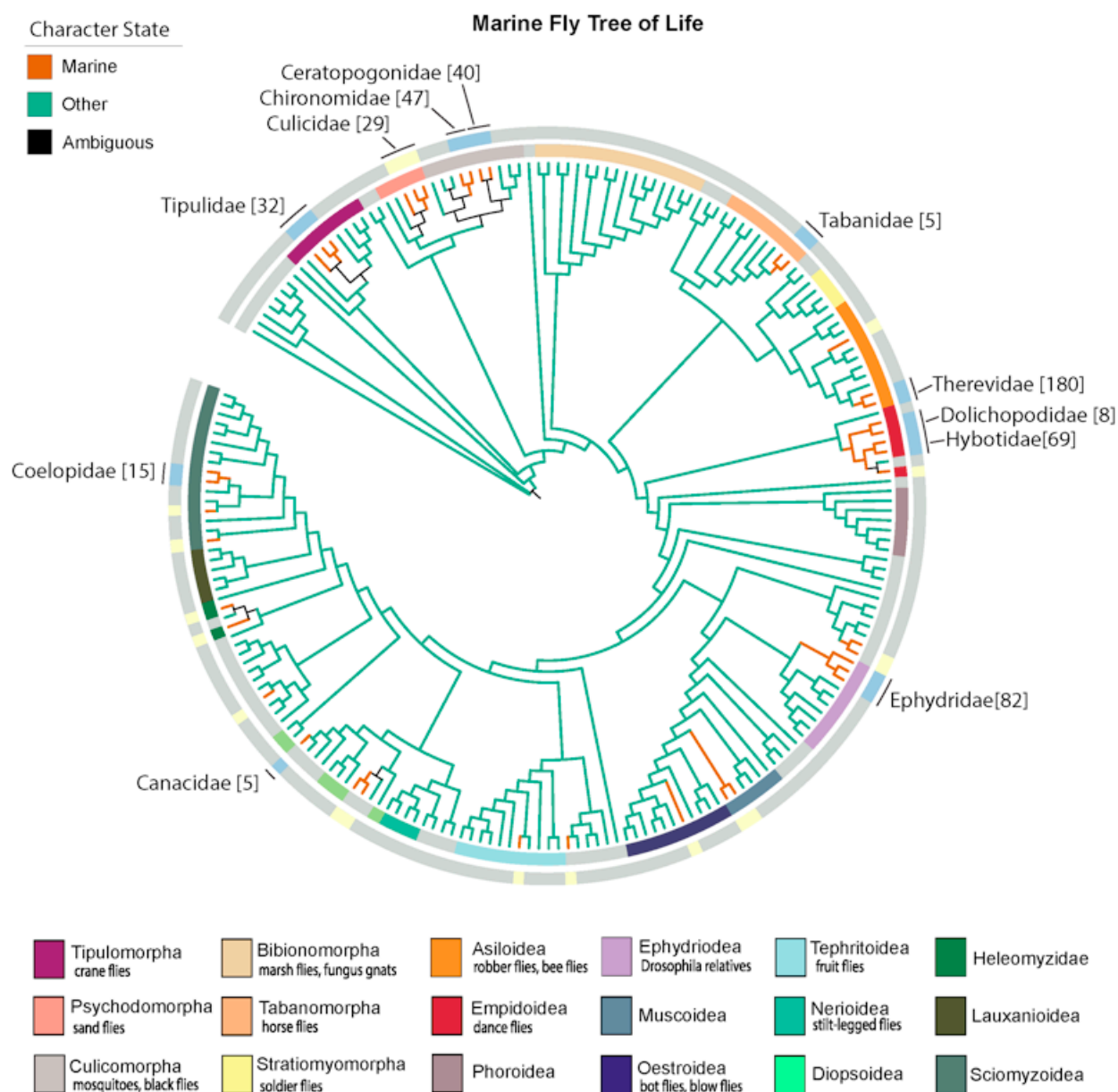
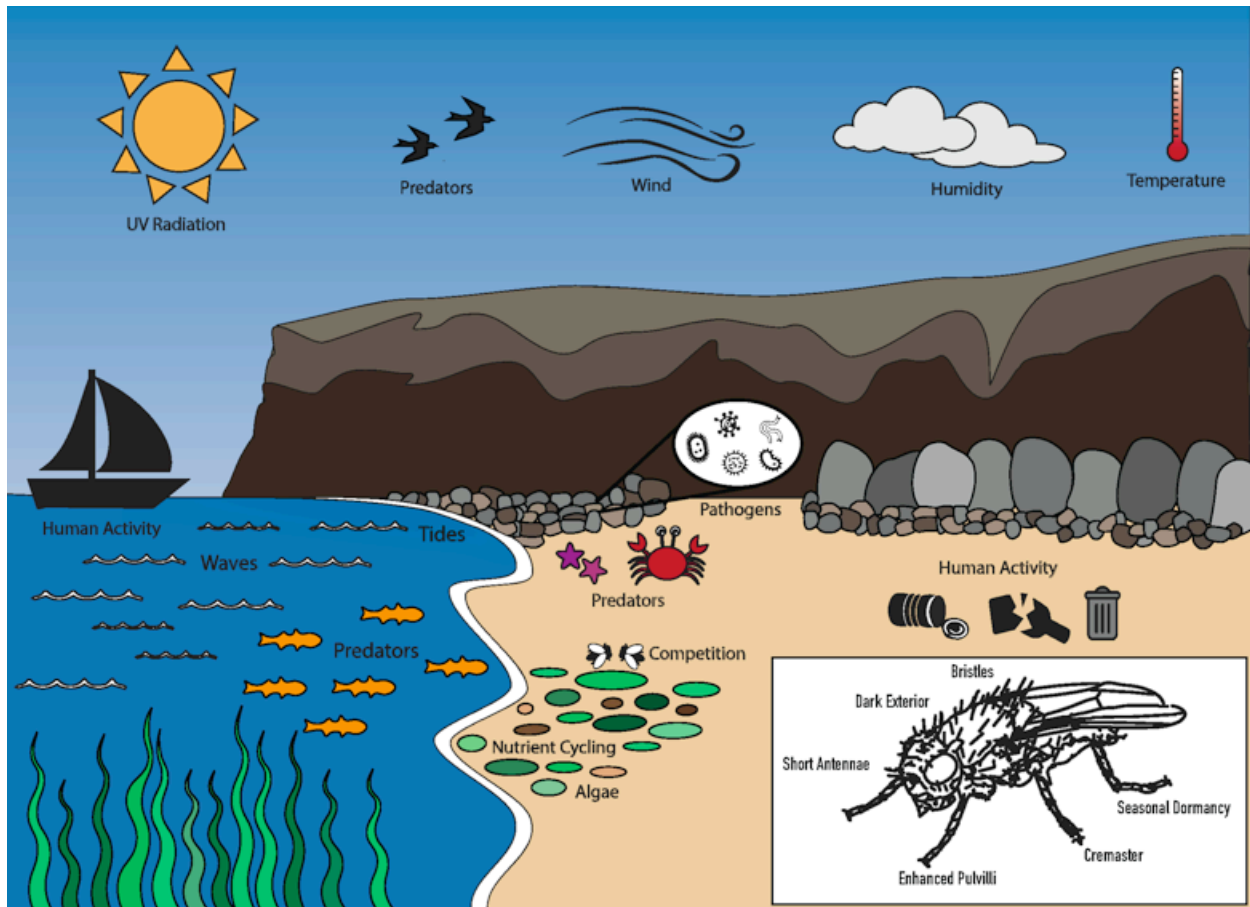


Figure 2.3: Figure 2.3: Stressors (abiotic and biotic) and physiological adaptations for marine flies are shown. Figure was adapted from (Brodin and Andersson 2009).



Chapter 3

Aquatic life history and blood-feeding: reconstructing their association in Diptera

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Transition between chapter 2 and 3

While Chapter 2 examined the evolution of marine dwelling Diptera, Chapter 3 examines the evolutionary history of aquatic Diptera and its possible relations to blood-feeding. Chapter 3 explores the presence of aquatic life history across the fly tree of life and looks for correlations between aquatic life history and blood-feeding.

Abstract

Aquatic Diptera are some of the most diverse and notable flies, impacting human health and providing ecosystem services. Despite the importance of aquatic Diptera, the evolution of aquatic life history and blood-feeding are not sufficiently understood. Here, we examine the evolution of aquatic life histories and their associations with blood-feeding across Diptera. Aquatic and blood-feeding Diptera are phylogenetically conserved across the fly tree of life. We found evidence that the most recent common ancestor of Diptera likely had an aquatic life history. We discuss the rise of blood-feeding flies and specifically speculate on its relation to aquatic life history. Blood-feeding behaviors often occur in fly lineages that also have aquatic life histories, but not significantly more often. Evidence suggests that, in some cases, adaptations to larval aquatic life history were a precursor for flies expanding their diets to take blood meals.

Introduction

Diptera (flies) are generally thought of as terrestrial insects, yet over 46,000 species of flies are aquatic or semiaquatic – more than in any other order of insects (Adler & Courtney, 2019). Aquatic Diptera occupy multiple habitats, varying across damp microhabitats on rocks in the intertidal zones, wet seaweed, water-filled containers, and full submersion in standing and running waters. They also play many significant ecological and societal roles as food resources, bio-control agents, bio-indicators, and medically relevant vectors of diseases (i.e. malaria, dengue, and yellow fever). Despite their importance, many aquatic Diptera have not yet been identified and described due to insufficient ecological knowledge and a lack of taxonomic and natural history investigations (Adler & Courtney, 2019; Courtney, 2019).

It is generally accepted, despite the lack of explicit tests, that early flies exhibited an aquatic life history (Bertone et al., 2008; Bertone & Wiegmann, 2009; Wiegmann, 2011). The earliest diverging extant fly families, Deuterophlebiidae and Nymphomyiidae, are aquatic, with larvae adapted to flowing freshwater aquatic environments (Wiegmann & Yeates, 2017). Closely related clades, including crane flies (Tipuloidea), have many lineages in which the larval stage spends considerable time in an aqueous environment. Similarly, all mosquitoes (Culicidae) have aquatic larvae and require water to lay their eggs (Dale & Breitfuss, 2009; J. R. Wallace, 2019). Larvae from these groups develop by eating particulate matter (detritus), grazing, or filtering algae or diatoms (Bertone et al., 2008; Labandeira, 2005).

It is not clear, however, if the most recent common ancestor of all flies had an aquatic life history. The closest known extant relatives to flies are the Siphonaptera (fleas) and Mecoptera (scorpion flies) (Wiegmann & Yeates, 2017). In contrast to flies, fleas are not associated with aquatic habitats, and often lay their eggs on or near their mammal or bird host. The majority of

mecopterans inhabit moist environments, although the adults are frequently associated with wooded habitats (Capinera, 2008). The mecopteran family Nannochoristidae have aquatic larvae with habits that are superficially similar to dipteran larvae. Considering that early diverging dipteran groups commonly have aquatic associations, it seems possible that the most recent common ancestor of extant flies shared an aquatic life history (Lehane, 2005).

Among the aquatic flies, blood-feeding flies such as mosquitoes (Culicidae), black flies (Simuliidae), and horseflies (Tabanidae) play significant roles in human disease as parasite vectors. Their considerable behavioral and morphological specializations that facilitate blood-feeding have been the subject of extensive research for the development of vector and biological control systems (Gibson & Torr, 1999). Across Diptera, blood-feeding has evolved independently, exhibiting at least 12 separate origins (Wiegmann et al., 2011). Despite the wealth of studies focusing on aquatic ecosystems and blood-feeding behaviors, little is known about the evolutionary history of these traits in Diptera and whether these traits have influenced one another.

This study aims to reconstruct the evolutionary history of aquatic life histories and blood-feeding across the fly tree-of-life, and to test for phylogenetic signal and correlated evolution of these traits. Here, we quantitatively ask the following questions: 1) Did the most recent common ancestor of all Diptera have an aquatic life history? 2) Are blood-feeding or aquatic life history evolutionarily dependent on one another? Or did these traits evolve independently in parallel? We address these questions using the family-level phylogeny of Wiegmann *et al.* (2011), which includes 212 taxa representing over 147 families of Diptera. We test the hypothesis that blood-feeding was more likely to evolve in aquatic flies than in species adapted to other environments.

Methods

Characters. We build upon a family-level molecular phylogeny of flies from a previously published study, Wiegmann *et al.* (2011). This source represents the most recent publicly available dataset that incorporates a comprehensive phylogeny of fly families. We compiled the dataset of 212 taxa representing approximately 147 fly families. Information about the ecological niches of each taxon was extracted from Adler and Courtney (2019) and Wiegmann *et al.* (2011), and was supplemented with data from primary literature, review publications, and personal observations (Adler & Courtney, 2019; Wiegmann et al., 2011). Because of their diverse habitats and lifestyles, aquatic Diptera remain challenging to classify. Idiosyncrasies in the usage of the word “aquatic” in the literature ranging from association with damp microhabitats to full dependence and submersion in water complicate accurate classifications. We coded aquatic characters at the level of family as a proxy for major lineages, as this classification level is widely discussed in Adler and Courtney (2019) and Wiegmann *et al.* (2011). Here, we define a fly family to be aquatic if at least one species has at least one life history stage associated with an aqueous environment. Generally, it is in the larval and pupal stages that flies are most frequently associated with an aqueous environment, but observations of adults with any linkage to the aquatic environment (beyond drinking or transient contact) were also included in the annotations. General categorizations of larval and adult ecological trajectories can be explored at the family or superfamily level without being entangled by the sub-patterns of exception at lower branches (Yeates & Wiegmann, 2005).

Sources we used to gather ecological data relating to blood-feeding include Grimaldi *et al.* (2005), Yuval (2006), and Wiegmann *et al.* (2011). For annotating blood feed behaviors, we coded blood-feeding behaviors based on classifications in Yuval (2006) and Grimaldi (2005). We narrow our definition of blood-feeding in our study to flies that blood-feed as part of their reproductive development. This definition excludes flies that feed on flesh, which may also include lipids, fats, sugars, and muscle tissue in addition to the blood meals, as part of their diet. Annotated tables can be examined in Appendix Table B.1, which list the number of fly families that are known to occupy aquatic environments and feed on blood. We coded each fly family, not the tip labeled species, for ecological traits: aquatic (Yes = 1/ No = 0) and blood-feeding (Yes = 1/No = 0).

Ancestral State Reconstruction of Aquatic Life History and Blood-feeding. We investigated the evolution of the aquatic life history and blood-feeding separately to assess how frequently each trait was gained or lost across the phylogeny. Using a phylogeny with annotated tips (the presence or absence of the fly family's aquatic life history or blood-feeding), we used PastML for ancestral state reconstruction (ASR) for discrete characters (Ishikawa *et al.*, 2019).

We used the marginal posterior probabilities approximation (MPPA) in PastML to perform ASR by maximum likelihood. For the character evolution models, analyses were performed based on the F-81 model, the Jukes Cantor model, and the estimate from tips (EFT) model. The F-81 model allows marginal posteriors to be inferred with an optimized scaling factor, while the Jukes Cantor model uses equilibrium frequencies where all frequencies between states are equal, instead of being estimated. The EFT model calculates the equilibrium frequencies based on the tip state proportions. Visualizations and analyses were created by PastML in zoomable html maps on the PastML webserver (Ishikawa *et al.*, 2019).

Stochastic Character Mapping. An alternative method for ancestral state reconstruction is stochastic character mapping, which provides information on the timing and the evolutionary transitions along the branches of a phylogeny (Huelsenbeck *et al.*, 2003; Nielsen, 2002). Stochastic character mapping can test the presence of aquatic life history as the ancestral state for the most recent common ancestor of flies. To infer possible histories of aquatic and blood-feeding associations along the branches in the fly phylogeny, we used the 'make.simmap' command in the Phytools package implemented in R (Bollback, 2006; R Core Team, 2013; Revell, 2012). We estimated the prior distribution on the root node of the tree based on the character states on the tips of the tree (π = "estimated"). In addition, we used a continuous-time reversible Markov model to fit to our Q matrix (Q = "empirical"). Ancestral states were estimated by an 'equal rates' (ER) model and an 'all rates different' (ARD) model, and then simulated 100 character histories.

Phylogenetic signal and Trait correlations. Phylogenetic signal offers insights into macroevolutionary processes and observed macroecological patterns, elucidating whether it would be difficult to infer evolutionary history of traits within the phylogeny (Hernandez *et al.*, 2013). We determined the presence of phylogenetic signal using the D statistic via the 'phylo.d' function in the R package "caper" (Fritz & Purvis, 2010; Orme *et al.*, 2013). The D statistic compares the observed phylogenetic signal in a binary trait with the signal under a continuous

Brownian motion model of trait evolution. A D-value equal to 1 supports the “no signal” model, where the observed trait has a phylogenetic random distribution across the tips of the phylogeny. $D > 1$ suggests that the trait is more overdispersed than expected at random. $D = 0$ supports the Brownian motion model, and a negative D-value indicates that the traits are strongly clumped (Fritz & Purvis, 2010).

For the trait correlation analysis, Pagel’s (1994) discrete variables compare the likelihood of the independent model to the correlated model of two binary traits (Pagel, 1994). This method characterizes evolutionary changes along the branch of the phylogenetic tree using a continuous-time Markov model. The independent model estimates four parameters, where each of the two variables can go through two evolutionary state transitions ($0 \rightarrow 1$; $0 \leftarrow 1$). In the dependent model, eight parameters are estimated for the four possible states of the two traits (States: (0,0), (0,1), (1,0), and (1,1); Figure 3.1). Changes from one state to another are defined by a transition rate parameter: values Q1:Q8 (Figure 3.1). Using the ‘fitPagel’ function carried out in phytools in R, we implemented these evolutionary models and then selected the best model via log-likelihood and weighted AIC scores (Pagel, 1994; Revell, 2012). The magnitude of the transition rates between the four character states can reveal the associations between those with aquatic life history and blood-feeding (Figure 3.1, (1,1)). Acquiring both traits (Figure 3.1, (1,1)) could have evolved from non-aquatic and non-blood-feeding flies (Figure 3.1, (0,0)) by first gaining an aquatic life history and then becoming blood-feeding (Figure 3.1, (0,1)), or by an alternative route of acquiring blood-feeding and then gaining an aquatic life history (Figure 3.1, (1,0)).

Results

Ancestral state reconstruction of aquatic life history. Flies with aquatic life histories are distributed widely throughout the order Diptera. Although an aquatic life history appears prominently in early diverging dipteran lineages, it also occurs across the fly tree in more recently diverging schizophoran lineages, such as within Sciomyzoidea, Oestroidea, and Muscoidea. As a result of combining information from Adler and Courtney (2019) and Wiegmann *et al.* (2011), we found that 32% (47/147 represented fly families) of fly families have at least one species that exhibits an aquatic life history, while the remaining 68% are restricted to terrestrial environments (Adler & Courtney, 2019; Wiegmann, 2011). Of the nematoceran families, 61% (16/26 families) showed an association with an aquatic habitat. The distribution of aquatic Diptera is shown in Figures 3.2 and 3.3, and a table of the family and its associations can be found in Appendix Table B.1.

Our ancestral state analyses showed evidence that the most recent common ancestor of flies likely had an aquatic life history. At least 30 times across the fly tree-of-life, aquatic life history in flies was independently reacquired. All models of evolution, F-81, EFT, and JC, showed consistent plausible scenarios of an aquatic ancestral state for extant Diptera (Figure 3.2). Posterior probability values from the ancestral state reconstruction analyses were globally consistent across F-81, EFT, and JC models of the tree in both non-brachyceran lineages including Psychodomorpha and Culicomorpha and brachyceran families. We can infer aquatic life history at the root of the phylogeny, i.e. the nodes subtending Deuterophlebiidae, Nymphomyiidae, and all other Diptera, (posterior probabilities 0.72 under F-81, 0.87 under JC and 0.90 under EFT) and at the internal nodes of higher lineages. These included the outgroup

node uniting mecopterans, siphonapterans, and Amphiesmenoptera, and within Diptera in the infraorders Tipulomorpha, Psychodomorpha, and Culicomorpha. In ambiguous cases across of the fly tree-of-life, ancestral aquatic life history was partially supported in the internal splits within Sciomyzoidea, Oestroidea, Opomyzoidea (between groups O and group N in Figure 3.2), and Muscoidea, ranging in posterior probabilities values from 0.47 to 0.72.

Ancestral state reconstruction of blood-feeding in Diptera. Six percent (10/147) of represented families exhibited blood-feeding behaviors (Figure 3.2). These families include Psychodidae, Corethrellidae, Culicidae, Ceratopogonidae, Simuliidae, Rhagionidae, Tabanidae, Hippoboscidae, Glossinidae, and Muscidae. The presence of blood-feeding is commonly seen in nematocerans (mosquitoes, sand flies, and etc.) and Tabanomorpha in the lower Brachycera (horse flies and snipe flies). Although percentages are generalizations of the recognized families, 80% (8/10) of blood-sucking families exhibit an aquatic life history. Lineages within Hippoboscoidea, such as families Hippoboscidae and Glossinidae, showed a lack of aquatic life history, but exhibited blood-feeding behaviors. In the subsection of acalyptratae, although known to exhibit diverse habitats, no known families of flies exhibited obligate blood-feeding.

Phylogenetic signal. To assess the tendency of whether related taxa are more similar to each other than they are to more distantly related taxa, we measured phylogenetic signals for both aquatic life history and blood-feeding. Aquatic life history was found to be phylogenetically clumped among families ($D = -0.67$; probability of resulting from random phylogenetic structure = 0 and probability resulting from Brownian phylogenetic structure 0.94). Similarly, we found blood-feeding to have phylogenetic patterning that is not significantly different from the Brownian expectation, but is extremely unlikely to occur randomly ($D = -1.21$; probability of resulting from random phylogenetic structure = 0 and probability resulting from Brownian phylogenetic structure 0.99). Related taxa are more similar for both aquatic life history and blood-feeding than would be expected by chance.

Stochastic Character Mapping. Stochastic character mapping confirmed the presence of aquatic life history as the ancestral state for Diptera, which is observed with a 0.93 PP (Figure 3.3). Within Neodiptera (which includes Brachycera and Bibionomorpha and does not include Culicomorpha, Psychodomorpha, and Tipulomorpha), aquatic life history was apparently lost but then regained independently in several lineages. For example, aquatic life history is found in Sciomyzoidea with nodes where values ranged from 0.44 PP to 0.93 PP. Tabanomorpha shared an aquatic life history with the ancestral character state value of 0.25 PP.

For blood-feeding, our reconstruction results revealed lack of blood-feeding in the higher levels of ancestral states across the fly tree, but showed prominence of blood-feeding in superfamilies such as Muscoidea and Hippoboscoidea. The presence of blood-feeding is reconstructed as an ancestral character state for Culicomorpha, which contains several blood-feeding mosquitoes (Culicidae) and black flies (Simuliidae) (0.19 PP) (See Figure 1 and 2). Blood-feeding is characteristic of a minority of the families sampled in the fly tree-of-life.

Independent vs. Dependent Trait Correlation. To assess the evidence for trait dependence between aquatic life history and blood-feeding, we performed a phylogenetic maximum likelihood correlation test, using Pagel's method (Pagel, 1994). The trait evolving first is considered more likely to promote the evolution of the subsequent trait (Griffin et al., 2019;

Pagel, 1994). Using the ‘phytools’ package in R, we examined independent and dependent models between two binary traits (Revell, 2012). The independent model was compared in log-likelihoods to the dependent models. Although the difference between the independent and the dependent model fell short of passing the likelihood-ratio test ($LR = 5.567$, $d.f. = 4$, $p > 0.05$), we found that the model where blood-feeding flies were more likely to have first acquired aquatic life history traits before they became blood-feeding had the highest relative model weight for the weighted AIC (0.56, See Table 2.1 and Appendix Table B.2).

Discussion

Phylogenetic signal. Aquatic life history is widely distributed and also found in clusters across the fly tree-of-life. Likewise, blood-feeding is often conserved, as shown through testing of phylogenetic signal (Figure 3.2). A possibility for this non-random phylogenetic distribution in aquatic life history might be that closely related families share similar larval environments and, thus, close relatives are likely to share an aquatic life history. For blood-feeding, the same can be said; perhaps there are genetic constraints or determinants for blood-feeding mouth parts of the adult flies.

Aquatic life history. Summarizing, in a large dataset of 212 taxa and over 147 families of flies, our ancestral state reconstruction analyses based on multiple models indicate that the most recent common ancestor of flies had an aquatic life history. The successful invasion of aquatic environments appears to have been facilitated by a number of preadaptations, which enable them to avert or minimize the negative effects of water exposure. Compared to other terrestrial insects, Diptera that exist in water for parts of their lives must evolve pathways for osmoregulation and respiration as well as alternate methods of locomotion to interact with their fluid environments (Adler & Courtney, 2019; Eriksen et al., 1996; Pak et al., 2021). To feed in water, the head and mouthparts of some aquatic fly taxa are heavily modified with, for instance, cephalic fans for filter feeding and predatory prehensile antennae (Wallace & Merritt, 1980). Aquatic Diptera larvae and pupae of multiple lineages have evolved other adaptations, such as silk, pseudopodia, and suckers to adhere to surfaces in flowing water. The development of cyclorrhaphan immatures allowed exploitation of a wide variety of environments from flesh to fruit, outside of feeding in the aquatic zone (Grimaldi et al., 2005).

Some of these adaptations to life in water have clearly influenced even terrestrial life stages. The intra-chronic meshwork of aeropyle openings on terrestrial eggs allows for breathing during wet conditions, such as during rain (Adler & Courtney, 2019; Hinton, 1969). This may also be the case in terms of sensory modalities. Gustatory and olfactory receptors suitable for detecting molecules in the air are not efficient in water. As insects evolved from marine crustacean ancestors, there was a major functional shift in insect sensory gene networks (Missbach et al., 2014). Thus, insects in which some life stages are aquatic (generally larvae in aquatic flies) and some stages are terrestrial likely have more complex genetic networks involved in olfaction and gustation than purely terrestrial insects (Almudi et al., 2020).

In addition, many species of aquatic Diptera inhabit and develop in microbe-rich environments (Deguenon et al., 2019). The bacterial community associated with aquatic Diptera is diverse and differs among species, life stages, and sexes (Adler & Courtney, 2019). Microbial

communities can influence a host's behavior, development, and speciation rates (Bordenstein & Theis, 2015), so diverse microorganism faunas divergent between terrestrial and aquatic flies may functionally alter fly behavior and morphology. For one species of adult mosquito, altered aquatic microfauna affect the development and biomass during the early instar stages (Duguma et al., 2017). Furthermore, a broad diversity of symbiotic gut fungi (trichomycetes) is associated with aquatic Diptera (Ferrington Jr et al., 2005). Similarly, some blood-feeding flies have endosymbionts that help them extract nutrients from their diet (i.e. tsetse flies and *Wigglesworthia*) (Geiger et al., 2018).

Our findings may aid in future studies by clarifying the coevolution of insect host and vector, such as mosquito-malaria associations (Hurd, 2007). Insects are known to carry thousands of bacterial symbionts, including parasites, mutualistic partners, and even protozoans - species that interact with each other but do not depend on each other for survival (Toth et al., 2006). For example, insights into the evolutionary aspects and spread of malaria can be provided by a deeper understanding of macro-evolutionary relationships between *Plasmodium* and their vectors, as well as with their vertebrate hosts (Lambrechts & Saleh, 2019). Further investigation of these relationships could be valuable for the exploration of symbiotic communities in aquatic Diptera. Characterizing transitions to and from aquatic life history in flies has implications for interpreting fly evolution, diet, morphology, and physiology, as well as their role as disease vectors (Yuval, 2006). How Diptera interacts with the aquatic environment in an evolutionary context remains unexplored, and has many implications for how they diversified and adapted to many ecological habitats.

Blood-feeding. Blood-feeding is often associated with whole vertebrate blood, which is a protein-rich resource, consisting mostly of protein hemoglobin, with small amounts of lipids, fats, and sugars (Harrison et al., 2021). Blood-feeding has evolved multiple times throughout Arthropoda, although less frequently than transitions between terrestrial and aquatic life histories. Distantly related blood-feeding flies have some curious parallels. For instance, blood-feeding Culicomorpha (mosquitoes and relatives), Psychodomorpha (sand flies and relatives), and Tabanomorpha (horse flies and snipe flies) similarly undergo an aquatic larval stage, while only adult females feed on blood (Ribeiro et al., 2010; Yuval, 2006). In addition, these groups also have similar reproductive strategies – most species mate in massive swarms, which may limit female mate choice (Yuval, 2006). Amongst calyptrate flies, blood-feeding behavior evolved independently in Muscoidea and Hippoboscoidea (Yuval, 2006). Generally all life stages of Hippoboscoidea are considered as blood-feeding (Colwell, 2001). Blood-feeding Muscidae, particularly *Stomoxys* and *Haematobosca* (stable flies), have terrestrial larvae and both male and female adults feed on blood (Ibáñez-Bernal et al., 2020).

The existence of an aquatic larval stage is a commonly observed similarity among lineages of blood-feeding flies. The effects of diet and ecological conditions in the larval stage can contribute immensely to the reproductive success of adult flies, and nutritional status is highly correlated to copulatory success (Gobbi et al., 2013; Kaspi et al., 2002; Perez-Staples et al., 2008). Aquatic larvae endure similar ecological conditions which may have selected for parallel adaptations that led to the evolution of blood-feeding behavior in adult flies. It is possible that lineages that commonly lay their eggs in aquatic breeding sites, e.g. Culicidae (mosquitoes) and Tabanidae (horse flies), developed strategies such as larval predation and

anautoxy (female reliance on blood-feeding for egg development) to circumvent ephemeral environmental conditions and/or to overcome nutritionally deficient diets associated with aquatic ecosystems.

Waage (1979) and Yuval (2006) hypothesized that the origin of dipteran blood-feeding evolved either from the expansion of a predatory diet to other species or as a result of close proximity to the host's habitat (Waage, 1979; Yuval, 2006). We see examples of this in rhagionids (snipe flies), where most of the lineages are predatory, but lineages of *Symphoromyia* and *Spaniopsis* have switched to blood-feeding (Imada & Kato, 2016; Wiegmann et al., 2000). There are also many examples of blood-feeding flies living in close proximity to their blood-meal hosts (mammal, frog, and bird) and the waste they produce (sweat, urine, saliva, feces, etc.) (Waage, 1979). Particularly for tabanids, extant blood-feeding flies visit flowers for nectar. The fly ancestors that used piercing-sucking mouthparts for plant feeding may have later transitioned to blood-feeding (Karolyi et al., 2014). Based on our results, we present another route where blood-feeding evolved out of dietary necessity due to the nutritional limitations of an aquatic life history, presumably to assist in securing protein for reproductive success in an environment where resources are temporal or scarce.

Trait correlation analysis. In our trait correlation analysis, our results demonstrated an ambiguous link between an aquatic life history and blood-feeding; transitions to blood-feeding tend to be preceded by acquiring an aquatic life history in many models, but not significantly. This pattern is perhaps not surprising, given that not all aquatic Diptera are blood-feeding. However, for many blood-feeding Diptera, it seems the aquatic life history must have evolved prior to blood-feeding, as we found 80% percent of blood-feeders also share an aquatic life history. Particularly for the blood-feeding trait and less so for aquatic life history, this suggests that these traits are heavily compatible with genetic determination or constraints upon morphology, such as blood-sucking mouth parts and vermiform body plans. Further analyses can isolate the core specific genetic factors among more sampled taxa.

In comparison, few groups of flies that blood-feed do not have aquatic larvae. For example, the larvae of tsetse flies (Glossinidae) develop within the mother before burrowing in soil and pupating immediately (Barclay & Vreysen, 2011). The mother imbibes blood for the slow development of a single larva within its uterus (Barclay & Vreysen, 2011; Yuval, 2006). Internal pupation provides shelter and a guaranteed food source (Rogers & Randolph, 1985).

In addition, given our definition of blood-feeding, families such as Carnidae, Oestridae, Calliphoridae, Sarcophagidae, Chloropidae, and Helomyzidae were not scored as blood feeders. We observe that larval haematophagy is more common among the schizophorans flies than in lower Diptera. While these families are generally parasitic, it is not clear if blood is their only food or whether other nutrients from other tissue or decaying matter is necessary for development. For example, larvae and pupae of the genus *Carnus* (Carnidae) are ectoparasites of bird nestlings. Unlike the mobile blood-feeding adult mosquitoes, these blood feeders are confined to a near-closed environment, feeding on carrion, faeces, and other secretions (Iwasa et al., 2014; Václav et al., 2016). In other families, like Calliphoridae and Sarcophagidae, many species cause myiasis by dispersing their eggs or larvae onto animal flesh or different organs and tissues, causing bacterial contamination and an immune response by the host (de Azeredo-Espin

& Lessinger, 2006). Other schizophoran flies that feed on vertebrate blood usually have specific host species (e.g. keds, nasal bot flies, *Batrachomyia* frog parasites), whereas mosquitoes and horse flies tend to be generalists or specialized on broader groups, like birds (Grimaldi et al., 2005). In many of these cases, the timing and development of emergence of these flies relies heavily on securing shelter and acquiring enough nutrients to survive to adulthood, but not necessarily requiring a blood meal singularly. Blood or a high protein meal could be part of their essential diet, but further investigation would be necessary to disentangle whether specific diet treatments affect development and reproduction of these blood-feeding flies.

The patterns are more clear when we consider other routes for evolutionary transitions. While plant-feeding evolved multiple times in Diptera, we observe no known blood-feeding flies with terrestrial phytophagous larvae (Wiegmann et al., 2011). Few to no blood-feeding flies with larvae that parasitize other insects, with the exception of keds and bat flies (Hippoboscoidea), are seen in which all life stages feed on vertebrate blood. The same can be said for fungivory. It is known that proteins are required for growth and development of eggs. Sources of protein are scattered in nature, as in pollen, films of yeast, and bacteria, yet protein in its most concentrated forms occur in other animals (Grimaldi et al., 2005). This strongly suggests that the repeated evolution of blood-feeding flies with aquatic larvae is not random.

Given the strong evidence that the ancestral fly was aquatic, it may be that an aquatic life history helped pave the way for flies to become blood feeders and vectors for some of the most deadly diseases to humans. The bodies of blood-feeding dipterans are often the sites of sexual reproduction for microbial parasites (O'Donoghue, 2017). In addition, an adult wandering fly finding a large mobile vertebrate host for a blood meal is a feat of sensory coordination. Hematophagous flies use visual cues, heat, and carbon dioxide and other expelled gasses to find their hosts (Gerry et al., 2008). Strong positive selection and gene duplication could solve the conundrum of aquatic larvae requiring olfactory and gustatory receptors with different functionalities than terrestrial adults. This accelerated tempo could then have been co-opted in adult blood-feeding flies. Comparative genetic studies between blood-feeding flies and their relatives are needed, as well as comprehensive comparisons of gut microbiota.

Bridging ecological histories and phylogenetic elements advances our understanding of the evolution of specific traits, despite the number of exceptions to the rule in diverse groups such as flies. Diptera provide a useful study system for understanding the ecological divergence between terrestrial and aquatic environments. Mapping features on phylogenetic hypotheses has shown that flies have repeatedly made ecological shifts (Wiegmann et al., 2011; Wiegmann & Yeates, 2017), with the most recent common ancestor most likely exhibiting an aquatic life history. Future research may reveal whether the propensity for blood feeding in aquatic flies has a genetic basis. Improved taxonomic sampling is needed to establish the history of specific transitions for each group of blood-feeding flies. Improved sampling would also allow for precise sister group comparisons between blood-feeding flies and their relatives, and aquatic and terrestrial flies to explore evolutionary rate shifts. Further understanding of the ecological basis for blood-feeding in flies may help the biological approaches for pest and disease control. As phylogenetic studies continue to refine the fly tree-of-life, and ecological characters become better understood, future investigations can further test hypotheses of trait evolution and correlation in aquatic and blood-feeding Diptera.

Figure 3.1: Diagram showing the rates of change between the states between aquatic to blood feeders through the Q1- Q8 values. The thickness of arrows is proportional to an increased rate.

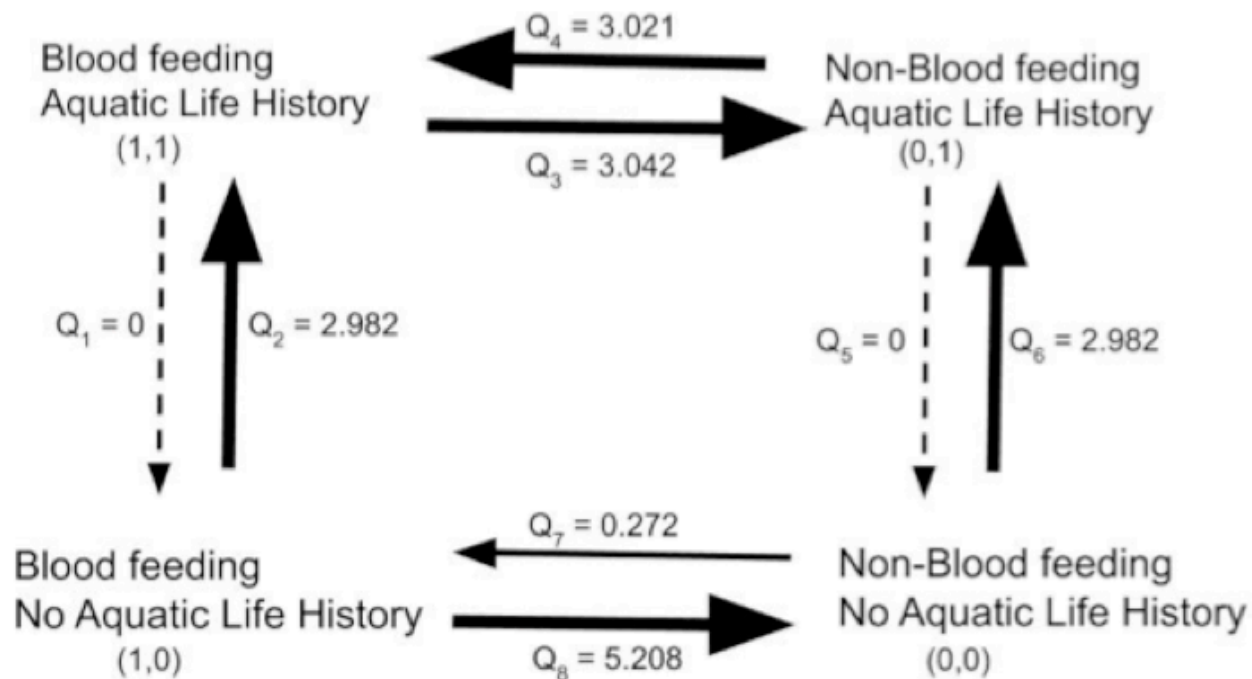


Figure 3.2: Ancestral state reconstruction for discrete characters from PastML constructed via MPPA under F-81 model of evolution. The black branch lengths represent segments of the tree where the estimate of the state is ambiguous (either aquatic, blood-feeding or other), e.g. if larval biology is unknown (Ishikawa et al., 2019; Letunic & Bork, 2021).

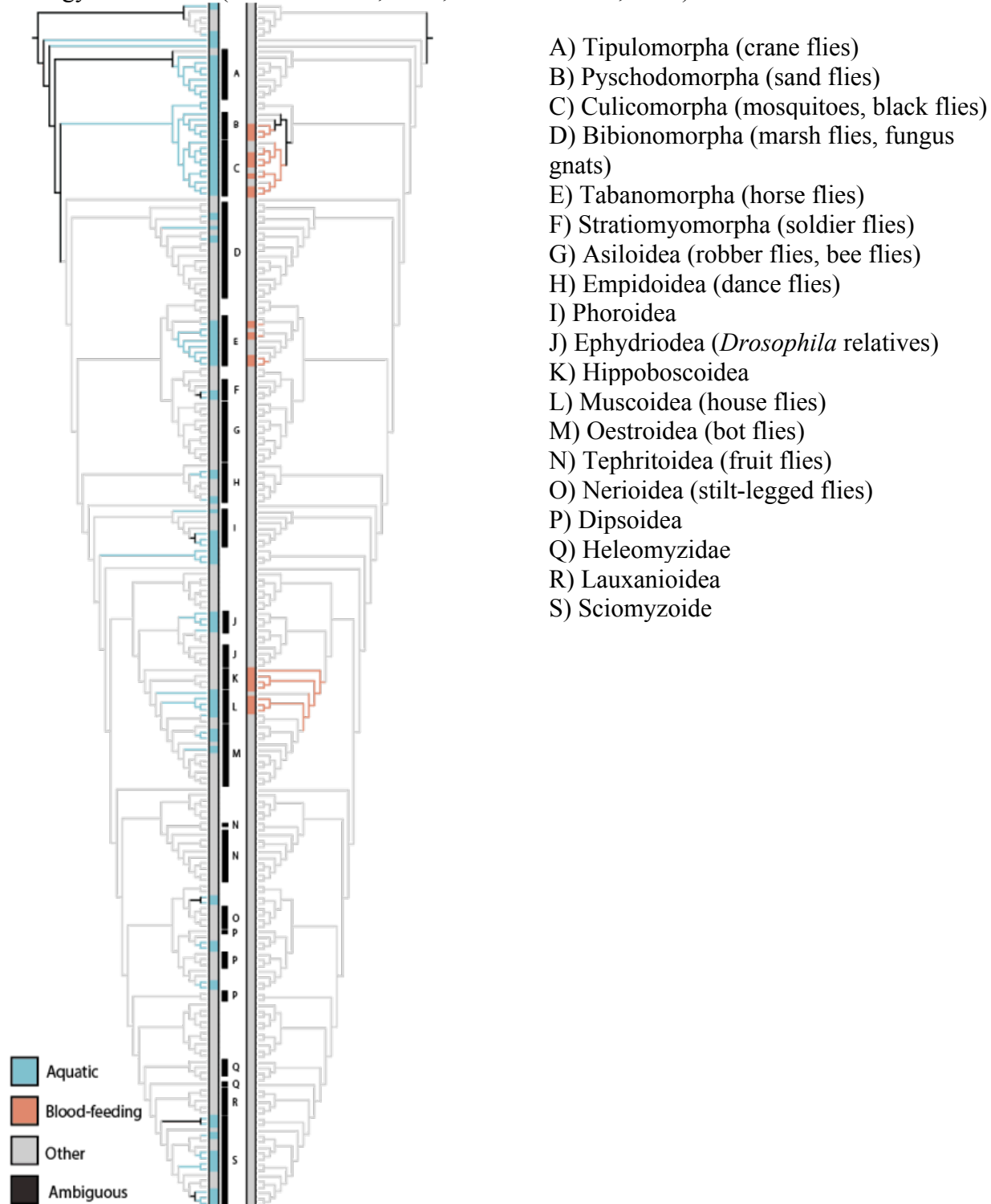


Figure 3.3: Stochastic Character Mapping of aquatic life history with a focus on the backbone of the fly phylogeny based on Wiegmann *et al.* (2011). Pie charts at nodes represent ancestral states that were calculated as the marginal posterior probability (PP) of each possible discrete character state.

Aquatic Fly Phylogeny

- Aquatic Life History
- Other

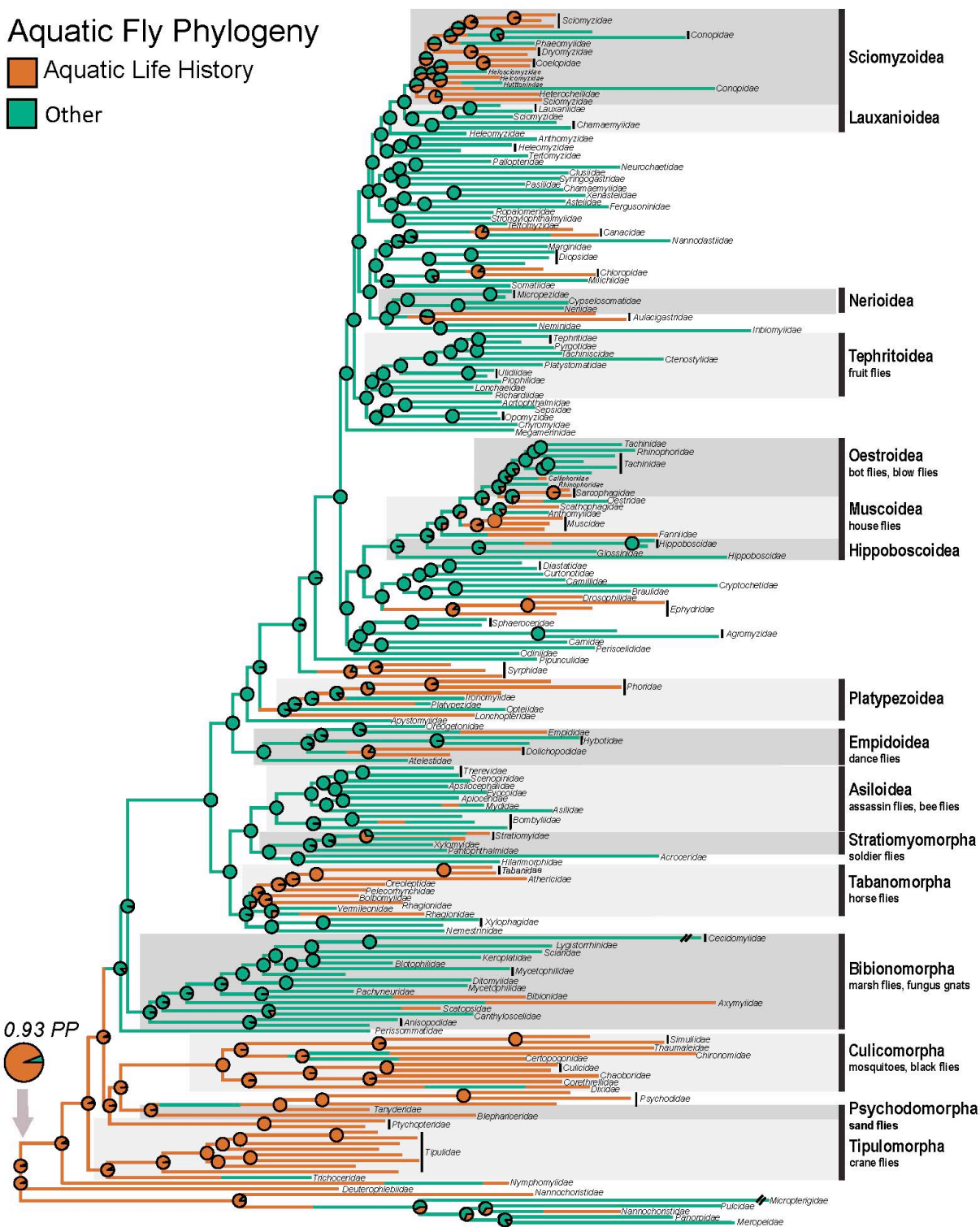


Table 2.1: Trait Correlation Models and AIC scores

Models	Independent	aquatic life history is dependent on blood feeders	blood feeders is dependent on aquatic life history	aquatic life history and blood feeders are dependent on each other
AIC	297.0316	298.9927	295.4688	299.3366
Weighted AIC	0.2580	0.0967	0.5636	0.0815

Conclusion

This dissertation builds on our understanding of aquatic Diptera, how they adapt to saltwater conditions in marine environments, and evolutionary associations with blood-feeding. We find patterns when combining ecological data with phylogenies. In the first chapter, we witnessed a single transition between marine to freshwater within lineages of the beach fly (Canacidae: *Procanace*) phylogeny. Then, we observed the prevalence of marine flies across the fly tree of life. Although initially believed to be rare occurrences, we found that marine flies are widely distributed across the Diptera phylogeny. Finally, we explored the evolution of aquatic life history and tested whether aquatic life history is associated with blood-feeding. Although not statistically significant, we found most blood feeding flies share an aquatic life history. Overall, our collective understanding of the evolutionary processes that lead flies to adapt to the aquatic environment is still in its infancy. Thus, building on our results here, future studies that add more ecological descriptions, taxon sampling, and phylogenies will clarify evolutionary patterns in these important and widespread insects.

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Appendix B. Supplementary Information for Chapter 3

Table B.1: Annotated Families

Not all infraorder or superfamilies are labeled, and annotations were made for families (not based on the species from the tips) that included one species that had an aquatic life history or blood feeding behavior. Species are based on the phylogeny from Wiegmann et al. (2011).

Infraorder or superfamilies	Family	Species list	Aquatic	Blood feed
	Scorpion Fly	<i>Merope_tuber</i>		
	Scorpion Fly	<i>Panorpa_sp</i>		
	Nannochoristidae	<i>Nannochorista_sp</i>	Yes	
	Pulicidae	<i>Ctenocephalides_felis</i>		
	Micropterigidae	<i>Micropteryx_calthella</i>		
	Nannochoristidae	<i>Microchorista_philpotti</i>		
	Deuterophlebiidae	<i>Deuterophlebia_coloradensis</i>	Yes	
	Nymphomyiidae	<i>Nymphomyia_dolichozeza</i>	Yes	
Tipulomorpha	Trichocercidae	<i>Trichocera_brevicornis</i>		
Tipulomorpha	Pediciidae	<i>Ula_elegans</i>	Yes	
Tipulomorpha	Limoniidae	<i>Dactylolabis_montana</i>	Yes	
Tipulomorpha	Limoniidae	<i>Hexatoma_longicornis</i>	Yes	
Tipulomorpha	Limoniidae	<i>Hoplolabis_armata</i>	Yes	
Tipulomorpha	Cylindrotomidae	<i>Liogma_nodicornis</i>	Yes	
Tipulomorpha	Limoniidae	<i>Antocha_obtusa</i>	Yes	
Tipulomorpha	Tipulidae	<i>Dolichozeza_americana</i>		
Tipulomorpha	Tipulidae	<i>Tipula_abdominalis</i>	Yes	
	Ptychopteridae	<i>Bittacomorpha_sp</i>	Yes	
	Ptychopteridae	<i>Ptychoptera_sp</i>	Yes	
Psychodomorpha	Blephariceridae	<i>Edwardsina_gigantea</i>	Yes	
Psychodomorpha	Tanyderidae	<i>Protoplasa_fitchii</i>	Yes	
Psychodomorpha	Psychodidae	<i>Clogmia_albipunctata</i>	Yes	Yes
Psychodomorpha	Psychodidae	<i>Lutzomyia_longipalpis</i>	Yes	Yes
Psychodomorpha	Psychodidae	<i>Phlebotomus_duboscqui</i>	Yes	Yes
Culicopmorpha	Dixidae	<i>Dixa_submaculata</i>	Yes	
Culicopmorpha	Corethrellidae	<i>Corethrella_amazonica</i>	Yes	Yes
Culicopmorpha	Chaoboridae	<i>Chaoborus_americana</i>	Yes	
Culicopmorpha	Culicidae	<i>Toxorhynchitesamboinensis</i>	Yes	Yes
Culicopmorpha	Culicidae	<i>Anopheles_gambiae</i>	Yes	Yes
Culicopmorpha	Ceratopogonidae	<i>Stilobezzia_antennalis</i>	Yes	Yes
Culicopmorpha	Chironomidae	<i>Chironomus_tepperi</i>	Yes	

Culicopmorpha	Thaumaleidae	<i>Androprosopa_americana</i>	Yes	
Culicopmorpha	Simuliidae	<i>Simulium_paynei</i>	Yes	Yes
Culicopmorpha	Simuliidae	<i>Parasimulium_crosskeyi</i>	Yes	Yes
	Perissommatidae	<i>Perissomma_mcalpinei</i>		
Bibionomorpha	Anisopodidae	<i>Olbiogaster_sackeni</i>		
Bibionomorpha	Anisopodidae	<i>Sylvicola_fenestralis</i>		
Bibionomorpha	Canthyloscelidae	<i>Synneuron_decipiens</i>		
Bibionomorpha	Scatopsidae	<i>Coboldia_fuscipes</i>	Yes	
Bibionomorpha	Axymyiidae	<i>Axymyia_furcata</i>	Yes	
Bibionomorpha	Bibionidae	<i>Bibio_longipes</i>	Yes	
Bibionomorpha	Pachyneuridae	<i>Cramptonomyia_spenceri</i>		
Bibionomorpha	Mycetophilidae	<i>Manota_sp</i>		
Bibionomorpha	Ditomyiidae	<i>Symmerus_annulatus</i>		
Bibionomorpha	Mycetophilidae	<i>Diadocidia_ferruginosa</i>		
Bibionomorpha	Mycetophilidae	<i>Pseudobrachypeza_bulbosa</i>		
Bibionomorpha	Bolitophilidae	<i>Bolitophila_sp</i>		
Bibionomorpha	Keroplastidae	<i>Arachnocampa_flava</i>		
Bibionomorpha	Sciaridae	<i>Bradysia_tilicola</i>		
Bibionomorpha	Lygistorrhinidae	<i>Lygistorrhina_sanctaecatharinae</i>		
Bibionomorpha	Cecidomyiidae	<i>Mayetiola_destructor</i>		
Bibionomorpha	Cecidomyiidae	<i>Lestremiinae_sp</i>		
	Nemestrinidae	<i>Trichophthalma_sp</i>		
	Xylophagidae	<i>Xylophagus_abdominalis</i>		
	Xylophagidae	<i>Exeretonevra_angustifrons</i>		
Tabanomorpha	Rhagionidae	<i>Rhagio_hirtis</i>	Yes	Yes
Tabanomorpha	Vermileonidae	<i>Vermileo_opacus</i>		
Tabanomorpha	Rhagionidae	<i>Chrysopilus_thoracicus</i>	Yes	Yes
Tabanomorpha	Bolbomyiidae	<i>Bolbomyia_nana</i>	Yes	
Tabanomorpha	Pelecorrhynchidae	<i>Glutops_singularis</i>	Yes	
Tabanomorpha	Oreoleptidae	<i>Oreoleptis_torrenticola</i>	Yes	
Tabanomorpha	Athericidae	<i>Atherix_variegata</i>	Yes	
Tabanomorpha	Tabanidae	<i>Tabanus_atratus</i>	Yes	Yes
Tabanomorpha	Tabanidae	<i>Haematopota_pluvialis</i>	Yes	Yes
	Hilarimorphidae	<i>Hilarimorpha_mentata</i>		
	Acroceridae	<i>Ogcodes_basalis</i>		
Stratiomyomorpha	Pantophthalmidae	<i>Pantophthalmus_bellardii</i>		
Stratiomyomorpha	Xylomyidae	<i>Xylomya_parens</i>		
Stratiomyomorpha	Stratiomyidae	<i>Actina_viridis</i>	Yes	
Stratiomyomorpha	Stratiomyidae	<i>Hermetia_illucens</i>	Yes	
Asiloidea	Bombyliidae	<i>Mythicomyia_sp</i>		

Asiloidea	Bombyliidae	<i>Paracosmus_sp</i>	
Asiloidea	Bombyliidae	<i>Bombylius_major</i>	
Asiloidea	Asilidae	<i>Asilus_crabroniformis</i>	
Asiloidea	Mydidae	<i>Mydas_clavatus</i>	
Asiloidea	Apioceridae	<i>Apiocera_haruspex</i>	
Asiloidea	Evocoidae	<i>Evocoa_chilensis</i>	
Asiloidea	Apsilocephalidae	<i>Apsilocephala_sp</i>	
Asiloidea	Scenopinidae	<i>Prorates_sp</i>	
Asiloidea	Therevidae	<i>Acrosathe_novella</i>	
Asiloidea	Therevidae	<i>Phycus_kroeberi</i>	
Empidoidea	Atelestidae	<i>Atelestus_pulicarius</i>	
Empidoidea	Dolichopodidae	<i>Parathalassius_candidatus</i>	Yes
Empidoidea	Dolichopodidae	<i>Neurigona_quadrifasciata</i>	Yes
Empidoidea	Hybotidae	<i>Ocydromia_glabricula</i>	
Empidoidea	Hybotidae	<i>Hybos_culiciformis</i>	
Empidoidea	Empididae	<i>Clinocera_caerulea</i>	Yes
	Oreogetonidae	<i>Oreogeton_scopifer</i>	
	Apystomyiidae	<i>Apystomyia_elinguis</i>	
Platypezoidea	Lonchopteridae	<i>Lonchoptera_uniseta</i>	Yes
Platypezoidea	Opetiidae	<i>Opetia_nigra</i>	
Platypezoidea	Platypezidae	<i>Paraplatypeza_velutina</i>	
Platypezoidea	Ironomyiidae	<i>Ironomyia_nigromaculata</i>	
Platypezoidea	Phoridae	<i>Sciadocera_rufomaculata</i>	Yes
Platypezoidea	Phoridae	<i>Phora_sp</i>	Yes
Platypezoidea	Phoridae	<i>Megaselia_scalaris</i>	Yes
Syrphoidea	Syrphidae	<i>Microdon_tristis</i>	Yes
Syrphoidea	Syrphidae	<i>Episyrphus_balteatus</i>	Yes
Syrphoidea	Syrphidae	<i>Rhingia_nasica</i>	Yes
Syrphoidea	Pipunculidae	<i>Cephalops_longistylis</i>	
	Odiniidae	<i>Neoalticomerus_seamansi</i>	
	Perisclidinae	<i>Perisclis_sp</i>	
	Carnidae	<i>Meoneura_sp</i>	
	Agromyzidae	<i>Cerodontha_dorsalis</i>	
	Agromyzidae	<i>Phytomyza_ilicicola</i>	
	Sphaeroceridae	<i>Spelobia_bifrons</i>	
	Sphaeroceridae	<i>Pseudocollinella_humida</i>	
Ephydroidea	Ephydridae	<i>Coenia_palustris</i>	Yes
Ephydroidea	Ephydridae	<i>Psilopa_polita</i>	Yes
Ephydroidea	Ephydridae	<i>Hydrellia_griseola</i>	Yes
Ephydroidea	Drosophilidae	<i>Drosophila_melanogaster</i>	Yes

Carnoidea	Braulidae	<i>Braula_coeca</i>		
Carnoidea	Cryptochetidae	<i>Cryptochetum_sp</i>		
Ephydroidea	Camillidae	<i>Camilla_sp</i>		
Ephydroidea	Curtonotidae	<i>Curtonotum_helvum</i>		
Ephydroidea	Diastatidae	<i>Campichoeta_punctum</i>		
Ephydroidea	Diastatidae	<i>Diastata_fuscula</i>		
Hippoboscoidea	Hippoboscidae	<i>Paratrichobius_longicrus</i>		Yes
Hippoboscoidea	Glossinidae	<i>Glossina_morsitans</i>		Yes
Hippoboscoidea	Hippoboscidae	<i>Crataerina_hirudinus</i>		Yes
Hippoboscoidea	Hippoboscidae	<i>Ornithomyia_avicularia</i>		Yes
Muscoidea	Fanniidae	<i>Fannia_canicularis</i>	Yes	
Muscoidea	Muscidae	<i>Drymeia_alpicola</i>	Yes	Yes
Muscoidea	Muscidae	<i>Musca_domestica</i>	Yes	Yes
Muscoidea	Muscidae	<i>Stomoxys_calcitrans</i>	Yes	Yes
Muscoidea	Anthomyiidae	<i>Delia_radicum</i>		
Muscoidea	Scathophagidae	<i>Scatophaga_stercoraria</i>	Yes	
Oestroidea	Oestridae	<i>Cephenemyia_phobifer</i>		
Oestroidea	Sarcophagidae	<i>Sarcophaga_bullata</i>	Yes	
Oestroidea	Sarcophagidae	<i>Peckia_alvarengai</i>	Yes	
Oestroidea	Rhinophoridae	<i>Phyto_melanocephala</i>		
Oestroidea	Calliphoridae	<i>Cochliomyia_macellaria</i>	Yes	
Oestroidea	Tachinidae	<i>Gymnosoma_nudifrons</i>		
Oestroidea	Tachinidae	<i>Siphona_flavifrons</i>		
Oestroidea	Tachinidae	<i>Tachina_grossa</i>		
Oestroidea	Tachinidae	<i>Exorista_larvarum</i>		
Oestroidea	Rhinophoridae	<i>Axinia_zentae</i>		
Oestroidea	Tachinidae	<i>Phania_funesta</i>		
Diopsoidea	Megamerinidae	<i>Megamerina_sp</i>		
Sphaeroceroidea	Chyromyidae	<i>Gymnochiromyia_sp</i>		
Opomyzoidea	Opomyzidae	<i>Geomyza_tripunctata</i>		
Opomyzoidea	Opomyzidae	<i>Opomyza_florum</i>		
	Sepsidae	<i>Sepsis_cynipsea</i>		
Carnoidea	Acartophthalmidae	<i>Acartophthalmus_nigrinus</i>		
Tephritoidea	Richardiidae	<i>Richardia_teevani</i>		
	Lonchaeidae	<i>Lonchaea_polita</i>		
Tephritoidea	Piophilidae	<i>Mycetaulus_sp</i>		
Tephritoidea	Ulidiidae	<i>Melieria_omissa</i>		
Tephritoidea	Ulidiidae	<i>Herina_palustris</i>		
Tephritoidea	Platystomatidae	<i>Rivellia_syngenesiae</i>		
Tephritoidea	Ctenostylidae	<i>Nepaliseta_ashleyi</i>		

Tephritoidea	Tachiniscidae	<i>Tachinisca_cyaneiventris</i>	
Tephritoidea	Pyrgotidae	<i>Cardiacera_nr._miliacea</i>	
Tephritoidea	Tephritidae	<i>Ceratitis_capitata</i>	
Tephritoidea	Tephritidae	<i>Trupanea_bullocki</i>	
Carnoidea	Inbiomyiidae	<i>Inbiomyia_mcalpineorum</i>	
Opomyzoidea	Neminidae	<i>Nemula_longarista</i>	
Opomyzoidea	Aulacigastridae	<i>Aulacigaster_sp</i>	Yes
Opomyzoidea	Aulacigastridae	<i>Cyamops_nebulosa</i>	Yes
Nerioidea	Neriidae	<i>Telostylinus_sp</i>	
Nerioidea	Cypselosomatidae	<i>Rhinopomyzella_sp</i>	
Nerioidea	Micropezidae	<i>Compsobata_cibaria</i>	
Nerioidea	Micropezidae	<i>Cnodacophora_sellata</i>	
Diopsoidea	Somatiidae	<i>Somatia_aestiva</i>	
Carnoidea	Milichiidae	<i>Paramyia_nitens</i>	
Carnoidea	Chloropidae	<i>Incertella_albipalpis</i>	Yes
Carnoidea	Chloropidae	<i>Thaumatomyia_notata</i>	Yes
Diopsoidea	Diopsidae	<i>Teloglabus_sp</i>	
Diopsoidea	Diopsidae	<i>Sphyracephala_brevicornis</i>	
Diopsoidea	Diopsidae	<i>Cyrtodiopsis_dalmanni</i>	
Opomyzoidea	Marginidae	<i>Margo_sp</i>	
Sphaeroceroidea	Nannodastiidae	<i>Azorastia_mediterranea</i>	
Carnoidea	Canacidae	<i>Procanace_dianneae</i>	Yes
Carnoidea	Canacidae	<i>Tethinosoma_fulvifrons</i>	Yes
Diopsoidea	Tanypezidae	<i>Neotanypeza_sp</i>	
		<i>Strongylophthalmyia_pengelly</i>	
Diopsoidea	Strongylophthalmyiidae	<i>i</i>	
	Ropalomeridae	<i>Willistoniella_pleuropunctata</i>	
Opomyzoidea	Fergusoninidae	<i>Fergusonina_turneri</i>	
Opomyzoidea	Asteiidae	<i>Asteia_amoena</i>	
Opomyzoidea	Xenasteiidae	<i>Xenasteia_shalam</i>	
Carnoidea	Australimyziidae	<i>Australimyza_sp</i>	
	Psilidae	<i>Chamaepsila_hennigi</i>	
	Syringogastridae	<i>Syringogaster_sp</i>	
Opomyzoidea	Clusiidae	<i>Clusia_lateralis</i>	
Opomyzoidea	Neurochaetidae	<i>Neurochaeta_sp</i>	
Tephritoidea	Pallopteridae	<i>Palloptera_umbellatarum</i>	
Opomyzoidea	Teratomyzidae	<i>Teratomya_sp</i>	
Helomyzidae	Heleomyzidae	<i>Trixoscelis_sp</i>	
Helomyzidae	Heleomyzidae	<i>Suillia_variegata</i>	
Opomyzoidea	Anthomyzidae	<i>Anthomyza_gracilis</i>	

Helomyzidae	Heleomyzidae	<i>Heteromyza atricornis</i>	
Lauxanioidea	Chamaemyiidae	<i>Parochthiphila coronata</i>	
Lauxanioidea	Chamaemyiidae	<i>Cremifania nearctica</i>	
Lauxanioidea	Celyphidae	<i>Spaniocelyphus umsinduzi</i>	
Lauxanioidea	Lauxaniidae	<i>Lyciella decipiens</i>	
Lauxanioidea	Lauxaniidae	<i>Minettia flaveola</i>	
Sciomyzoidea	Sciomyzidae	<i>Natalimyza sp</i>	Yes
Sciomyzoidea	Heterocheilidae	<i>Heterocheila buccata</i>	Yes
Sciomyzoidea	Conopidae	<i>Stylogaster sp</i>	
Sciomyzoidea	Huttoninidae	<i>Huttonina glabra</i>	
Sciomyzoidea	Helcomyzidae	<i>Helcomyza mirabilis</i>	Yes
Sciomyzoidea	Helosciomyzidae	<i>Neosciomyza luteipennis</i>	
Sciomyzoidea	Coelopidae	<i>Lopa convexa</i>	Yes
Sciomyzoidea	Coelopidae	<i>Coelopa vanduzeei</i>	Yes
Sciomyzoidea	Dryomyzidae	<i>Dryomyza anilis</i>	Yes
Sciomyzoidea	Dryomyzidae	<i>Oedoparena glauca</i>	Yes
Sciomyzoidea	Phaeomyiidae	<i>Pelidnoptera nigripennis</i>	
Sciomyzoidea	Conopidae	<i>Physocephala marginata</i>	
Sciomyzoidea	Conopidae	<i>Myopa sp</i>	
Sciomyzoidea	Sciomyzidae	<i>Pherbellia annulipes</i>	Yes
Sciomyzoidea	Sciomyzidae	<i>Neolimnia obscura</i>	Yes
Sciomyzoidea	Sciomyzidae	<i>Tetanocera ferruginea</i>	Yes

Table B.2: Trait correlation analysis

Trait	Correlation test log Likelihood	LRT
Aquatic - Blood-feeding	Independent log lik: -144.5158 Dependent log lik: -141.7344	5.5627; p = 0.0619