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UNDERSTANDING THE HORSE FLY SPECIES AND SPECIES RELATIONS OF THE T*abanus nigrovittatus* COMPLEX ALONG COASTAL LOUISIANA

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Entomology

by
Darrius M. Davis
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ABSTRACT

The Dipteran family Tabanidae is among the most diverse families of insects that is comprised of approximately 144 genera with 4455 described species (Pape et al. 2011). Members of the family Tabanidae have been studied for well over 100 years in Louisiana. Hine (1906, 1907) conducted the first surveys of the members of the tabanid family and noted the presence of 14 species. Jones and Bradley (1923) supplemented Hine's observations with their own inventory of horse flies across four parishes and found an additional 14 species that were not recorded by Hine. Subsequently, Tidwell (1970, 1973) conducted a survey of horse flies of Louisiana and constructed dichotomous keys for identification of 87 species across eleven genera. Leprince et al. (1991) completed a survey of horse flies captured in mixed bottomland hardwood regions in St. Landry Parish and Lafourche Parish and reported the seasonal abundances of 14 species found within the two study locations. While these surveys yielded valuable information, there has been no comprehensive survey of horse flies along the Louisiana coast. The purpose of this study was to establish inventories of the horse flies of tidal marshes of Louisiana which range from freshwater to the high salinity zones. The study was initiated to describe the spatial and temporal occurrence of the greenhead horse fly, *Tabanus nigrovittatus* and potential sibling species relations along coastal Louisiana using morphometric techniques, DNA barcoding methods, and phylogenetic trees. Monthly adult tabanid collections were made with canopy traps in tidal marshes of Barataria Bay in Plaquemines Parish and Caillou Bay in Terrebonne Parish in intermediate (~3ppt), brackish (~8ppt), and saltmarsh (~16ppt) locations. Three species of tabanids (*T. acutus, T. hinellus* and *C. flavidus*) were collected along with *T. nigrovittatus* within the estuaries. Specimens of *T. acutus* were collected primarily during crespuscular and nocturnal hours. Population abundance of the different *Tabanus* species varied

among salinity zones. The number of collected specimens of *T. hinellus* decreased with increasing salinity while specimens of the *T. nigrovittatus* complex were collected at both High and Low salinities sites, dependent upon the estuary and time. In addition to seasonal distribution data, genetic barcoding of the four species was completed. The cytochrome oxidase I (CO1) subunit was found to be the locus with the highest resolution power to make species-level designations. Previously unidentified larvae and males were successfully matched to the genetic sequences of morphologically identified females of the different species. The *T. nigrovittatus* complex populations were bivoltine through multiple seasons. Then, *T. nigrovittatus* complex specimens collected from early and late season and from the different salinity zones were used to measure the genetic diversity within the *T. nigrovittatus* complex by using morphometrics and phylogenetic analysis. The morphometric analyses showed that total body length of specimens of the *T. nigrovittatus* complex were higher in the first generation of the year compared to the second. Phylogenetic analysis of the *T. nigrovittatus* complex showed that there are three clades of greenheads in the estuaries of Louisiana among the salinity zones. One of the clades was found in High salinity zones and was genetically similar to *T. nigrovittatus* specimens from Massachusetts. A second clade was native to the lower salinity zones. Additionally, flies from the third clade fit the description of *T. conterminus*. The findings from this study provide more insight into the *T. nigrovittatus* complex along the Gulf Coast that includes the addition of *T. conterminus*. The species identity of the freshwater member of the *T. nigrovittatus* complex remains to be determined.

INTRODUCTION

The Dipteran family Tabanidae is among the most diverse families of insects that is comprised of approximately 144 genera with 4455 described species (Pape et al. 2011). Tabanid distribution ranges reach as far north as the Bering Strait and as far south as the southern portions of Argentina , with the greatest species diversity within the Neotropical regions (Mullens 2019, Wilkerson and Coscaron 1984). Representatives of the family can be found on every continent except Antarctica (Pape et al. 2011).

Tabanids are very diverse and complex groups of invertebrates with thousands of described species globally. Population studies on tabanids have yielded valuable results on tabanid behaviors and their impacts on and within their environments. Historically, tabanids have been considered important livestock pests. Foil et al. (1984) studied tabanids as vectors of equine infectious anemia and compared relative abundances of adult females within the study area to virus transmission events in a prospective study, and correlated peak abundance of different species to virus transmission events. Husseneder et al. (2016) showed that the greenhead horse fly, *Tabanus nigrovittatus,* is an indicator species of marsh health following the Deepwater Horizon Oil Spill by comparing relative abundances and genetic structure of fly populations at locations that were affected by the oil spill to those that were not impacted. Conspecific or even undescribed coastal species potentially could be used as additional bioindicator models of marsh health; therefore, a comprehensive survey of the horse flies of coastal Louisiana and their spatial distributions is needed.

Members of the family Tabanidae have been studied for well over 100 years in Louisiana. Hine (1906) studied horse flies with a middorsal stripe and noted adult horse fly species present in Louisiana, such as *Tabanus quinquevittatus* and *Tabanus fuscicostatus*.

Among some of the collected species with a middorsal stripe were species that Hine (1907), at the time, questioned as indigenous to Louisiana such as *Tabanus nigrovittatus*. Jones and Bradley (1923) supplemented Hine's observations with their own inventory of horse flies across East Baton Rouge, Madison, St. James, and Orleans parishes, and found an additional 14 species that were not recorded by Hine. Subsequently, Tidwell (1970, 1973) conducted a survey of horse flies of Louisiana and constructed dichotomous keys for identification.

Tidwell (1970) investigated larval and adult taxonomic relationships of horse flies in linking their ecological and distribution. Three years later, Tidwell (1973) conducted a comprehensive review of the horse flies of Louisiana based on systematic relationships. Tidwell also provided an inventory of the species that were observed during his studies and provided ecological descriptions and biological information of the adult and larval forms of each of the species. Tidwell described the dominant species within specific vegetation regions of Louisiana, Leprince et al. (1991) completed a survey of horse flies captured in mixed bottomland hardwood regions in St. Landry Parish and Lafourche Parish and reported seasonal abundances of 14 species found within the two study locations. While these surveys yielded valuable information, there has been no comprehensive survey of horse flies along the Louisiana coast.

In the Nearctic and Neotropical regions, two large complexes of flies of the genus Tabanus exist. The first is the lineola complex that consists of adults with multiple horizontal striations on their green-ground eyes. Morphologically, all individuals of the lineola complex have median stripes with nearly always dorsolateral stripes along the abdomen. The second complex, acknowledged as the *T. nigrovittatus* complex, consists of adults that are characterized by fairly medium size with a single eye-band on a green background in life. Morphological characters of individuals of this second complex includes middorsal striping along the abdomen

with faint yellow to white palpi. *Tabanus nigrovittatus* was placed within a complex with five other species along the Gulf Coast which include *T. conterminus* Walker, *T. fulvilineis* Philip, *T. fuscicostatus* Hine, *T. mularis* Stone, *T. nigrovittatus* Macquart, and *T. quinquevittatus* Wiedemann (Goodwin 1994). However, only two of the six species within the complex, *Tabanus nigrovittatus* and *T. conterminus*, are considered sympatric along the Atlantic Coast (Hine 1906).

There has been no previous study examining the diversity of the *T. nigrovittatus* complex within the different salinity zones of the tidal estuaries of the Gulf of Mexico. Therefore, the goal of this study was to conduct a comprehensive survey of the spatial and temporal occurrence of horse flies in and along coastal Louisiana through two objectives. The first objective was to survey horse fly species present along coastal Louisiana through time near Spartina marshes by trapping with canopy traps along the coastline and within the coastal marshes. The second objective was to describe the spatial and temporal occurrence of the greenhead horse fly, *Tabanus nigrovittatus* and potential sibling species relations along coastal Louisiana using morphometric techniques, DNA barcoding methods, and phylogenetic trees.

CHAPTER 1. LITERATURE REVIEW

1.1 Biology of Tabanidae

The Dipteran family Tabanidae is among the most diverse families of insects comprised of approximately 144 genera with 4455 described species (Mullens 2019). Tabanid distribution ranges reach as far north as the Bering Strait and as far south as the southern portions of Argentina, with the greatest species diversity within the Neotropical regions (Mullens 2019, Wilkerson and Coscaron 1984). Representatives of the family can be found on every continent except Antarctica (Pape et al. 2011).

Members of Tabanidae can lay large egg masses ranging from 200-500 eggs per mass. Hine (1907) reported finding brownish masses of tabanids reaching nearly 500 eggs. Hine (1907) observed *Chrysops flavidus* to have deposited around 200 or more eggs on a single leaf. Generally, most adult females oviposit egg masses on low-lying vegetation close to moist soil, suitable for larval development. Tabanid larvae develop within the soil and are considered to be apex predators in their environment. Tabanid larvae have been found to consume a wide array of food sources ranging from fungi to larger invertebrates, although Chrysops larvae feed more readily on smaller invertebrates such as Chironomidae (Tidwell 1973, Axtell 1976). Immature tabanids develop in a variety of locations and substrates from dry to wet including tree holes, drainage ditches and margins of ponds (Axtell 1976). Upon reaching the pupal stage, larvae travel closer to the soil surface for emergence. Upon emergence, females are capable of flight and mating. Adults can be found in different mixed-hardwood forests to open prairies and swamps (Tidwell 1973).

The biology of male tabanids has not been studied to the extent than for females, but mating behaviors have been observed regarding male tabanids. Bailey (1948) reported male

hovering behavior at inches above vegetation or ground level, and when a mate is coupled, copulation lasts no longer than a few minutes. This hovering behavior is purely male-related and is believed to take place hours after emergence since males live much shorter lives than females (Bailey 1948). Bailey observed small masses of male *T. nigrovittatus* swarming, particularly in the early morning hours prior to female flight activity. Environmental conditions such as temperature and wind direction are acknowledged as playing a role in the presence of males and the windward direction at which they align themselves in hovering behavior. Rockel and Hansens (1970) suggested that males of certain species emerge slightly prior to female emergence peaks as part of the possible mating swarms since mortality in males was found to increase in the days following the female emergence. This observation supports those made by Bailey (1948) as they both state that male lifespan is relatively short in comparison to females.

Emergence of four commonly collected species in New Jersey, *Chrysops fulginosus, C. atlanticus, Tabanus nigrovittatus* and *T. lineola*, were studied using emergence traps by Rockel and Hansens (1970). Emergence traps were used in determining sex ratios, emergence sites and dates as well as population densities at various locations. They found that emergence of *T. nigrovittatus* occurs from soil in close proximity to high water levels where they previously found the species to dominate as larvae.

Catts and Olkowski (1972) studied the mating behaviors of *Chrysops fuliginosus* in salt marshes where flight activity, behavior and mating incidence in males and females were observed. Hovering behavior was observed in males as they awaited a suitable partner for copulation. Climatic conditions were also explained in terms of mating; males displayed pursuit activity between air temperatures of 18-24^{o}C. After copulation, males were observed feeding on flowering plants while females sought suitable hosts for blood-feeding.

1.2 Tabanid Feeding

Females of most species of tabanids are hematophagous and anautogenous which means they require a blood meal for egg development. Wilson et al. (1966) showed that the presence of carbon dioxide resulted in attractant of tabanids to their hosts for females of 18 Tabanus species, two Chrysops species and *Leucotabanus annulatus* (Wilson et al 1966).

Female tabanids are classified as economically important pests because they serve as the most important insects of mechanical transmission of disease agents and inflict blood loss and pain associated with their bites, primarily due to their large blood meals and feeding behavior (Mullens 2019). A comprehensive review of disease agents transmitted by tabanids was made by Krinsky (1976) who evaluated the routes of transmission in addition to the disease agents transmitted by numerous species of horse and deer flies around the world. Of the over-200 references within the review, 188 tabanid species were named in reference to the 32 recorded tabanid-transmitted animal diseases. Tabanids have been proven to be biological vectors of five agents of diseases including *Loa loa*. More importantly, the number of pathogens mechanically transmitted is large, with eighteen agents of disease including equine infectious anemia virus, hog cholera virus and protozoa such as *Trypanosoma evansi* (Krinksy 1976, Foil 1989).

Mullens and Gerhardt (1979) observed females of 19 tabanid species feeding on cattle. Feeding sites and tabanid behavior was based on the genera of tabanids present. The color of the animal did not affect feeding numbers nor feeding site. Mullens and Gerhardt (1979) also suggested that perhaps the feeding sites for each species correlated to the differences in mouthpart lengths. Members of Chrysops fed more around the head and neck regions where hair is shorter while females of Tabanus species fed more in areas with denser patches of hair.

Hybomitra species fed more heavily on the lower portions of the cattle. Mullens and Gerhardt (1979) also suggested that perhaps efficacy of trapping methods could be influenced by different species preferred feeding sites. For example, bottom-entry traps such as the Manitoba canopy trap could target more of the fly species that feed on lower portions of the cattle such as Hybomitra species. Conversely, the high-feeding species of Chrysops and Tabanus, which feed more on the upper portions and head region of cattle, could be collected using different trapping methods that target upper regions.

Investigations by Magnarelli and Anderson (1980) support the claims made by Mullens and Gerhardt (1979) with the study showing feeding behavior of 17 species of tabanids. In addition to this, feeding behavior or engorgement of the flies were also observed. Members of Chrysops showed continuous engorgement once feeding began at a site. Preference for the feeding sites in Chrysops species were primarily within the head and neck areas of livestock. Tabanus species had more disrupted feedings, with specimens witnessed to feed on the back, side and legs of cattle and move within these locations until completely engorged (Magnarelli and Anderson 1980).

Foil et al. (1984) studied tabanids as vectors of equine infectious anemia and compared relative abundances of adult females within the study area to virus transmission events in a prospective study in which they correlated seasonal peak abundance of different species to transmission events over a one-year period. Foil (1989) discussed the different routes of transmission of infectious agents by tabanids to livestock and humans. The review demonstrates the concepts of transmission by tabanids as vectors such as different vector behavior among different tabanid species.

The horse flies, which is a generic term for tabanids other than deer flies, within this family are a serious nuisance to livestock. In contrast, deer flies (Chrysops spp.) attack humans more frequently and are described biological vectors of the agents of tularemia and loiasis (Foil 1989). Tabanids can cause major blood loss in livestock during extreme cases due to feeding time and population densities. It is estimated that even a small population of 50 flies can engorge on one animal over a 10-hour period and uptake 300ml of blood (Hansens 1979). Although control methods exist, they do not suppress tabanid populations within any given area for long-term. Hansens (1979) suggest that the use of box and canopy traps for control had been made successful with adult female horse flies. Repellants such as DEET helps suppress Chrysops attack and ethyl hexanediol reduces biting efforts in deer flies.

Foil and Hogsette (1994) suggested partial control methods that could be employed for adult horse fly population suppression. Control methods were placed into four categories: chemical, cultural, mechanical and biological. Chemical methods such as pyrethroid sprays were reported to provide livestock with partial protection by controlling up to 20% of tabanid populations. Cultural methods such as having selective grazing areas away from heavily wooded areas have also been suggested to aid in reducing tabanid impacts in some areas. Mechanical control using traps was reported to provide short-term control of tabanid activity (Foil and Hogsette 1994). Biological control does occur and may be effective when predators such as dragonflies and the horse guard wasp, *Phanurus tabaniyorus*, are around cattle populations (Hine 1907, Foil and Hogsette 1994).

1.3 The Greenhead Horse Fly Species Complex

In the Nearctic and Neotropical regions two large complexes of flies of the genus Tabanus exist. Fairchild (1983) documented an extensive list of 84 species of the lineola

complex, in which adults all exhibit a green-ground eye with a lateral median pale stripe along with upper and lower extended stripes that appear purple in life. All individuals have median stripes with nearly always dorsolateral stripes along the abdomen. Additional features of the complex include a bicolored tibiae, hyaline or tinted wings, browned wing venation.

The second large complex of flies consists of adults that are characterized by fairly medium size with a single eye-band on a green background in life. Middorsal striping along the abdomen with faint yellow to white palpi. This complex is acknowledged as the *T. nigrovittatus* complex. Along the Gulf Coast in the Nearctic region, six species of horse flies are considered to belong to this complex consisting of *Tabanus conterminus, T. fulvilineis* Philip, *T. fuscicostatus, T. mularis, T. nigrovittatus,* and *T. quinquevittatus* (Goodwin 1994). There are several taxonomic references to the larval descriptions of each of these flies. Teskey (1969) described the larvae of *T. nigrovittatus* and *T. quinquevittatus*. Freeman (1987) provided larval descriptions of *T. conterminus* and Tidwell (1973) provided descriptions of immature *T. fusicostatus*. The complex is comprised of both coastal and inland species depending upon where larvae develop. Larvae of *T. conterminus* and *T. nigrovittatus* represent coastal species since they have been found in salt marsh sediments (Freeman 1987). Larvae of *T. fuscicostatus*, *T. mularis,* and *T. quinquevittatus* are acknowledged to have similar larval morphological characteristics that include similarity in color, pubescence patterns and anal segments but are all inland species that live in damp, semi-arable soil (Goodwin 1994). Unfortunately, a larval description for *T. fulvilineis* has yet to be made although types of this specimen have been collected along the Gulf Coast from Texas to northern portions of Florida (Philip 1957).

Goodwin (1994) placed *T. nigrovittatus* within a complex with five other species along the Gulf Coast, but only two of the six species *Tabanus nigrovittatus* and *T. conterminus*, are

sympatric with one another along the Atlantic Coast. According to Hine (1906), *T. conterminus* and *T. nigrovittatus* were two species that resembled each other very closely along the Atlantic coast with only the coloration of the thorax and total body length to distinguish the two species. Hansens (1979) stated that the saltmarsh greenhead, *T. nigrovittatus*, ranges from Nova Scotia to Florida along the Atlantic and from Florida to Texas along the Gulf Coast. *Tabanus conterminus* has only been recorded within the salt marshes of the Atlantic, in Massachusetts to New Jersey and in Georgia (Freeman and Hansens 1972, Graham and Stoffolano 1983, Freeman 1987).

Philip (1962) described *Tabanus eadsi* as a species based upon specimens collected along the south coast of Texas. Since *T. eadsi* has the common greenhead characters such as the single eye band, bicolored legs, and yellowish pleural piles, it was placed within the *T. nigrovittatus* complex. Specimens of *T. eadsi* was thought to be sympatric with *T. texanus*. Philip (1962) collected only a few specimens of *T. eadsi*, therefore comparisons of the two species was reviewed by using museum specimens of *T. texanus* from the Ohio State University's collection. Due to differences in color characteristics in the femora and palpi of specimens, Philip determined that these were two different species. Philip (1962) compared *T. eadsi* to *T. quinquevittatus* and stated that *T. eadsi* was morphologically closer to *T. quinquevittatus* than to *T. nigrovittatus* based upon the coloration of the pleural piles and genae. Similarly, Tidwell (1973) placed *T. eadsi* and *T. quinquevittatus* within the same couplet of his key while *T. nigrovittatus* separated elsewhere. Philip (1962) also mentioned the subspecies *T. nigrovittatus fulvilineis* as a coastal species that differs from *T. quinquevittatus* through additional characteristics such as whiter pleural pile, pale palpi and yellow femora, but this subspecies may require further descriptions. Goodwin (1994) stated that the relationship between *T. fulvilineis* and the remainder of the species within this complex remains to be determined since immatures

of this species are not well-documented. There has not been an attempt towards distinguishing immatures of *T. fulvilineis,* but it is recognized as an independent taxon that it only known from Florida and Mississippi (Goodwin 1994).

Jones and Anthony (1964) provided the description for *T. quinquevittatus* and stated that there was a possibility that this species occurs in Florida as a part of the *T. nigrovittatus-quinquevittatus* complex. Jones and Anthony acknowledged that *T. quinquevittatus* was morphologically close to *T. nigrovittatus* but the latter occurs in coastal areas, inferring *T. quinquevittatus* as an inland species. This was supported by their description of adult activity by stating that outbreaks were confined to low meadows and adjacent to high-ground animal pastures in New York, which would not be located in close proximity of a coastal area such as *T. nigrovittatus*. However, Sutton and Carlson (1997) showed that there was likely one or more undescribed species within the complex, and Nalen et al. (2015) documented *T. quinquevittatus* to have a Florida distribution.

1.4. *Tabanus nigrovittatus-T. conterminus* Relationship

Freeman and Hansens (1972) extracted tabanid larvae from salt marsh soil from New Jersey using various different techniques. The use of larvicides, floatation methods, digging and drying marsh substrate were among the major techniques used to extract larvae from the soil. During the rearing process, Freeman and Hansens (1972) found that there were some larvae that appeared to be different from both *T. nigrovittatus* and *T. lineola*. After rearing those larvae to adults, it became apparent that the adults matched Hine's description of *T. conterminus*. Although larvae were collected through all extraction techniques, it became more of interest of the location where the larvae were collected. The cluster of questionable larvae were found more

abundantly in creek banks while *T. nigrovittatus* larvae were found more abundant in the open marsh soils.

In a follow-up study, the larvae found in the creek banks of Freeman and Hansens (1972) were conclusively that of *T. conterminus*. Freeman (1987) produced the first morphological study of immatures of *T. conterminus* in comparison to those of *T. nigrovittatus*. Descriptions of the larvae and pupae of *T. conterminus* as well as a descriptive key to separate larvae commonly found in salt marshes along the Atlantic were provided in the report. The last instar larvae of *T. conterminus* were 25-33mm long and creamy-white in appearance as opposed to beige or amber for larvae of *T. nigrovittatus*. Striations on the thoraces and some abdominal segments of immatures of *T. conterminus* were different than found in *T. nigrovittatus*. Dorsolateral markings along with pseudopodial pubescence also was different between the two species. Freeman (1987) stated that often the dorsolateral markings and anal segment pattern will differentiate the larvae of *T. conterminus* and *T. nigrovittatus*. The shape of the callus tubercules of the pupae of the two species also were different. The larvae of *T. conterminus* were collected in Massachusetts and Georgia which provided a record of the farthest south that this species was known to occur.

With the suggestion that there were multiple distinct taxa existing within salt marshes of New Jersey, Jacobson et al. (1981) performed an electrophoretic survey, using proteins of individuals of the populations of horse flies from four locations within the New Jersey area. These researchers observed a deviation from Hardy-Weinberg equilibrium among two groups of flies based on differences in allele frequencies and loci. The flies exhibited a size difference with one group of flies to be slightly larger than the second group. These researchers concluded that there were at least two possible sympatric species due to the lack of gene flow between the two groups within New Jersey's coastal marshes.

Graham and Stoffolano (1983) stated that the distribution range of *T. conterminus* extended the farthest north to Massachusetts. Burger et al. (1985) concluded that the correct name for the horse fly found conspecific to *T. nigrovittatus* should be *T. conterminus*. *Tabanus simulans* was an older name given to *T. conterminus*. Currently, *T. simulans* is not an accepted species. Sofield et al. (1984), showed that *T. simulans* holotype was more closely related to *T. nigrovittatus* while the lectotype *T. conterminus* represented the second species within the complex. Burger et al. (1985) also suspected that individuals of *T. conterminus* may occur from Nova Scotia to Florida, and which overlapped with the distributional range of *T. nigrovittatus*.

Sofield et al. (1984) used morphometric analyses that suggested that *T. conterminus* was a cryptic species present among the horse flies collected in New Jersey marshes. They used a total of 15 head characters for discriminate analysis among the two species. Measurements from these characters were used to place each fly in one of the two species; four characters together, total body length and three head characters, grouped the flies into either species with complete discrimination (Sofield et al. 1984).

Subsequently, Sofield et al. (1985) investigated the size and seasonal distributions of both *T. conterminus* and *T. nigrovittatus* in salt marshes of New Jersey. Using protein electrophoresis, samples of flies collected from five study sites were identified to species based on alleles that encoded enzymes found in both species similar to the results of Jacobson et al. (1981). A total of 292 flies were collected but only 18 of those were *T. conterminus*, with *T. nigrovittatus* as the remainder. Mean body lengths of *T. nigrovittatus* and *T. conterminus* were found to be 8.44-12.44mm and 11.58-14.01mm respectively. Additionally, *T. nigrovittatus* body lengths were found to be different among the collection locations, but all specimens of *T. conterminus* were similar in body lengths among collection sites. Sofield et al. (1985) also found that as the season

progressed, *T. nigrovittatus* body length decreased. The seasonal occurrence of populations of *T. nigrovittatus* was longer than that of *T. conterminus*. Sofield et al. (1985) suggested that *T. nigrovittatus* had prolonged periods of adult emergence and referred to previous literature of the possibility of bivoltism. In contrast, *T. conterminus* populations reached peak abundance in mid-July with a rapid decline in August suggesting a univoltine season,

Graham and Stoffolano (1983) recorded major differences among the egg mass structures of both *T. nigrovittatus* and *T. conterminus* in the field and laboratory in Massachusetts. They compared egg laying site preferences, coloration and number of eggs per mass between the smaller *T. nigrovittatus* and larger *T. conterminus*. Structurally, *T. nigrovittatus* was recorded to deposit double-tiered, gray egg masses while *T. conterminus* deposited single-layered, tan masses. Egg masses laid by *T conterminus* were longer than *T. nigrovittatus* in both the field and lab even though these two species were both observed to lay egg masses towards the tips of the blades of *Spartina alterniflora* on the face of the blade.

Sutton and Carlson (1997) used cuticular hydrocarbon analysis (CHC) on 151 flies to show the presence of three distinct chromatotypes of the greenhead horse fly, *T. nigrovittatus*, along the Atlantic coast. The body length of the flies also was recorded. Flies with a CHC of Type I was evidently *T. conterminus*, since the majority of those flies had total body lengths of greater than 14mm. Flies from Nova Scotia within the study had a Type II chromatotype while Type III were from Virginia (Sutton and Carlson 1997). Thus, chromatotype II was considered to be *T. nigrovittatus* whereas Type III flies were not assigned a species identification.

Sakolsky et al. (1999) utilized the methods of species discrimination by both Sofield et al. (1984) and Sutton and Carlson (1997) to distinguish flies within their study area in Massachusetts. CHC analyses and morphometric models were used on a small subsample of 15

flies. Of the 15 horse flies, four flies were conclusively determined as *T. conterminus* within both models while two other flies were concluded to be *T. conterminus* through canonical scoring but not by chromatotype. The researchers concluded that CHC analyses helps to identify most flies correctly and that canonical scoring may not be sufficient enough to determine the species of flies. Canonical scoring did not match up well with morphometric models which showed that there was clearly two separate species based on total body length alone.

1.5 Surveys of the Horse Flies of Louisiana

Hine (1906) was the first to report the presence of horse flies with a middorsal stripe, such as *Tabanus quinquevittatus* and *Tabanus fuscicostatus* in Louisiana. Subsequently, Hine (1907) provided a report of the horse flies found in Louisiana and provided descriptions of adult and immature stages for 39 species from the genera Chrysops and Tabanus within the family Tabanidae. Seven of the thirty-nine species, *Chrysops brunneus, C. pudicus, Tabanus costalis, T. melanocerus, T. nigrovittatus, T. quinquevittatus*, and *T. weidemann*. occurred in coastal areas. Hine noticed a distin

ct difference in the abundance of horse flies relative to vegetational regions, stating higher relative abundances around wooded areas. Included within his report, Hine (1907) provided defining characters of two genera, Chrysops and Tabanus, as well as the listed the species within the genera.

Jones and Bradley (1923) supplemented Hine's observations with an inventory of horse flies of Louisiana found in East Baton Rouge, Madison, St. James, and Orleans parishes, and found an additional 14 species from three genera that were not recorded by Hine. There were 53 species of tabanids recorded from Louisiana by Hine (1907) and Jones and Bradley (1923) by 1923. Three species, *Tabanus flavus, T. uniformis* and *T. turbidus*, recorded by Jones and

Bradley (1923) were also reported to be crepuscular. Although Jones and Bradley (1923) added more species to the known list of horse flies within Louisiana, they did not record any additional coastal species to supplement Hine's 1907 report.

Although the presence of 53 species in Louisiana was described, Wilson (1963) was the first researcher to establish seasonal distribution and abundance of different species of adult tabanids. Wilson used a mare as a bait animal to collect the horse flies and recorded seasonal occurrence of the most abundant three or four species collected in each of four Louisiana parishes (St. Landry, Tensas, West Baton Rouge and St. Helena) over a three-year period from 1960-1962. A total of 28 species representing 5 genera of horse flies were recorded during the three years of this study. Wilson (1963) established the first comparisons of horse fly species abundances and compositions in lowland and upland areas and showed three peaks of emergence which occurred from mid-May through mid-July in lowland areas. In upland areas, five peaks occur over a five-month period of May through September. The highest peaks of abundance for both localities occur mid-June (Wilson 1963).

Tidwell (1970) investigated larval and adult taxonomic relationships of horse flies in Louisiana and compared the ecological aspects of horse flies by dividing his study areas into six vegetational regions. Collections of adult horse flies were made using various methods that included sweep netting and using dry ice blocks placed on cars and Malaise traps as bait for the flies (Tidwell 1970). Tidwell provided taxonomic keys for 10 genera and 29 species of Tabanidae larvae as well as 13 genera and 100 species of adult tabanids (Tidwell 1970 &1973). Larvae were extracted from a variety of substrates including dead wood, tree holes, mud and plant roots. The larvae were reared to the adult stage to properly determine species. During rearing attempts, Tidwell found that horse fly larvae were predatory and consumed a diversity of

provided food sources. Anatomical structures of the larvae were used in determining diagnostic larval characteristics for each species.

Leprince et al. (1991) completed a survey of horse flies captured in mixed bottomland hardwood regions of St. Landry Parish and Lafourche Parish in Louisiana. They used an improved and more effective method of trapping by using canopy traps baited with dry ice Hribar et al. (1991). Leprince et al. (1991) reported seasonal abundances of 14 species found within the two study locations over the course of the season from early-March to mid-October. However, there has been no comprehensive survey of the seasonality of horse fly species along the Louisiana coast.

In addition to tabanid seasonal patterns being important to recognize relative to mechanical transmission of agents of livestock disease, population studies on horse flies can yield information regarding environmental conditions and or changes. For example, Husseneder et al. (2016 & 2018) showed that the greenhead horse fly *Tabanus nigrovittatus* is an indicator species of marsh health following the Deepwater Horizon Oil Spill by comparing relative abundance and genetic structure of horse fly populations at locations that were impacted by the oil spill to locations that were not impacted. Husseneder et al. (2016) found that relative abundances of horse flies were estimated to be lower in oiled areas. Genetic bottlenecks were detected among the impacted populations through microsatellite genotyping; there were less family clusters and reduced migration among populations. In the follow-up study, Husseneder et al. (2018) reported signs of population recovery five years after the oil spill as genetic bottlenecks began to disappear and migration among populations began to contribute to rebounding effective population sizes at the formerly oiled areas.

1.6 Environmental Niches

In the coastal marshes of New Jersey, Schulze et al. (1975) examined *T. nigrovittatus* populations to understand the species' seasonal movements from environmental factors. Land use was also taken into account as traps were strategically placed from *Spartina alterniflora* marsh gradually inland. This was to further examine populations using the gradual transition from saline marshes to freshwater swampland inland. Abundance of *T. nigrovittatus* was found to be higher in areas where vegetation were more sparse and open. In addition, fly numbers were highest in traps that were lower to the ground, no more than three feet high. This suggested that *T. nigrovittatus* are low-fliers and prefer open vegetation that marshland provides the further out in distance from urban areas. This study also found that *T. nigrovittatus* is found more abundant during times of high sunlight/ low cloud cover. The preferential habitat of *T. nigrovittatus* suggests that this species is more tolerant of open saltmarshes.

Environmental niche distributions allow researchers to understand the ecology and life history of organisms among other information. In the case of pests such as Tabanus species, identifying species habitats is vital towards understanding how to both control and assess the species and environmental systems they inhabit. Davis (2019) used land cover and climatic variables in describing niches and distributions of species within their study range. Occurrence of species presence across the study region was measured using predictor variables including relative humidity, vegetation, and cloud cover.

In an environmental niche modeling study conducted by Davis (2019), Global Positioning System (GPS) technology was used in the southeastern US to identify environmental niches in which the most pervasive, abundant, and commonly encountered tabanids occupy. Records of the six modeled species, *T. fulvulus, T. lineola, T. subsimilis, T. quinquevittatus, T.*

sparus milleri, and *T. sulcifrons*, were used in determining distribution ranges using environmental conditions. Relative humidity was found to highly correlate with species distributions across the states. Unfortunately, GPS-data from some southern states including Louisiana and Georgia did not provide enough records of these species in order to establish distribution models.

In the coastal marshes of Louisiana, insect and plant communities were inventoried over a one-year period within two of Louisiana's largest estuarine systems (Aker 2020). A total of 71 insect families were collected across three salinity zones, designated as Low, Mid and High using sweep netting towards establishing baseline population data for those coastal marshes. In the study, Aker (2020) found strong correlations among insect communities relative to water salinities and the plant communities in which those insect communities existed. Feeding guilds and trophic-level interactions were also examined relative to water salinities. Aker (2020) established that there were distinct plant and insect communities that existed both across salinity zones and exclusive to one of the three zones. This study was the first of its kind to establish the population distribution data of Louisiana's coastal marshes. Unfortunately, more knowledge on insects not targeted within this study such as obligate blood feeders like tabanids is needed to add to the existing knowledge and database of species present as well as niches that insects occupy within those marshes.

1.7 DNA Barcoding

Identification methods for the genus Tabanus has been difficult due to the overlap and continuum in morphological characters associated with species and complexes. Many species-level characters such as features on the head or thorax of flies are observed in only one sex. In addition, those characters may differ based on preservation and age of the specimens.

Recently Morita et al. (2016) provided a phylogenetic analysis of 110 taxa of the family Tabanidae to understand historic, monophyletic lineages of the tribes and subfamilies of the group. Targeting seven gene fragments that included a 28S, cytochrome oxidase 1 (CO1), carbamoyl-phosphate synthase (CPS), a protein coding nuclear gene and lanyl-tRNA-synthetase, these researchers were able to generate concatenated nucleotide datasets for the tribes and subfamilies, including outgroups, of the family Tabanidae. From a phylogenetic standpoint, the data provided in this study provides the framework for the timing and diversification of the various linages. This study by Morita et al. (2016) provided a large foundation for future Tabanidae research since the modern approach of molecular analysis has provided a better glimpse of the evolutionary history associated with this large group. The results of the molecular systematic study was able to establish that this group of insects are very diverse, yet still require lots of attention in establishing concrete relationships of the group.

Molecular tools such as DNA barcoding was used by Davis (2019) to identify Tabanus species found across six states of the southeastern US. Through DNA sequences generated using primers for the CO1 subunit, nine clades were formed for forty horse fly species within a phylogenetic tree (Davis 2019). This study provided a large foundation and database for Tabanus sequences as horse fly species within this genus have been difficult to concretely sort into confident groups. DNA barcodes and methods from this study aids in the expansion of discovering species present at any locality as well as aids in identifying immature stages that are also difficult to identify and less encountered.

DNA barcoding has also been used outside of North America to study Tabanus bromius populations in Turkey, Croatia and Iran by Sanal Demirci et al. (2021). In addition to analyzing the genetic structure of the populations, these researchers used phylogeography to understand if

there were differences in the populations of T. bromius on a regional scale. The results of the study showed two main clades, one from Iran and the other from Turkey. The Turkey clade splits into two subclades that consist of the flies from Croatia. The findings of the this study by Sanal Demirci et al. (2021) shows that horse flies of the same species can still show great genetic diversity based on a geographical standpoint. In addition, phylogenetics used within this study clearly indicated that the structure of the phylogenetic analysis was influenced by environmental and climatic differences based on the regions in which the indivi-duals were native.

CHAPTER 2. A COMPREHENSIVE INVENTORY OF THE HORSE FLIES AND THEIR DISTRIBUTION ALONG COASTAL LOUISIANA

2.1 Introduction

The Dipteran family Tabanidae is among the most diverse families of insects consisting of over 4400 species across 144 genera and can be found on every continent with the exception of Antarctica (Pape et al. 2011). Adult females can be found in an array of habitats ranging from freezing tundras to bottomland hardwood forests and temperate coastal marshland. Most female members of the family Tabanidae are hematophagous and require a single blood meal to produce an egg mass (anautogenous). Subsequently, egg development normally takes a few days and oviposition also may take several days (Mullens 2019). However, there are a small number of species members that are autogenous, meaning that the first egg mass can be laid without a bloodmeal but subsequent egg development requires a bloodmeal. Tabanid larvae develop in both terrestrial and aquatic environments and are apex predators in their environment; they have been found to consume a wide array of food sources ranging from fungi to larger invertebrates (Axtell 1976). After emergence and mating, adult females seek out suitable oviposition sites or a blood-meal depending on physiological requirements.

Tabanids are both nuisance and economically important pests of humans and livestock. Tabanids are also considered to be important mechanical and biological vectors of disease agents due to many biological factors including their feeding behavior, vector size, and population densities (Foil 1989). Since most tabanid species are anautogenous, host-seeking behavior increases chances of disease agent transmission to susceptible hosts (Krinsky 1979). Larger tabanid species have been shown to ingest larger quantities of blood, but smaller tabanids still pose risk of transmission of pathogens to hosts (Foil 1989). Population densities also play a

factor in disease transmission events but tabanid control efforts are difficult (Krinsky 1979, Foil 1989).

Currently, area-wide control measures through the uses of insecticides are not possible for tabanids like those in the genus Tabanus since adults of many species inhabit large areas and using insecticides to control adults or immature stages would not be economically or environmentally friendly (Hansens 1979). Partial protective measures for humans do exist such as the use of repellents like DEET to reduce biting events deer flies (Hansens 1979). Coastal tabanids are considered nuisance pests of man and the nuisance caused has increased over time due to increased human activity such as recreational fishing and boating as well as camping within the coastal ranges of the greenhead horse fly, *Tabanus nigrovittatus*. Along the Atlantic coast of the United States, greenhead horse fly management efforts exist where fly activity affects the recreational industry along the coast. *Tabanus nigrovittatus* populations comprise up to 95% of the horse fly population along coastal marshland areas (Hansens 1979). Area-wide control for tabanids in general, and *T. nigrovittatus* in particular, has been difficult to achieve due to the extensive larval habitats and reproductive capacity of the flies. Flooding of marsh soil to control and suppress larval development has been studied, but that process was not efficient and could cause harm to areas of marshland (Hansens 1979). Currently, the use of traps that target adults is the preferred greenhead control method. Traps are used in greenhead control programs such as the Northeast Massachusetts Mosquito Control and Wetlands Management District and the Department of Public Works, Office of Mosquito Control in New Jersey & https://www.atlantic-county.org/mosquito-control/greenhead.asp). Large tabanid populations along the coast are acknowledged to be economically important due to human annoyance; and

taxonomic and biological research is needed to discover methods for suppression of *T. nigrovittatus* populations.

The Deepwater Horizon platform explosion in 2010 resulted in the release of 4.9 million barrels of oil into the Gulf of Mexico, and impacted a multitude of aquatic and terrestrial species. Following the oiling of coastal areas, there were numerous studies that sought to measure the impact this event had on the coast of the Gulf of Mexico with particular interest in the heavily impacted Louisiana coastal marshes. Members of multiple coastal bird species of various sizes died as a result of oiling (Haney et al. 2014). Many marine invertebrate species also were impacted as the oil penetrated the surface of the water as well as the water column. Terrestrial arthropod communities were also shown to have sustained dramatic impact due to the oil spill (McCall and Pennings 2012). Furthermore, genetic structure and population abundance of the greenhead horse fly, *Tabanus nigrovittatus*, was shown to have been impacted by the Deepwater Horizon Oil Spill (Husseneder et al. 2016 & 2018). The presence and seasonal occurrence of *T. nigrovittatus* was described in at least four areas along the Louisiana coastline (Husseneder et al. 2018). Within 5-6 years after the oil spill a population reboundvia immigration was observed, which mitigated the genetic bottlenecks caused by population crashes in oiled areas. The sensitivity of this species to oilingmakes *T. nigrovittatus* a viable bioindicator species of marsh health within its habitat (Husseneder et al. (2018).

Surveys of population abundances and temporal distribution of *T. nigrovittatus* have been conducted extensively along the Atlantic in marshes of New Jersey and Massachusetts to down the coast in North Carolina (Freeman and Hansens 1972; Dale and Axtell 1975). Freeman and Hansens (1972) studied population abundance focusing more on larval densities within the marsh soil using multiple collection methods including floatation, larvicide, and drying marsh soil. Dale

and Axtell (1975) described temporal distributions of multiple tabanid species numbers relative to environmental data such as light, wind and air temperature recorded on an hourly basis for over multiple days. They found that *T. nigrovittatus* abundance correlated heavily with days of intense light, little to no wind speed, and increased temperatures on average of 25°C (77°F).

Similar studies of population abundance and temporal distributions of *T. nigrovittatus* have not been as thorough along the Gulf Coast from Texas to Florida; although Husseneder et al. (2016) studied the population abundance and seasonal occurrence of *T. nigrovittatus* in limited locations in Louisiana. There have been studies conducted on inland tabanid species in various regions of Louisiana. A comprehensive survey of the horse flies of Louisiana was conducted by Tidwell (1973) but the seasonal distribution and detailed ranges of coastal species were not described. Leprince et al. (1991) completed a survey of horse fly seasonal abundances and temporal distributions of species in inland areas of Louisiana, but similar studies have not been conducted for coastal Louisiana. Thus, a comprehensive inventory of the horse flies of coastal Louisiana is warranted.

The purpose of this current study was to survey and identify the tabanid species that exist along the coast by using canopy traps deployed along the coastline and within the estuaries relative to selected salinity zones. Objective one of this study was to use trapping methods to supplement data collected from the study sites used by Husseneder et al. (2018) and describe the spatial distribution of *T. nigrovittatus* and other species along the entire coastline of Louisiana. A second objective of this study was to use trapping methods to establish the spatial and temporal occurrence of the species of tabanids that are native to the coastal estuaries of Louisiana. A third objective was to establish DNA barcoding sequence data for use in identifying and providing confirmation of native coastal tabanid species identification as adults and immatures.

2.2 Materials and Methods

Sampling Overview

Terrestrial canopy trap sampling was used to establish the presence and temporal patterns of *T. nigrovittatus* in 2010, 2011 and an inventory of species collected along the Louisiana coast in 2018 and 2019. Canopy traps also were used to collect adult female tabanids found within the estuaries as confirmation of their native origin as well as their spatial and temporal distribution. Male tabanids were collected in light traps as a component of a survey of nocturnally active insects within the estuaries. Tabanid larvae were collected in previous surveys of immature tabanids as part of a larger project to identify the impacts of the DWH spill and then stored in ETOH.

Adult Collecting Methodology

Canopy traps baited with CO_2 and a black ball were the primary traps used for capture of adult female tabanids (Hribar et al. 1991). The traps were deployed for a minimum of two hours when possible. Specimens were collected in plastic bags that were attached to each catch container and the bags were labelled according to site, date, and trap duration. The specimens were then transported to the lab on dry ice and stored at -80ºC until being processed. Specimens then were sorted on a cold plate and identified to species using keys and descriptions provided by Tidwell (1970, 1973), Hine (1906), and Sofield et al. (1984). During species verification of specimens, individuals designated as *T. nigrovittatus* met the following criteria: total body length of 10.7-13.8mm and the abdomen yellowish sublaterally with a pale median stripe, along with yellowish palpi, a brown basal callus, and a grayish-yellow mesonotum (Table 2.1). Specimens identified as *Tabanus nigrovittatus* were differentiated between previously acknowledged coastal species that have eye patterns similar to *T. nigrovittatus*. That group of flies will be referred to as

specimens of the *T. nigrovittatus* complex. (Table 2.1). The number of specimens per species per location was recorded and all specimens were then stored at -20 ºC.

Table 2.1. Valid species of the *Tabanus nigrovittatus* complex that have been recorded within coastal areas and with the potential of inhabiting Louisiana's coastal waters. Species described here are acknowledged as valid species per the Integrated Taxonomic Information System's species reports (www.itis.gov). Embolden phrases represent characters used to differentiate *T. nigrovittatus* from the other greenheads.

Species	Key Characters						Original Description	Range
	Size	**Color**	**Thorax**	**Abdomen**	**Eyes**	**Palpi**		
Tabanus eadsi	10-12mm	yellowish and brown	yellowish, w/o stripes	brownish w/middorsal pale stripe and **two submedian stripes**	single band on green ground	Darker yellow	Philip 1962	Coastal areas of TX and LA
Tabanus quinquevittatus	13-16mm	predominately yellow; yellow-brown	dull-golden; yellow-grayish	brown with median parallel-sided yellowish stripe	green w/ one reddish purple stripe; two green bands in life	Creamy white	Wiedemann 1821	Texas, Arkansas; most of the 1/2 eastern US as far west as panhandle of TX
Tabanus nigrovittatus	9-16mm; 9-11mm	yellowish and brown	gray w/faint yellow tinge	yellow brownish w/ median pale stripe	green w/ one reddish purple band	Whitish with faint yellow	Macquart 1847	Nova Scotia to Texas
Tabanus conterminus	12-14mm; "5.5-6.5 lines*" = ~11.6-13.7mm	"colored much like nigrovittatus"	steel gray; "chest gray, clothed w/short hoary hairs; breast hoary, clothed w/ white hairs"	Yellow-brownish; **broad at chest and narrows posteriorly**	**"bronzed, parted above by a rather broad interval"**	Yellowish-white with black hairs	Walker 1850	Massachusetts to Georgia

*Walker 1850 provided the original description of *T. conterminus*. In his findings, Walker used lines to measure specimens. Each line was about 2.1mm and were therefore converted to millimeters here.

Terrestrial Trapping

Thirteen canopy trap inland sites, with multiple locations at select sites, were accessible by terrestrial transportation were selected along coastal Louisiana within wildlife management areas, state parks, and private lands (Figure 2.1). In addition, six sites accessible by boat in two of Louisiana's estuaries were used (Figure 2.2). All inland trap sites were selected based upon road access to sites and with tidal marshes within sight. These sites all were within 8 kilometers of the Coastwide Reference Monitoring System's (CRMS) stations (https://lacoast.gov/crms_viewer/Map/CRMSViewer). Monthly trap collections were made during the months of April through October in 2010, 2011, 2018 and 2019.

Permits for collecting adult horse fly were obtained from the Louisiana Department of Wildlife and Fisheries (LNHP-18-078 and WDP-19-070). Verbal permission to collect horse flies was provided by representatives of the grounds at Avery Island.

Lateral Coastline Collection (Figure 2.1)

In 2010 and 2011, canopy traps were deployed at four road sites set along a 32km stretch of road in Plaquemines Parish (Figure 2.1: Site 7: 29.62213 N, 89.95350 W; 29.55636 N, 89.88911 W; 29.51589 N, 89.76131 W; 29.46136 N, 89.68525 W). Three road sites also were used in St. Bernard Parish (Site 8: 29.82301 N 89.75553 W; 29.84533 N 89.73544 W; 29.83852 N 89.68786 W).

In 2018, trap sites were in Vermilion Parish at Palmetto Island State Park (Site 1: 29.864815 N, 92.133160 W), in Iberia Parish at Avery Island (Site 2: 29.914755 N, 91.904717 W), in Terrebonne Parish at Mandalay Bay Wildlife Refuge (Site 6: 29.550313 N, 90.790517 W), in Orleans Parish at Pelican Pointe Marina (Site 11: 30.131792 N, 89.761642 W) and in Tangipahoa Parish at Joyce Wildlife Management Area (Site 13: 30.397268 N, 90.428923 W).

Four canopy trap sites were used in St. Mary Parish, two at Cypremort Point State Park (Site 3: 29.733001 N, 91.847474 W and 29.738246 N, 91.853289 W), one at Burns Point Park (Site 4: 29.574588 N, 91.537936 W), and one at Lake End Park (Site 5: 29.727196 N, 91.178900 W). Two canopy trap sites were in St. Bernard Parish in Delacroix (Site 9: 29.758193 N, 89.783849 W) and Hopedale (Site 10: 29.821478 N, 89.608583W). There were two trap sites in St. Tammany Parish at Fontainebleau State Park (Site 12: 30.336416 N, 90.045593 W and 30.336576 N, 90.045967 W).

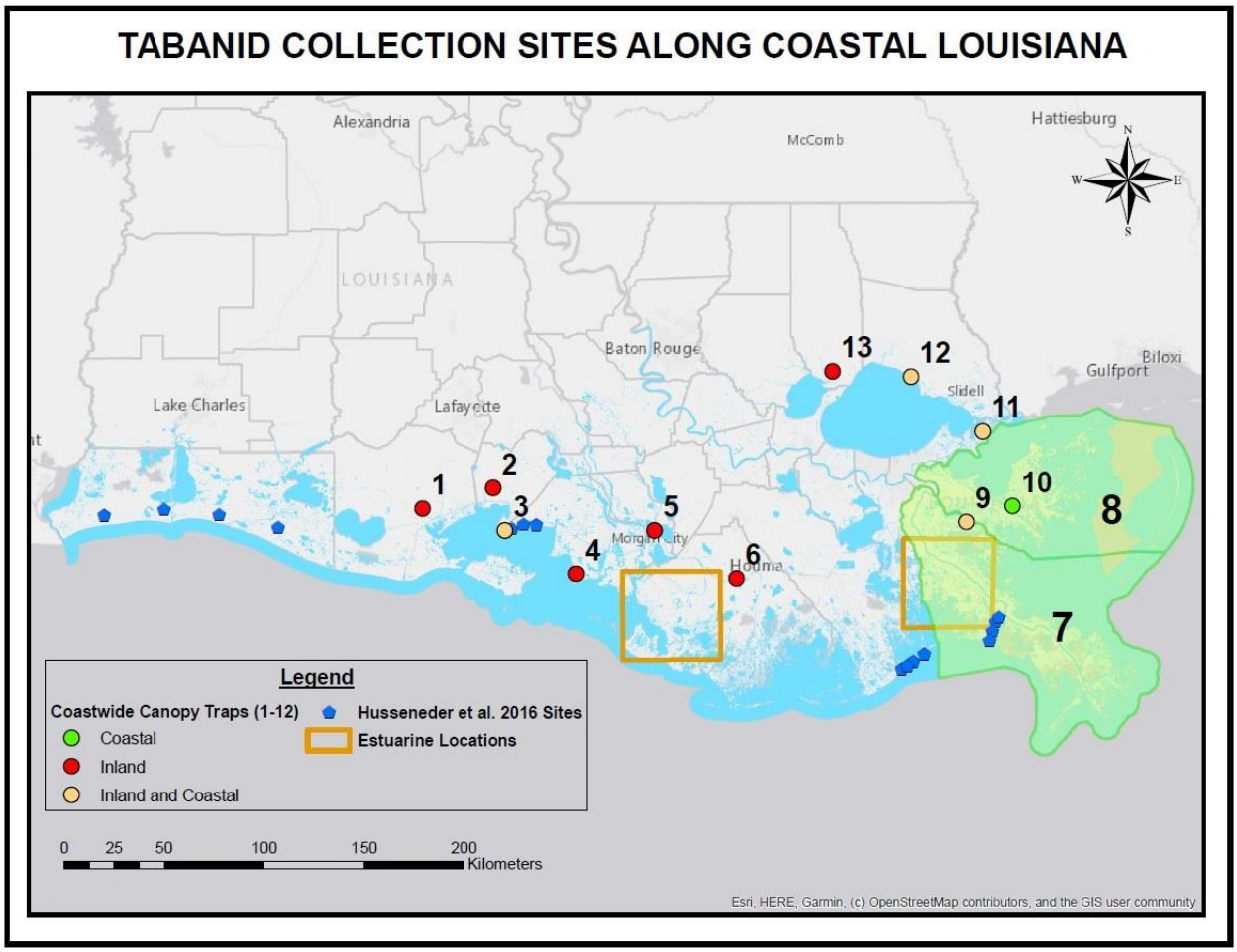

Figure 2.1 The thirteen tabanid canopy trap collection sites along the coastline used in this study. Eighteen canopy traps were deployed in ten parishes at thirteen sites. Sites 1: Palmetto Island State Park (Vermilion), 2: Avery Island (Iberia), 3: Cypremort Point State Park (x2) (St. Mary), 4: Burns Point Park (St. Mary), 5: Lake End Park (St. Martin), 6: Mandalay Bay Wildlife Refuge (Terrebonne), 7: Plaquemines Parish, 8: St. Bernard Parish, 9. Delacroix (St. Bernard), 10. Hopedale (St. Bernard), 11: Pelican Point Marina (Orleans), 12: Fontainebleau State Park (x2) (St. Tammany), and 13: Joyce Wildlife Management Area (Tangipahoa). Figure also shows estuary canopy trap areas, Barataria and Caillou Bay, as well as canopy trap sites of Husseneder et al. 2016 that performed previous coastal tabanid trapping. The estuarine locations are of Barataria and Caillou Bay. Canopy traps defined as coastal collected specimens of *T. nigrovittatus*, inland traps collected species that were not *T. nigrovittatus* and were considered as inland species. Coastal and inland trap sites collected *T. nigrovittatus* along with inland species.

Water Salinity Data

Monthly water salinity data, collected from the Hydrographic Monthly downloadable data of the CRMS stations from the Coastal Protection and Restoration Authority website (https://cims.coastal.louisiana.gov/DataDownload/DataDownload.aspx?type=hydro_monthly),

were used to generate water salinity ranges nearby each of the canopy trap sites on land. Monthly data over the four-year period, 2016-2019, of the closest CRMS stations within 8km of the nearest canopy trap location were used to generate salinity ranges of each trap location. Boxplots were generated using salinity data from the nearest CRMS station to the canopy trap site and color-coded based on presence/absence of coastal species.

Tidal Marsh Collections

In 2019, adult tabanid collections were made in tidal marshes of Barataria Bay in Plaquemines Parish and Caillou Bay in Terrebonne Parish (Figure 2.2). Collection sites, which were only accessible by boat, were selected based on 4 years (2016-2019) of monthly salinity data from the CRMS stations. These study sites also were used in other invertebrate census studies such as those of Aker (2020) and Rayle (2021). The marsh island sites were in salinity zones classified as intermediate (~3ppt), brackish (~8ppt), and saltmarsh (~16ppt) communities, and subsequently will be referred to as within Low, Mid and High salinity zones (Figure 2.2). There were three trap sites at each of the three salinity zones within both bays, totaling eighteen sites (Figure 2.2).

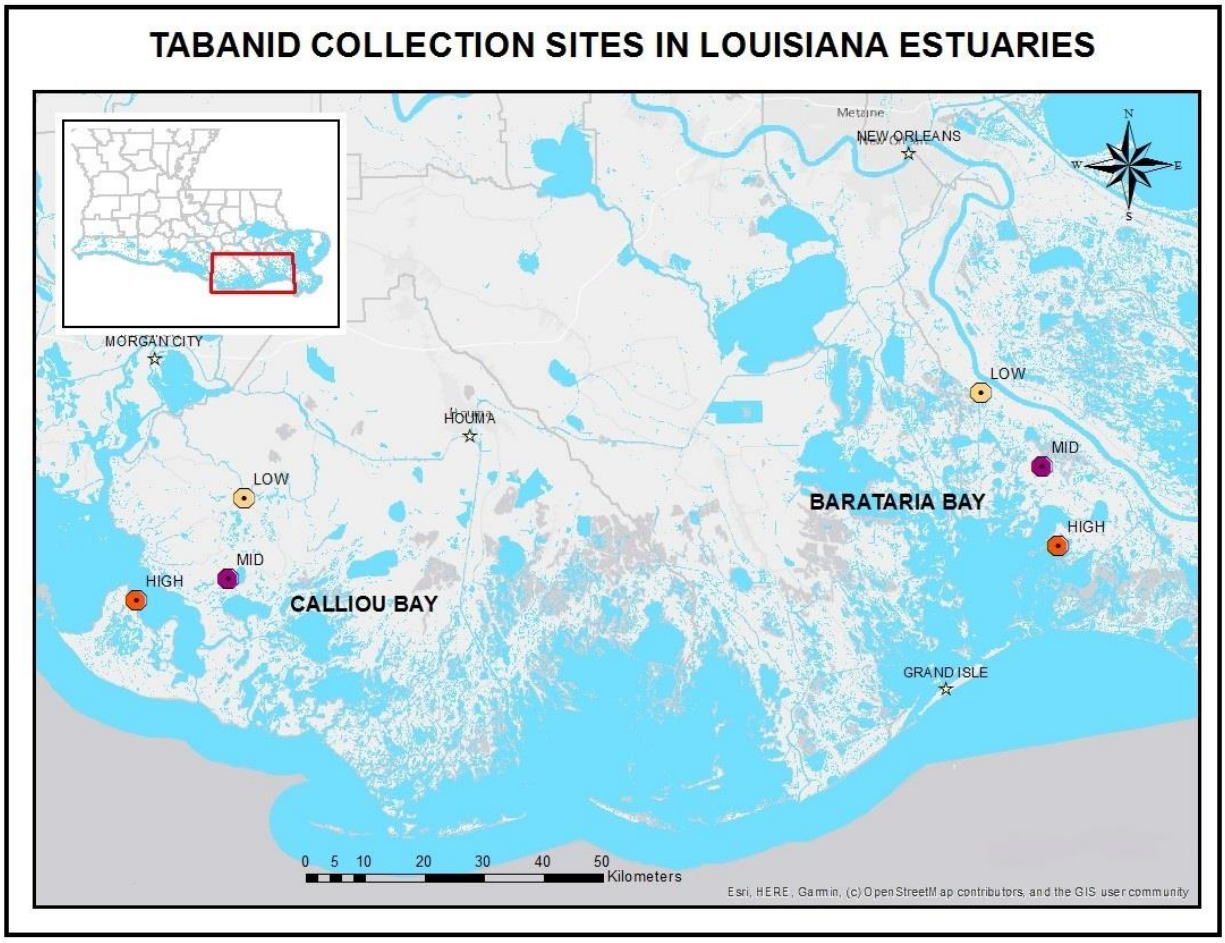

Figure 2.2. Horse fly canopy trap collection sites within Caillou and Barataria Bay. There are three salinity zones in each estuary: Low, Mid, and High; the sites were chosen based on the Coastwide Reference Monitoring System stations' data. Three canopy traps were deployed at each of the salinity zones for a total of eighteen canopy traps across both of the bays.

Two trapping strategies were used: diurnal only collections and diurnal/overnight collections. For diurnal collections, traps were deployed during the day and collected after at least two hours and/or before sunset (1930 CST). For diurnal/overnight, traps were deployed during the day and the collection containers were collected and replaced near sunset; the second collection was made the next day. Since catch containers were collected the following day, overnight species presence and abundance was calculated using adjusted trap hours of 0630 CST and 1930 CST for sunset and sunrise respectively.

Collections for diurnal species presence and abundance surveys were made seven times, at least once a month at all salinity zones, in both bays in 2019. In Barataria Bay, collections were made on 16 April (Julian Day (JD) 106), 16 May (JD 136), 10 June (JD 161), 17 July (JD 198), 9 August (JD 221), 28 August (JD 240) and 11 September (JD 254). Diurnal collections made in Caillou Bay were performed on 30 May (JD 150), 23 June (JD 174), 9 July (JD 190), 1 August (JD 213), 16 August (JD 228), 3 September (JD 246), and 3 October (JD 276).

Diurnal/Overnight canopy trap surveys also were made in both bays at all salinity zones. Collections were made in 2018 and 2019 and data from both years were combined to generate species presence and abundance at each salinity zone. Three overnight collections were made in Barataria Bay once in May, July and August of 2018. In 2019, three additional overnight collections were made once in April (JD 106-107), May (JD 136-137) and June (JD 161-162). In Caillou Bay, overnight collections were made twice in 2018 in May and July and twice in 2019 in May (JD 150-151) and June (JD 147-175).

Tabanus nigrovittatus Temporal Distribution

Canopy trap data from 2010 and 2011 population abundance surveys were used to construct seasonal abundance figures for *Tabanus nigrovittatus* east and west of the Mississippi River in Plaquemines Parish and St. Bernard Parish. The three highest canopy trap catches were used in calculating average catch per trap day for each of the parishes. Seasonal abundance data were used from multiple seasons to generate a complete seasonal distribution of *T. nigrovittatus* along the coastline.

The average numbers of *T. nigrovittatus* per hour captured in 2019 from May to October at Caillou and Barataria Bay at each salinity zone were log (x+1) transformed to normalize the data prior to statistical analyses. First, a one-way ANOVA was used to analyze all possible

comparisons of the means among the six sites. Tukey-Kramer adjustments were used to separate the means and compare p-values among the ANOVAs to determine statistical differences in population abundances at each of the sites.

Larval Tabanid Collection

Tabanid larvae were collected from sediment samples taken from five study locations along coastal Louisiana in 2016 and 2017 (Figure 2.1); these same study locations were used by Husseneder et al. (2016). Sediment collection and flotation methods described by Bhalerao (2018) were used to collect larvae from substrate. Marsh substrate samples were removed from the five-gallon buckets used to store the substrate and placed into containers filled with water and mixed with rock salt. Marsh substrate was then broken up, washed and searched for a few minutes until floating larvae were collected. Larvae were placed in individual vials with 95% ethanol and were later transferred to 70% ethanol. Larval tabanid collections were made six times at Cypremort Point in St. Mary Parish and Rockefeller Wildlife Refuge in Cameron Parish in 2016. Marsh substrate collections were made on 11 April (JD 102), 20 June (JD 172), 20 July (JD 202), and 20 September (JD 264) at Rockefeller Wildlife Refuge. Collections were made on 23 June (JD 175) and 2 August (JD 215) at Cypremort Point in 2016. Five collections were made in 2017, three at Cypremort Point on 11 April (JD 101), 17 May (JD 137), and 7 June (JD 158) and two at Grand Bayou on 20 April (JD 110) and 19 June (JD 170).

Light Traps

Insect collections were made monthly from May 2018 through June 2019 using CDC light traps (John W. Hock Co., Gainesville, FL). Traps were placed on PVC pipes 1.5 m off the ground before dusk and collected after dawn. Three trap types (incandescent, incandescent baited

with 2 kg dry ice, and ultraviolet light) were used at each salinity level for a total of nine traps per collection for each bay. Traps were placed at each of the three collection sites within each of the six estuarine locations (Low, Mid and High salinity zones in both Barataria Bay and Caillou Bay), and trap type was rotated among sites after each collection. Overnight collections were made twelve times at Barataria Bay and eleven times at Caillou Bay.

Specimens were placed on dry ice, transported back to campus and stored at -80C until processed. Subsequently, individual adult tabanids were sorted, pinned, and identified to gender and species level using the keys of Tidwell (1973), and then stored at -20C.

DNA Barcoding

After coastal tabanid abundance surveys were completed, all specimens were identified to species level using dichotomous keys by Tidwell (1973) and Goodwin and Drees (1996). Females of each of the four coastal tabanid species, *Tabanus acutus*, *T. hinellus*, *T. nigrovittatus* and *Chrysops flavidus* reported to exist within the estuaries by Tidwell (1973) and Goodwin and Drees (1996) were selected for DNA barcoding. Only a subset of flies of each coastal species was used during the DNA barcoding process. Those specimens were selected based on fully distinguished morphological features for their species. Fourteen specimens of *T. nigrovittatus*, twelve specimens of *T. hinellus*, ten of *T. acutus*, and seven of *C. flavidus* were selected for barcoding. Eleven male tabanids collected from light traps and eleven tabanid larvae collected from marsh sediment also were used for DNA analysis. In order to capture intraspecific genetic variation, the female tabanids of the same species were selected from different locations within the surveys.

Prior to selecting loci to use within this project, a GenBank reference search (10/21/2020) yielded limited reference sequence records of the coastal species that were expected to occur

within the estuarine study areas. The database contained 23 *Tabanus nigrovittatus* sequences across three loci. Two of the sequences were for each of the 18S ribosomal RNA gene regions (KU321600.1 & KT222915.1) and one for the partial cytochrome oxidase subunit (KT381971.1). The 20 remaining sequences were of cloned *T. nigrovittatus* microsatellite sequences and not useful for DNA barcoding. There were no sequence references in NCBI GenBank for *T. hinellus* and *T. acutus*. There were three partial sequences for *Chrysops flavidus* across three genes: a partial AATS1 gene (KM243607.1), a partial CO1 gene (KM243498.1), and a 28S RNA partial sequence (KM243402.1).

Due to lack of representation in GenBank for the coastal species and to test which locus or combination of loci had the best species resolution for Sanger sequencing, all three loci previously used for *T. nigrovittatus* were chosen for analysis in the current study (Table 2.2). The loci consisted of two genomic 18S rRNA gene regions amplified by the primer pairs 18Sai/18Sbi (982bp) (Whiting et al. 1997) and NF1/18Sr2b (412bp) (Porazinska et al. 2009) and a mitochondrial cytochrome oxidase CO1 amplified by primer pair LCO1490/HCO2198(687bp) (Cywinska et al. 2010). All 43 female specimens were sequenced at those three loci, but due to poor sequence quality and repeated sequence failures, that yielded 113 sequences. Mitochondrial cytochrome oxidase CO1 region, i.e., the locus that provided the greatest resolution power among the three loci used to sequence female tabanids, was chosen to sequence the male tabanids as well as the larvae of unknown species.

DNA was extracted from the thoraces of the adults and the abdominal midsection (1^{st} – 7^{th} abdominal section) of the larvae. The head, wings and abdomen of the adults were kept and stored as voucher samples at -20C. The heads and anal regions of the larvae, including the first and last ring of pseudopodia, were stored as voucher samples in 70% ethanol at -20C.

For DNA extraction, a cocktail mixture of proteinase K and Buffer ATL was made for the lysis step in the DNeasy Blood & Tissue Kit (Qiagen Cat No. /ID: 69506, Qiagen, Germantown, MA). Aliquots were taken from the cocktail and transferred into individual 2mL microcentrifuge tubes for each dissected thorax for the adults and abdominal midsection of the larvae. The thoraces were then homogenized within the tubes using a pestle. All homogenized samples were then lysed overnight at 56C. DNA was then extracted from each sample using the DNeasy Blood & Tissue Kit (Qiagen Cat No. /ID: 69506, Qiagen, Germantown, MA) following the manufacturer's protocol. DNA concentrations were determined using Qubit 4 Fluorometer (ThermoFisher Scientific) with the Qubit dsDNA BR Assay Kit (Invitrogen) resulting in a concentration range of 3-35 ng/ul. The resulting DNA was then purified using the QIAquick PCR Purification Kit (Qiagen) using the manufacturer's protocol.

PCR was performed using OneTaq 2X Master Mix with Standard Buffer (Cat. No. M0284S, New England Biolabs, Ipswich, MA) using manufacturer's protocol of 12.5ul OneTaq MasterMix, 8.5ul nuclease free water, 0.5ul of 10nM/uL each of forward and reverse primers and 3ul template DNA to reach the standard reaction setup of 25ul. Thermal cycler (Bio-Rad) conditions were specific for each locus with the addition of an initial denaturation step of 94°C for 2 minutes and a final elongation of 68°C for 5 minutes (Table 2.2).

Gel electrophoresis was performed on the PCR products in a 1% agarose gel against the GeneRuler 1kb DNA Ladder (ThermoFisher Scientific) to ensure amplification of PCR products with the correct size at a concentration range of 20-60ng/uL. Agarose gel results were digitally analyzed for size and concentration using imaging software on an E-Gel Imager System with UV Light Base (ThermoFisher Scientific). Following confirmation of amplicons and DNA

purification bidirectional Sanger sequencing was performed by the LSU Genomics Facility, Baton Rouge, LA using an ABI 3130xl Genetic Analyzer.

After bidirectional Sanger sequencing results were received, sequences were edited in Geneious Prime 2020.2.4. Forward and reverse sequences were trimmed with an error probability of 0.04 and were then assembled, using the De Novo Assemble option, into single contigs for each locus and each individual. Sequences were clipped to the longest common sequence length for each locus: 672 bp and 417 bp for the 18S rRNA genes (18Sai/18Sbi and NF1/18Sr2b respectively) and 429 bp for the CO1 gene. Sequences of the three loci were then manually concatenated. For phylogenetic tree construction based on single and concatenated loci, contigs from each locus, along with reference sequences of top matches in the NCBI GenBank Database, were aligned using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/), kmer4_6 (iteration 1) and pctid_kimura (subsequent) for distance measure; UPGMB for clustering method; pseudo for tree rooting; CLUSTALW for sequence weighting scheme with output format as Pearson/FASTA. All alignments were saved in PHYLIP format and imported into IQTree (Los Alamos Lab USA https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html), to produce maximum likelihood trees using bootstrap analysis with 1000 replicates. The sequences of *Chrysops flavidus* were used as the outgroup for all generated trees.

Inter- and intraspecific pairwise similarities were produced by aligning all sequences for each species for each locus into a multiple align file and calculating the percent sequence identity. Distance matrices were searched for the lowest and highest similarities to generate a range of intraspecific variability for each species and locus. Average pairwise similarity along with standard deviation were calculated using distance matrices.

Table 2.2. Loci and primer pairs used for DNA barcoding along with PCR conditions for each.

Locus	Region	Primers	PCR conditions
18Sai/18Sbi (982 bp) (Whiting et al. 1997)	Nucleotide range 391-1421 of 18S rRNA gene	5’ CCTGAGAAACGGCTACCACATC 3’/ 5’ GAGTCTCGTTCGTTATCGGA 3’	Denaturation at 94^{o}C for 30s, annealing at 52^{o}C for 45s and extension at 68^{o}C for 1min15s for 34 cycles.
NF1/18Sr2b (412 bp) (Porazinska et al. 2009)	400 bp from the 3’ end of 18S rRNA gene	5’ GCCTCCCTCGCGCCATCAGGGTGGTG CATGGCCGTTCTTAGTT 3’/ 5’ GCCTTGCCAGCCCGCTCAGTACAAAG GGCAGGGACGTAAT 3’	Denaturation at 94^{o}C for 30s, annealing at 54^{o}C for 45s and extension at 68^{o}C for 30s for 34 cycles.
LCO1490/HCO 2198 (687 bp) (Cywinska et al. 2010)	Nucleotide range 1490-2198 of mitochondrial cytochrome oxidase I gene	5' GGTCAACAAATCATAAAGATATTGG 3'/ 5' TAACTTCAGGGTGACCAAAAAATCA 3'	Denaturation at 94^{o}C for 30s, annealing at 46^{o}C for 45s and extension at 68^{o}C for 45s for 34 cycles.

2.3 Results

Inventory of Coastal Species

Over the course of the study, twelve species of horse and deer flies representing four genera of Tabanidae were collected across nine coastal Louisiana parishes (Table 2.3). Eleven of the twelve species were captured among the terrestrial traps along the coast. Five species, *Chlorotabanus crepuscularis, Leucotabanus annulatus, Tabanus nigripes, T. petiolatus* and *T. stygius*, were only captured at terrestrial trap sites. Specimens of eight species captured at the terrestrial sites were found at the same localities as *T. nigrovittatus* (Table 2.3). Five species, *Chrysops flavidus, Tabanus acutus, T. atratus, T. americanus* and *T. hinellus*, were captured along with *T. nigrovittatus* at the coastal marsh sites. The three species collected along with *T. nigrovittatus* within the estuaries were *T. acutus, T. hinellus* and *C. flavidus*,

The only member of the genus Chrysops that was collected along the coastline within this study was *C. flavidus,* which was captured strictly within the estuaries of Barataria and Caillou Bay (Figure 2.2 and Table 2.3). Specimens of this species were more frequent at Low and Mid salinity zones within the estuaries. During trapping days when the majority of the species captured were *T. nigrovittatus* and *T. hinellus*, specimens of *C. flavidus* made up for about 10-15% of the total catch in one canopy trap.

Of the twelve species captured in this study, *T. nigrovittatus* was the most commonly collected species. A total of 2880 specimens of *T. nigrovittatus* were captured in the estuaries among all salinity zones in both bays during the field season of 2019. The second most abundant species was *T. hinellus* with 129 specimens collected. Only 48 specimens of *T. acutus* were captured in both bays across all salinities in 2019.

Of the twelve species captured in this study, nine have been described as inland species. For *Chlorotabanus crepuscularis,* which is a crepuscular/nocturnal species, only two individuals were captured at Joyce Wildlife Management Area over the course of this study. *Leucotabanus annulatus* was another species of low occurrence; only three specimens were collected at Lake End Park and in St. Mary Parish.

Tabanus lineola is the inland morphotype and sister species of the coastal species *T. hinellus*. *Tabanus lineola* was captured at sites surrounded by wooded area. This species was found to be the dominant species captured in forested areas in locations where the coastal species *T. nigrovittatus* was absent. In four trapping attempts at Joyce W.M.A and Lake End Park, a combined total of 233 *T. lineola* individuals were captured at both sites while no specimens of *T. nigrovittatus* were captured.

Tabanus nigripes, T. petiolatus and *T. stygius* specimens were all captured closer to bottomland hardwood forested areas and areas with closer proximity to freshwater. At Lake End Park and Burns Point Park among other areas, eighteen female *T. nigripes* were captured along with ten *T. petiolatus* specimens and five *T. stygius* specimens over the course of this study during five trapping events, but *T. nigrovittatus* specimens were collected.

Some specimens collected during this study were similar in appearance to *T. nigrovittatus* but had a slightly different grayish thorax and the total body lengths were greater than the specified *T. nigrovittatus* size range; therefore, these flies were not included in any analysis within this chapter but are analyzed in detail in Chapter 3.

Table 2.3. Species of Tabanidae captured within and along coastal Louisiana in the 2018 and 2019 field seasons.

Species	Canopy Trap Site	Localities/Parishes
Chrysops		
flavidus *	*Estuaries*	Terrebonne and Plaquemines
Chlorotabanus		
crepuscularis	*13*	Tangipahoa
Leucotabanus		
annulatus	*3*	St. Mary
Tabanus		
acutus *	*Estuaries*	Terrebonne and Plaquemines
atratus	*1, 2, 3, 4, 12, 13 and estuaries*	Iberia, Plaquemines, St. Mary, St. Tammany, Tangipahoa, Terrebonne, and Vermilion
americanus	*2, 11, 12 and estuaries*	Iberia, Orleans, Plaquemines, St. Tammany, and Terrebonne
hinellus *	*3, 4, 7, 8, 9, 10, 12 and estuaries*	Plaquemines, St. Bernard, St. Mary, St. Tammany, and Terrebonne
lineola	*1, 2, 4, 5, 6, 10, 11, 12 and 13*	Iberia, Orleans, St. Bernard, St. Mary, St. Tammany, Tangipahoa, Terrebonne and Vermilion
nigripes	*1, 2, 3, 4, 6, and 12*	Iberia, St. Mary, St. Tammany, Terrebonne and Vermilion
nigrovittatus *	*3, 7, 8, 9, 10, 12 and estuaries*	Jefferson, Orleans, Plaquemines, St. Bernard, St. Mary, St. Tammany, and Terrebonne
petiolatus	*1, 2, 6 and 13*	Vermilion, Iberia, Terrebonne and Tangipahoa
stygius	*1, 3, 11 and 13*	St. Mary, St. Tammany, Tangipahoa, and Vermilion

Asterisk (*) denotes coastal species. Species recorded in each parish where at least one representative member was captured. Canopy trap site numbers can be found on the map of Figure 2.1. Captured flies were identified using identification methods by Tidwell 1970 and 1973.

Tabanus nigrovittatus Habitats

Water salinity was calculated using monthly data from CRMS stations over four years (2016-2019) at each station. Results show the occurrence of *T. nigrovittatus* in seven areas with intermediate-brackish to saline water salinities within 0.1-20.5 parts per thousand (ppt) (Table 2.4 and Figure 2.3). In six areas with fresh to fresh-intermediate water salinities, within 0-6.7 ppt, specimens of *T. nigrovittatus* were not collected.

Table 2.4. Water salinity range (ppt) recorded by CRMS stations within 8 kilometers of canopy trap sites using monthly data over a four year period (2016-2019).

Trap	Location	Latitude/Longitude	Water Salinity (ppt)	*T. nigrovittatus*	Julian Day of Collections	Year of Collection
1	Palmetto Island State Park	29.864815 N, 92.133160 W	0-5.5	No	135, 141, 177, 199, 218	2018
2	Avery Island	29.914755 N, 91.904717 W	0.7-6.7	No	135, 141, 177, 199, 218	2018
3	Cypremort Point State Park	29.733001 N , 91.847474 W	0.1-10.6	Yes	135, 141, 177, 199, 218	2018
4	Burns Point Park	29.574588 N, 91.537936 W	0.2-2.8	No	133, 156, 170, 205	2018
5	Lake End Park	29.727196 N, 91.178900 W	0.1-0.7	No	133, 156, 170, 205	2018
6	Mandalay Bay Wildlife Refuge	29.550313 N, 90.790517 W	0-1.9	No	133, 156, 170, 205	2018
7*	Plaquemines Parish	Across the Parish	1.9-20.5	Yes	110, 125, 130, 177, 180, 194, 201, 231, 246, 273	2010 & 2011
8*	St. Bernard Parish	Across the Parish	0.6-7	Yes	111, 125, 138, 174, 194, 202, 210, 232, 238, 257, 274	2010 & 2011
9	Delacroix	29.758193 N, 89.783849 W	0.2-7.9	Yes	148, 172, 205	2018
10	Hopedale	29.821478 N, 89.608583 W	1.7-18.2	Yes	148, 172, 205	2018
11	Pelican Pointe Marina	30.131792 N, 89.761642 W	0.9-6.8	Yes	148, 172, 205	2018
12	Fontainebleau State Park	30.336416 N, 90.045593 W	0.2-2.9	Yes	128, 142, 170, 212	2018
13	Joyce W.M.A.	30.397268 N, 90.428923 W	0.2-3.2	No	128, 142, 170, 212	2018

*In both parishes, 3-4 traps were placed across each. In Plaquemines Parish, traps were placed at: 29.30325 N, 89.67677 W; 29.3952 N, 89.67031W; 29.62213 N, 89.95350 W; and 29.51589 N, 89.76131 W. In St. Bernard Parish, traps were placed at: 29.82301 N, 89.75553 W; 29.83852 N, 89.68786 W; and 29.81929 N, 89.61348 W.

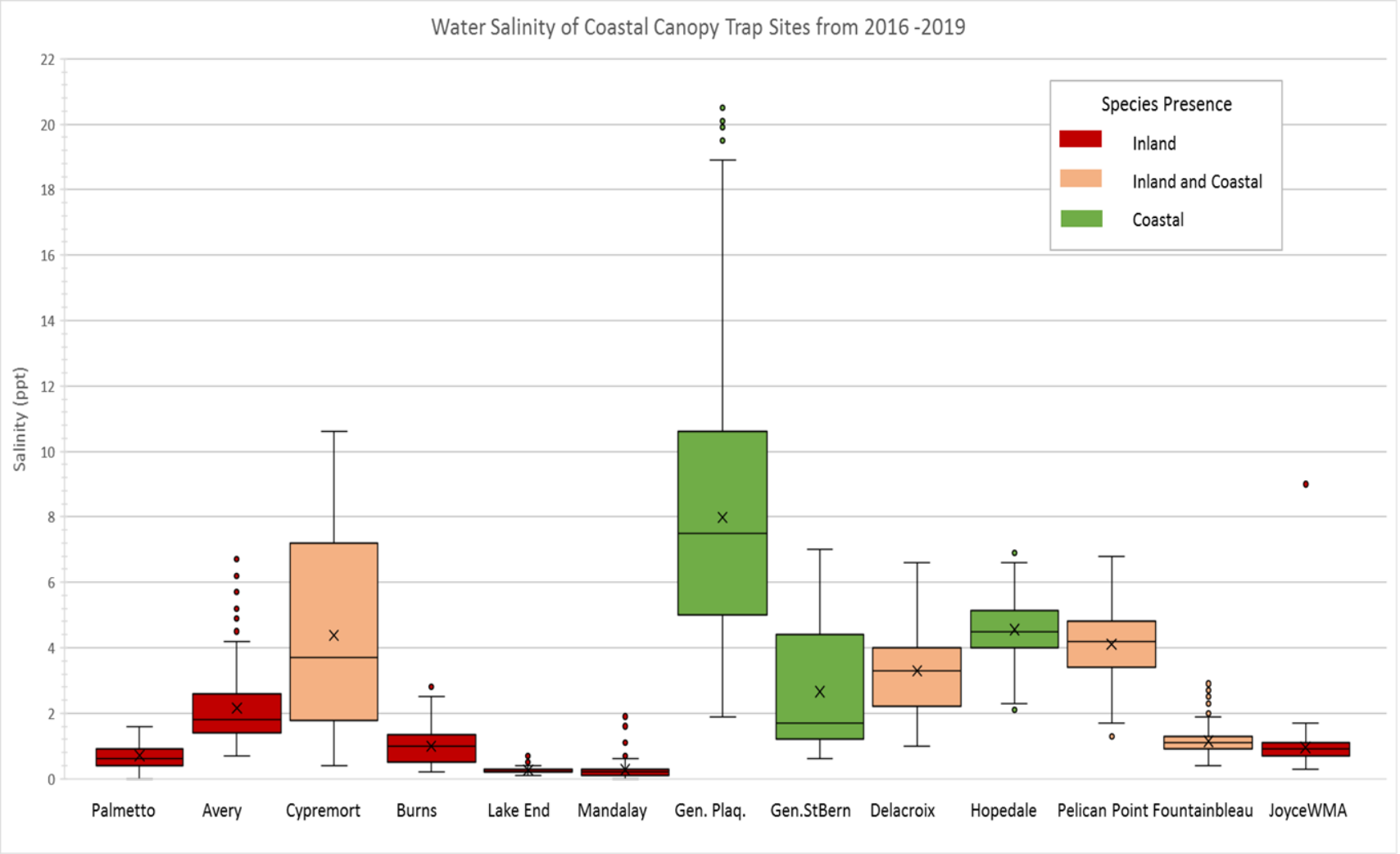

Figure 2.3. Water salinity range of the thirteen canopy trap locations generated using monthly salinity data over a four-year period (2016-2019) from the nearest CRMS station to the canopy trap. Sites color-coded based on inland and/ or coastal species presence at each location.

Tabanus nigrovittatus Temporal Distribution

The seasonal patterns of *T. nigrovittatus* data collected across Plaquemines and St. Bernard Parishes in 2010 and 2011 were similar (Figures 2.4 and 2.5). Although specimens of *T. nigrovittatus* were found continuously throughout the season, there was a large abundance peak that occurred during the 2011 seasons at both localities from mid-April to mid-May (Figures 2.4 and 2.5). Unfortunately trapping attempts did not begin until mid-season in 2010 (following the oil spill) so data for the same period in the early season could not be analyzed. However, data from both years, beginning in mid-June through the remainder of the season show continued emergence and collection of horse flies along the coast.

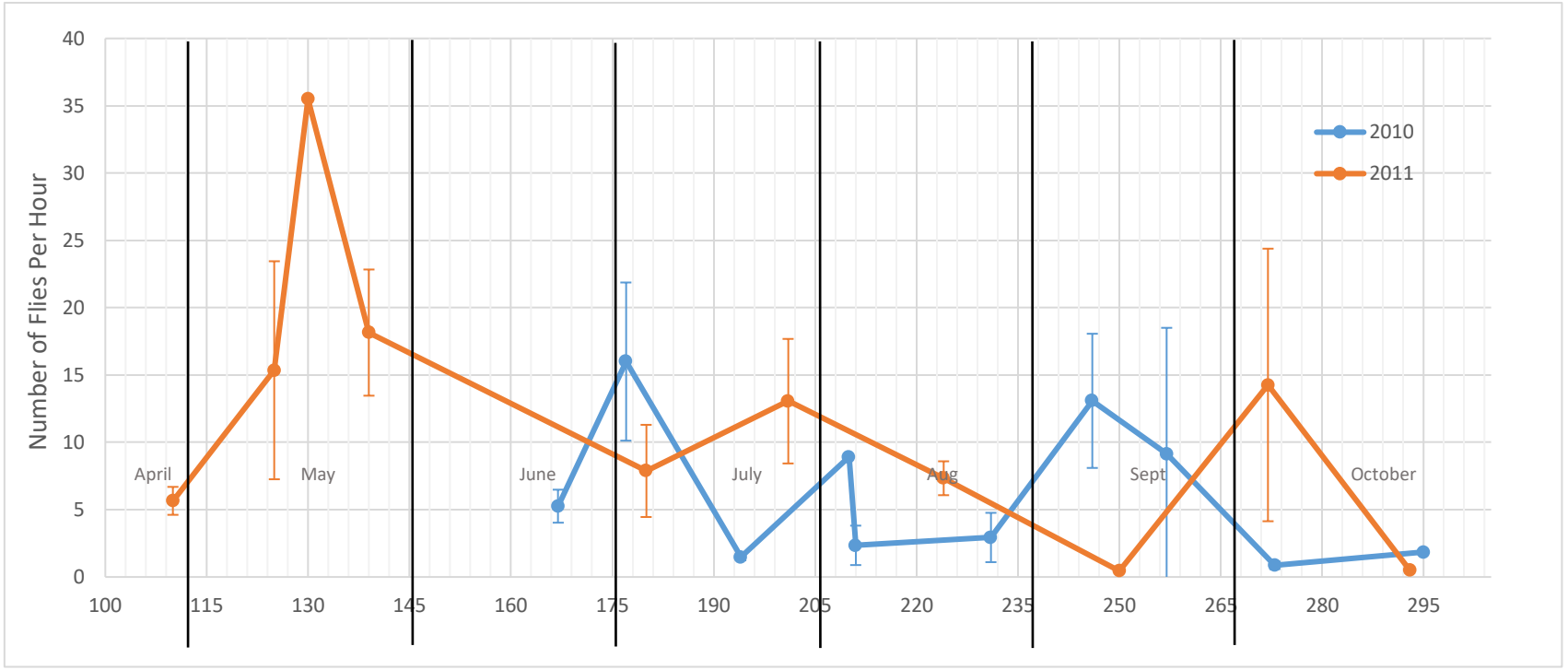

Figure 2.4. Average number of *Tabanus nigrovittatus* trapped (flies/hour/trap) in Plaquemines Parish in 2010 and 2011. Standard error was used for each of the traps. Standard error bars were an average from the total catch of traps for each respective day.

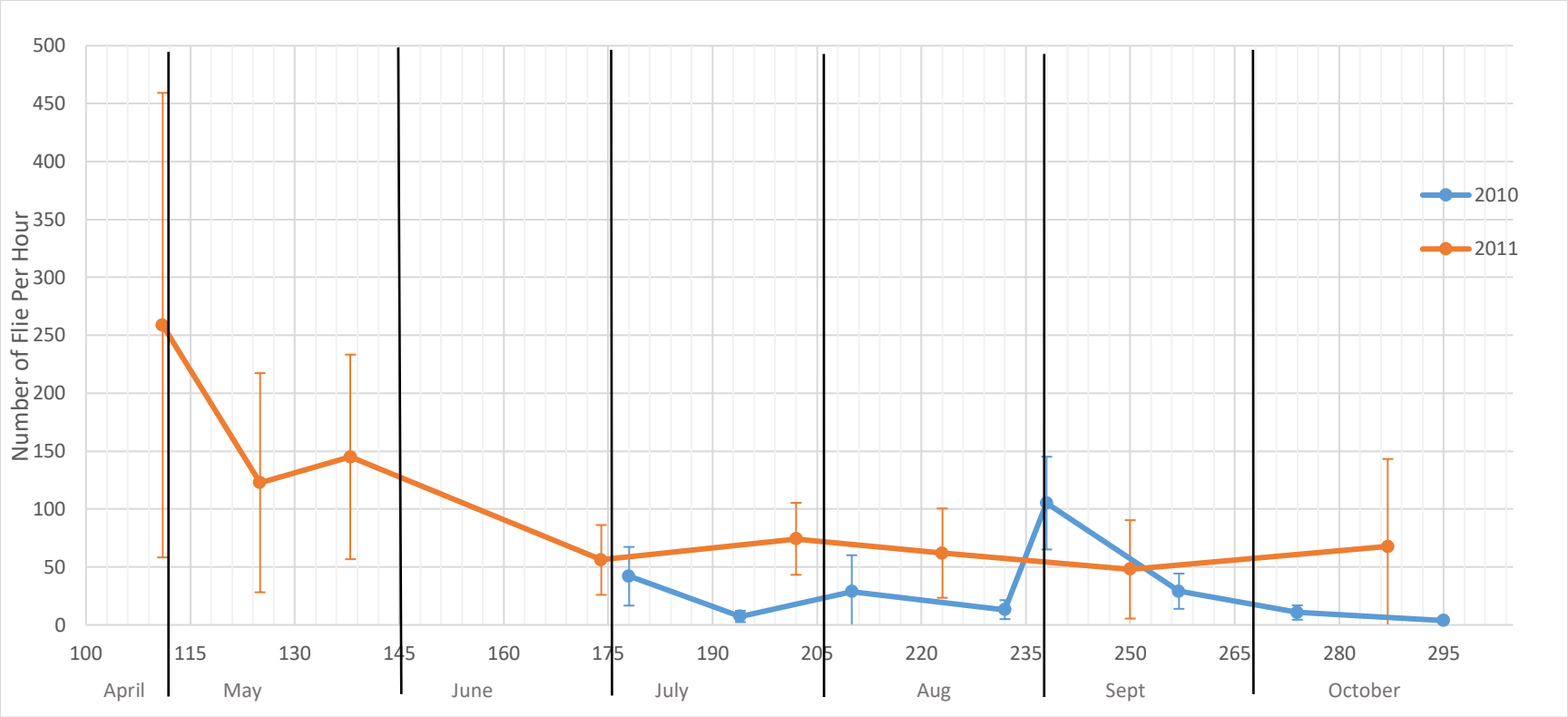

Figure 2.5. Average number of *Tabanus nigrovittatus* trapped (flies/hour/trap) in St. Bernard Parish in 2010 and 2011. Standard error was used for each of the traps. Standard error bars were an average from the total catch of traps for each respective day.

Tabanid Larvae

Sixty-two tabanid larvae were extracted from marsh substrate collected from two field sites, Cypremort Point and Grand Bayou of Husseneder et al. 2018, over the 2016-2017 period with 28 of those collected in 2016 and 34 larvae collected in 2017. Eleven of the 28 (39%) larvae from 2016 were collected at Cypremort Point on 2 August (JD 215). Only 2-5 larvae were extracted from marsh substrate during each of the other larval collection days. In 2017, nine of the 34 (26%) larvae were collected at Cypremort Point on 11 April (JD 101). At the following collection days, 5-8 larvae were extracted per day between Grand Bayou and Cypremort Point.

Light Traps

A total of 209 male and female *T. nigrovittatus* were captured in light traps from the estuaries of Barataria and Caillou Bay from July 2018-June 2019 (Figure 2.2). The majority (118 or 56%) of those flies were captured in the month of May with the next highest capture of only nine flies in October (Figure 2.6). Of the flies captured in May, 56 of those were males and 53 of which were captured in the Mid-salinity zone (Figure 2.7).

Twenty-seven males were captured each in traps with incandescent lights as well as in incandescent light traps baited with dry ice. Only two were captured in ultraviolet traps. The majority (87% or 56) of male individuals were captured in May. Sixty–two female *T. nigrovittatus* were captured in May; thirty-nine females were captured in traps with incandescent lights baited with dry ice at the Mid salinity sites, twenty-two were captured in incandescent light traps, ten captured were in one trap at the Mid salinity zone. There was only one female captured in an ultraviolet trap.

Of the flies captured in October, six males were captured in the high salinity zone, three each found in incandescent light traps and traps baited with dry ice. Only three females were captured in October at the Low salinity sites with traps baited with dry ice.

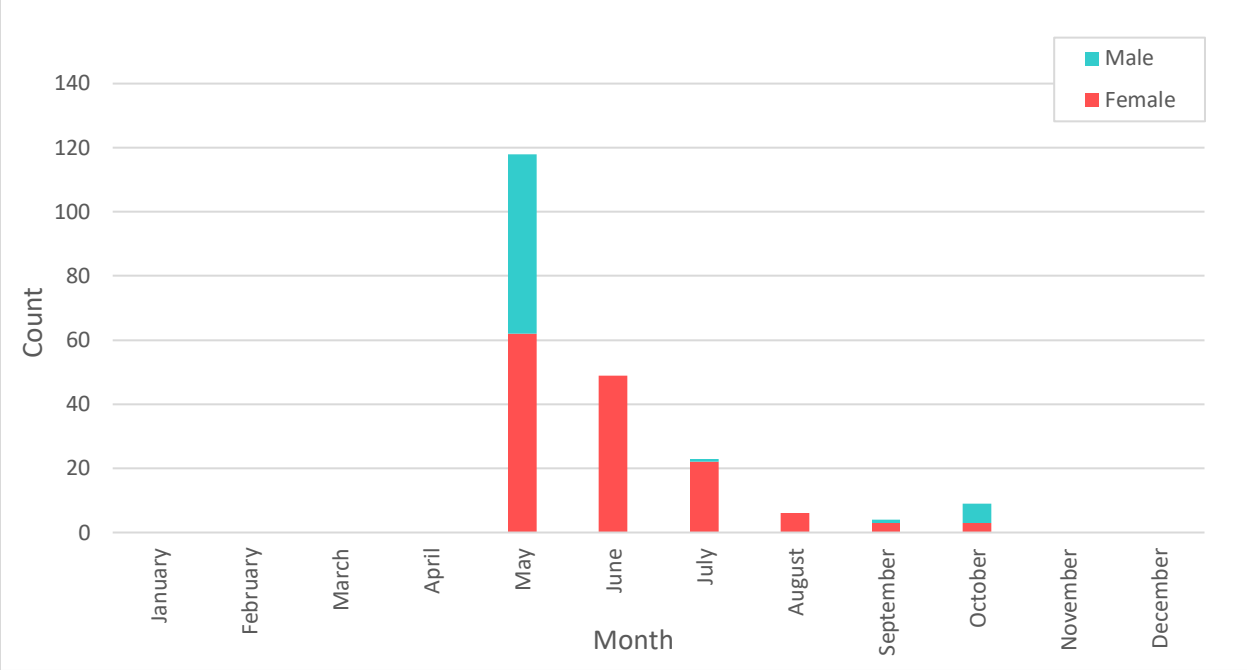

Figure 2.6. The number of male and female *Tabanus nigrovittatus* captured in light traps at Caillou and Barataria Bay over the course of July 2018-June 2019.

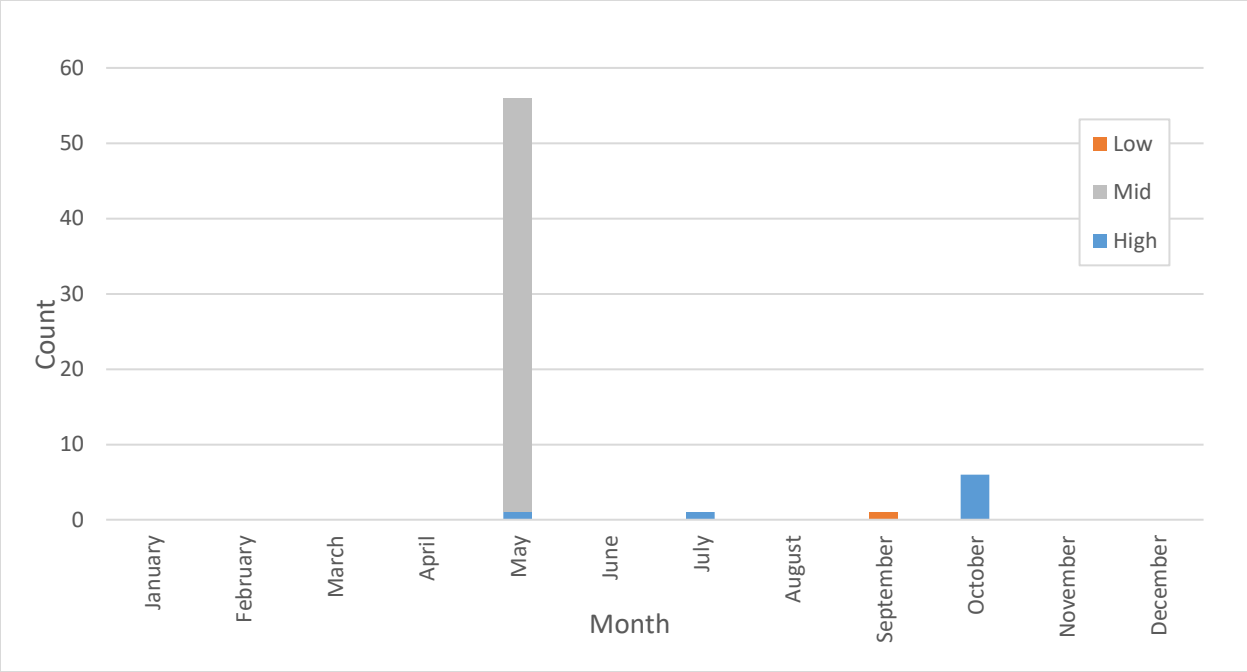

Figure 2.7. The abundance of male *Tabanus nigrovittatus* captured within different salinity zones in Caillou and Barataria Bay over the course of July 2018- June 2019.

Tidal Marsh Horse Fly Collections

Diurnal vs Overnight Coastal Canopy Trap Surveys

Only specimens of *Tabanus nigrovittatus* and *T. hinellus* were captured within the estuaries during diurnal hours. The relative abundance of *T. nigrovittatus* remained relatively constant as salinity increased . In contrast, as salinity increased, *T. hinellus* abundance decreased overall with 29 collected at Low and 10 collected each at Mid and High salinity . Specimens of *T. acutus* were collected within the three salinity zones along with *T. nigrovittatus* and *T. hinellus* during nocturnal collections. During those collections, *T. acutus* abundance decreased as salinity increased. There were 132 specimens collected at the Low salinity sites, 104 specimens collected at the Mid salinity sites, and only 16 specimens collected at the High salinity sites . There was a similar trend for *T. hinellus* populations during the overnight collections. As salinity

increased, *T. hinellus* catches decreased from 10 individuals at Low, 7 in the Mid and none collected at the High salinity sites. However, this trend was not apparent in diurnal collections.

Tabanus nigrovittatus Abundance by Salinity

A two-factor ANOVA was conducted to compare the rate that flies were trapped between salinity zones within both bays. The natural log of the calculated average number of flies per hour for each trap day was used in the analysis. There was a significant effect of both bay ($F_{(1, 110)} = 9.461$, $p = 0.003$) and the interaction of salinity and bay ($F_{(2, 110)} = 11.608$, $p < 0.001$) on the average number of *T. nigrovittatus* collected per hour. The collection rate of *T. nigrovittatus* varied by salinity when looking at the individual bays, but not when they were considered together ($F_{(1, 110)} = 1.850$, $p = 0.162$).

Post-hoc comparisons using the Tukey HSD test indicated significant differences of abundances among the salinity zones at both locations (Table 2.5). Shorthand identifications were used for both locations, C for Caillou and B for Barataria, along with their respective salinity trap site. Significantly fewer individuals were collected per hour at B-Low than B-High ($p = 0.041$), C-Low ($p < 0.001$), and C-High ($p = 0.029$), significantly more individuals were collected at C-Low than B-Mid ($p = 0.006$), and significantly more individuals were collected at C-Mid than C-Low ($p = 0.001$). The highest number of flies per trap was collected in the Low salinity zone at Caillou and the lowest number was collected at Barataria Low salinity zone. The *T. nigrovittatus* catch at High and Mid salinity zones were not statistically different amongst one another between the locations.

Table 2.5. Average number of *Tabanus nigrovittatus* trapped (flies/hour) by location and salinity in 2019 compared using a two-factor ANOVA. Collection rates that share a letter were not significantly different based on the results of a Tukey HSD post-hoc test.

Location	Salinity	Mean ± SE	Mean (log x+1) ± SE
Caillou	Low	7.88 ± 1.74	1.88 ± 0.22 [a]
	Mid	2.88 ± 0.73	1.12 ± 0.17 [bc]
	High	4.69 ± 0.90	1.61 ± 0.14 [ab]
Barataria	Low	1.68 ± 0.54	0.89 ± 0.15 [c]
	Mid	4.29 ± 0.98	1.48 ± 0.19 [bc]
	High	7.22 ± 1.89	1.87 ± 0.28 [ab]

Letters indicate statistical differences (p-values less than or equal to 0.05 Tukey Kramer post hoc analysis.

Tabanus nigrovittatus Temporal Distribution by Salinity

The temporal occurrence of *T. nigrovittatus* across all three salinity zones in both bays for the study period was examined. Historical water salinities used to select the study sites between the two bays were comparable among the salinity zones. Salinities at the Low zones ranged from 3.3-6 ppt with an average of 5.2 ppt. The Mid zones ranged from 6-10.4 ppt with an average of 9.1 ppt. Historical water salinity between the bays at the High zones were 12-15.8 ppt with an average of 14 ppt. However, at the time of the current study, water salinities did not deviate much between the zones and bays; therefore, the collection sites were not altered.

In Barataria Bay, *T. nigrovittatus* abundance increased around mid to late-June and decreased through July and early-August until numbers began to climb again around mid-August into September (Figure 2.8). This trend was exhibited throughout the different salinity zones at Barataria Bay; however, there was a sharp increase in abundance in the High salinity zone in September. In Caillou Bay, *T. nigrovittatus* abundances exhibited a similar trend, at the Low and Mid salinities where abundances were high around late May to early-June and declined through

July and early-August. Number of flies collected per hour later increased again in late-August through early-September and begin to decline thereafter (Figure 2.9). Much like in Barataria, there was an increase in abundance at the Low salinity zone in Caillou Bay in late-August.

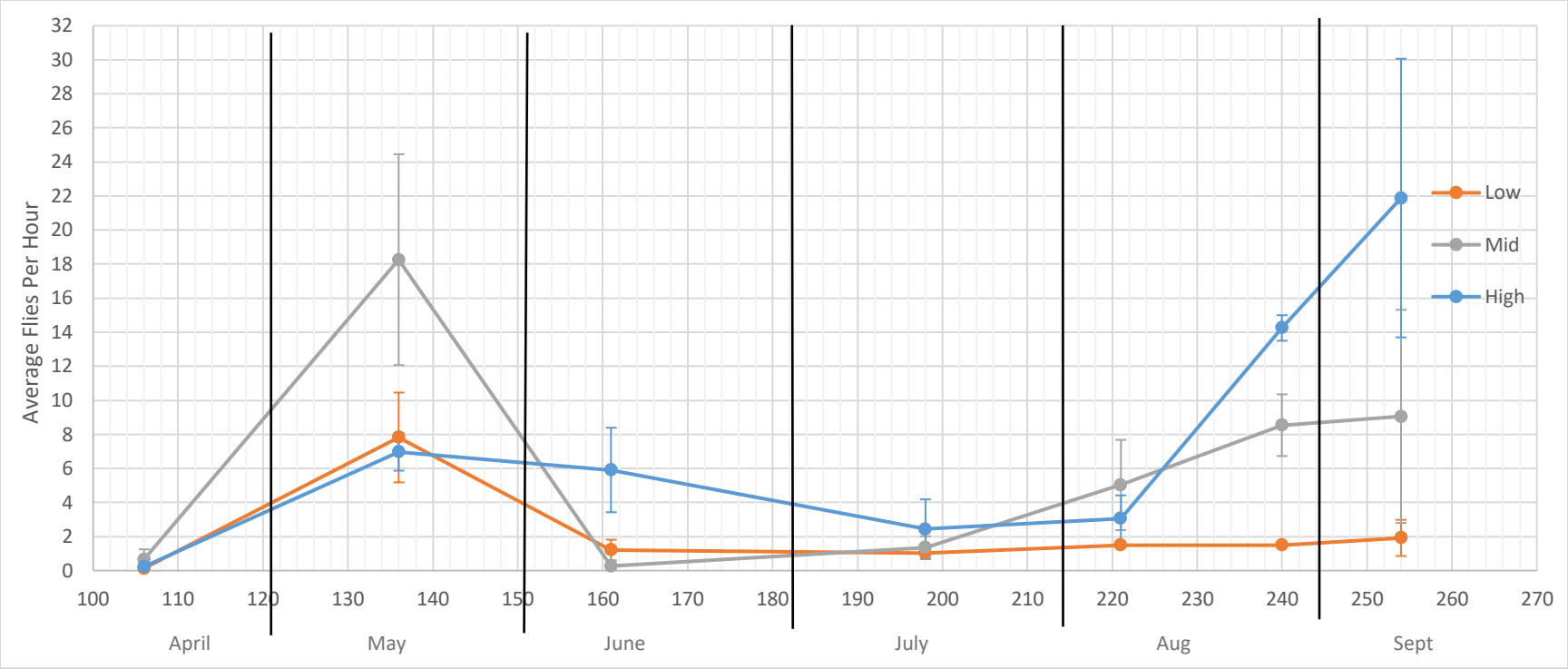

Figure 2.8. Average number of *Tabanus nigrovittatus* (flies/hour/Julian day) trapped in Barataria Bay across the three salinity levels: Low, Mid, and High in 2019. Standard error was used for each of the trapping days. Standard error bars were an average from the total catch of traps for each respective day at each salinity zones.

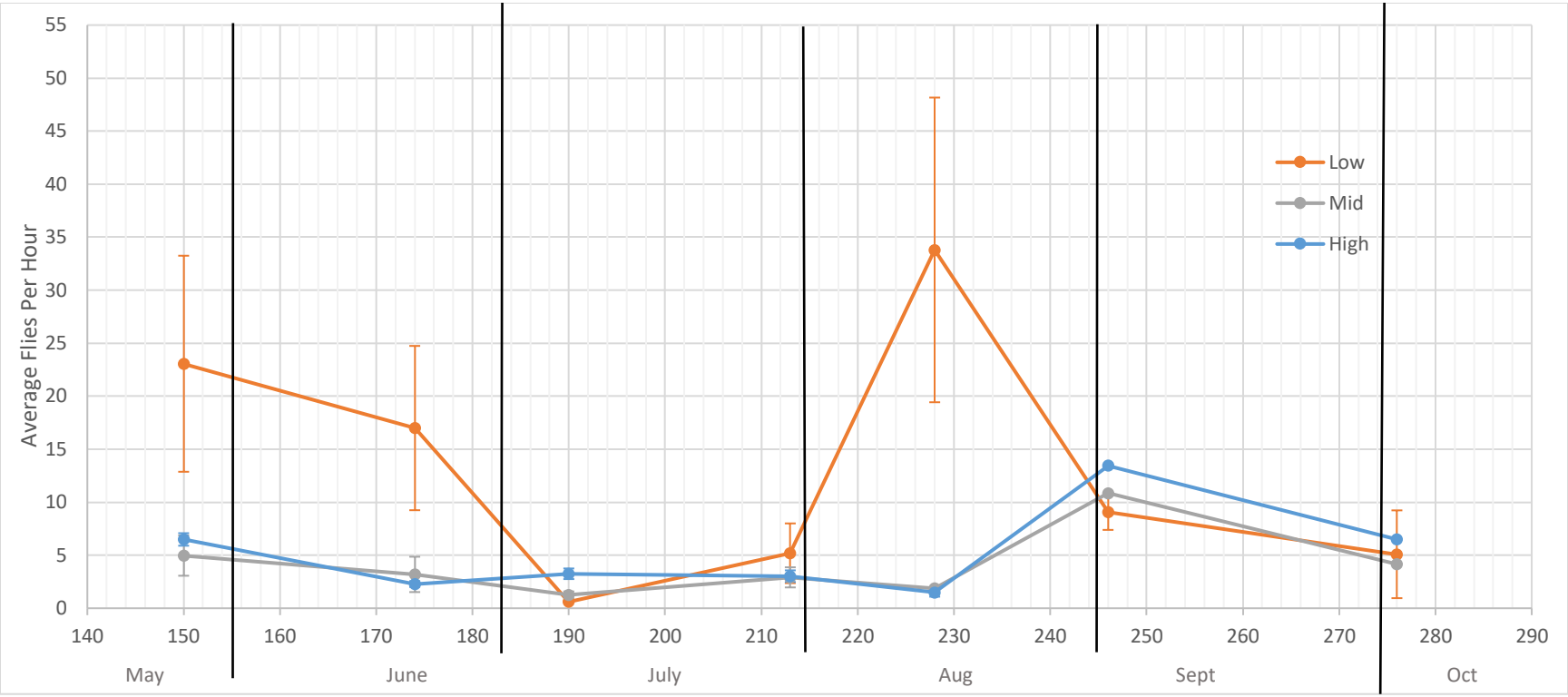

Figure 2.9. Average number of *Tabanus nigrovittatus* (flies/hour/Julian day) trapped in Caillou Bay across the three salinity levels: Low, Mid, and High in 2019. Standard error was used for each of the trapping days. Standard error bars were an average from the total catch of traps for each respective day at each salinity zones.

DNA Barcoding

As previously mentioned, during the process of species designations, there were specimens that resembled that of *T. nigrovittatus* but lacked the full descriptive characters for the species. Those specimens were larger in size and had longer total body length as well as a more grayish thorax than the yellow thorax descriptive of *T. nigrovittatus*. Since no species designation was given to these flies, they were not included in any of the analyses made within this chapter but will be considered in Chapter 3. The morphologically identified specimens of each of the known species used within this current study were collected from various localities along the coast and from different collection days (Table 2.6)

Since there was little species representation in the NCBI GenBank database for the species identified and sequenced within this study, all species sequences generated across the three loci were submitted to NCBI GenBank to expand the database of information pertaining to the sequences of these species.

Table 2.6. Collection dates and locations of the specimens used for DNA sequencing and phylogenetic tree analysis.

Species	Specimen Number	Collection Date	Location
Tabanus nigrovittatus	1	10/4/2016	Cameron
Tabanus nigrovittatus	1a	5/30/2018	Caillou
Tabanus nigrovittatus	1b	5/30/2018	Caillou
Tabanus nigrovittatus	2	10/4/2016	Cameron
Tabanus nigrovittatus	2a	5/30/2018	Caillou
Tabanus nigrovittatus	2b	5/30/2018	Caillou
Tabanus nigrovittatus	3	10/4/2016	Cameron
Tabanus nigrovittatus	4	9/25/2017	Cypremort
Tabanus nigrovittatus	5	9/25/2017	Cypremort
Tabanus nigrovittatus	6	9/25/2017	Cypremort
Tabanus nigrovittatus	7	9/25/2017	Cypremort
Tabanus nigrovittatus	8	5/16/2018	Caillou

Table cont'd

Species	Specimen Number	Collection Date	Location
Tabanus nigrovittatus	9	5/16/2018	Caillou
Tabanus nigrovittatus	10	5/16/2018	Caillou
Tabanus hinellus	1	7/13/2016	Cypremort
Tabanus hinellus	2	7/13/2016	Cypremort
Tabanus hinellus	2b	8/24/2016	Cameron
Tabanus hinellus	3	7/13/2016	Cypremort
Tabanus hinellus	4	8/8/2018	Barataria
Tabanus hinellus	5	8/8/2018	Barataria
Tabanus hinellus	6	8/8/2018	Barataria
Tabanus hinellus	7	7/8/2017	Cameron
Tabanus hinellus	8	7/8/2017	Cameron
Tabanus hinellus	9	7/8/2017	Cameron
Tabanus hinellus	10	7/8/2017	Cameron
Tabanus hinellus	69	7/27/2018	Barataria
Tabanus acutus	1	5/24/2018	Barataria
Tabanus acutus	2a	7/27/2018	Barataria
Tabanus acutus	3	5/24/2018	Barataria
Tabanus acutus	4	5/24/2018	Barataria
Tabanus acutus	5	8/8/2018	Barataria
Tabanus acutus	6	8/8/2018	Barataria
Tabanus acutus	7	8/8/2018	Barataria
Tabanus acutus	8	5/30/2018	Caillou
Tabanus acutus	9	5/30/2018	Caillou
Tabanus acutus	10	5/30/2018	Caillou
Chrysops flavidus	1	5/30/2018	Caillou
Chrysops flavidus	2	5/30/2018	Caillou
Chrysops flavidus	3	5/30/2018	Caillou
Chrysops flavidus	4	5/30/2018	Caillou
Chrysops flavidus	8	7/27/2018	Barataria
Chrysops flavidus	9	7/27/2018	Barataria
Chrysops flavidus	10	7/27/2018	Barataria
Unknown	Unknown Larva 1	6/7/2017	Cypremort
Unknown	Unknown Larva 2	6/7/2017	Cypremort
Unknown	Unknown Larva 3	6/19/2017	Grand Bayou
Unknown	Unknown Larva 4	6/19/2017	Grand Bayou
Unknown	Unknown Larva 5	4/11/2017	Cypremort
Unknown	Unknown Larva 6	4/20/2017	Grand Bayou
Unknown	Unknown Larva 7	5/17/2017	Cypremort
Unknown	Unknown Larva 8	7/20/2016	Cypremort
Unknown	Unknown Larva 9	7/20/2016	Cypremort
Unknown	Unknown Larva 10	9/20/2016	Cameron
Unknown	Unknown Larva 11	9/20/2016	Cameron

Table Cont'd

Species	Specimen Number	Collection Date	Location
Unknown	Unknown Male 1	May	Barataria
Unknown	Unknown Male 2	May	Barataria
Unknown	Unknown Male 3	May	Barataria
Unknown	Unknown Male 4	May	Barataria
Unknown	Unknown Male 5	July	Caillou
Unknown	Unknown Male 6	July	Caillou
Unknown	Unknown Male 7	July	Caillou
Unknown	Unknown Male 8	June	Barataria
Unknown	Unknown Male 9	October	Caillou
Unknown	Unknown Male 10	October	Caillou
Unknown	Unknown Male 11	September	Barataria

There were no existing species sequence references in GenBank for *T. acutus* and *T. hinellus*. Therefore, the top matches in GenBank for specimens of both of these species were different species and the sequence similarities to the references in GenBank were lower than the intraspecific similarities (Table 2.7). The specimens of *T. acutus* had a 98.75% similarity to a previously submitted *T. nigrovittatus* sequence, using the 18Sai/18Sbi locus. In addition, using LCO1490/HCO2198, the *T. acutus* specimens of this study matched to existing Hybomitra sequences and a Tabanus species sequence (Table 2.7). *Tabanus hinellus* specimens, when compared against GenBank sequences, paired to a *Haematopota pluvialis*, Tabanus species sequence, and *T. similis* for 18Sai/18Sbi, NF1/18Sr2b, and LCO1490/HCO2198 loci respectively (Table 2.7). For *C. flavidus*, there were no reference sequences for either of the 18S loci but there was a CO1 subunit reference to an existing barcode sequence for *C. flavidus* submitted prior to this study. As stated previously, there were reference sequences for *T. nigrovittatus* in NCBI GenBank for each of the three loci.

There was a 99.34-100% identity match for specimens of *T. nigrovittatus*, at both 18S loci, to the *Tabanus nigrovittatus* reference sequences (KT222915.1 & KU321600.1) (Table 2.7). For all but one individual, percent similarity of CO1 subunit sequences of specimens of *T.*

nigrovittatus in this study matched 98.15-99.59% to an existing *T. nigrovittatus* sequence. The top matches for one individual, *T. nigrovittatus* 2a, was to three specimen of an unidentified *Tabanus* species (MF837932.1, MG163497.1, KP047275.1) but with only 95.89% identity for all three references (Table 2.7). Overall, the ranges of intraspecific pairwise identities for *T. nigrovittatus* were on average 99.84% (range: 99.21-100%), 99.91% (range: 99.52-100%) and 99.04% (range: 98.25-100%) for the 18Sai/18Sbi, NF1/18Sr2b and CO1 loci respectively.

Table 2.7. Percent sequence identity of specimens of morphologically identified coastal tabanid species using the mitochondrial CO1 and two 18S ribosomal RNA gene regions. Intraspecific percent identity represents sequence similarity among specimens of the same morphologically identified species. Top Match Percent Identity column displays the highest sequence similarity to preexisting genomic sequences within the GenBank database (10/21/2020) along with the top match accession number(s).

ID	Gene	Primer Pair	Accession Number	Closest Match	Percent Identity	Match Accession Number
C. flavidus 1	18S	ai/bi	MW531927.1	*Chrysops niger*	*97.84*	AF073889.1
C.flavidus 2	18S	ai/bi	MW531923.1	*Chrysops niger*	*97.88*	AF073889.1
C.flavidus 3	18S	ai/bi	MW531924.1	*Chrysops niger*	*97.78*	AF073889.1
C.flavidus 4	18S	ai/bi	MW531925.1	*Chrysops niger*	*97.88*	AF073889.1
C. flavidus 8	18S	ai/bi	MW531928.1	*Chrysops niger*	*97.84*	AF073889.1
C.flavidus 9	18S	ai/bi	MW531926.1	*Chrysops niger*	*97.75*	AF073889.1
C.flavidus 10	18S	ai/bi	MW531929.1	*Chrysops niger*	*97.37*	AF073889.1
T.acutus 1	18S	ai/bi	MW531950.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 3	18S	ai/bi	MW531951.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 4	18S	ai/bi	MW531952.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 5	18S	ai/bi	MW531953.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 6	18S	ai/bi	MW531954.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 7	18S	ai/bi	MW531955.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 8	18S	ai/bi	MW531956.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 9	18S	ai/bi	MW531957.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.acutus 10	18S	ai/bi	MW531958.1	*Tabanus nigrovittatus*	*98.75*	KT222915.1
T.hinellus 1	18S	ai/bi	MW531940.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 2	18S	ai/bi	MW531941.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 3	18S	ai/bi	MW531942.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 4	18S	ai/bi	MW531943.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 5	18S	ai/bi	MW531944.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 6	18S	ai/bi	MW531945.1	*Haematopota pluvialis*	*99.55*	KC177294.1

Table Cont'd

ID	Gene	Primer Pair	Accession Number	Closest Match	Percent Identity	Match Accession Number
T.hinellus 7	18S	ai/bi	MW531946.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 8	18S	ai/bi	MW531947.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 9	18S	ai/bi	MW531948.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.hinellus 10	18S	ai/bi	MW531949.1	*Haematopota pluvialis*	*99.55*	KC177294.1
T.nigrovittatus 1	18S	ai/bi	MW531930.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 1A	18S	ai/bi	MW531931.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 2	18S	ai/bi	MW531932.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 2A	18S	ai/bi	MW531933.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 3	18S	ai/bi	MW531934.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 5	18S	ai/bi	MW531935.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 6	18S	ai/bi	MW531936.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 7	18S	ai/bi	MW531937.1	*Tabanus nigrovittatus*	*100*	KT222915.1
T.nigrovittatus 9	18S	ai/bi	MW531939.1	*Tabanus nigrovittatus*	*99.34*	KT222915.1
T.nigrovittatus 10	18S	ai/bi	MW531938.1	*Tabanus nigrovittatus*	*100*	KT222915.1
C.flavidus 1	18S	NF1/18Sr2b	MW531884.1	*Chrysops niger*	*98.77*	AF073889.1
C.flavidus 2	18S	NF1/18Sr2b	MW531885.1	*Chrysops niger*	*98.93*	AF073889.1
C.flavidus 3	18S	NF1/18Sr2b	MW531886.1	*Chrysops niger*	*98.93*	AF073889.1
C.flavidus 4	18S	NF1/18Sr2b	MW531887.1	*Chrysops niger*	*98.93*	AF073889.1
C.flavidus 5	18S	NF1/18Sr2b	MW531888.1	*Chrysops niger*	*98.85*	AF073889.1
C.flavidus 7	18S	NF1/18Sr2b	MW531889.1	*Chrysops niger*	*98.92*	AF073889.1
C.flavidus 8	18S	NF1/18Sr2b	MW531890.1	*Chrysops niger*	*98.93*	AF073889.1
C.flavidus 9	18S	NF1/18Sr2b	MW531891.1	*Chrysops niger*	*98.93*	AF073889.1
C.flavidus 10	18S	NF1/18Sr2b	MW531892.1	*Chrysops niger*	*98.93*	AF073889.1
T.acutus 3	18S	NF1/18Sr2b	MW531905.1	Tabanus sp.	*99.76*	MK714120.1
T.acutus 4	18S	NF1/18Sr2b	MW531906.1	Tabanus sp.	*100*	MK714120.1
T.acutus 5	18S	NF1/18Sr2b	MW531907.1	Tabanus sp.	*100*	MK714120.1

Table Cont'd

ID	Gene	Primer Pair	Accession Number	Closest Match	Percent Identity	Match Accession Number
T.acutus 6	18S	NF1/18Sr2b	MW531904.1	Tabanus sp.	*99.76*	MK714120.1
T.acutus 7	18S	NF1/18Sr2b	MW531908.1	Tabanus sp.	*100*	MK714120.1
T.acutus 8	18S	NF1/18Sr2b	MW531909.1	Tabanus sp.	*100*	MK714120.1
T.acutus 9	18S	NF1/18Sr2b	MW531910.1	Tabanus sp.	*100*	MK714120.1
T.acutus 10	18S	NF1/18Sr2b	MW531911.1	Tabanus sp.	*100*	MK714120.1
T.hinellus 1	18S	NF1/18Sr2b	MW531893.1	Tabanus sp.	*95.95*	MK714120.1
T.hinellus 2	18S	NF1/18Sr2b	MW531894.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 3	18S	NF1/18Sr2b	MW531895.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 4	18S	NF1/18Sr2b	MW531896.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 5	18S	NF1/18Sr2b	MW531897.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 6	18S	NF1/18Sr2b	MW531898.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 7	18S	NF1/18Sr2b	MW531903.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 8	18S	NF1/18Sr2b	MW531899.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 9	18S	NF1/18Sr2b	MW531900.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 10	18S	NF1/18Sr2b	MW531901.1	Tabanus sp.	*96.37*	MK714120.1
T.hinellus 69	18S	NF1/18Sr2b	MW531902.1	Tabanus sp.	*96.37*	MK714120.1
T.nigrovittatus 1	18S	NF1/18Sr2b	MW531913.1	*Tabanus nigrovittatus*	*100*	KU321600.1
T.nigrovittatus 1A	18S	NF1/18Sr2b	MW531922.1	*Tabanus nigrovittatus*	*99.75*	KU321600.1
T.nigrovittatus 2	18S	NF1/18Sr2b	MW531914.1	*Tabanus nigrovittatus*	*100*	KU321600.1
T.nigrovittatus 3	18S	NF1/18Sr2b	MW531915.1	*Tabanus nigrovittatus*	*100*	KU321600.1
T.nigrovittatus 4	18S	NF1/18Sr2b	MW531912.1	*Tabanus nigrovittatus*	*99.75*	KU321600.1
T.nigrovittatus 5	18S	NF1/18Sr2b	MW531916.1	*Tabanus nigrovittatus*	*100*	KU321600.1
T.nigrovittatus 6	18S	NF1/18Sr2b	MW531917.1	*Tabanus nigrovittatus*	*100*	KU321600.1
T.nigrovittatus 7	18S	NF1/18Sr2b	MW531918.1	*Tabanus nigrovittatus*	*100*	KU321600.1
T.nigrovittatus 8	18S	NF1/18Sr2b	MW531919.1	*Tabanus nigrovittatus*	*100*	KU321600.1
T.nigrovittatus 9	18S	NF1/18Sr2b	MW531920.1	*Tabanus nigrovittatus*	*100*	KU321600.1

Table Cont'd

ID	Gene	Primer Pair	Accession Number	Closest Match	Percent Identity	Match Accession Number
T.nigrovittatus 10	18S	NF1/18Sr2b	MW531921.1	*Tabanus nigrovittatus*	*100*	KU321600.1
C. flavidus 1	CO1	LCO1490/HCO2198	MW532708.1	*Chrysops flavidus*	*99.59%*	KM243498.1
C. flavidus 2	CO1	LCO1490/HCO2198	MW532704.1	*Chrysops flavidus*	*99.39%*	KM243498.1
C. flavidus 3	CO1	LCO1490/HCO2198	MW532706.1	*Chrysops flavidus*	*99.59%*	KM243498.1
C. flavidus 4	CO1	LCO1490/HCO2198	MW532705.1	*Chrysops flavidus*	*99.39%*	KM243498.1
C. flavidus 8	CO1	LCO1490/HCO2198	MW532707.1	*Chrysops flavidus*	*99.59%*	KM243498.1
C. flavidus 9	CO1	LCO1490/HCO2198	MW532709.1	*Chrysops flavidus*	*100.00%*	KM243498.1
C. flavidus 10	CO1	LCO1490/HCO2198	MW532710.1	*Chrysops flavidus*	*99.80%*	KM243498.1
T. acutus 1	CO1	LCO1490/HCO2198	MW532724.1	Hybomitra sp. (x3) | Tabanidae sp.	96.11%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 2a	CO1	LCO1490/HCO2198	MW532721.1	Hybomitra sp. (x3) | Tabanidae sp.	96.31%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 3	CO1	LCO1490/HCO2198	MW532722.1	Hybomitra sp. (x3) | Tabanidae sp.	96.31%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 4	CO1	LCO1490/HCO2198	MW532725.1	Hybomitra sp. (x3) | Tabanidae sp.	96.11%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 5	CO1	LCO1490/HCO2198	MW532726.1	Hybomitra sp. (x3) | Tabanidae sp.	96.11%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 6	CO1	LCO1490/HCO2198	MW532727.1	Hybomitra sp. (x3) | Tabanidae sp.	96.11%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 7	CO1	LCO1490/HCO2198	MW532728.1	Hybomitra sp. (x3) | Tabanidae sp.	96.11%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 8	CO1	LCO1490/HCO2198	MW532729.1	Hybomitra sp. (x3) | Tabanidae sp.	96.11%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 9	CO1	LCO1490/HCO2198	MW532723.1	Hybomitra sp. (x3) | Tabanidae sp.	96.31%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. acutus 10	CO1	LCO1490/HCO2198	MW532730.1	Hybomitra sp. (x3) | Tabanidae sp.	96.11%	MF832733.1 | MF831701.1 | JF868996.1 | HM381280.1
T. hinellus 1	CO1	LCO1490/HCO2198	MW532713.1	*Tabanus similis*	*97.54%*	KR439470.1
T. hinellus 2	CO1	LCO1490/HCO2198	MW532711.1	*Tabanus similis*	*97.33%*	KR439470.1
T. hinellus 2b	CO1	LCO1490/HCO2198	MW532714.1	*Tabanus similis*	*97.54%*	KR439470.1

Table Cont'd

ID	Gene	Primer Pair	Accession Number	Closest Match	Percent Identity	Match Accession Number
T. hinellus 3	CO1	LCO1490/HCO2198	MW532715.1	*Tabanus similis*	*97.54%*	KR439470.1
T. hinellus 4	CO1	LCO1490/HCO2198	MW532720.1	*Tabanus similis*	*97.33%*	KR439470.1
T. hinellus 5	CO1	LCO1490/HCO2198	MW532716.1	*Tabanus similis*	*97.54%*	KR439470.1
T. hinellus 6	CO1	LCO1490/HCO2198	MW532712.1	*Tabanus subsimilis*	*97.54%*	HQ944977.1
T. hinellus 8	CO1	LCO1490/HCO2198	MW532717.1	*Tabanus similis*	*97.54%*	KR439470.1
T. hinellus 9	CO1	LCO1490/HCO2198	MW532718.1	*Tabanus similis*	*97.54%*	KR439470.1
T. hinellus 10	CO1	LCO1490/HCO2198	MW532719.1	*Tabanus similis*	*97.54%*	KR439470.1
T. nigrovittatus 1a	CO1	LCO1490/HCO2198	MW532741.1	*Tabanus nigrovittatus*	*99.38%*	KT381971.1
T. nigrovittatus 1b	CO1	LCO1490/HCO2198	MW532737.1	*Tabanus nigrovittatus*	*98.97%*	KT381971.1
T. nigrovittatus 2a	CO1	LCO1490/HCO2198	MW532731.1	Tabanus sp.	*95.89%*	MF837932.1 | MG163497.1 | KP047275.1
T. nigrovittatus 2B	CO1	LCO1490/HCO2198	MW532735.1	*Tabanus nigrovittatus*	*99.38%*	KT381971.1
T. nigrovittatus 3	CO1	LCO1490/HCO2198	MW532732.1	*Tabanus nigrovittatus*	*98.56%*	KT381971.1
T. nigrovittatus 4	CO1	LCO1490/HCO2198	MW532736.1	*Tabanus nigrovittatus*	*98.97%*	KT381971.1
T. nigrovittatus 5	CO1	LCO1490/HCO2198	MW532738.1	*Tabanus nigrovittatus*	*99.59%*	KT381971.1
T. nigrovittatus 6	CO1	LCO1490/HCO2198	MW532734.1	*Tabanus nigrovittatus*	*98.15%*	KT381971.1
T. nigrovittatus 7	CO1	LCO1490/HCO2198	MW532733.1	*Tabanus nigrovittatus*	*98.56%*	KT381971.1
T. nigrovittatus 8	CO1	LCO1490/HCO2198	MW532739.1	*Tabanus nigrovittatus*	*99.59%*	KT381971.1
T. nigrovittatus 9	CO1	LCO1490/HCO2198	MW532740.1	*Tabanus nigrovittatus*	*99.59%*	KT381971.1

The phylogenetic tree generated based on the concatenated sequences of the three loci further displayed the differences between the genera and species within this study. A concatenated tree was first constructed to provide the highest resolution power for specimens within this study. All specimens of the respective species formed their own unique clades along the tree (Figure 2.15). To further investigate the resolution power of each of the three loci, we decided to construct separate phylogenetic trees for each of the three loci to determine if any single locus had sufficient resolution power for species distinction. Using sequences of the 18Sai/18Sbi locus, all specimens within each designated species formed their own clades separate from other species (Figure 2.10). Reference sequences used within the tree were the top matches from NCBI GenBank with the highest percent identity match to the specimens from this study. All of the *C. flavidus* specimens of this study matched on average 99.81% similar (range: 99.4-100%) to one another at the 18Sai/18Sbi locus (Table 2.8). Interspecific percent identities of the *T. acutus* and *T. hinellus* specimens were 100% at the 18Sai/18Sbi locus (Table 2.8). All specimens of *T. acutus* matched 98.75% to the preexisting *T. nigrovittatus* sequence in the GenBank database (Table 2.7).

The subsequent phylogenetic tree was constructed using sequences at the second 18S locus, NF1/18Sr2b. Specimens of *C. flavidus* all matched again to a *C. niger* sequence in GenBank (Figure 2.11). All identified specimens here formed a clade of their own once again at this locus. However, the *T. acutus* specimens matched to a barcoded Tabanus species sequence from GenBank. The *T. nigrovittatus* specimens all matched to a previously barcoded *T. nigrovittatus* sequence in GenBank.

A phylogenetic tree of the third locus, CO1, provided the highest species resolution among the three loci (Figure 2.12). The preexisting *C. flavidus* sequence in the GenBank

database matched to the *C. flavidus* specimens in this study. The *T. acutus* specimens of this study equally matched to three Hybomitra sequences and a general Tabanus species sequence that were all formerly in the database. *Tabanus hinellus* specimens from this study provided a match to *T. similis* and *T. subsimilis* in the database and the *T. nigrovittatus* specimens of this study matched to a previously barcoded *T. nigrovittatus* sequence as well as a general Tabanus species sequence (Figure 2.12).

Table 2.8. Range, means and standard deviation (S.D.) of intra- and interspecific pairwise identities (%) of the four species across the three loci.

		C. flavidus			*T. acutus*			*T. hinellus*			*T. nigrovittatus*		
		Range	*Mean*	*S.D.*	*Range*	*Mean*	*S.D.*	*Range*	*Mean*	*S.D.*	*Range*	*Mean*	*S.D.*
18S ai/bi	*C. flavidus*	99.4-100	99.81	0.04									
	T. acutus	96.79-97.5	95.78	0.09	100	100	0						
	T. hinellus	96.52-97.28	95.7	0.09	98.64	98.64	0	100	100	0			
	T. nigrovittatus	96.06-97.39	97.07	0.19	97.63-98.64	98.53	0.09	98.42-99.32	99.23	0.07	99.21-100	99.84	0.09
18S NF1	*C. flavidus*	99.79-100	99.97	0.01									
	T. acutus	95.65-95.88	95.82	0.01	99.52-100	99.88	0.02						
	T. hinellus	92.81-93.95	93.91	0.03	95.2-96.37	96.47	0.69	98.54-100	99.83	0.14			
	T. nigrovittatus	94.69-94.92	94.88	0.01	97.83-98.31	98.21	0.02	94.5-95.88	95.73	0.11	99.52-100	99.91	0.02
CO1	*C. flavidus*	99.07-100	99.47	0.08									
	T. acutus	90.68-91.38	90.84	0.04	99.77-100	99.89	0.01						
	T. hinellus	88.11-89.28	88.9	0.12	91.61-92.54	91.94	0.05	98.37-100	99.44	0.31			
	T. nigrovittatus	88-89.51	88.6	0.21	91.72-93.47	93.47	0.23	88.69-90.68	89.8	0.36	98.02-100	99.04	0.4

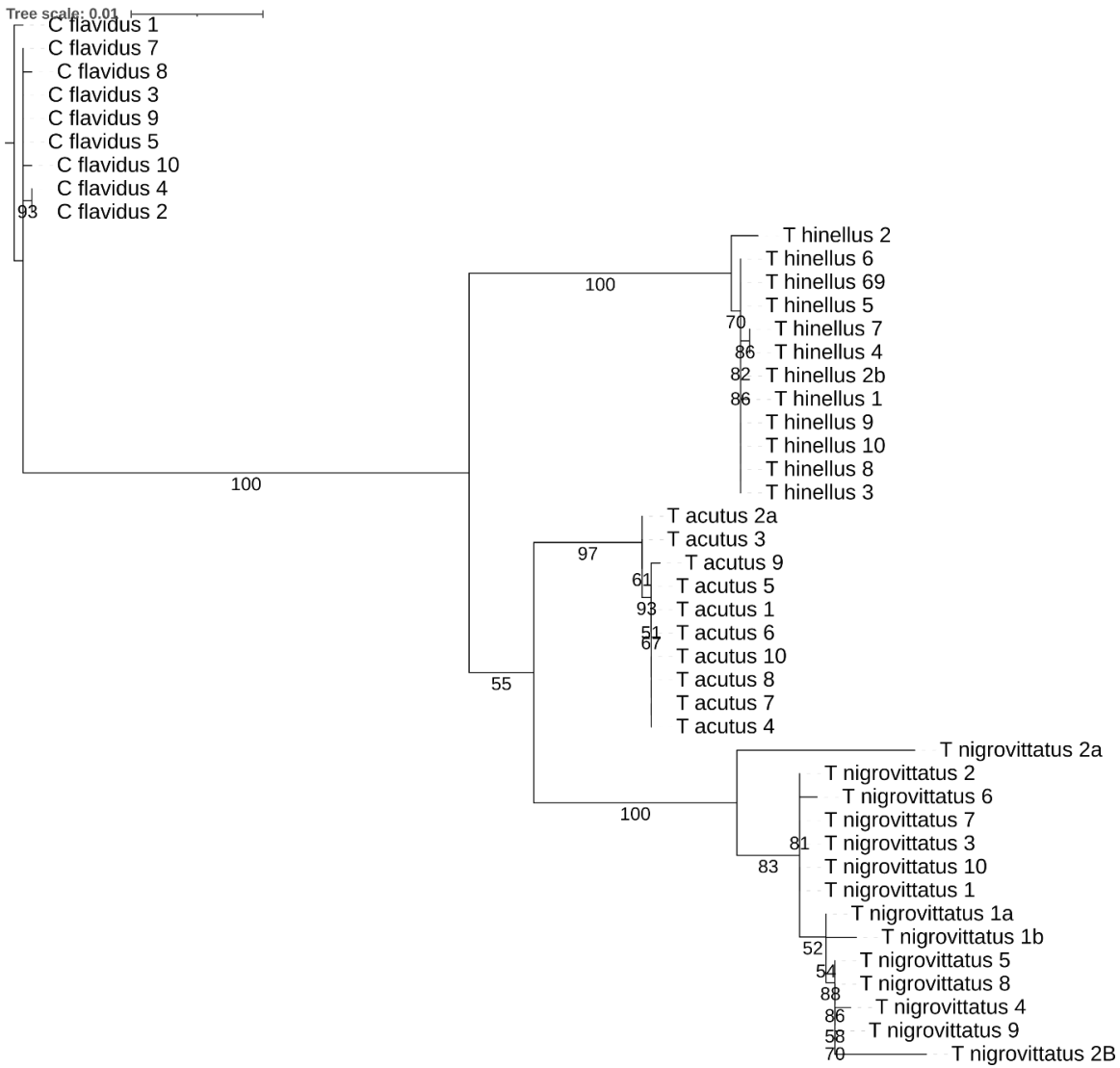

Figure 2.10. Maximum likelihood phylogenetic tree constructed based on concatenated sequences of two regions of the 18S rRNA genes and the CO1 gene, from tabanid species acknowledged to occur along coastal Louisiana. Tree scale is a representative of the average number of nucleotide substitutions per site. Bootstrap values are the confidence values for each site across 1000 replications.

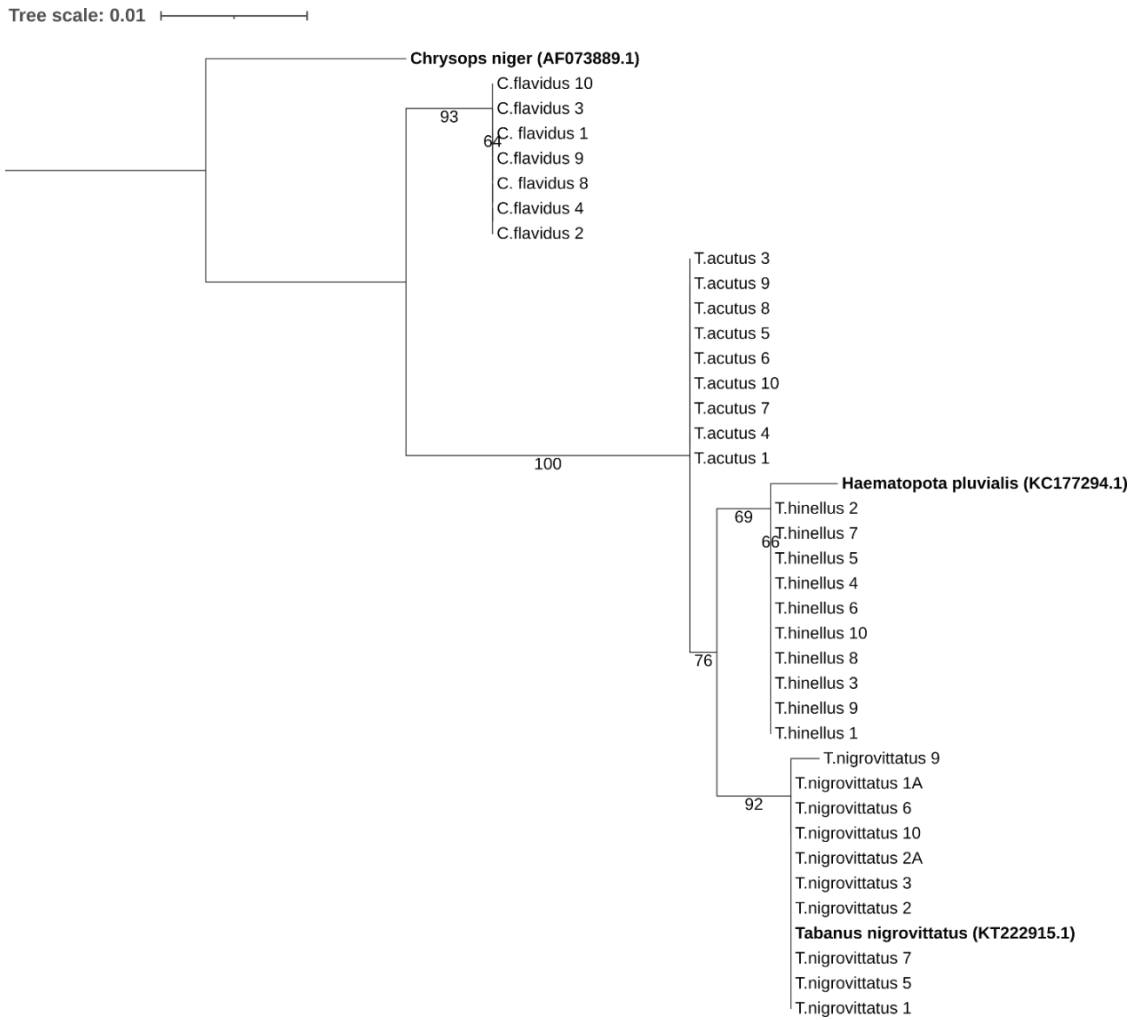

Figure 2.10. Maximum likelihood phylogenetic tree constructed based on genetic sequences generated using the 18Sai/18Sbi locus of females of the known coastal tabanid species that occur along coastal Louisiana. Reference sequences retrieved from NCBI GenBank are embolded. Tree scale is a representative of the average number of nucleotide substitutions per site. Bootstrap values are the confidence values for each site across 1000 replications.

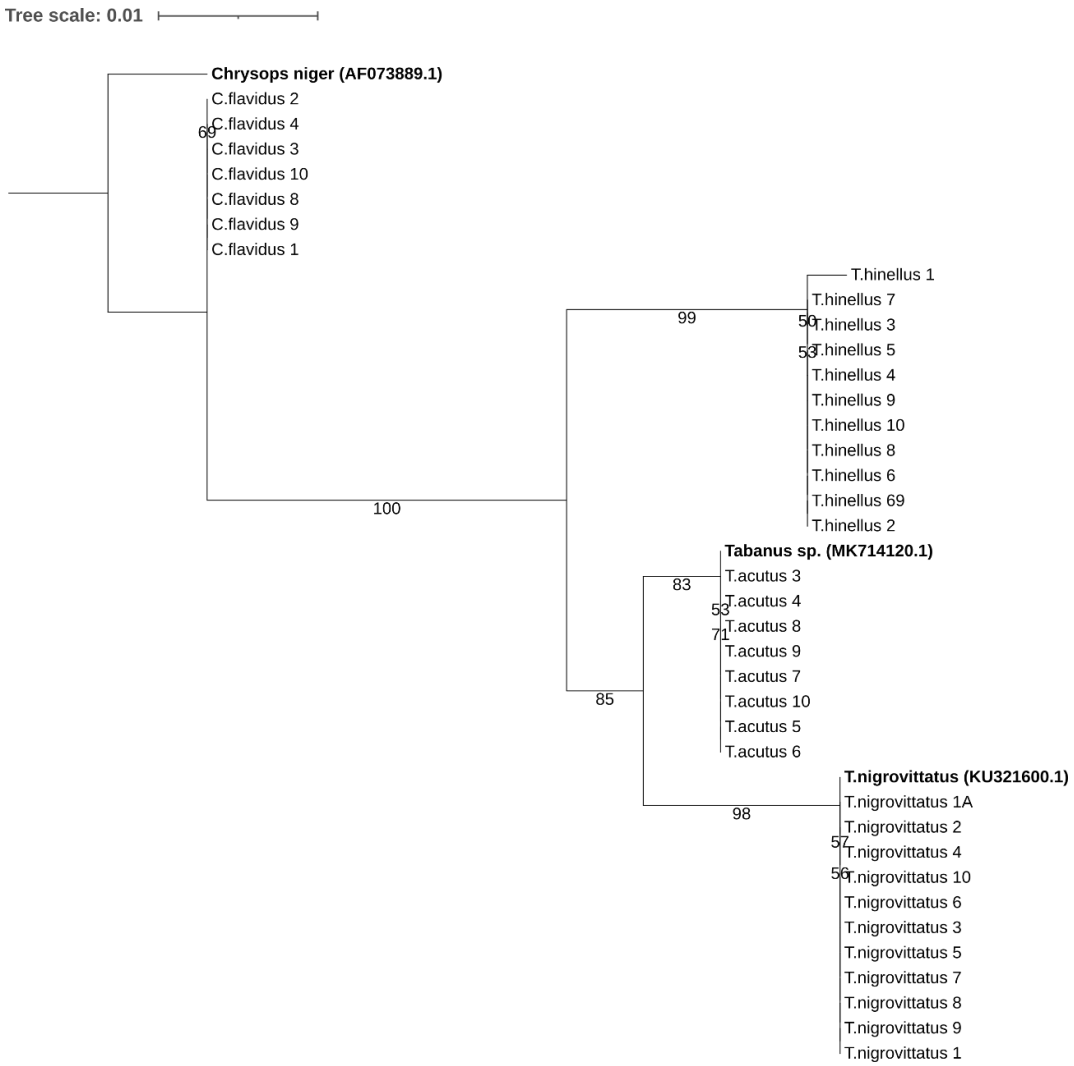

Figure 2.11. Maximum likelihood phylogenetic tree constructed based on genetic sequences generated using the 18S NF1/18Sr2b locus of females of the known coastal tabanid species that occur along coastal Louisiana. Reference sequences retrieved from NCBI GenBank are embolded. Tabanus sp with “sp.” abbreviated for species. Tree scale is a representative of the average number of nucleotide substitutions per site. Bootstrap values are the confidence values for each site across 1000 replications.

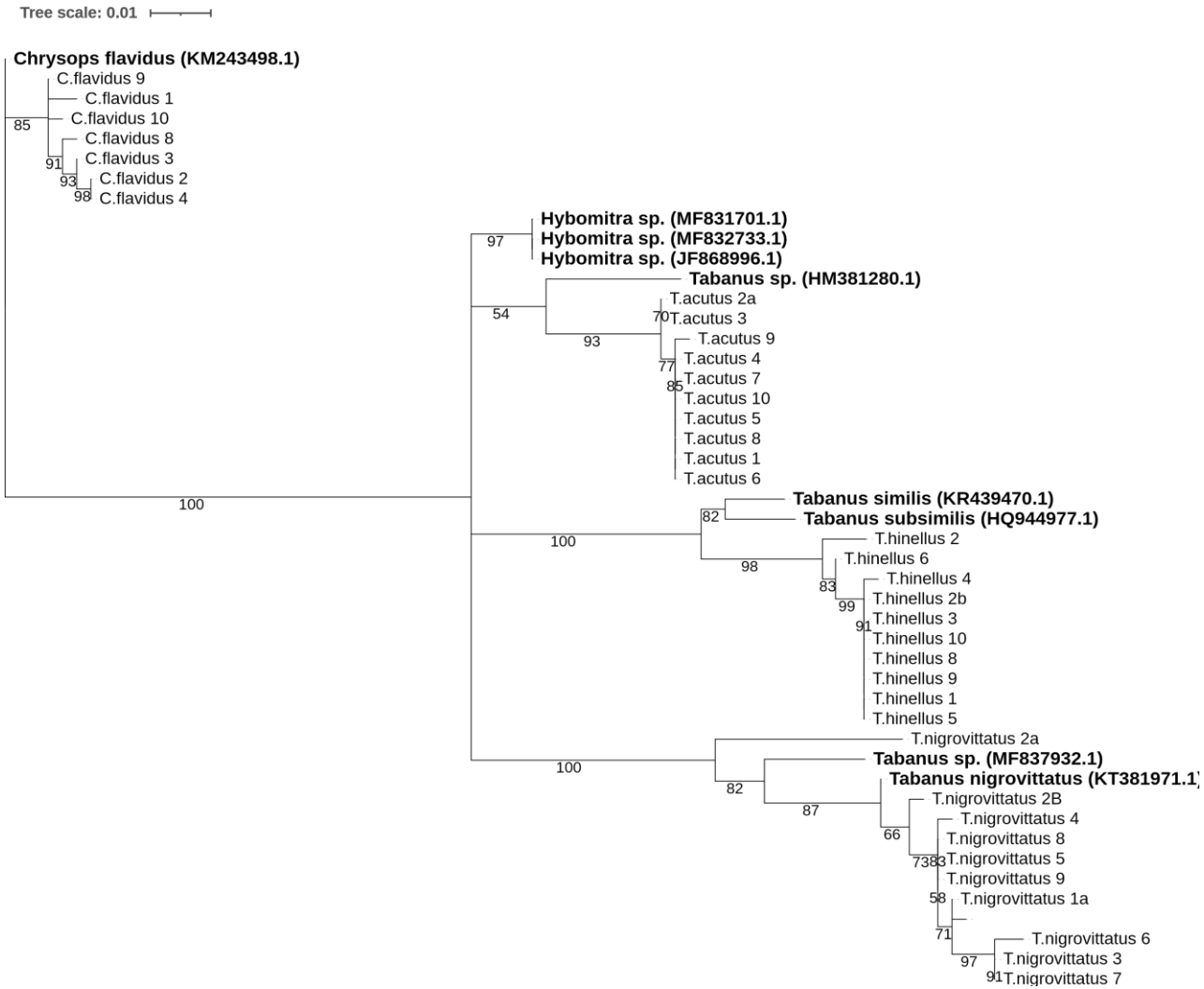

Figure 2.12. Maximum likelihood phylogenetic tree constructed based on genetic sequences generated using the LCO1490/HCO2198 locus of females of the known coastal tabanid species that occur along coastal Louisiana. Reference sequences retrieved from NCBI GenBank are embolded. Tabanus and Hybomitra sp with "sp." abbreviated for species. Tree scale is a representative of the average number of nucleotide substitutions per site. Bootstrap values are the confidence values for each site across 1000 replications.

Consensus sequences generated for all specimens of each of the four species were compared among each of the three loci. Alignments of all 18Sai/18Sbi sequences showed that there were numerous mutations dispersed across a 140 base pair section of the entire 982 base pair sequence between the species. *Chrysops flavidus* had a total of 16 mutations that differentiated this species from the genus Chrysops from the three species of the genus Tabanus. Those mutations are as follows: two insertions, two deletions, five transitions, and seven transversions along the region (Figure 2.13). Among the three Tabanus species, *Tabanus acutus* had 9 deletions and only one transition when compared against both *T. hinellus* and *T. nigrovittatus* individually. Only one transition and two transversions separate *T. nigrovittatus* and *T. hinellus* (Figure 2.13).

Comparisons of the consensus alignments made from the NF1/18Sr2b locus showed that most mutations were dispersed across a 69bp sequence region that genetically distinguished the four species. Unlike the 18Sai/18Sbi locus, NF1/18Sr2b distinguishes the Chrysops species with only 10 mutations from the Tabanus species which were five deletions, three transitions and two transversions. This locus does however provide better species resolution power than 18Sai/18Sbi among the three Tabanus species, *T. acutus, T hinellus* and *T. nigrovittatus*. There were thirteen mutations that distinguished *T. acutus* from *T. hinellus* with three transitions, four transversions and six deletions along the *T. hinellus* sequence region (Figure 2.14). Similarly, the six deletions and four transversions also separated *T. hinellus* from *T. nigrovittatus* as well as four transitions. Six total mutations distinguished *T. acutus* and *T. nigrovittatus* which were two transitions and four transversions (Figure 2.14).

The differences displayed between the inter- and intraspecific ranges among the loci varied but provided enough distinction to prove that the resolution powers among the loci are all

different (Table 2.8). The CO1 locus, LCO1490/HCO2198, provided the highest inter- and intraspecific variations among the three loci. The variances displayed among the sequences were dispersed across the entire sequence with no concentrated region of differences like 18Sai/18Sbi and NF1/18Sr2b (Figure 2.15). The 18Sai/18Sbi locus provided very little distinction between the three Tabanus species in which there were less evident mutations among them although the locus still provided enough resolution power to distinguish between the two genera Chrysops and Tabanus. The NF1/18Sr2b locus provided a pairwise similarity range that was more distant at the species level but the CO1 locus provided the greatest range with much better distinctions both across the genera and among each species (Table 2.7). The CO1 locus provided the best overall resolution based on the large difference between inter- and intraspecific data; therefore, the CO1 locus was used to identify the male tabanids and the larvae.

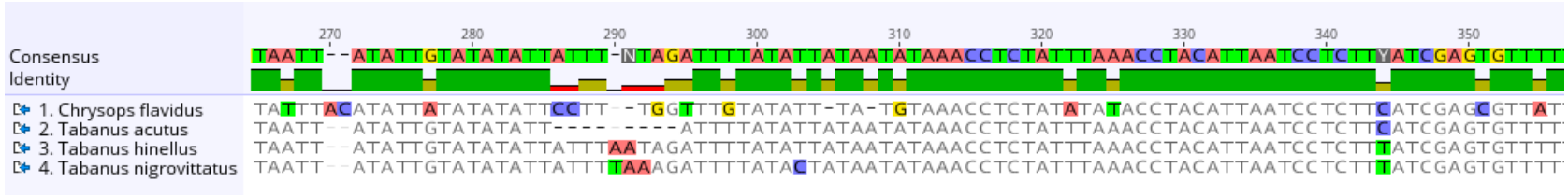

Figure 2.13. Multiple alignment file of the consensus sequences generated from the specimens of each species at the 18S ai bi locus. Colors indicate base differences and "–" indicates insertions/deletions among each of the four coastal tabanid species. Colors coded by base: A=red, C=blue, G=yellow and T=green.

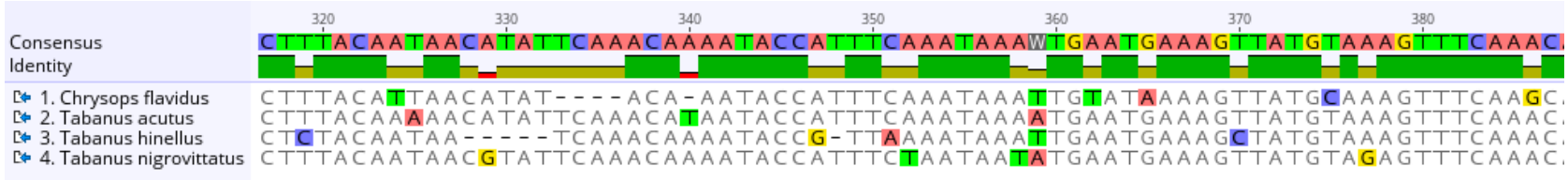

Figure 2.14. Multiple alignment file of the consensus sequences generated from the specimens of each species at the 18S NF1 locus. Colors indicate base differences and "–" indicates insertions/deletions among each of the four coastal tabanid species. Colors coded by base: A=red, C=blue, G=yellow and T=green.

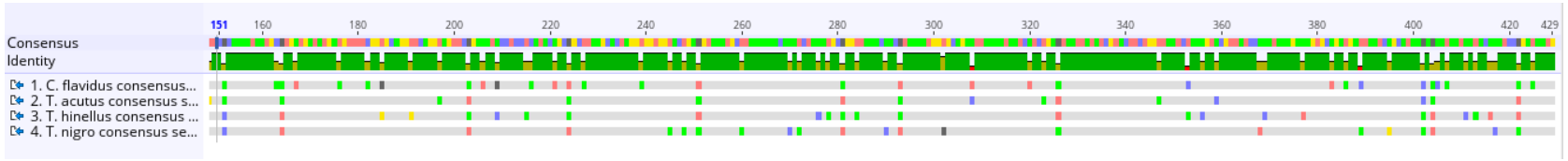

Figure 2.15. Multiple alignment file of the consensus sequences generated from the specimens of each species at the CO1 locus. Colors indicate base differences and "–" indicates insertion/deletions among each of the four coastal tabanid species. Colors coded by base: A=red, C=blue, G=yellow and T=green.

Female specimens of the coastal species were submitted to NCBI GenBank (January 25, 2021) as species references. Following the submission, a phylogenetic tree using the submitted sequences as references was constructed using DNA sequences of males and larvae collected within this study. In addition to those sequences, the top match tabanid sequences found in GenBank prior to submissions were also used. The phylogenetic tree clustered six of the eleven males to the sequences of females morphologically identified as *T. nigrovittatus*. The remaining five males were clustered with the sequences of female *T. hinellus* specimens and were therefore identified as *T. hinellus* (Figure 2.16). Eight of the eleven larvae grouped with the sequences of the morphologically identified *T. nigrovittatus* females. Two larvae grouped with the *T. acutus* and one larva clustered with the *C. flavidus* female specimens that were morphologically identified and barcoded in this study (Fig. 2.19, Table 2.8).

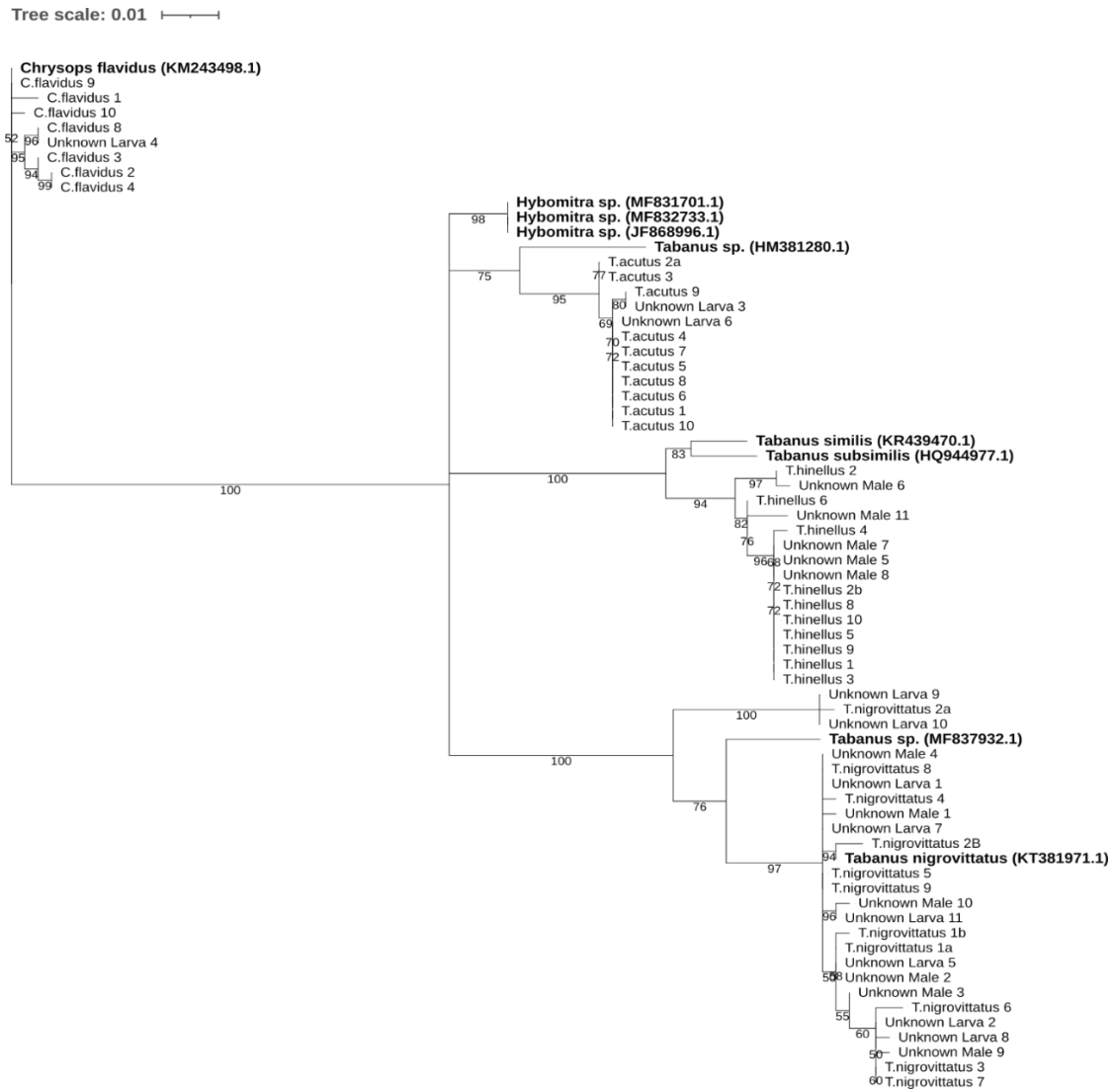

Figure 2.16. Maximum likelihood phylogenetic tree constructed based on genetic sequences generated using the LCO1490/HCO2198 locus of the known coastal tabanid species that occur along coastal Louisiana as well as unidentified tabanid larvae and males collected within the study. Additional sequences retrieved from NCBI GenBank are embolded. Tabanus and Hybomitra sp with "sp." abbreviated for species.

Table 2.9. Top species matches of the collected tabanid males and larvae against CO1 sequences in the GenBank database following sequence submissions of the four coastal tabanid species (1/30/2021).

ID	Gene	Primer Pair	Closest Match	Percent Sequence Identity	Match Accession Number
Unknown Larva 1	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	100	MW532738.1 | MW532739.1 | MW532740.1
Unknown Larva 2	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	100	MW532732.1 | MW532733.1
Unknown Larva 3	CO1	LCO1490/HCO2198	*Tabanus acutus*	100	MW532723.1
Unknown Larva 4	CO1	LCO1490/HCO2198	*Chrysops flavidus*	100	MW532707.1
Unknown Larva 5	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	100	MW532741.1
Unknown Larva 6	CO1	LCO1490/HCO2198	*Tabanus acutus*	100	MW532730.1
Unknown Larva 7	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	100	MW532740.1
Unknown Larva 8	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.76	MW532733.1
Unknown Larva 9	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.76	MW532731.1
Unknown Larva 10	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.76	MW532731.1
Unknown Larva 11	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.76	MW532740.1
Unknown Male 1	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.07	KT381971.1
Unknown Male 2	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	100	MW532741.1
Unknown Male 3	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.76	MW532741.1
Unknown Male 4	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.76	MW532739.1
Unknown Male 5	CO1	LCO1490/HCO2198	*Tabanus hinellus*	100	MW532719.1
Unknown Male 6	CO1	LCO1490/HCO2198	*Tabanus hinellus*	99.76	MW532711.1
Unknown Male 7	CO1	LCO1490/HCO2198	*Tabanus hinellus*	100	MW532718.1
Unknown Male 8	CO1	LCO1490/HCO2198	*Tabanus similis*	97.44	KR439470.1
Unknown Male 9	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.76	MW532733.1
Unknown Male 10	CO1	LCO1490/HCO2198	*Tabanus nigrovittatus*	99.52	MW532738.1
Unknown Male 11	CO1	LCO1490/HCO2198	*Tabanus subsimilis*	97.19	GU803795.1

2.4 Discussion

Inventory of Coastal Species (2018 and 2019)

In this study, specimens of twelve species of Tabanidae representing four genera including Tabanus, Chrysops, Chlorotabanus and Leucotabanus were captured within c coastal marshes and longitudinally along Louisiana (Table 2.3). Hine (1907) and Tidwell (1973) both provided an overview of tabanids within the state of Louisiana while Jones and Bradley (1923) and Leprince et al. (1991) provided inventories of species present in the selected regions of Louisiana. Jones and Bradley (1923) sampled areas around Baton Rouge and found three of the same species as discovered in this study, *Chrysops flavidus*, *Tabanus atratus* and *T. lineola*. Leprince et al. (1991) also provided temporal distributions of seventeen species of six genera in certain areas in inland Louisiana. Seven of those species (*Chlorotabanus crepuscularis, Leucotabanus annulatus, T. americanus, T. atratus, T. lineola, T. nigripes* and *T. stygius*) were also collected within this study. However, the current study is the first to provide an inventory of species present along the coastline and within coastal marshes of Louisiana.

Six of the twelve species collected in this study were collected within the estuaries: *C. flavidus, T. acutus, T. atratus, T. americanus, T. hinellus* and *T. nigrovittatus.* While *T. hinellus* and *T. nigrovittatus* were captured both within the estuaries and the terrestrial sites, *T. acutus* was only captured within the estuaries. The presence of these three species within the estuaries is consistent with previous species descriptions (Hine 1907, Tidwell 1973, Goodwin and Drees 1996). *Chrysops flavidus* was the only other species collected strictly at estuarine sites. The remaining two species, *T. americanus* and *T. atratus*, can be found within the estuaries, but as strong fliers these large flies are common at many inland locations in Louisiana; thus the capture of the adult flies does not mean they are native to the estuaries (Table 2.3).

Members of the species *Tabanus lineola* were the most commonly collected flies at all inland sites. Tidwell (1970) suggested that *T. lineola* belongs to a complex of species including its sister species *T. hinellus* and other species including *T. similis* and *T. subsimilis*. Tidwell (1970) also reported collecting *T. lineola* within all of the major ecological regions but most often within the bottomland hardwood forests of the inland portions of Louisiana and that it is replaced by *T. hinellus* in coastal marshes. In a more recent study, Davis (2019) used environmental niche models and showed that *T. lineola* and *T. subsimilis* were the two most widely distributed species across the eastern US with predicted presence ranging from northern Louisiana and Arkansas to the northeast into Massachusetts. Data presented within this study supports the observations of Tidwell (1970) and Davis (2019) since *T. lineola* was not found at any of the estuarine sites but the counterpart, *T. hinellus,* was collected within the bay systems. Tidwell (1970) also found *T. hinellus* almost exclusively in coastal marsh regions.

Water Salinity

In the current study, there were four locations of the traps located longitudinally along the coastline where both coastal species, *T. nigrovittatus* and *T. hinellus,* were collected alongside specimens of the inland species *T. lineola*. Cypremort Point State Park (Site 3 in St. Mary Parish), Delacroix (Site 9 in St. Bernard Parish), Pelican Point Marina (Site 11 in Orleans Parish) and Fontainebleau State Park (Site 12 in St. Tammany Parish), were the four terrestrial locations of mixed coastal and inland species collections. In those collections, specimens of *T. nigrovittatus* were the most abundant. The presence of the three species or at least *T. lineola* and one of the coastal species in certain areas may be a result of differences in water salinities or larval habitats in proximity to the trapping sites. Tidwell (1970) reported that although *T. lineola* is collected inland, there are morphological intermediates between it and *T. hinellus* that are

sometimes collected inland from coastal areas. *Tabanus lineola* larvae have been collected in bottomland hardwood forests of St. Landry and Tangipahoa Parishes, much like the habitat where adults were collected within this study (Tidwell 1973). Since the overlapping flight range of the two species can cover different larval habitats, it is not uncommon to collect both species in the same trap during a trapping event. This is the case in areas where water salinities can change due to many environmental factors including tide levels and currents, such as at trap sites 10 and 12 at Hopedale and Fontainebleau State Park, respectively (Figures 2.1 and 2.6).

Although *T. hinellus* and *T. nigrovittatus* are both considered to be coastal species, their larval habitats are not necessarily the same. For example, *T. nigrovittatus* was not collected at Site 4 (Burns Point Park) while *T. hinellus* was present (Figure 2.1). Burns Point Park is located within the closest proximity of the Atchafalaya River which is a freshwater tributary that extends into the Gulf of Mexico with potentially varied low salinity habitats not compatible with *T. nigrovittatus* larval requirements, but could favor those of *T. hinellus*. Direct salinity ranges have not been previously studied for these species, but *T. nigrovittatus* is known to be collected around *Spartina alterniflora* marshes. Spartina plant communities dominate Louisiana's marsh systems and have been found to make up more ground cover as salinity increases (Aker 2020). For example, Aker (2020) found *Spartina alterniflora* as the dominant species at the High salinity zones comprising up 93.2% ground cover in those low-marsh areas.

Tabanid Larval Collections (2016 and 2017)

Tabanus nigrovittatus larvae are top level invertebrate predators within the food web of marsh ecosystems. As such, the presence of larvae can be indicative of past and current marsh health. In the current study, *T. nigrovittaus* larvae were found in different localities from Cameron to St. Mary and Plaquemines Parish and also found during multiple consecutive months

from April - July as well as in September (Table 2.6). Mullens (2019) stated that tabanid maturity from egg to adult is dependent on species and can be accomplished in as little as 6 weeks. Mullens (2019) also stated that pupation periods vary by species but is believed to last between 6-21 days. More specifically, Thompson et al. (1979) reared *T. nigrovittatus* larvae under laboratory conditions and was able to get larvae to maturity in 6-8 weeks. Although egg to pupa development was relatively short, the pupae within the study did not emerge until the following year as adults. Since it is known that tabanid larvae can take from 3 months up to two years to emerge (Axtell 1976, Thompson et al. 1979, Mullens 2019), the collected larvae from both years, 2016 and 2017, show that the marsh environments along the coast were healthy and suitable for the apex predator within the environment for extended periods.

Tabanus nigrovittatus Temporal Distribution (2010-11, 2019)

Following the Deepwater Horizon Oil Spill, Husseneder et al. (2016) observed similar patterns of seasonal abundance in *T. nigrovittatus* populations within unaffected collection locations in western Louisiana that were found during 2010 and 2011 in St. Bernard and Plaquemines Parish (Figures 2.4 & 2.5). During the tabanid collections in 2019, the same patterns of abundance were observed across the salinity zones in Barataria and Caillou Bay (Figures 2.10 and 2.11). There are two notable periods of abundance during the season where flight activity of females increases. The first flies appear early within the season during mid-May to mid-June with small, continued emergence throughout the season. A second "generation" appears during the latter portion of the season from early-August into late-September but prior to the fall/winter period in the absence of adult populations, when no flight activity occurs

Schulze et al. (1975) examined *T. nigrovittatus* populations in coastal New Jersey to understand the species' seasonal flight activity from environmental factors. The study began

during the 1973 season and was repeated in 1974. Both season collections did not begin until early July and ended mid-August. Similarly, Sofield et al. (1985) conducted a population abundance study on *T. nigrovittatus* in New Jersey that began in Mid-June and ended in late-August. They stated that the distribution of the *T. nigrovittatus* complex is from mid-June to mid-September with the most productive month of July in New Jersey (Sofield et al. 1985). It is apparent that the season at which flies occur in the upper Atlantic marshes is far shorter than the flight season along the Gulf Coast, from mid-April through early-October. However, the short flight season that occurs in the northeast is typically the result of one cohort as there is only one general peak of abundance later in the season, while on the Gulf Coast the season is long enough to support two peaks of abundance. Furthermore, there still remain unresolved questions of whether the second peak that occurs in the South is associated with development of larvae produced from eggs of the spring peak females or of another cohort of flies produced from the previous year. Most tabanids overwinter as larvae and tabanid abundances across the years may be a result of environmental conditions that affect adult emergence (Mullens 2019). These reasons could reflect the continued occurrence of adults throughout the season as larval development occurs throughout one year into the next year which coincides with the thought of two subpopulations present during a season. The first subpopulation comes as overwintering larva that emerge during the spring. The second comes as oviposition from adults during the spring with emergence during the late summer which results in the two “generations”. This pattern, during favorable environmental conditions, can continue perpetually which would make *T. nigrovittatus* a bivoltine species.

Light Traps (Males)

Canopy traps baited with dry ice (CO_2) and a black ball normally do not capture male tabanids. In the current study, we found that light traps were more effective in capturing males than canopy traps. The two trap types that caught the most males were incandescent traps with and without dry ice. Since UV traps were not as effective, future efforts to collect males should use incandescent apparently without the need of dry ice. Since males are not blood feeders, the lack of attraction provided by dry ice is expected; presence or absence of dry ice made no difference.

Of all captured males, 88% were collected in May and another 10% were collected in October. The timeframes at which the males were collected fall within the two temporal abundance peaks of the captured females. An emergence study conducted by Rockel and Hansens (1970) indicated that the number of *T. nigrovittatus* males was nearly equal to the females during the peak of the season in July. Furthermore, fewer males were collected in the days following peak emergence in July although the females were consistently collected through September. Rockel and Hansens (1970) also stated that males will typically live for a few days, perhaps up to over a week while females can live up to two weeks. Rockel and Hansens (1970) suggested that males emerge slightly prior to females' emergence and this is evident within the observed distribution in this study. We report for the first time that mating swarms potentially occur around the two female abundance peaks which has not previously been described.

Estuary Collections-Diurnal/Overnight (2019)

Diurnal and overnight collections also were conducted as part of the temporal distribution surveys across the salinity zones. Abundance of *T. nigrovittatus* populations was the highest among all species across all salinity zones (Fig 2.10), while specimens of *T. hinellus* were

collected more at lower saline sites. During the diurnal collections, both *T. hinellus* and *T. nigrovittatus* were captured while *T. acutus* was not. Tidwell (1973) reported that *T. acutus* is active during daylight and also after dark. In this study, overnight collections established that *T. acutus* is primarily active during crepuscular-nocturnal periods in all salinity zones (Figure 2.8). Therefore, we concluded that *T. acutus* is a crepuscular/nocturnal species.

Among the four species found within the estuaries of this study, *T. nigrovittatus*, was the most abundant. As *T. nigrovittatus* has been previously used as a model species to show the impacts and recovery of marsh systems from oiling by Husseneder et al. (2016 and 2018), the current study aids to expand on those findings in establishing baseline data of the species within different salinity zones which has not been previously studied. Although the model species *T. nigrovittatus* was a valuable tool as a bioindicator, the use of the other two species, *T. hinellus* and *T. acutus* as co-bioindicators would be of little value to use independent of *T. nigrovittatus* due the differing but overlapping occurrences. In addition, larval characteristics and habitats for these two species have not been studied as well as those of *T. nigrovittatus*. Additional insight to larval habitats and diet would also have to be taken into account if using *T. hinellus* and *T. acutus* as indicators of marsh health.

DNA Barcoding

Prior to this study, the only DNA sequence records available within NCBI GenBank for determining or confirming tabanid species that occur within the estuaries were those of *T. nigrovittatus* (Bhalerao 2018). Since there were very few representative sequences for *T. nigrovittatus* and *C. flavidus* and none for *T. hinellus* and *T. acutus*, reliable, morphologically identified individuals were used to establish genetic identities with intra- and interspecific

variances. In addition, those sequences were used to confirm the male and larval identities and to understand the phylogenetic relationship among the species.

Until recently, due to the diversity of the genus Tabanus and the many morphological characters of the species that make up the group, identification of specimens can be difficult depending upon resources. Recently, accurate and dependable use of molecular tools such as DNA barcoding has proven successful in identifying and grouping Tabanus species through phylogenetics by Davis (2019). Davis (2019) used the CO1 subunit to identify 40 Tabanus species from specimens collected from six states in the Southeastern United States. Nine major clades were identified from the 40 horse fly species; the authors also established "complexes" of those species. One of those complexes involved greenheads, but only one *T. nigrovittatus* was included in the phylogenetic tree. Davis (2019) provided the baseline for using DNA barcoding and molecular identifications to expand the library of the species within the group. This current study adds to the database by providing additional species that were not identified in the former study. Ultimately, comparisons of described species in different areas may lead to expanding knowledge of those species' distributional ranges and possible detections and descriptions of new species.

Species identifications of male and immature stages of tabanid species can be more difficult than the identification of adult females. Davis (2019) suggested that the use of DNA barcoding could improve the ability to identify male and larval tabanids. The current study demonstrates that both males and larvae of multiple species can be successfully identified through DNA barcoding and phylogenetic analyses with the use of reference sequences that are now in GenBank. Through this molecular gateway of identifying species by well-established DNA barcodes, detailed temporal and spatial surveillance for tabanids at all life stages is

achievable. In particular, additional support for identification of the life stages of *T. nigrovittatus* aids in understanding the particular marsh types that the species inhabit.

The DNA sequencing conducted on the larvae collected within this study showed that eight of the eleven larvae matched to the barcoded bioindicator species of marsh health, *T. nigrovittatus* (Table 2.8). Those larvae were collected throughout the year supporting evidence that *T. nigrovittatus* larvae develop within and upon the soil substrates and emerge when reaching maturity (Thompson et al. 1979, Mullens 2019). The remaining three larvae were identified as two *T. acutus* larvae and one *C. flavidus* larva. Nucleotide BLAST sequence results and clustering of the males collected with the light traps in this study along the phylogenetic trees, detected two reported species (Table 2.9). The two species, *T. nigrovittatus* and *T. hinellus*, were expected to be collected during the trapping attempts as they have previously been collected along coastal Louisiana.

Sequences obtained using both 18S loci (18Sai-18sbi and NF1-18Sr2b) helped identify the intraspecific relationship between the Chrysops and Tabanus species (Table 2.7). Consensus sequence alignment comparisons also show the numerous differences between the two genera as there are more transversions, insertions and deletions between the Chrysops and Tabanus using 18Sai-18Sbi over NF1-18Sr2b (Figures 2.13 and 2.14). However, this was no surprise as many studies have shown the 18S loci to be very effective in sorting out genetic sequences of different genera. In addition to the genera-level differences made at the CO1 locus using the LCO1490-HCO2198 primers, species-level genetic differences also can be made more clearly. Pairwise identities at this locus also provided a much broader range of interspecific identities that were still greater than the intraspecific ranges, which when compared to the other loci, is more

effective in the analysis of relationships between the three Tabanus species and the coastal Chrysops species.

The relationship between the species using the concatenate of the three loci point to the idea that *T. acutus* and *T. nigrovittatus* are more closely related to one another than to *T. hinellus*. However, using the loci with the highest resolution power within this study, CO1, the phylogenetic tree shows that the three species all evolved from the same common ancestor. There remains more to be analyzed in determining the actual phylogeny of these species but the phylogenetic results of this study are unique and the first of their kind to analyze the relationship between the four coastal tabanid species, *Chrysops flavidus, T. acutus, T. hinellus* and *T. nigrovittatus* across multiple loci.

The use of DNA barcoding has proven to be a very effective tool in making species designations of Tabanus species (Davis 2019). Data produced within this study aids in the identification of the four coastal tabanid species on a large scale through the identities of multiple specimens of the same species as well as targeting multiple gene regions. Molecular identifications such as those reported in this study show that these tools can aid and supplement traditional morphological identification methods particularly in separating similar or more closely related species within species complexes. The eye bands of pinned tabanids rapidly disappear and the eye bands are critical for identification of many species and in the case of identifying museum specimens. DNA barcoding allows for more certain species confirmations in collected specimens over the typical morphometric methods since body parts and other characters can be damaged or missing in historical specimens. This form of direct identification using barcoding also aids in understanding species complexes that are known to exist such as those of *T. hinellus* and *T. nigrovittatus*. There is difficulty in properly identifying species within

complexes where specimens of different species strongly resemble one another. Species specific sequences generated here are the foundation to understanding the species themselves as far as inhabitance and ecology but they also provide the baseline data in understanding possible species complexes such as the *T. nigrovittatus* complex along coastal areas.

CHAPTER 3. GENETIC DFFERENCES WITHIN THE TABANUS NIGROVITTATUS SPECIES COMPLEX OF COASTAL LOUISIANA

3.1 Introduction

The saltmarsh greenhead horse fly, *Tabanus nigrovittatus*, is responsible for approximately 95% of the tabanid attacks of man along coastal areas on the Atlantic seaboard ranging from Nova Scotia to Florida and along the Gulf Coast to Texas (Hansens 1979). Females of *T. nigrovittatus* are autogenous which means that they are capable of producing their first egg mass, ranging on average from 150-170 eggs per mass, without a bloodmeal (Bolser and Hansens 1974; Graham and Stoffolano 1983). Egg masses oviposited by *T. nigrovittatus* are generally two-tiered masses composed of gray eggs (Graham and Stoffalano 1983). The larvae are apex predators that develop on or within sediments in Spartina marshes consuming a wide array of food sources ranging from fungi to larger invertebrates including tabanid larvae (Axtell 1976, Stoffolano 1979, Bhalerao 2018). The high reproductive rate, autogeny and efficient larval development combine to contribute to large sustainable populations in certain locations.

Previous studies in New Jersey have suggested that *T. nigrovittatus* populations may be bivoltine (Sofield 1985). Data generated from Chapter 2 within this current study supports this idea since there were two general abundance peaks across multiple seasons (Fig. 2.4 and 2.5). However, there are no studies to show whether the existence of two separate populations of *g*reenhead horse flies represents two generations of single species or genetically separated clades. There have been multiple studies that suggested that *T. nigrovittatus* is part of a complex of species in certain locations. Philip (1962) placed *T. eadsi* within the *T. nigrovittatus-quinquevittatus* complex along with *T. texanus*, stating that *T. eadsi* and *T. texanus* are geographical segregated, but Thompson and Pechuman (1970) found that their ranges were identical. Burger et al. (1985) suggested that *T. conterminus* occurs within the same

distributional range of *T. nigrovittatus*, from Nova Scotia to Florida and possibly along the Gulf Coast. Goodwin (1994) suggested a group of at least six species, *Tabanus conterminus, T. fulvilineis, T. fuscicostatus, T. mularis, T. nigrovittatus,* and *T. quinquevittatus,* comprised the *T. nigrovittatus* complex. Adults in the *T. nigrovittatus* complex can be separated through body lengths and various dorsal patterns including abdominal markings (Table 2.1), but the body length ranges described for those species overlap. Of the species suggested to be in the complex, only four are coastal species in the United States: *T. nigrovittatus*, *T. texanus*, *T. eadsi* and *T. conterminus*. Although the possibility exists that these species are sympatric along the Gulf Coast, the possible existence of cryptic species within or contributing to the seasonal peaks of *T. nigrovittatus* abundance has not been studied.

Hine (1906) observed two species (*T. nigrovittatus* and *T. conterminus*) along the Atlantic coast that closely resembled one another with only the total body length and thoracic coloration distinguishing the two. Sofield et al. (1984) used species-diagnostic isozymes to differentiate between specimens of *T. conterminus* and *T. nigrovittatus* collected in coastal New Jersey. Sofield et al. (1984) then followed the isozyme analyses with morphological measurements and designated four physical characters: dorsal width of frons, width of the scape, head width and total body length, to speciate specimens of both species in the field. Sakolsky et al. (1999) used cuticular hydrocarbon analyses (CHC) on a series of *T. nigrovittatus* complex specimens with individuals in a range of sizes and combined that with morphometric comparisons and ultimately challenged the concept of the validity of the species *T. conterminus*. However, Sakolsky et al (1999) did not use any genetic comparisons in their attempts to identify *T. conterminus* out of *T. nigrovittatus* specimens. There have been no recent studies that confirm the presence of *T. conterminus* along the Gulf Coast.

Stone (1938) recognized *T. nigrovittatus*, *T. simulans* and *T. conterminus* within the same complex using specimens collected from Florida. Sutton and Carlson (1997) used cuticular hydrocarbon (CHC) analyses to show the existence of three chromatotypes within a series of flies of the *T. nigrovittatus* complex collected in marshes along the Atlantic Coast including Nova Scotia, Massachusetts, Delaware, Virginia, South Carolina and Florida. Two of the chromatotypes, Type II and Type III, were the most abundant within the study while only 11% (or 16 of 152 individuals) of Type I flies were present. Type I flies all had body lengths within the range of 10.2mm-15.6mm with the majority of body length of 14mm or more. Type I flies were all assumed to be *T. conterminus*. The body length ranges of Type II and Type III flies overlapped considerably (~8mm-14mm+), but the mean body length of Type III flies was smaller than Type II flies. Type II flies were designated as *T. nigrovittatus* based on morphological characters that also were consistent with descriptions from Hine (1906). No species name was associated with Type III flies (Sutton & Carlson 1997).

Previous studies such as those performed by Sofield et al. (1984), Sutton and Carlson (1997) and Sakolsky et al. (1999), have indicated possible existence of cryptic and/or unidentified species that are placed under *T. nigrovittatus* along the Atlantic Coast, but none have been based upon modern genetic comparisons. Since the distributional range of *T. nigrovittatus* extends from Nova Scotia to Florida and along the Gulf Coast, focus on the variability of this species complex found along the central Gulf Coast is warranted. Furthermore, there has been no previous study examining the diversity of the *T. nigrovittatus* complex within the different salinity zones of the tidal estuaries of the Gulf of Mexico. Therefore, the first objective of this study was to conduct longitudinal surveys of the *T. nigrovittatus* complex in different bays and different salinity zones in Louisiana's estuaries and measure the body length

of the captured specimens. A second objective was to measure the genetic diversity within the *T. nigrovittatus* complex by using morphometrics and phylogenetic analysis of DNA barcode sequences of specimens collected from different estuaries and salinity zones. Given the possibility of finding more than one species in the *T. nigrovittatus* complex within this study, specimens identified as *Tabanus nigrovittatus* relative to eye color pattern and color of thorax will be referred to as either *T. nigrovittatus* complex or greenheads prior the conclusion section.

3.2 Materials and Methods

Tabanid Collection

In 2018 and 2019, tabanid collections described in Chapter 2 were made in two estuaries of coastal Louisiana, Barataria Bay in Plaquemines Parish and Caillou Bay in Terrebonne Parish using canopy traps (Hribar et al. 1991). Adult horse flies from three salinity zones: Low , Mid and High were collected. All individuals collected in the 2018 and 2019 estuary surveys were pinned on Styrofoam cutouts and stored in 9 X 7 ¾ X 2 1/8 MailMaster Mailing Kraft -Boxes (Cat. No. PK98, Mason Box, Pawtucket, RI) based on collection day for each trip. The boxes were stored at -20^{o}C to keep safe for downstream applications. Individuals were sorted on cold plates and identified to species using morphological characters defined by Tidwell (1973) and Goodwin and Drees (1996).

In 2019 and 2020, greenheads were collected from Orleans, Massachusetts by the Cape Cod Mosquito Control Greenhead Fly Control District and shipped overnight to the campus of Louisiana State University. Specimens were stored at -20C and were later identified to species on cold plates. All flies within those collections were adult females. A total of twenty-five flies from those collections were selected for analysis within this study.

Morphometrics

Following identification, the total body lengths of the flies in the *T. nigrovittatus* complex were made using two methods: digital calipers and a digital imaging system. Body length of each fly was recorded into a spreadsheet with cells respective to the location of the individual pinned flies within the boxes. This was created for ease of access to specific individuals for future applications. The three categories of length were used to categorize the flies; flies less than 10.7mm, flies within the range of 10.7-13.8mm and flies that were larger than 13.8mm (Table 3.1). The categories were defined from previously published morphometric analysis by Burger (1985) that define *T. nigrovittatus* body length between 10.7 and 13.8mm. Although this body length range was established for *T. nigrovittatus*, it is not exclusive of other species. The flies were designated based on collection location and salinity zones and were further grouped into "Early" and "Late" season flies. Flies designated as Early season were collected from April through June. Flies classified as Late season specimens were collected from August through October. Flies collected in the month of July were omitted from the time periods to minimize overlap of possible early and late cohorts if they exist. Then, three individuals were selected from each category using a random number generator totaling a target group of 108 individuals. However, there were some categories, such as those with flies larger than 13.8mm, that contained less than three flies and those categories were not included. Therefore, there were only 72 individuals available for analyses (Table 3.1).

Flies obtained from Massachussetts were measured and a group of 25 individuals designated within the *T. nigrovittatus* complex with body lengths ranging from 7.9mm to 12.6mm were selected. There were no flies from Massachusetts that were larger than 13.8mm.

A labeling system was created for the categories of flies. The first letter within each identifier represented the bay of collection, Barataria (B) or Caillou (C). The second letter represented the salinity zone of collection: Low (L), Mid (M), or High (H). The third identifier was the timeframe or "season" at which the individual was collected: Early (E) or Late (L). The last numerical value was given as the measurement of the total body length for that individual.

During the process of identification and measurements, there were a series of ten flies collected within the Mid salinity zones of Caillou Bay in 2019 that did not fit the description of *T. nigrovittatus* because they were over 10.7-13.8mm and had a grayish thorax. These flies were also selected for further analysis but were not included as part of the measurement procedure. These flies were labeled as TCLA, for Terrebonne (T), Caillou (C), and Louisiana (LA) followed by the measurement of the total body length of that fly.

Total Body Length Measuring Methods

A 6-inch digital display caliper (BioQuip Products, Rancho Dominguez, CA) was used to measure pinned specimens by hand. Measurements made with the digital calipers were recorded to the nearest tenth of a millimeter. Each specimen was removed from the boxes, carefully measured from head to abdomen with the digital calipers, and then returned to the original place in the series of flies. To counteract the possibility of user biases such as sight, dexterity (of the calipers) and accuracy of the measurements, multiple persons measured the same flies using this method and the duplicate measurements were compared.

Prior to the utilization of the imaging system, a prepped Microsoft Excel spreadsheet was created to process fly measurements once all fly measurements were recorded. Pinned flies were adjusted so that the flies were on the same vertical plane within each box. This process was to ensure that all fly measurements would be reflected accurately and prevent skewing during

measuring. Digital imaging of all specimens was performed using the Dun, Inc. BK PLUS Imaging System that was custom designed and developed for Dr. Nathan Lord, Department of Entomology, Louisiana State University. Images of each whole box of pinned flies were taken using CaptureOnePro software version 11.3.1. Characteristics of each image was recorded within the file name such as zoom/magnification, lens size, and box name for reference in post-processing. In some instances, multiple photos were taken of the same camera frame and the images were stacked into Zerene Stacker version 1.04 software to render one functional image. Post-processing defined within this investigation was the process of making initial measurements then transferring those measurements into a finalized and functional Microsoft Excel spreadsheet to use the data for future applications.

All fly measurements using finalized images were performed using Adobe Photoshop CS6 Extended version 13.0.1 with the ruler tool. Measurements began by zooming onto a series of flies, selecting the clearest anterior point of the head of each fly and dragging the measuring tool to the very posterior of the abdomen and allowing the software to record the measurements. Using the zoom tool did not alter results as the program auto-corrected for zoom during tabanid measuring. Measurements were recorded in Adobe Photoshop to five decimal places as text (.txt) files. When all measurements were complete, the recorded measurements were transposed into Microsoft Excel so that numbers in each cell were representative of the fly locations within each box. All measurements were rounded to two decimal places in post processing. Manual input of the metadata for each of the individual collections were input into the spreadsheet once all measurements were imported and finalized.

Comparison of the Measuring Methods

A paired t-test was conducted on fly measurements made by both measuring methods to compare the accuracy between digital caliper measurements and measurements recorded using the digital imaging system. Since multiple individuals measured the same flies using the digital caliper, by hand, an average of the timeframes taken to measure those flies were used in the comparison.

Population Differences

Salinity data collected from Coastal Reference Monitoring Stations from 2014 to the end of 2017 for the six zones combined for the two bays were compared using a Welch Test. Results of the Welch test, ($F(5, 126.26) = 61.99$, $p < 2.2e\text{-}16$), indicated that there were differences among the salinity zones but not between zones of Barataria and Caillou Bay. Therefore, the bay systems were combined for analysis during the population abundance comparisons within this study.

Two Factor ANOVAs followed by Tukey HSD post hoc tests were performed to test the effects of salinity zone and seasonality (Early/Late) on tabanid length for groups of flies measuring less than 10.7 mm and flies with measurements in the range of 10.7mm to 13.8mm.

DNA Barcoding

Flies to be selected for DNA sequencing were grouped using designations such as the total body length of each individual (flies less than 10.7mm; 10.7-13.8mm; flies larger than 13.8mm), estuary in which the individuals were collected (Barataria or Caillou), the salinity zone of collection (Low, Mid or High) as well the time of year of collection (Early or Late). Thus, there were thirty-six categories of flies (Table 3.1); three individuals from each category with at

least three flies were selected for use in DNA sequencing. Additionally, the series of the ten larger flies from Caillou Bay in Louisiana and the 25 flies obtained from Massachusetts were selected for sequencing. The genetic sequences were to be used in identifying potential cryptic species such as those proposed by Sofield et al. (1984) and Sutton and Carlson (1997).

The thorax of each fly was used for DNA extractions as described in Chapter 2. The CO1 locus amplified with the LCO1490/HCO2198 primer pair, used in Chapter 2, was sequenced within this investigation since use of that locus provided the greatest resolution power for species designations. The methods for DNA extraction, PCR reactions, sequencing and the construction of phylogenies were identical to those used to barcode the coastal species in Chapter 2. The species *Tabanus acutus* which was barcoded in Chapter 2 was used as an outgroup for the generation of the phylogenetic trees within this chapter.

Fisher's Exact test was used to test for statistical differences of the proportions of flies from different clades that occurred in the three salinity zones and by season.

3.3 Results

Morphometrics

Horse fly body length measurements ranged from 8.7mm to 15.3mm in Caillou Bay and 8.9mm to 14.8mm in Barataria Bay (Figures 3.1 and 3.2). The body length measurements of flies both above and below the published range for *T. nigrovittatus,* 10.7mm to 13.8mm (Burger 1985), were compared by the salinity zones in which they were collected. Flies smaller than 13.8 mm were captured at all salinity zones across both bays while flies larger than 13.8mm were found only at the Low and High salinity zones in both bays (Table 3.1). However, as previously stated, there were larger flies at Mid-salinity that were not included in the morphometric comparison.

Table 3.1. Number of horse flies captured in the salinity zones of Caillou and Barataria Bay in 2019 designated as *T. nigrovittatus* complex that fall within and out of the reported body length range, of 10.7-13.8mm.

	Flies Less than 10.7				≥ 10.7 - ≤ 13.8				Flies Larger than 13.8			
	Barataria		**Caillou**		**Barataria**		**Caillou**		**Barataria**		**Caillou**	
	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Low	3	8	4	43	205	31	206	146	1	0	4	4
Mid	2	107	3	1	132	144	25	9	0	0	0	0
High	4	28	4	13	102	170	28	19	1	0	1	4

Measuring Methods

Time Comparison

The average timeframe taken to measure a subset of 160 specimens between lab assistants, individually, using digital calipers was an hour and 15 minutes. This process included removing each pinned specimen from the box, carefully measuring without damaging the specimen (i.e. head breaking or cutting the abdomen with the caliper jaws) and recording the measurements directly into a Microsoft Excel spreadsheet. Using this method, each fly took about thirty seconds to process.

Using the Dun, Inc. BK PLUS Imaging System, the preparation of a spreadsheet prior to any measurements taken, required the user to take about 5 minutes to set-up. Quality assurance of flies to be on the same plane also took an additional twenty minutes. Depending on if multiple images had to be taken, five to ten minutes was used to take pictures prior measurements. Approximately 20 minutes was taken to measure the 160 specimens which equates to about eight flies per minute. Measurements were then recorded for the flies and the process to transfer those measurements onto a spreadsheet took an additional ten minutes for the user. Manual input of the

metadata for each collection added five to ten minutes after measuring before generating a functional spreadsheet for each of the collections. In total, 65-75 minutes were used to generate a functional, usable spreadsheet of recorded measurements.

Accuracy Comparison

The statistical comparisons of measurements made using the two methods show that there was no difference in the method of measure (p=0.6575, df=165 and t-statistic=0.4441) (Table 3.2). The standard deviation of difference between the two measuring methods was 0.034 mm which suggests little difference in using either method (Table 3.2).

Table 3.2. Statistical comparison of the accuracy of the two measuring methods used within this investigation. There was no significance between the two methods (paired-t-test, t= 0.4441, P=0.6575, n= 166, df:165).

Measuring Method	Mean	Median	Minimum	Maximum	Range	St. Dev.
Imaging System	11.57	11.54	9.36	13.72	4.36	0.90
Digital Calipers	11.58	11.60	9.60	13.60	4.00	0.87

Population Differences Based on Morphometrics

There were statistical differences in body lengths of flies among the salinity zones and season (Early vs Late) in certain populations (Table 3.3 and 3.4). For flies smaller than the expected body length for *T. nigrovittatus* (10.7 mm), there was a significant effect of salinity ($F(2, 217) = 7.787$, $p < 0.001$), but not season ($F(1, 217) = 3.276$, $p = 0.072$) with no interaction of salinity and time on length ($F(2, 217) = 1.499$, $p = 0.226$) (Table 3.3, Fig 3.1). For tabanids with a body length within the expected size range of *T. nigrovittatus* between 10.7 and 13.8 mm there was a significant effect of salinity on length ($F(2, 1213) = 18.600$, $p < 0.001$) and of season on length ($F(1, 1213) = 230.87$, $p < 0.001$), but no significant effect of the interaction between salinity and time on length ($F(2, 1213) = 1.574$, $p = 0.208$) (Table 3.4, Fig 3.2).

Regardless of where flies are collected, a decrease in body size over the span of the entire season was evident. The flies collected in the Early collections were larger than those collected in the Late collections for both flies measuring less than 10.7mm and those with body lengths that measured 10.7-13.8mm across all three salinity zones (Table 3.3 and 3.4).

Table 3.3 Differences in mean body lengths of tabanids less than 10.7 mm by site (Low Salinity, Mid Salinity, and High Salinity) and time (Early/Late).

Flies less than 10.7			
Site	Time	Number of Specimens	Mean Length ± SE
Low	Early	7	12.36 ± 0.03 [a]
	Late	51	11.78 ± 0.05 [a]
Mid	Early	5	12.09 ± 0.06 [b]
	Late	111	11.49 ± 0.09 [b]
High	Early	8	12.11 ± 0.06 [b]
	Late	41	11.68 ± 0.05 [b]

Differences were compared using a Two-Factor ANOVA followed by Tukey HSD post hoc tests. Mean body lengths followed by the same letter were not significantly different.

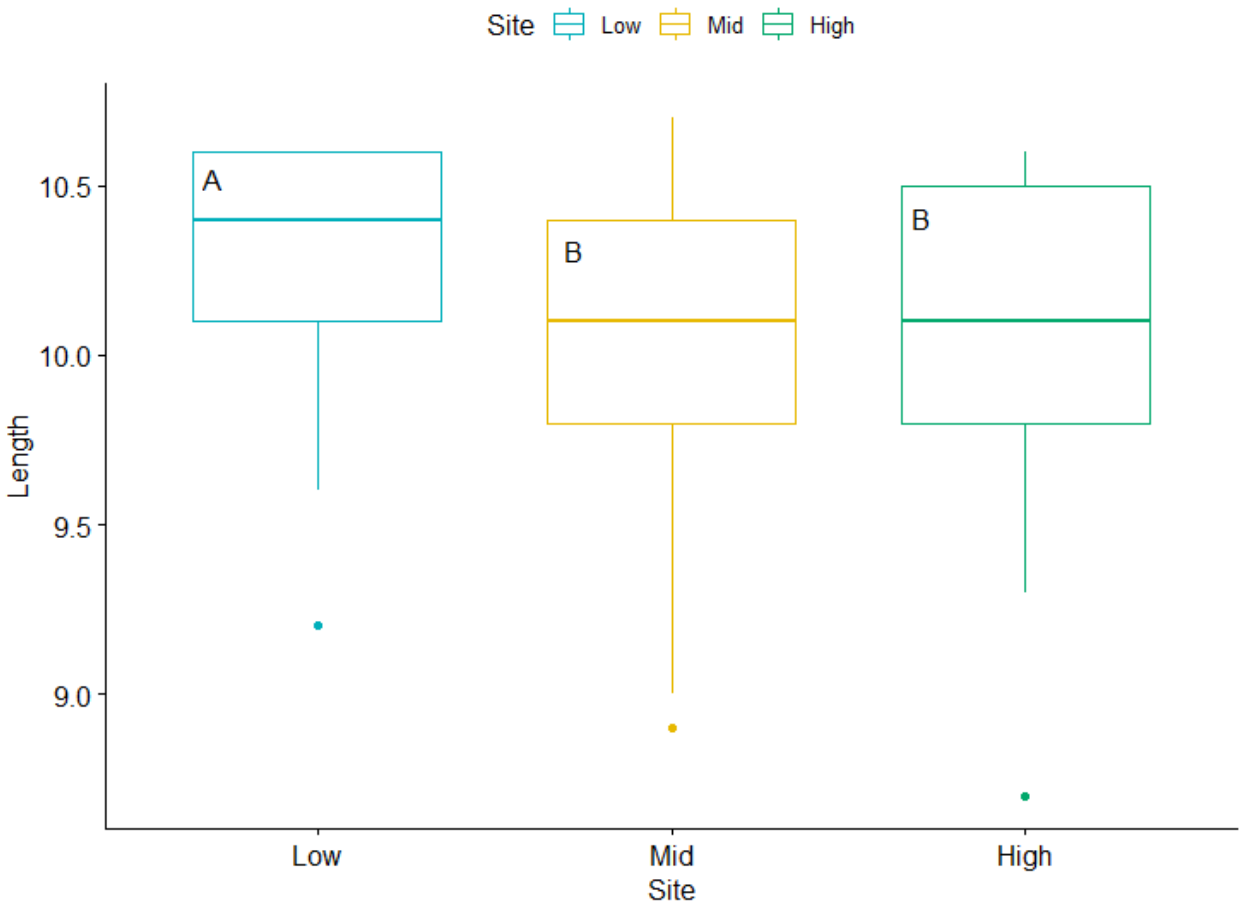

Figure 3.1. Differences in mean body lengths of tabanids less than 10.7 mm by salinity zone (site) combined among Barataria and Caillou Bay. Flies from the Low salinity zone were significantly larger than those from Mid and High salinityThere was a significant effect of salinity on total body length of flies among the three salinity zones. Seasonality on body length nor the comparison of seasonality and time interactions on body lengths were significantly different.

Table 3.4. Differences in mean body lengths of tabanids between 10.7 and 13.8 mm by site (Low Salinity, Mid Salinity, and High Salinity) and time (Early/Late).

Flies 10.7 - 13.8			
Site		Number of Specimens	Mean Length ± SE
Low	Early	411	10.37 ± 0.09 [a]
	Late	179	10.30 ± 0.05 [b]
Mid	Early	157	10.44 ± 0.10 [c]
	Late	153	10.05 ± 0.04 [d]
High	Early	130	10.13 ± 0.15 [c]
	Late	189	10.10 ± 0.07 [bd]

Differences were compared using a Two-Factor ANOVA followed by Tukey HSD post hoc tests. Mean body lengths followed by the same letter were not significantly different .

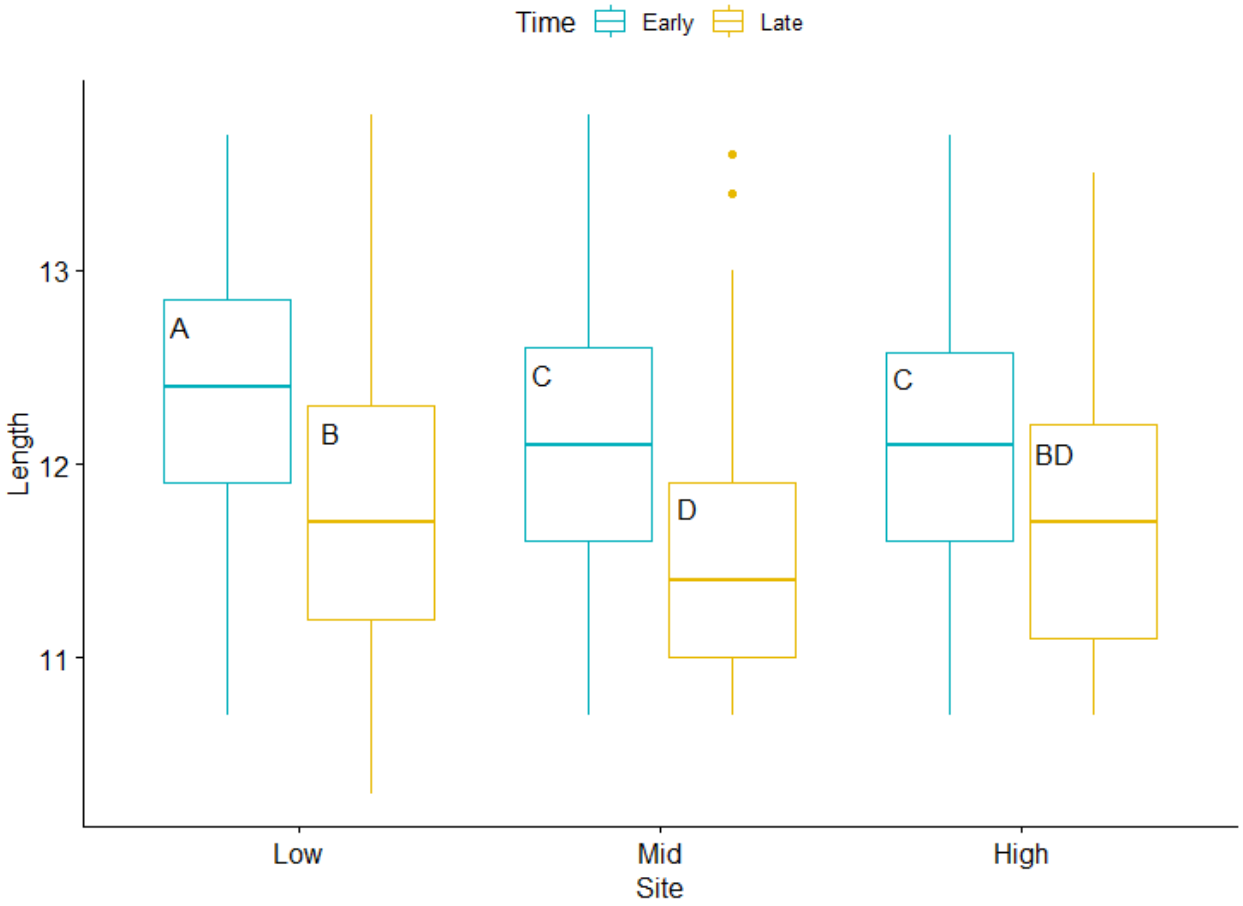

Figure 3.2. Differences in mean body lengths of tabanids less than 10.7 mm by site combined among Barataria and Caillou Bay. There was a significant effect of salinity on length and of season on length, but no significant effect of the interaction between salinity and time on length.

Temporal Distribution

Histograms of the fly measurements were constructed for each salinity zone within each bay to determine if there were geographic or seasonal differences in body length. Flies smaller than 10.7mm were captured during both early and late season at all three salinities in both bays. In Caillou Bay, although flies less than 10.7mm were collected throughout the season, they were more abundant in the later portion of the season at the Low and High salinities (Figure 3.3). There were fewer flies that measured less than 10.7mm collected in Caillou Bay at the Mid sites

with more collected in the Early season (Figure 3.3). Smaller flies were collected in higher abundances in the later portion of the year throughout all salinities in Barataria Bay (Figure 3.4) with the highest abundance collected in the Mid salinity zone (Figure 3.4).

Flies collected with body lengths within the range of 10.7mm to 13.8mm were the most abundant in this study. In Caillou Bay, greenheads within that range were collected during both Early and Late season across all salinity zones. The highest abundance of flies measuring 10.7-13.8mm collected in Caillou Bay were collected in the Low salinity zones. In Barataria Bay, patterns normal distribution of flies within the *T. nigrovittatus* size range was collected at all salinities during both portions of the season, and the late flies were smaller than the early flies in each zone (Figure 3.4).

Flies larger than 13.7mm were captured later in the year in Low and High salinities in Caillou Bay (Figure 3.3). No flies larger than 13.8mm were collected at any of the Mid salinity sites in the late season at Caillou Bay. However, all of the specimens from the series of flies collected in Caillou Bay that had a grayish thorax were found in the Mid-salinity zone during the early portion of the year measured at or greater than 14mm. The flies measuring more than 13.7mm collected in Barataria Bay occurred in the earlier portion of the season at the Low and High salinity sites (Figure 3.4).

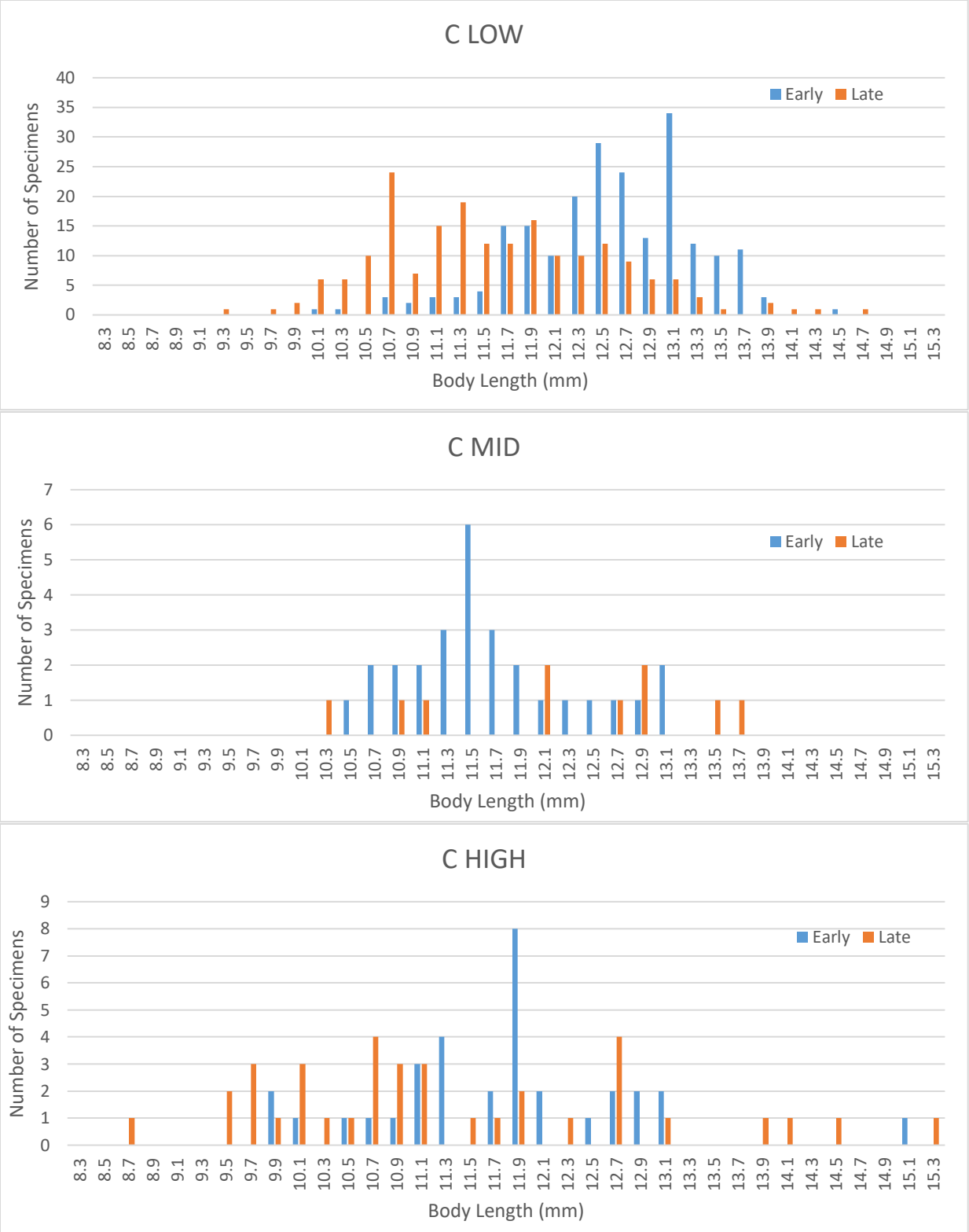

Figure 3.3. Body length of horse flies captured at each salinity zone in Caillou Bay in 2019 by seasonality (Early/Late). Early season from April through June. Late season defined as August through October.

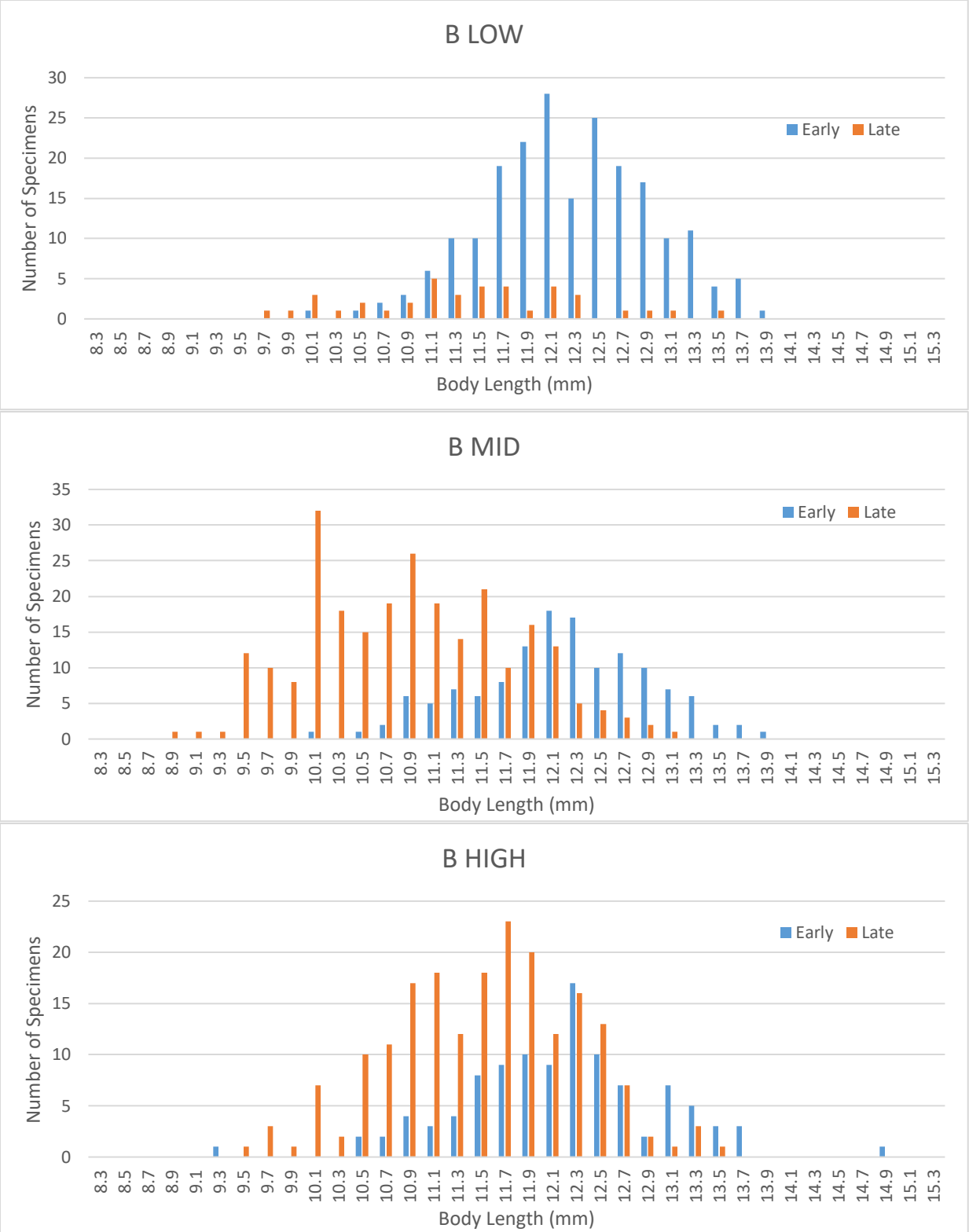

Figure 3.4. Body length of horse flies captured at each salinity zone in Barataria Bay in 2019 by seasonality (Early/Late). Early season from April through June. Late season defined as August through October.

DNA Sequencing

A phylogeny of all specimens selected for genetic analysis within this study was constructed (Figure 3.5). The phylogenetic tree consisted of 112 CO1 sequences that were generated from 7 *T. acutus* (outgroup), 6 male *T. nigrovittatus*, 8 *T. nigrovittatus* larvae, 7 TCLA flies, 23 flies from Massachusetts, and 61 individuals chosen from the random number generator of the flies from Louisiana. The phylogeny of all specimens was comprised of four unique clades in addition to the outgroup *T. acutus* clade (Figure 3.5). After the phylogenetic tree was generated, the clades within the tree were given reference initials. Clade M was made up of flies from Massachusetts along with one fly collected from Barataria in the Mid salinity zone. Clade TN was comprised of individuals identified as greenheads primarily from the High salinity zones. Clade X was assigned to all flies identified as greenheads that were in a separate clade from TN and found primarily in the Low and Mid salinity zones. Clade TC was made up mostly of the flies that were collected in Louisiana that fit previous descriptions of *T. conterminus* plus two specimens from Massachusetts.

Consensus sequences generated using the specimens of each designated clade were compared to visualize the numbers and locations of mutations separating individuals from different clades. The alignments of the sequences showed that there were numerous mutations dispersed across a 429 base pair sequence from the LCO1490/HCO2198 locus. All mutations were nucleotide switches with no insertions nor deletions along any of the consensus sequences (Fig. 3.6)

Among the clades generated along the phylogenetic tree, Clade TN and M were the most closely related clades. Branch lengths along the tree showed that these two clades are the most recent split from a common ancestor. In addition, they shared the strongest sequence similarity

with an average pairwise sequence identity of 97.84% (range 95.4-98.36) (Table 3.5). Furthermore, there are few mutations between the two clades with six transitions and two transversions.

The next closely related clades are M and X with average similarity of 95.60% (range: 93.01-96.38). Differentiation between these clades consists of twelve transitions and five transversions. Clade TN and X shared a slightly lower similarity of 95.11 (range: 94.02-96.38) with twelve transitions although in different locations along the sequences. In addition, Clade TN and X exhibit six transversions between the two consensus sequences. From an ancestral standpoint, the branch lengths depicted along the phylogenetic tree were similar between Clades TN and M as they related to Clade X. Therefore, we concluded that the former two clades share the same common ancestor with a divergence at some point in time where Clade X is derived.

The most distant clades were Clade TC vs TN with only 94.78% similarity (range 94.16-95.33) between the consensus sequences based on eleven transitions and eight transversions (Figure 3.6). Between Clade TC and X, there were fourteen transitions and six transversions with an average sequence identity of 95.42% (range: 94.51-96.26) Between Clade TC and M, there were twelve transitions and five transversions with an average similarity of 95.43% (range: 93.3-96.03) (Figure 3.6 and Table 3.5).

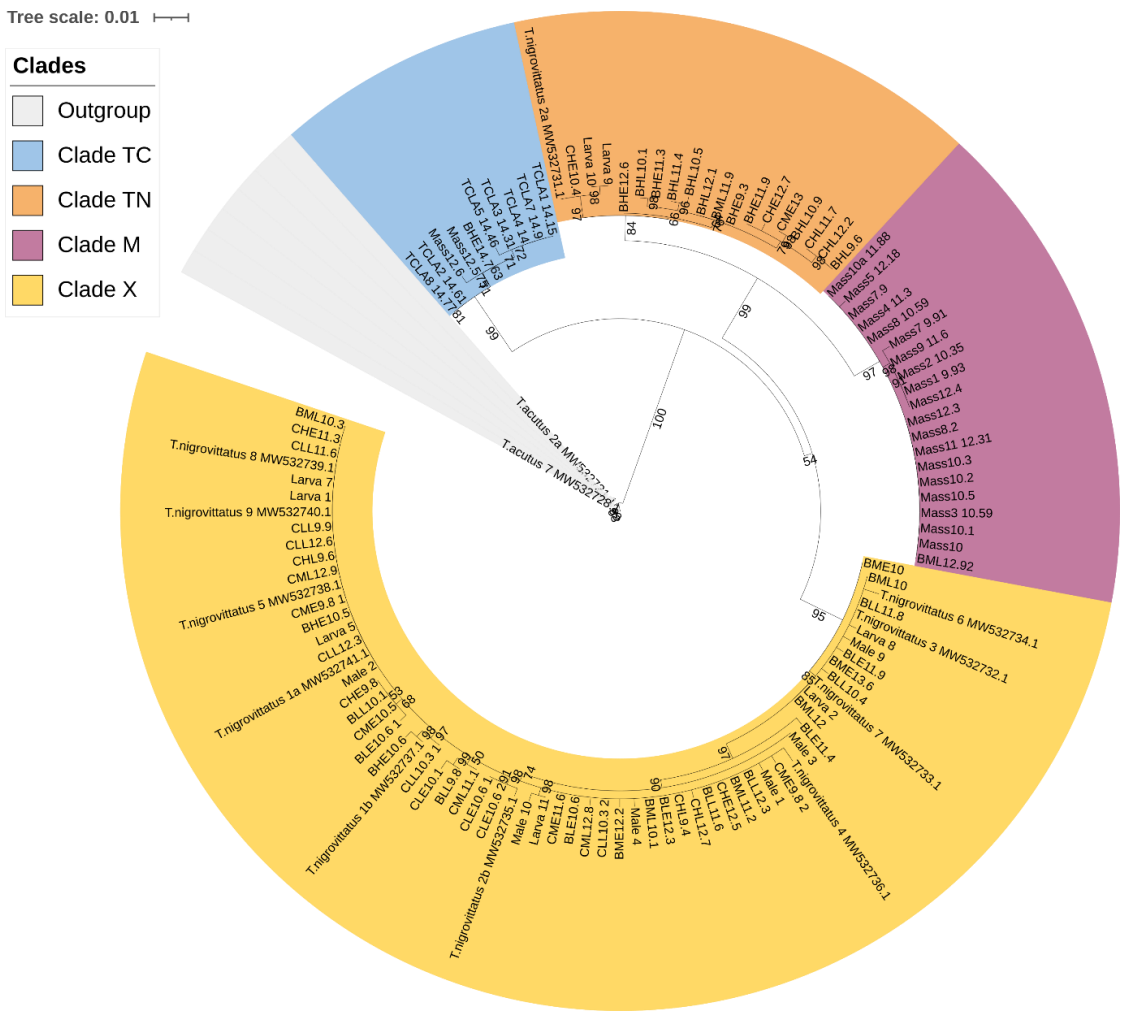

Figure 3.5. Maximum likelihood phylogenetic tree constructed using CO1 (LCO1490/HCO2198 primer pair) sequences generated from individuals primarily collected from the High saline zones of Louisiana, Clade TN. The greenhead specimens from the Low and Mid saline zones make up Clade X including the previously sequenced males and larvae. Specimens from Cape Cod Massachusetts make up the majority of Clade M. Specimens collected from Louisiana with body length greater than 13.8mm and that fit the description of *T. conterminus* make up the majority of Clade TC.. Tree scale is a representative of the average number of nucleotide substitutions per site. Bootstrap values are confidence values for node supports across 1000 replications.

Table 3.5. Pairwise comparisons of the sequence similarities (%) of the five clades formed from the specimens used for DNA sequencing

	T. acutus			Clade TC			Mass			Clade TN			Clade X		
	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Min	Mean	SD
T. acutus	99.77-100	99.89	0.01												
Clade TC	91.84-92.77	92.30	0.06	99.07-100	99.61	0.07									
Mass	90.29-92.31	92.02	0.19	93.30-96.03	95.43	0.26	96.97-100	99.53	0.47						
CladeTN	91.84-92.77	92.23	0.05	94.16-95.33	94.78	0.06	95.40-98.36	97.84	0.29	98.60-100	99.37	0.15			
Clade X	91.30-93.24	92.38	0.15	94.51-96.26	95.42	0.12	93.01-96.38	95.60	0.30	94.02-96.38	95.11	0.13	97.27-100	99.12	0.33

Previously barcoded specimens of *T. acutus* were used as the outgroup. Clade TN was made up of specimens from the morphometric analysis of flies from Louisiana including a barcoded *T. nigrovittatus* specimen from Chapter 2 that was submitted to GenBank. Clade X was made up of the remaining specimens from the random number generator as well as the remaining barcoded *T. nigrovittatus*. Clade M was made up of flies obtained from Massachusetts. Flies larger than 13.8mm, or the threshold for *T. nigrovittatus* range made up Clade TC.

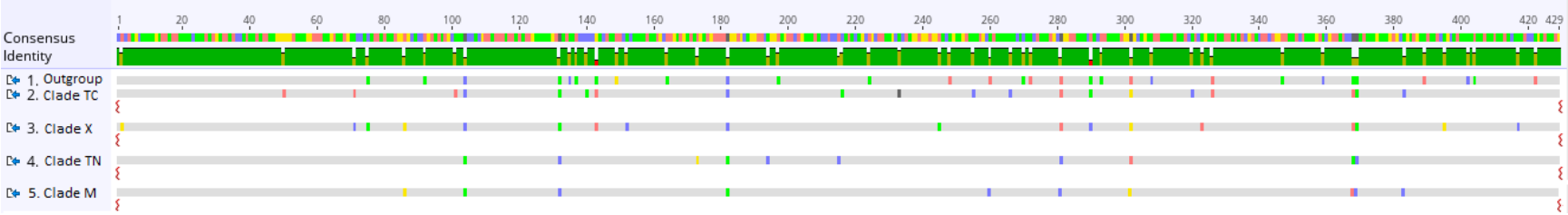

Figure 3.6. Multiple alignment file of the consensus sequences generated from the specimens of each designated clade. Colors indicate base differences and are coded as such: A=red, C=blue, G=yellow and T=green.

Further analyses were performed on the flies from the *T. nigrovittatus* complex collected from Louisiana o(Clades TN and X) to determine if the clades could be separated based on salinity zones in which the specimens were collected. Clade TN consisted of seventeen individuals that, although varied in total body length from 9.3mm to 12.92mm, were collected in the High saline zones predominately in Barataria Bay. Only five of the eighteen flies of Clade TN were from Caillou Bay and of those flies, all but one were collected in the High salinity zones (Figure 3.5). Only one of the barcoded *T. nigrovittatus* complex specimens clustered with the remaining specimens from Clade TN along with two larvae that were previously sequenced. The remaining barcoded individuals fell within Clade X along with all male tabanids and the remaining larvae (Figure 3.5).

There were significant differences between the proportions of flies from the three salinity zones found in Clades TN and X. There were significant differences among the proportions of flies collected in the Low salinity zone with more in Clade X; $p = 0.0042$ and High with more in Clade TN; p - 0.0035. (Table 3.6). There were no flies in Clade TN collected from the Low sites while 21 (48.83%) of the flies that comprised Clade X were collected in the Low zones (Figure 3.5). Fourteen (87.5%) flies from Clade TN were collected in High salinity but only seven (16.27%) of flies from Clade X were collected in the High salinity sites (Figure 3.5). There were no statistical differences in the percentage of flies collected from the Mid salinity zones between Clade TN (12.5%) and Clade X (34.88%).

In addition to the proportion differences between the clades based on salinity zones, we also analyzed the proportion of individuals, based on the time of year, early or late, in which flies were collected from each clade. Of the flies that make up Clade TN, 41.17% were collected in the early season whereas 46.51% of the flies that make up Clade X were collected during the

early season(Table 3.6). Inversely, the remainder of the flies that make up Clade TN and X, 58.2% and 53.48%, were collected in the late season. Overall, both clades had flies from both periods of the season and equally distributed throughout the season although there were fewer total flies that make up Clade TN.. There also were more flies in Clade TN than Clade X that were within the body range of *T. nigrovittatus*, 70.58% vs 51.16% respectively. In turn, Clade X had a higher percentage of flies of smaller body lengths 48.83% at 11.07 ± 1.18 than Clade TN (29.41% at 11.35 ± 1.17).

Table 3.6. Total number of flies from each salinity zone that make up Clades TN and X, selected from the morphometric analysis. Statistical differences of the proportions of flies was compared using Fisher's exact test. Significance between proportions of flies at the Low and High salinity zones based on P-values.

	Total Number of Flies	Mean Length ± SD	Low Salinity*	Percent at Low*	Mid Salinity	Percent at Mid	High Salinity*	Percent at High*
Clade TN	16	11.35 ± 1.17	0	0.00%	2	12.50%	14	87.50%
Clade X	43	11.07 ± 1.18	21	48.83%	15	34.88%	7	16.27%

Asterisk (*) denotes significant difference. P-value at Low Salinity was 0.0042. P- value at High Salinity was 0.0035.

3.4 Discussion

In comparison of the two morphometric measurement methods used in this study, there were no significant differences between the accuracy or labor time between them. However, there are different benefits for using either method. Prior literature detailing the comparisons of these two techniques has not been well-documented. The use of digital imaging systems to perform measurements on microscopic objects is extensive in the field of engineering for small circuits and electronic components but has not been used extensively with larger organisms such as the tabanids. In this study, digital imaging was a better system to utilize when measuring large series of flies as well as documenting images of specimens and their distinct characters for future reference. In the process of data input, error was eliminated since digital measures were recorded within a table. The use of digital calipers to record measurements by hand was just as accurate and more applicable to small series of specimen collections.

Size distributions of the flies used in the morphometric analyses were plotted in histograms to visualize potential differences in body length relative to salinity zone and on a temporal scale (Figures 3.3 and 3.4). In Barataria Bay, there were clear patterns of a species of greenheads with a spring and fall population, and that the flies in the fall were smaller than in the spring. This same pattern was observed in the Low salinity zone of Caillou Bay. There were very few flies captured in Barataria Low and Caillou Mid salinity zone in the fall which reduced the observation of differences between fall and spring population specimen size. The occurrence of a small number of larger flies, found in the High zones of Caillou, that were within the size range of *T. conterminus* along with the other greenheads, presented a pattern of two species present in fall and spring; thus it is likely that both species have two generations per year. Therefore, the data support the concept that the species in the *T. nigrovittatus* complex found within this study

likely are bivoltine in Louisiana. The morphometric results of the seasonal differences of tabanids of this study support a hypothesis that the smaller body size of *T. nigrovittatus* complex specimens in the fall could be attributed to a shorter development period for eggs deposited in the spring compared to those from the fall. Sofield et al. (1985), suggested that *T. nigrovittatus* may be at least partially bivoltine in New Jersey. Additionally, Sofield et al. (1985) conducted a size and seasonal distribution survey on *T. nigrovittatus* in New Jersey and found that although the average body range of *T. nigrovittatus* was 8.4-12.4 mm, specimens of this species collected later in the season were generally smaller than those collected at the beginning of the season. Since body size does differ relative to season, care should be taken to measure specimens from different collection periods to establish the size range of a multivoltine species. The collection of males in both spring and fall reported in Chapter 2 is strong evidence of this phenomenon.

Flies with total body lengths less than 10.7mm were predominately collected in the late season in both bays (Table 3.4). There were 20 flies (10%) less than 10.7mm collected early in the season compared to 200 flies collected later. In Caillou Bay, the majority of specimens less than 10.7 mm were found in the High salinity zone (Figure 3.1). In Barataria Bay, smaller specimens were more frequently collected in the Mid and High salinity zones (Figure 3.2). These data further support the concept that eggs deposited by females in the spring develop into smaller adults than their parents, likely due to a shorter growth period. Sutton and Carlson (1997), in their study on chromatotypes of greenheads from the Atlantic Seaboard, reported that flies of the Type III chromatotype were the smallest. Of the three chromatotypes they found, type III was left as an undescribed species which represented only 11% of the total number of flies examined. There was considerable overlap in the size range of Type II, which was designated as *T.*

nigrovittatus, and Type III. Sutton and Carlson (1997) suggested that Type III range could extend into the Gulf Coast.

Previous studies in the salt marshes of the Upper Atlantic have described the temporal occurrence of greenheads to be from mid-June to early/mid-September (3 months) with July being the month of greatest abundance (Schulze et al. 1975, Sofield et al. 1985). The season described in the Upper Atlantic is much shorter than the pattern recorded within this study along the Gulf Coast in Chapter 2. The seasonal occurrence for *T. nigrovittatus* described within this study was from mid-April to early October (6 months) over the course of multiple years. The life span of adult female greenheads ranges from 1-2 months. Emergence of greenheads in Louisiana or in the Upper Atlantic is not synchronous (Figures 2.10, 2.11, Sofield et al. 1985). A pattern of asynchronous emergence peaking mid-season in the Upper Atlantic would account for a three-month -univoltine season while asynchronous emergence with spring and fall peaks along the Gulf would account for a bivoltine season.

The difference in seasonal length between the two geographical areas provide an explanation to the observation of at least two abundance peaks along the Gulf in comparison to just a single peak in certain locations along the Atlantic. However, it is unknown whether the second peak in Louisiana, is the same species that occurs in the first abundance peak or if the second peak along the Gulf is of a cryptic species. The latter would point to possible undescribed, cryptic species that have been suspected to occur along the Atlantic Coast such as chromatotype III or an undescribed species along the Gulf Coast (Sutton and Carlson 1997). The predominance of late collections of flies less than 10.7mm could represent two different scenarios if there is a single cryptic form that is the smaller specimens or chromatotype III from Sutton and Carlson (1997). There could be a single generation of the smaller flies that is in the

late season or there could be a similar trend for a cryptic with larger flies occurring early followed by a second generation of smaller flies similar to the flies in the *T. nigrovittatus* size range (Figure 3.3, 3.4; Table 3.4).

This was the first study on the longitudinal occurrence of the *T. nigrovittatus* complex within different salinity habitats within extensive coastal estuaries. If there are different types of greenheads in these different habitats that do not key out to species other than *T. nigrovittatus*, then genetic studies to establish their relationships are warranted, and that was the subject of the objective 2 studies.

The phylogenetic tree which was comprised of 112 CO1 sequences revealed that there were 3 distinct clades of the flies from the *T. nigrovittatus* complex collected in coastal Louisiana (Figure 3.5) The lines of evolutionary descent depicted within the phylogenetic tree of all specimens within this study suggest that there was once a common ancestral lineage of which Clades TC, TN, and X were all part of. The lineage of the three of the clades showed an evolutionary change at some point in time that began with a common ancestor and later diverged with those clades as descendants. The phylogenetic tree alone suggests that there are possibly multiple species since the nodes would represent genetic traits that are representative of the clades.

Clade TC was the least similar clade to the other clades, with an average pairwise identity of 94.75% to TN and 95.42% to both M and X (Table 3.5). Consensus sequence alignments also showed that Clade TC had the greatest amount of base pair differences representing the highest genetic difference when compared to the other clades. Given the morphological characters such as having larger body lengths and gray thoraces, the flies of Clade TC collected in Louisiana

conclusively were not *T. nigrovittatus*. Previous records of flies resembling *T. nigrovittatus* with a gray thorax have been classified as *T. conterminous (ref?)*.

The locality at which the specimens from Clade TC were collected aligns with previous collections of *T. conterminus* in the Upper Atlantic. Previously, adults of *T. conterminus* were collected at sites that were closer inland than the sites where higher numbers of *T. nigrovittatus* were collected (Sofield et al. 1985). During this study, the larger specimens were collected in the Mid salinity zones of Caillou Bay during the early portion of the year. The larvae of *T. conterminus* that were discovered by Freeman and Hansens (1972) and later described by Freeman (1987), were collected more abundantly in ditch and creek banks where the tall form of *Spartina alterniflora* thrives. This conforms to the vegetation that appeared in Caillou Bay Mid salinity sites where the adults of Clade TC were collected.

There were fewer flies of Clade TC collected than specimens that were identified as *T. nigrovittatus*. Previous studies that compared population abundance of *T. nigrovittatus* and *T. conterminus* in the Northeast found that adults of *T. conterminus* were far less abundant. Sofield et al. (1984) reported a sample size of 116 *T. conterminus* versus the 2185 *T. nigrovittatus* specimens collected in one study, with the *T. conterminus* specimens accounting only for 5% of the catch. A year later, Sofield et al. (1985) collected 18 *T. conterminus* (6.5%) compared to 274 *T. nigrovittatus*, in New Jersey. Within this study, the larger specimens accounted for only 2% of the flies collected between those of *T. nigrovittatus* and *T. conterminus* size ranges.

Along with probable larval habitat and population size differences in Clades TC, as well as the different morphological characters, the flies in Clade TC were genetically different from *T. nigrovittatus*. Two flies collected in Massachusetts were placed in Clade TC which clearly indicates that the flies in TC are *T. conterminus.* Final confirmation that flies of Clade TC are *T.*

conterminus remains until type specimens identified as *T. conterminus* by earlier workers are examined.

Clades TN and M had the highest similarity among all clades with the average similarity of 97.82%. Furthermore, the alignment sequences show that there were less differences between Clades TN and M than there were between Clade X and Clade TC. Geographically, *T. nigrovittatus* has been collected in areas with high water salinity (Figure 2.3; Table 2.4); salinities of the immediate marshes in the Upper Atlantic would be higher than those along the Gulf Coast. The locality of Cape Cod, where the flies from Massachusetts from this study originated, extends into the Atlantic Ocean whereas there are tributaries and diversions that direct fresher water into the Gulf prior to encountering higher salinity water. Clade TN specimens were predominately collected in the High salinity zones of Barataria Bay (Figure 2.2). In addition, there were two larvae and a previously barcoded adult *T. nigrovittatus* sequence, from chapter 2, within the clade. The *T. nigrovittatus* sequence was derived from an individual that was collected from saltmarsh habitats of Barataria Bay. The two larvae in Clade TN were collected in Cameron Parish at the Rockefeller Wildlife Refuge at the very edge of the Gulf (Figure 2.1). The salinity zones in which flies from Clade TN and Clade M were collected as well as the close genetic match suggest that the specimens of both clades are members of the species *T. nigrovittatus*.

Mating behavior of *T. nigrovittatus* has not been extensively studied, but hovering behavior of males prior to breeding has been documented. Males are known to hover around the larval habitats. In this study, the males of the *T. nigrovittatus* complex were collected from Barataria and Caillou Bay primarily in the Mid salinity zones. The clustering of all males that

were sequenced fell into Clade X; females of Clade X were primarily collected in the Low and Mid salinity zones.

Larvae from Clade X were mainly collected from Cypremort Point State Park, which is located near Vermilion Bay (Figure 2.1). Average water salinity for Vermilion Bay during the time of this study ranged from 2-6ppt (brackish water) (https://nwis.waterdata.usgs.gov/nwis). n contrast, the two larvae from Clade TN that were collected in Plaquemines Parish in saltmarsh salinities ranging from 10-20ppt (https://nwis.waterdata.usgs.gov/nwis).

Finding larvae placed in Clade TN suggests that *T. nigrovittatus* or Clade TN, is native to high salinity zones. Finding larvae and males assigned to Clade X suggests that this type is native to low and mid-salinity zones. Larvae of *T. nigrovittatus* are known to grow in salt water marshes or at least brackish waters (Freeman and Hansens 1972, Freeman 1987). The food web to support tabanid larval growth within the high salinity zones is plentiful. Aker (2020) and Rayle (2021) found an abundance of macrofauna available at high salinity zones in marsh sediment. With previous findings of *T. nigrovittatus* larvae in marshes of the Upper Atlantic as well as along coastal Louisiana (Husseneder et al. 2016) as well as recent studies of the macrofauna available as food sources within Louisiana's marshes, the clustering of the two larvae in Clade TN would support the idea that *T. nigrovittatus* is native to high-saline marsh.

Clade X had differences when compared to the remaining clades. There is a clear distinction between clades TN and X of flies from Louisiana based on salinity gradients as TN specimens were found in the High salinity zones while X specimens were found in more brackish waters. There was a significant difference in the percentage of flies of clades TN and X when comparing salinity zones, particularly in Low vs High zones (Table 3.6). Flies collected from the Low zones accounted for 48.83% of the flies in Clade X with only 16% from collected

in High. There were no flies in Clade TN from the Low zones, and 82.35% of the flies from Clade TN were collected in the High salinity zones. There were no statistical differences in the percentage of flies collected from the Mid salinity zones between Clade TN (12.5%) and Clade X (34.88%). Since TN was not found in Low where the majority of X were collected, and that an equivalent amount of individuals from both clades were found in Mid; these data trend toward X having a low salinity origin and TN having a high salinity origin. These observations support the existence of two "types" relative to the dominance of the salinity zones where the flies were collected between the two clades. Approximately half of the flies in both Clades X and TN were collected in both early and late periods. Therefore, no evidence was found to support the concept that the seasonal population peaks in different salinity zones represent a cryptic species outside of flies in Clades X and TN.

Through the analysis of the phylogenetic tree, the pairwise comparisons and the base pair alignments among each of the clades, we conclude that there are at least three types or clades of greenheads in Louisiana. The cuticular hydrocarbon analysis by Sutton and Carlson (1997) pointed toward the existence of two described species and a third chromatotype along the Atlantic seaboard. The third chromatotype discovered by Sutton and Carlson (1997) may have been individuals of Clade X within this study, but retrospective comparison is not possible.

Previously, Philip (1962) described *T. eadsi* as a member of the *T. nigrovittatus* complex as a yellowish and brown fly with a single-banded green eye in life with a body length of 9-12mm (Table 2.1). The geographic range of *T. eadsi* has been described as within coastal areas of Texas and Louisiana which is included the range of *T. nigrovittatus* which has a larger range in body length and larger reported geographical range. The closely related descriptions between *T. nigrovittatus* and *T. eadsi* are differentiated by the abdomen of *T. eadsi* possessing two darker

longitudinal submedian stripes (Philip 1962). Although both species have yellow palpi, *T. eadsi* varies from yellow to a mixture of yellow and black while palpi of *T. nigrovittatus* are always more yellowish (Philip 1962, Tidwell 1970). Temporal distribution of the smaller individuals of *T. nigrovittatus* were predominately collected in the latter half of the season at all salinities in both bays. However, previous literature has not recorded the collection of *T. eadsi* during the later months of the season and Philip (1962) suggested that continued collections of *T. eadsi* may reveal that it is an early season fly.

With the close similarity and geographical location reported for *T. eadsi*, we cannot rule out that it was a part of the *T. nigrovittatus* complex observed within this study. The specimens collected in this study have been retained as voucher specimens for comparison to *T. eadsi* as well as other described species in the *T. nigrovittatus* complex. Thus, voucher specimens from various museum collections will have to be examined before concluding the identification of the flies in Clade X.

The sampling design of this study, using periodic surveys for greenheads throughout the salinity zones of two major bays, provided data that when combined with previous studies, points toward the existence of three distinct clades of flies in the greenhead complex within the tidal estuaries of Louisiana. Flies that fit the description of *T. conterminus* were collected along the Gulf Coast for the first time. Two flies that were collected in Massachusetts that were within the lower limits of the size range of *T. conterminus* (Sutton and Carlson 1997). Since *T. conterminus* is a described species, comparison of available vouchers from Louisiana to vouchers of *T. conterminus* from collections made by previous authors should provide enough information to confirm that *T. conterminus* does exist in Louisiana. Individuals of the clade designated as TN were from High salinity zones and absent from the Low salinity zone in Caillou. The flies

considered to be *T. nigrovittatus* from Cape Cod Massachusetts were genetically similar to the flies in Clade TN. Thus, the saltmarsh greenhead horse fly, *T. nigrovittatus*, should be considered as native to the high salinity saltmarsh regions of Louisiana, which typically are the habitat of smooth cordgrass, *Spartina alterniflora*. Flies placed in Clade X were captured in the Low salinity zone of Caillou in the absence of flies from Clade TN. The seasonality of flies in Clade X was also bivoltine with the members of the fall population being smaller than the spring population. If voucher specimens for this clade do not match vouchers from other greenhead species that have been described, the species designation will depend upon morphological comparisons between members of the X and TN clades and subsequent confirmation by sequencing.

CONCLUSIONS

This study was the first to provide an inventory of species present along the coastline and within coastal marshes of Louisiana. In this study, specimens of twelve species of Tabanidae with members of four genera including Tabanus, Chrysops, Leucotabanus and Chlorotabanus were collected within and around coastal Louisiana. Members of the *T. nigrovittatus* complex were the most abundant and dominated in relative species abundance across the salinity zones within the estuaries. Flies within the established *T. nigrovittatus* body range of 10.7-13.8mm were most abundant and were more frequently collected in the early portion of the season.

Results of adult abundance data and water salinity data showed the occurrence of specimens of the *T. nigrovittatus* complex in several areas with intermediate-brackish to high-saline waters. In areas with fresh to fresh-intermediate water salinities, specimens of *T. nigrovittatus* were not collected. The results of this study show that specimens of *T. nigrovittatus* can be collected in inland traps in close association to high saline waters.

Over the course of multiple seasons, there were two notable periods of abundance of *T. nigrovittatus*; the first peak occurred mid-May to mid-June and the second from early-August into late-September. Furthermore, seasonal patterns within the different salinity zones were similar. These observations indicate that members of the *T. nigrovittatus* complex can have more than one generation per year.

Comparison of diurnal and overnight collections established that *T. acutus* is primarily active during crepuscular-nocturnal periods in all salinity zones. Specimens of *T. hinellus* and the *T. nigrovittatus* complex were primarily active in diurnal periods. Abundance of female *T. hinellus* decreased with increasing salinity; individuals were most abundant in the Low salinity zone with fewer in the Mid zones and none in the High zones.

Male *T. nigrovittatus* complex specimens were collected most abundantly in light traps during the early portion of the season in May with the next highest capture rate in October. The majority of male tabanids were collected in incandescent light traps while those baited with dry ice and ultraviolet traps were not as effective. The majority of males were collected within the Mid salinity zones of the estuaries.

This was the first study on the longitudinal occurrence of the *T. nigrovittatus* complex within different salinity habitats within extensive coastal estuaries. Data collected from the morphometric analyses showed that total body length of flies of the *T. nigrovittatus* complex were higher in the first generation of the year when compared to the fall generation.

The use of DNA barcoding for the four coastal tabanid species (*Chrysops flavidus, Tabanus acutus, T. hinellus* and *T. nigrovittatus*) frequently found within the estuaries, targeting multiple gene regions was successful. Comparisons of the DNA sequences made from the three loci showed that those of the 18S locus, 18Sai/18Sbi and NF1/18Sr2b are conclusive enough to differentiate between the genera of Tabanidae along with a few mutations that can distinguish the three species of Tabanus. However, the CO1 locus used within this study yielded the highest differentiation between the genera and species for females, males and immatures within this investigation. These genetic results are the first to show the relatedness between species and across genera for coastal tabanid species. Molecular identifications such as those reported in this study show that these tools can aid and supplement traditional morphological identification methods particularly in separating species complexes and identifying larvae.

Through the analysis of the phylogenetic tree, the pairwise comparisons and the base pair alignments among each of the clades, we concluded that there are at least three types or clades of greenheads in Louisiana. All larvae within the phylogenetic tree, identified as *T. nigrovittatus,*

were collected from two sites. Those in Clade X were collected at Cypremort State Park in Vermilion Bay where average water salinities ranged from 2-6ppt (brackish water). Immatures of Clade TN also were collected from sites in Plaquemines Parish where water salinities ranged from 10-20ppt (saltmarsh). This, in conjunction with adult distribution data of the two clades (TN and X) within the *T. nigrovittatus* complex suggested that there is a clade native to low and mid-saline zones while the other is native strictly to high saline zones.

Flies (Clade TC) that fit the description of *T. conterminus,* based on morphological characters, abundance, and area of collection were found along the Gulf Coast for the first time. Final confirmation that flies of Clade TC are *T. conterminus* remains until type specimens identified as *T. conterminus* are directly compared.

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Vita

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