

ABSTRACT

Phenotypic Plasticity: Temporal, Spatial and Behavioral Effects on Wing Morphology of Damselflies in Central Texas

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In winged animals, flight morphology must be adapted for optimum behavioral efficiency. Behaviors such as foraging, predator avoidance, and mating are strongly influenced by wing morphology and influenced by environmental conditions in many species. Geometric morphometric methods analyze and visualize subtle variations in wing shape. This study examined environmental and behavioral effects on wing shape and wing size in both sexes of multiple damselfly species over several flight seasons in central Texas. Wing size is a proxy for body size of damselflies. For *Argia sedula*, comparisons were made 1) between adults collected early in the season versus those collected late in the season, 2) between adults collected at different locations, and 3) among adults collected during several flight seasons at the same locations. Significant differences in wing shape and size occurred between seasons and between years, but not between locations. Using these findings, I broadened this study to examine temporal effects on wing size and wing shape of both sexes in eight damselfly species. Analyses indicated significant differences in wing shape and wing size between early and late

flight seasons in every comparison, including seven populations of four species. Damselflies emerging early in the flight season were significantly larger than those emerging later in the flight season. In contrast, significant shape and size differences between years occurred in only one of six species. Finally, I examined wing morphology associated with gender by comparing 1) mated and unmated damselflies of both sexes from three species, and 2) males and females from ten species. Significant differences in wing shape and size occurred between mated and unmated damselflies in one of nine populations sampled. As expected, significant differences in wing shape and wing size occurred between males and females in nineteen of the twenty comparisons made from ten species. These results suggest that differences in seasonal and annual environmental conditions frequently influence wing shape and body size in both sexes of multiple damselfly species. This work presents an original, comprehensive study of environmental and behavioral effects on wing morphology using geometric morphometric techniques.

Phenotypic Plasticity: Temporal, Spatial, and Behavioral Effects on Wing Morphology of
Damselflies in Central Texas

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DEDICATION

To my husband Tommy, son Michael, daughter Teresa, and grandson Hunter
Thank you for your constant love and support

To Momma and Daddy
Thank you for everything

CHAPTER ONE

Introduction

General Overview

Wings represent a key morphological adaptation that contributes to the remarkable evolutionary success of flying animals, including insects. Flight affords many clear advantages when performing life functions. After adult emergence from an aquatic habitat, damselflies are critically dependent on their ability to fly (Bybee et al. 2016). Wings vary greatly in size, shape, and function among damselflies, and these differences in wing form and function translate into differences in flight performance. Natural selection may favor animals that are agile, fast, and efficient flyers with efficient ability to disperse, migrate, avoid predators, forage for resources, and search for mates (Kingsolver & Pfennig 2007; Outomuro et al. 2016). Studies among insect orders have shown the evolution of several different functional strategies, such as the relationships between wing-beat frequency, body weight, wing area, and wing shape (Petavy et al. 1997). Differences in wing morphology are often interpreted as the result of adaptive evolution driven by natural selection, within the limits of evolutionary constraints (Outomuro et al. 2016). Several characteristics making damselflies excellent model organisms for ecological studies include: 1) extensive phenotypic and ecological diversity

2) complex aquatic/terrestrial life cycles, and 3) comparatively large adults that facilitate observations in the wild (Bybee et al. 2016).

Shape and Shape Analysis

The shape of an object is all the geometric information that remains when location, scale, and rotational effects are filtered out (Webster & Sheets 2010). Some morphological traits of shapes can be quantified by single measurements, however if multiple aspects of geometric shape variation are quantified, a subtler picture emerges (Klingenberg 2010). Such a geometric morphometric approach is a powerful tool to evaluate correlations between shape and environmental variables (Lorenz et al. 2017). Studying wing shape using geometric morphometrics allows for the analysis and visualization of shape with great precision (Outomuro et al. 2013). Geometric morphometrics represents organismal forms (shapes) by assigning landmarks, which are points located precisely on all forms and establish a clear one-to-one correspondence between all specimens included in a study (Klingenberg 2010). Landmarked specimens are subjected to Procrustes superimposition to remove differences in location, size, and orientation (Webster & Sheets 2010). Geometric morphometrics differs from other applications of multivariate statistics in that most results from the geometric analyses can be visualized as shape changes and interpreted anatomically (Klingenberg 2010). Visualizations are based mainly on two principles: display of the relative displacements of corresponding landmarks in different shapes, or by showing the deformation of a regular grid, an outline or a surface that is interpolated from the shape change. These two methods can also be combined (Klingenberg 2013). Geometric morphometrics has been used in studies to distinguish between species (Yee et al. 2009; Lee & Lin 2012), to

assign organisms to taxonomic groups (Sadeghi & Dumont 2014), to distinguish between female damselfly morphs of a species (McTavish et al. 2012), to differentiate individuals of territorial contests (Bots et al. 2010), as well as to explore morphological variations resulting from environmental factors (Gibbs et al. 2011; Van Dyck & Wiklund 2002; Bots et al. 2009).

Developmental Plasticity

Genetically similar organisms reared under different conditions can display quite distinct characteristics (Stearns 1989). Phenotypic plasticity, the ability of a genotype to produce different phenotypes in different environments, is of primary interest to evolutionary biologists. Plasticity is an alternative to genetic change allowing populations to adapt to changing environmental conditions (Debat et al 2003). Developmental programs are not fundamentally altered, but either rates or degrees of expression of the developmental program may be modified (Smith-Gill 1983). Epigenetic effects, developmental interactions among cells, tissues and their environment, are of key importance as they provide flexibility in phenotypic plasticity. Phenotypic plasticity, the component of variation induced by environmental effects, can be of key evolutionary importance. Plasticity is based on epigenetic effects; an environmental stimulus can alter developmental processes so giving rise to a difference in the adult shape (Van Dyck & Wiklund 2002). Environmental stimuli can be physical factors, such as temperature, or complex stimuli, such as diet (Klingenberg 2010). Because the environment is variable, having only fixed, invariant traits is unlikely to maximize fitness. Better matching of phenotypes to environments can be achieved by phenotypic plasticity, which is the ability

of one genotype to produce different phenotypes in response to environmental conditions (Viney & Diaz 2012).

Environmental Factors and Behavior Affect Wing Shape

Environments are dynamic and vary in quality over space and time, both within and across individual generations (Viney & Diaz 2012). Fitness of an organism comes by surviving and reproducing in its environment and outcompeting other individuals. This fitness which depends on a phenotype being better matched to this environment (Viney & Diaz 2012). Flight performance results from interactions among morphology (i.e. wings), behavior, and the biological and physical environment (Outomuro et al. 2013). Insect flight is influenced by morphological traits, thus selection on flight performance is expected to favor different optima for flight morphology at different seasons (Van Dyck & Wiklund 2002). Damselflies exhibit a complex life cycle where individuals grow only as juveniles. Age and size at maturity must be optimized during larval development (Hammerschmidt et al. 2012).

Studies have consistently shown seasonal and habitat differences in morphology of insects. Two species of *Drosophila* that grew and developed under different temperature regimes resulted in extensive variations of adult size (i.e. body weight, thorax and wing length) and flight parameters (i.e. wing beat frequency) with significant differences between species (Petavy et al. 1997). Rainfall levels and temperature during development significantly contributed to variations in body mass and forewing loading in the butterfly *Pararge aegeria* (Gibbs et al. 2011). In *P. aegeria*, three developmental pathways with different temperature and photoperiod regimes, result in early spring adults, late spring adults, and summer adults. Significant variations in flight morphology

were found between butterflies from each seasonal cohort (Van Dyck & Wiklund 2002). Seasonal variations were also found in the damselflies *Enallagma cyathigerum* (Bots et al. 2009) and *Calopteryx virgo* (Gallesi et al. 2016). For the butterfly *Melitaea cinxia*, sampled from five sites, thorax mass was larger in males sampled at sites with the most fragmentation (Norberg & Leimar 2002). Using geometric morphometrics, significant wing shape variations were detected in the damselfly *Calopteryx virgo* over different latitudes (Outomuro & Johansson 2011).

Studies have shown that variations in morphology can also be associated with insect behaviors, such as mating. Odonates have traditionally been used as models of sexual conflict, sperm competition, and sexual selection in relation to color polymorphisms. Their fidelity to territorial sites, diverse reproductive behavior, and phenotypic variation make them excellent systems for field studies (Bybee et al. 2016). Sexual dimorphism is consistent among damselfly species (Bots et al. 2009; Outomuro & Johansson 2011; Gallesi et al. 2016). In contrast, studies between mated and unmated damselflies have shown widely varying results. In *Lestes viridis*, one study found mated males were smaller than unmated males (DeBlock & Stoks 2007), while Swillen et al. (2009) found that mated males had larger thorax mass than unmated males. *Coenagrion scitulum* mated and unmated males varied significantly in only one trait tested, flight endurance (Therry et al. 2014). Likewise, *Coenagrion puella* mated and unmated males significantly differed in body fat, only one of several traits compared (Gyulavari et al. 2014). *Lestes sponsa* mated males had shorter, broader wings than unmated males (Outomuro et al. 2016). In a study with *Chalcolestes viridis*, flight endurance was consistently favored in mated males, while conflicting results were found between mated

and unmated males for body mass and wing shape (Gyulavari et al. 2017). Wickman (1992) found that flight design was related to mating system, but not to mating status, among species of butterflies.

Study Objectives

My overall objective was to compile a comprehensive study of environmental and behavioral effects on wing morphology using geometric morphometric techniques. My specific objectives were to 1) make comparisons a) between adults collected early in the season versus those collected late in the season, b) between adults collected at different locations, and c) among adults collected during several flight seasons at the same locations for *Argia sedula*, 2) to examine temporal effects on wing size and wing shape of both sexes in eight damselfly species, and 3) to examine wing morphologies associated with gender by comparing a) mated and unmated damselflies of both sexes from three species, and b) males and females from ten species. Specimens were field collected over multiple years at several locations to provide an extensive data set including wide representation of taxa for this study, as well as to provide the above mentioned variables to test.

Summary of Chapter Contents

This document contains five chapters, including this introductory chapter. Chapter Two, employs geometric morphometric techniques to present variations in wing morphology resulting from seasonal differences annual differences, and habitat differences in the damselfly *Argia sedula*. Chapter Two (Environmental Effects on Wing Shape and Wing Size of *Argia sedula* (Odonata: Coenagrionidae), S. Stewart wrote and D. Vodopich edited the manuscript, S. Stewart analyzed the data and performed work in

the laboratory, S. Stewart and D. Vodopich collected damselflies in the field and conceived the study. Chapter Three expands results from Chapter Two with samples from eight species to examine environmental effects on wing morphology resulting from differences between seasons and among years. Chapter Four examines behaviorally driven variations in wing morphology of ten damselfly species by comparing wings collected from mated and unmated damselflies and from males and females for evidence of sexual selection on wing shape and size. Chapter Five summarizes these studies and suggests future work to explain morphological variations by coupling ecological and genetic studies.

CHAPTER TWO

Environmental Effects on Wing Shape and Wing Size of *Argia sedula* (Odonata: Coenagrionidae)

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Introduction

Wing shape greatly influences flight performance, energetic costs, and maneuverability of flight (Gyulavári et al., 2017; Lee & Lin, 2012), all of which in turn affect fitness (Breuker, Gibbs, Van Dongen, Merckx, & Van Dyck, 2010; Outomuro, Adams, & Johansson, 2013). Behavioral activities such as avoiding predators, foraging, defending territories, mating, and locating oviposition sites depend on efficient flight (Westfall & May, 2006; Breuker et al., 2010; Outomuro et al., 2014). Thus, flight morphology in winged insects is likely under strong selection to optimize these activities specifically involving staying aloft, maneuverability, and thrust (Betts & Wootten, 1988; Dellicour et al., 2017). All of these actions depend on the shapes of individuals' bodies and wings (Sadeghi & Dumont, 2014).

Variations in morphologies result from developmental and environmental processes producing differences among individuals and populations (Hoffman, Collins, & Woods, 2002). Environmental conditions, as well as genetic make-up, affect and constrain these differences (Outomuro & Johansson, 2011). Selection may operate on plastic traits such as flight morphology necessary for survival and reproduction (Breuker et al., 2010). Wing shape results from the combined and sometimes conflicting action of

natural and sexual selection pressures (Outomuro, Bokma, & Johansson, 2012) and from structural and aerodynamic constraints (Outomuro et al., 2013).

Developmental conditions dictate much of the morphological variation in the adult stage. For example, adult body size and shape depend on nutrients available to larvae as well as local environmental conditions including temperature and water quality (Corbet, 1999; Lee & Lin, 2012). Organisms that inhabit different environments (aquatic and terrestrial) as larvae and adults must optimize responses to selection pressures operating in each environment (Bybee et al., 2016). Notably, populations of the same damselfly species can live and develop in a considerable range of habitats with quite varied biotic and abiotic factors (Gibbs, Wiklund, & Van Dyck, 2010). For example, developmental responses to local factors and geographic variation contributed to significant differences in wing shape among Eurasian populations of the damselfly, *Calopteryx splendens* (Sadeghi & Dumont, 2014). Differences in subtle natural selective pressures among habitats can cause observable variations in wing shape (Outomuro & Johansson, 2011). Plasticity of wing development is advantageous for species occupation of varied environments and development of optimal flight design for local conditions (Breuker et al., 2010; Gibbs, Wiklund, & Van Dyck, 2010).

Environmental conditions vary in time, (e.g. season) as well as in space (e.g. habitat) conditions. Varied seasonal conditions should promote varied optima for flight morphologies (Van Dyck & Wiklund, 2002). Variation in flight morphology through the flight season is expected since 1) constraints during development often influence body size and wing morphology (Gibbs et al., 2010, Van Dyck & Wiklund, 2002), and 2) flight morphology is plastic in response to environmental conditions such as developmental

temperature (Westfall & May, 2006; Bots et al., 2009). For example, developmental temperatures influenced complex changes in *Drosophila* body shape with low temperatures resulting in longer wings (Pétavy, Morin, Moreteau, & David, 1997). Cooler seasonal temperatures allow longer flight in the butterfly *Pararge aegeria* (Gibbs et al., 2010). Each seasonal cohort of *P. aegeria* experienced different local weather conditions during larval development, and responded with within-year variation in adult phenotypes (Van Dyck & Wiklund, 2002).

Habitat conditions change annually as well as on a more or less predictable seasonal basis. Life cycle stages influenced by environmental changes may initiate morphological adaptations (Gallesi, Mobili, Cigognini, Hardersen, & Sacchi, 2016). For example, the variable length of larval life in damselflies depends largely on temperature and photoperiod (Westfall & May, 2006). This temporal environmental heterogeneity supports flexible development to produce the most adaptive phenotypes possible within environmental constraints. For example, butterfly morphologies are highly sensitive to weather and climate, particularly rainfall levels (Gibbs et al., 2010). Changes in wing-aspect ratio of *Drosophila melanogaster* have been related to environmental conditions, especially temperature extremes (Hoffman et al., 2005). Selection may promote plasticity of flight morphology and ensure that the development of these traits is flexible enough for rapid responses to varying conditions across space and time (Breuker et al., 2010).

The purpose of this study is to examine the sensitivity of damselfly developmental plasticity to variations in location, seasonality, and year-to-year habitat conditions. We use landmark-based geometric morphometrics to quantify and analyze wing shape in the damselfly *Argia sedula* (Hagen, 1861). Wing shape, a multivariate trait, can be examined

using Procrustes techniques in geometric morphometrics, which removes variation in orientation, position and scale but preserves shape (Bots et al., 2012; Hoffman et al., 2002; Ren, Bai, Yang, Zhang & Ge, 2017). Correction for the effects of allometry was accomplished using the residuals from a regression of shape on centroid size in subsequent analyses (Klingenberg, 2010). We searched for the effects of habitat, seasonality, and annual variation on wing shape and wing size of this damselfly. Specifically, our comparisons included differences in wing shape and wing size for 1) adults developing and emerging from three habitat types, river, stream, and wetlands, 2) adults emerging early in the flight season versus those emerging late in the flight season, and 3) adults emerging in different years from the same habitats.

Materials and Methods

Field collections and morphometric measurements

Argia sedula, Coenagrionidae, a common damselfly in the United States, ranges from Florida north to Pennsylvania, west to California, and south into Mexico. This wide distribution covers the southern two-thirds of the United States (Abbott, 2005).

Populations inhabit rivers with gentle current, and streams as well as ponds, lakes, and wetlands. Males have a predominantly black abdomen with bright blue rings while the females are a light brown (Paulson, 2011). Their reproductive life averages four to five days with a maximum of two weeks (Westfall & May, 2006). Our extensive collections of *A. sedula* from 2009–2015 included 977 males and 449 females from five locations and provided a database with seasonal, habitat, and annual dimensions (Table 2.1).

Various subsets of this database were selected to test our hypotheses. Selections of these

subsets were primarily based on adequate sample sizes ($n \geq 30$) for the hypotheses being tested.

Table 2.1. Localities, years, and sample sizes collected for females and males of the damselfly *Argia sedula*.

	Year	Females (n)		Males (n)	
Falls on the Brazos (FOB)	2009	40		60	
	2014	Total	= 132	Total	= 283
	2014	Early	= 34	Early	= 79
	2014	Late	= 30	Late	= 72
Lake Waco Wetlands	2009	Total	= 136	Total	= 257
	2009	Early	= 36	Early	= 64
	2009	Late	= 62	Late	= 124
	2010	118		130	
	2011	56		114	
Harris Creek	2015	0		49	
Nolan Creek	2015	0		44	
Totals		482		937	

To examine variations among wing shapes and wing sizes of adults developing and emerging from aquatic locations and environments, collections from four sites in Bell, Falls, and McLennan counties, Texas, USA were used. Falls on the Brazos (FOB), a site on the Brazos River (Falls County, 31.25 N, 96.92 W), has a rocky bottom and is approximately 150 m wide. Shoreline depth is 0.5 m, and velocity is 5 m/sec. Nolan Creek (Bell County, 31.04 N, 97.44 W) is approximately 25 m wide, shoreline depth 0.3 m, and velocity 2 m/sec. Harris Creek (McLennan County, 31.46 N, 97.29 W) has a width of approximately 8 m, a shoreline depth of 0.25 m, and intermittent flow of 0-1 m/sec. The Lake Waco Wetlands (McLennan County, 31.61 N, 97.30 W) covers 75

hectares with a residence time of eight days. Water flows through the Wetlands from the North Bosque River into Lake Waco.

To examine annual variations among wing shapes and wing sizes of adults collected from the same environment but different years, data subsets were used for each year from 2009-2011, and 2014-2015 collections.

To examine variations in wing shape and wing sizes of adults emerging early versus late in their flight season, data subsets from 2009 and 2014 and from two contrasting habitats were used. Early flight season (May-June) included adults whose larval development was primarily over winter. Late flight season (August-October) included adults whose larvae developed primarily in spring and summer temperatures.

Damselflies were netted, transported to the laboratory, killed using ethyl acetate, and weighed. Wings were spread and scanned at 800 dpi. Damaged wings were excluded from our analyses. To obtain shape data, sixteen homologous landmarks were designated on scans of the right forewings and digitized using tps.Dig2 software (Rohlf, 2015a) (Fig. 2.1). Landmarks were either at vein intersections or where veins reached the wing edge. Reliable and consistent landmarks were located on all wing images (McTavish, Smith, Guerrero, & Gering, 2012). Wing size was quantified as centroid size, which is the square root of the sum of the squared distances of the set of landmarks from their centroid (Outomuro et al., 2012). Wing size commonly and reliably estimates body size (e.g. abdomen length) (Outomuro & Johansson, 2011; Sadeghi, Adriaens, & Dumont, 2009). To evaluate measurement error, repeat measurements were taken for a subset of wings, and a Procrustes ANOVA was performed using MorphoJ software (Klingenberg, 2011). Results indicated that measurement error was not significant.

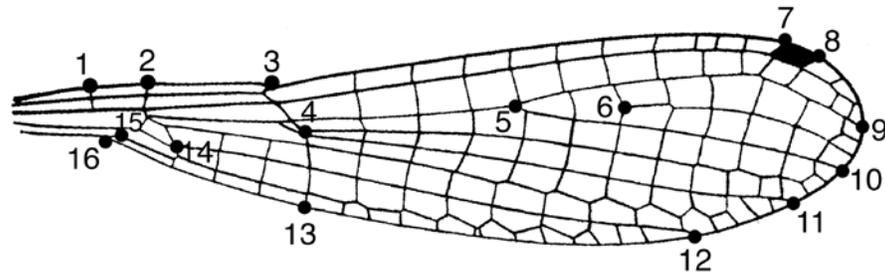


Figure 2.1. Right forewing of *Argia sedula* with landmark positions defining wing shape. All landmarks were consistent for both sexes. Modified from Westfall & May 2006.

Data analysis

Wing shape variations were analyzed using geometric morphometrics which rotates, translates, and scales landmark configurations into alignment via generalized least squares Procrustes superimposition (Klingenberg & McIntyre, 1998). Procrustes fit removes variation in scale, position, and orientation, but preserves shape (Outomuro & Johansson, 2011). To remove the allometric component of wing shape, a multivariate linear regression of the coordinates of the aligned configurations on centroid size was performed using MorphoJ (Klingenberg, 2011). Further analyses employed residuals from the regressions as the size-corrected shape variables (Klingenberg & McIntyre, 1998; Outomuro et al, 2013). Analyses were performed only on right forewings.

Given the unknown distribution of Procrustes coordinate data, we used Permutation Multivariate Analysis of Variance (PERMANOVA) with 10,000 random samples on size-corrected shape variables to assess F-ratio significance for variance

differences (Hoffmann et al., 2002; Slice, 2007). P-values were calculated from Wilks' lambda statistics. Comparisons of shape data were conducted using PAST software (Hammer, Harper, & Ryan, 2007) to assess whether differences in wing shape were associated with sampling location, seasonality, or annual variation (McTavish et al., 2012).

Principal components reduce data by summarizing multidimensional variation effectively in fewer dimensions (Klingenberg & McIntyre, 1998). For meaningful analyses, the first few principal components (PCs) should account for most of the total variation contained in a dataset. PCA of the size-corrected shape variables was conducted with MorphoJ (Klingenberg, 2011), which displayed patterns of variation as scatterplots and identified landmarks with the highest loading coefficients for positions most likely responsible for shape differences (Klingenberg & McIntyre, 1998). To visualize wing shape variations between groups, tps.Spln software (Rolf, 2004) was used to create deformation grids comparing the mean shape of the group with the mean for the merged groups (Outomuro & Johansson, 2011).

Mean centroid size of wings was calculated for each population to assess variation in overall wing size, and therefore overall body size. Correlation between wing centroid size and body size is quite high (Outomuro & Johansson, 2011). Centroid sizes were calculated using tpsRelw software (Rohlf, 2015b). A one-way ANOVA conducted with PAST tested significance of centroid size differences among populations, and was followed by Tukey's post hoc test to define pairwise differences (Hammer et al., 2007). Homogeneity of the variance and normality were tested by Levene's test and Shapiro-Wilk test, respectively (Sadeghi & Dumont, 2014).

Results

Early versus late flight seasons

Wing shape

Significant differences in wing shape were consistently found in all comparisons made between early emerging populations and late emerging populations. Wing shape varied significantly for early versus late flight seasons for compared populations in two years (2009, 2014), in two environments (Wetlands, FOB), and for both sexes. Other years and locations in our database had inadequate sample sizes ($n < 30$) for testing between early and late seasons. PERMANOVA on the size-corrected shape variables indicated that wing shapes of females from Wetlands 2009 ($F = 2.66$, $p = 0.03$) and FOB 2014 ($F = 9.17$, $p < 0.001$) varied significantly between early versus late seasons. Similarly, for males, wing shapes of adults from Wetlands 2009, ($F = 4.84$, $p < 0.001$) and FOB 2014 ($F = 17.52$, $p < 0.001$) varied significantly between early versus late flight seasons.

PCA on size-corrected shape variables indicated that the first two PCs accounted for 69.5% (females, 2009), 56% (males, 2009), 62.5% (females, 2014), and 54% (males, 2014) of the total variation in each comparison. Scatterplots visualized variation along the first two PCs for the comparisons between early and late season wing shapes (Fig.2.2). Deformation grids visualized the mean wing shape for each group (Fig. 2.3).

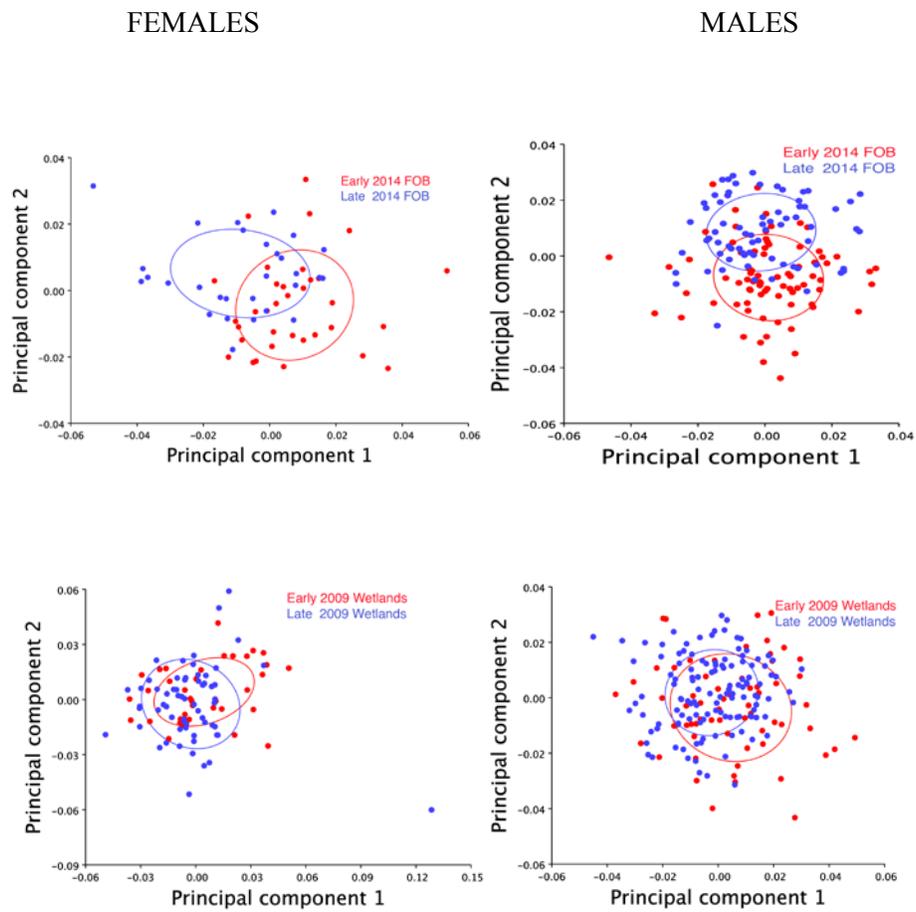


Figure 2.2. Scatterplots from PCA comparing wing shapes of females and of males collected early in the flight season with those collected late in the flight season in Wetlands 2009 and FOB 2014. Ellipses represent 50% frequency.

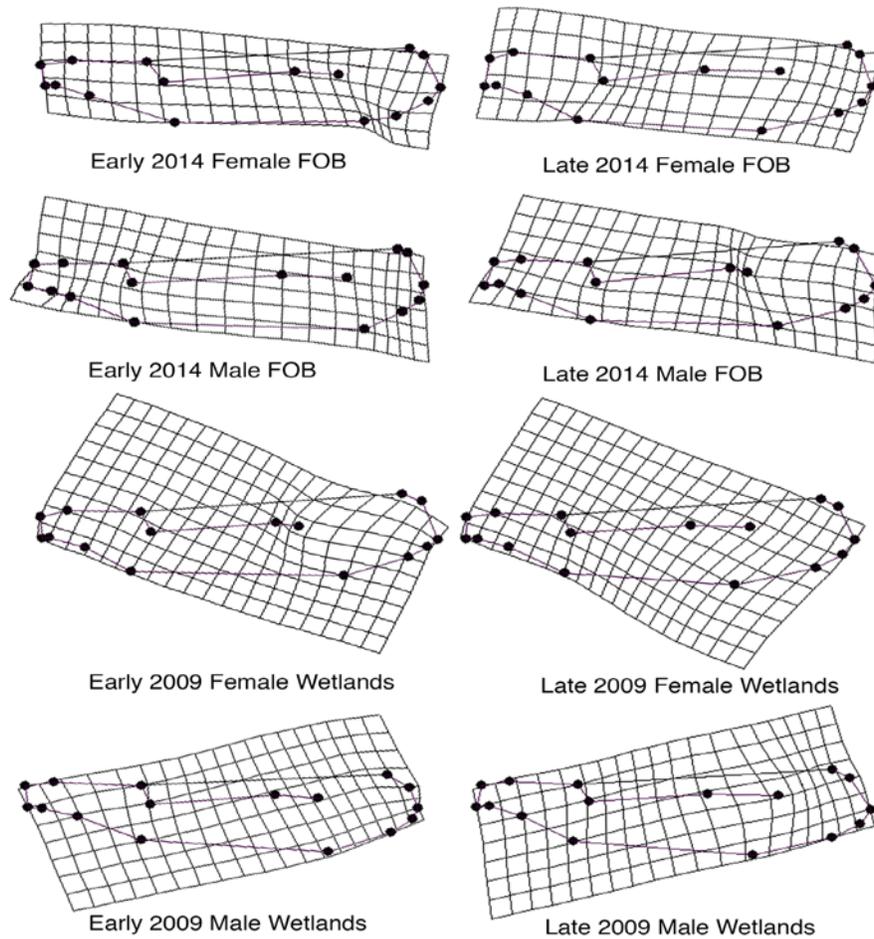


Figure 2.3. Variation of wing shape between females and males collected early versus those collected late in the flight seasons of Wetlands 2009 and FOB 2014. The deformation grids indicate the mean shape of each group in relation to the overall mean of the groups being compared. Minimal increases in factor level were occasionally used to highlight differences.

Centroid size

Centroid sizes as measures of overall wing sizes differed significantly for adults from early versus late flight seasons for populations compared from two years (2009, 2014), in two environments (Wetlands, FOB), and both sexes. For both males and females, wings of early emergers were larger than wings of late emergers. ANOVA indicated that wings of females sampled early season (centroid $\bar{x} = 893.27$) were

significantly larger than wings of females sampled late season (centroid $\bar{x} = 854.42$) from Wetlands 2009 ($F = 12.28$, $p < 0.001$). Wings of females sampled early season (centroid $\bar{x} = 944.72$) were significantly larger than wings of females sampled late season (centroid $\bar{x} = 848.99$) from FOB 2014 ($F = 13.61$, $p < 0.001$) (Fig. 2.4).

Wings of males sampled early season (centroid $\bar{x} = 832.98$) were significantly larger than wings of males sampled late season (centroid $\bar{x} = 805.03$) from Wetlands 2009 ($F = 27.20$, $p < 0.001$). Wings of males sampled early season (centroid $\bar{x} = 909.48$) were significantly larger than wings of males sampled late season (centroid $\bar{x} = 801.55$) from FOB 2014 ($F = 76.27$, $p < 0.001$) (Fig. 2.4).

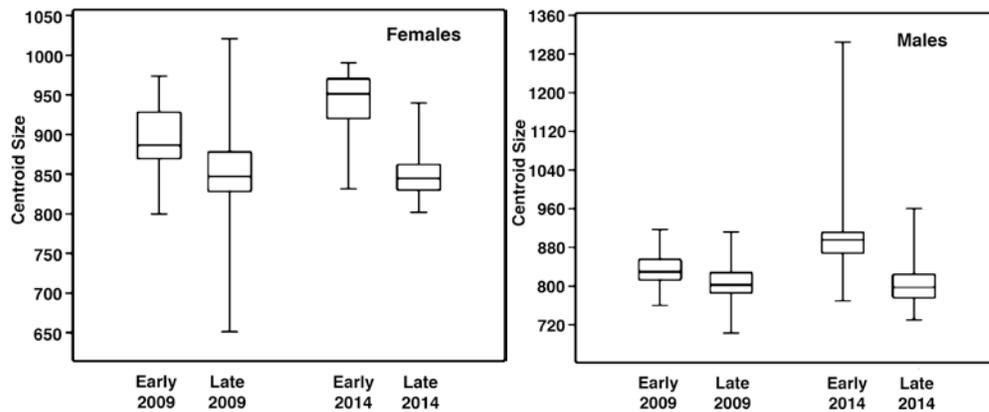


Figure 2.4. Mean centroid sizes of females and males collected early and those collected late in the flight seasons of Wetlands 2009 and FOB 2014. Note difference in y-axes.

Variation among habitat types

Wing shape

Variation in wing shape was between female populations sampled from two locations (Wetlands, FOB), and between male populations sampled from four locations (Wetlands, FOB and Harris Creek, Nolan Creek). No significant differences were found

between any locations sampled for both males and females. PERMANOVA on the size-corrected shape variables indicated that wing shapes of females from Wetlands 2009 did not differ significantly from wing shapes of females from FOB 2009 ($F = 2.27$, $p = 0.057$).

Wing shapes of males sampled at Wetlands 2009 did not differ significantly from those sampled at FOB 2009 ($F = 1.19$, $p = 0.29$). Likewise, wing shapes of males sampled at Harris Creek 2015 did not differ significantly from those of males sampled at the similar stream habitat Nolan Creek 2015 ($F = 0.70$, $p = 0.64$).

The first two components of the PC analysis of size-corrected shape variables accounted for 57% of the total variation in each of the comparisons between locations. Variation along the first two PCs was shown in scatterplots for each comparison of locations (Fig. 2.5). Deformation grids visualized the mean wing shape for each group (Fig. 2.6).

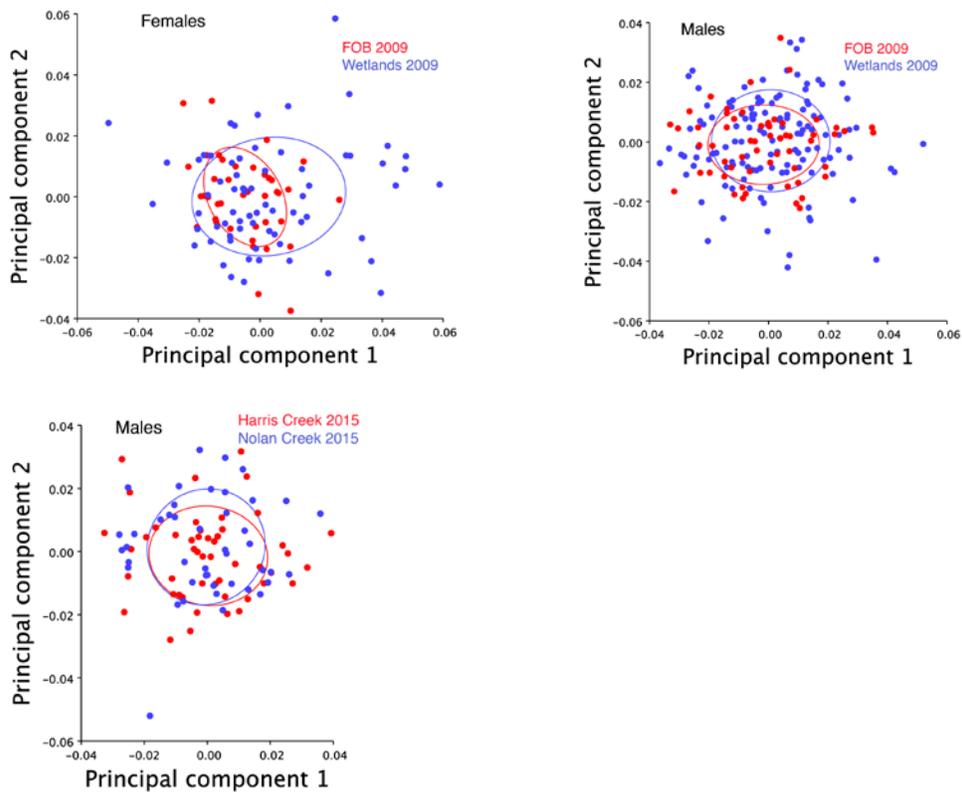


Figure 2.5. Scatterplots from PCA comparing wing shapes of females and males collected at Wetlands 2009 and FOB 2009; and males collected in Harris Creek 2015 and Nolan Creek 2015. Ellipses represent 50% frequency.

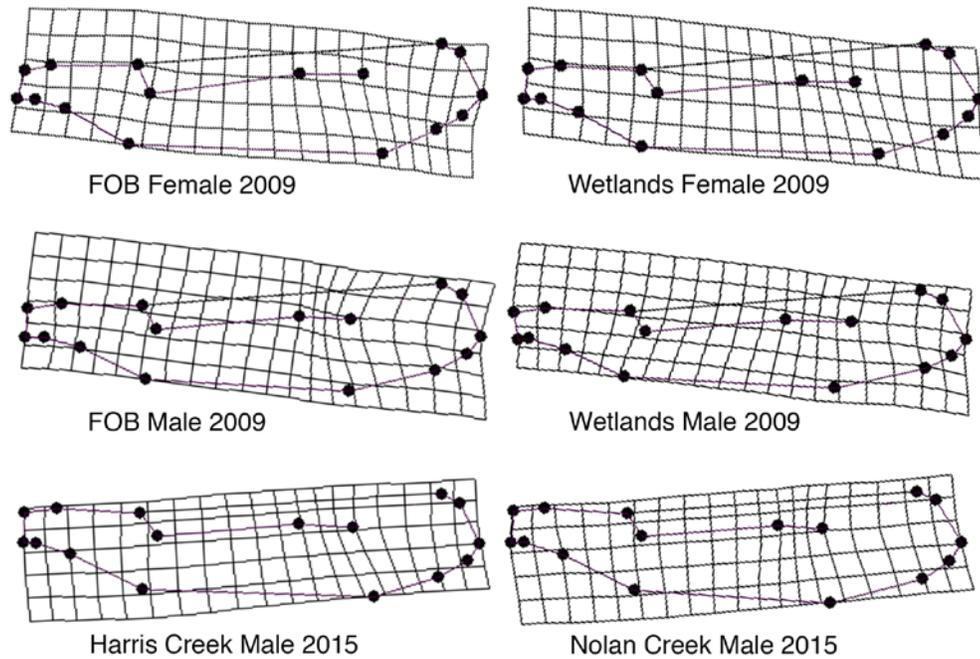


Figure 2.6. Variation of wing shape between females and males collected at Wetlands 2009 and FOB 2009; and males collected at Harris Creek 2015 and Nolan Creek 2015. The deformation grids indicate the mean shape of each group in relation to the overall mean of the groups being compared. Minimal increases in factor level were occasionally used to highlight differences.

Centroid size

Differences in wing centroid sizes were compared for females sampled from two locations (Wetlands, FOB) and for males sampled from four locations (Wetlands, FOB, and Harris Creek, Nolan Creek). Significant differences were found only in the male populations collected in 2009, with males sampled from the Wetlands larger than those sampled at FOB. ANOVA indicated that wing centroid sizes of females from Wetlands 2009 (centroid $\bar{x} = 878.28$) were not significantly different from those from FOB 2009 (centroid $\bar{x} = 869.66$) ($F = 0.666$, $p = 0.416$) (Fig. 2.7). Centroid sizes of males from

Wetlands 2009 (centroid $\bar{x} = 826.52$) were significantly larger than that of males from FOB 2009 (centroid $\bar{x} = 802.88$) ($F = 15.12$, $p < 0.001$) (Fig. 2.7). Wing centroid sizes of males from Harris Creek 2015 (centroid $\bar{x} = 831.00$) were not significantly different from those of males from Nolan Creek 2015 (centroid $\bar{x} = 838.31$) ($F = 1.19$, $p = 0.278$).

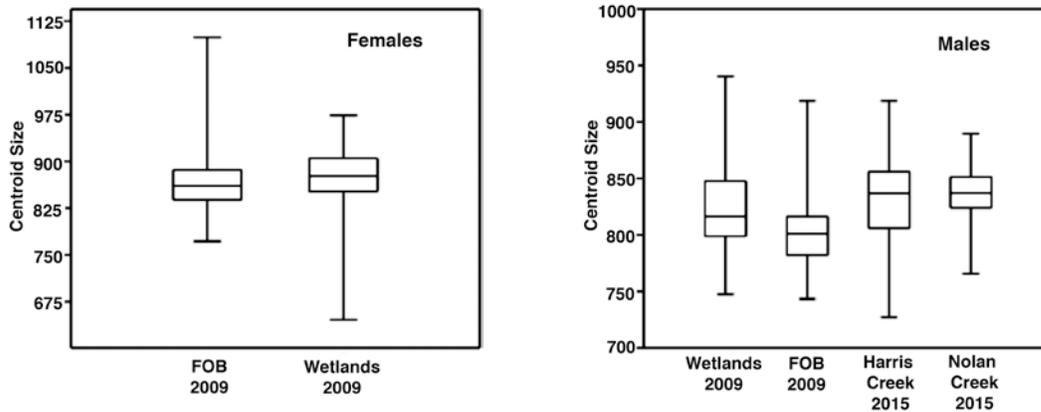


Figure 2.7. Mean centroid sizes of females and males collected in Wetlands 2009 and FOB 2009; and of males collected at Harris Creek 2015 and Nolan Creek 2015. Note difference in y-axes.

Annual variation

Wing shape

Variation in wing shape for females and males was examined for collections from 2009 and 2014 at FOB and from 2009, 2010, and 2011 at the Wetlands. Significant differences in shape were found in comparisons between male populations and between female populations sampled from FOB and from the Wetlands. For samples from FOB 2009 versus FOB 2014, PERMANOVA on the size-corrected shape variables revealed significant differences between wing shapes for females ($F = 2.76$, $p = 0.02$) and for males ($F = 5.83$, $p < 0.001$).

Females and males were also sampled from Wetlands in consecutive years 2009, 2010, and 2011. Mean wing shapes for females were significantly different among years (PERMANOVA, $F = 4.33$, $p < 0.001$), as were wing shapes for males (PERMANOVA, $F = 8.36$, $p < 0.001$). In pairwise comparisons between years, female wing shapes were significantly different between 2009, 2010 ($p < 0.001$) and 2010, 2011 ($p < 0.001$), but not significantly different between 2009, 2011 ($p = 0.09$). Wing shapes of males showed a similar pattern with significant differences between years 2009, 2010 ($p < 0.01$) and 2010, 2011 ($p < 0.01$), and no significant differences between years 2009, 2011 ($p = 0.08$).

PCA on size-corrected shape variables showed that the first two PCs accounted for 54% (females, 2009 and 2014), and 56% (males, 2009 and 2014) of the total variation in wing shape. The first two PCs accounted for 60% (females, 2009 and 2010), 51% (females, 2010 and 2011), 66% (females, 2009 and 2011) and 57.5% (males, 2009 and 2010), 58% (males, 2010 and 2011), 54% (males, 2009 and 2011) of the total variation in wing shape. Scatterplots illustrate variation along PC1 and PC2 (Fig. 2.8). Deformation grids visualize the mean wing shape for each group (Fig. 2.9), (Fig. 2.10).

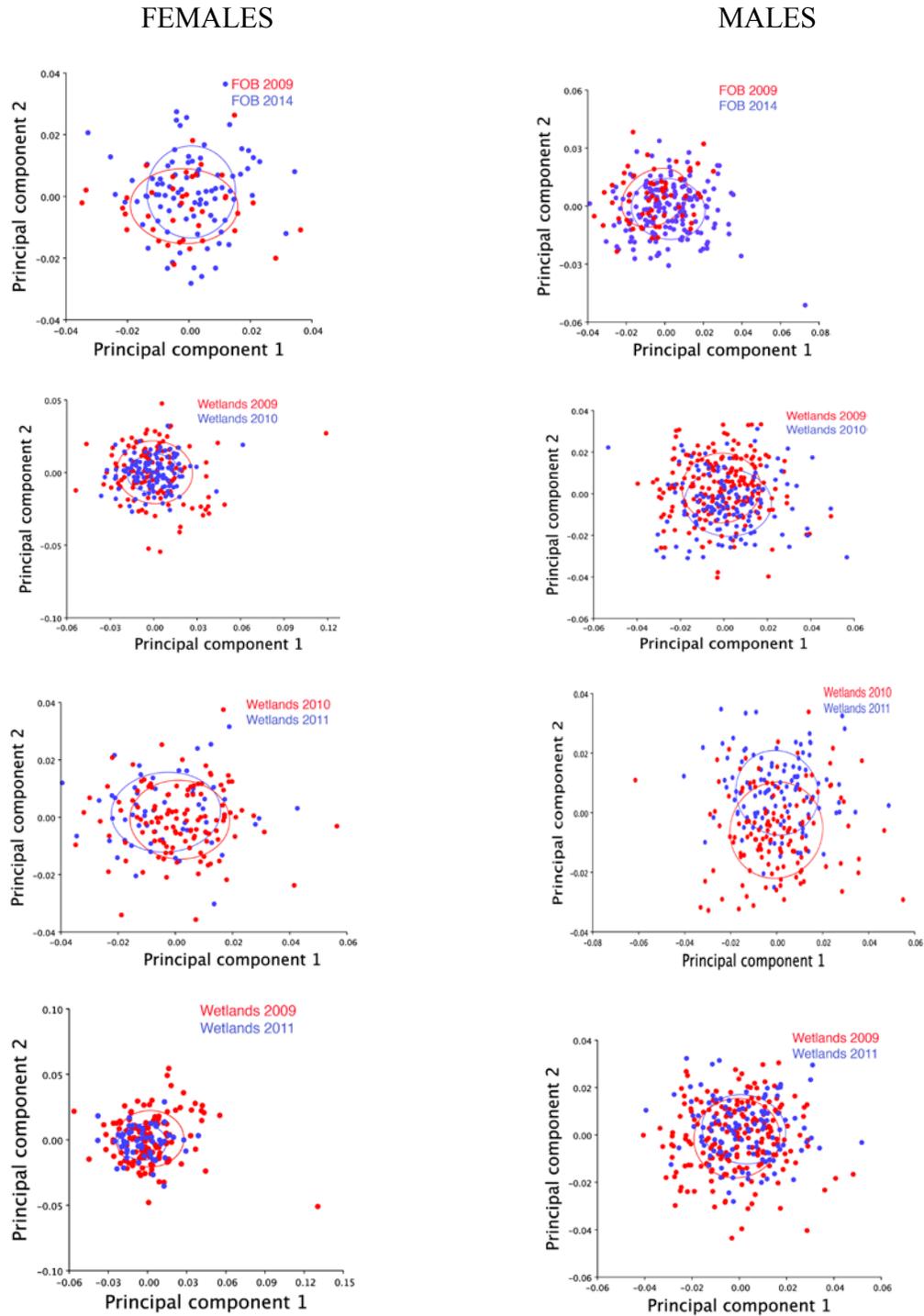


Figure 2.8. Scatterplots from PCA comparing wing shapes of females and males collected at Wetlands 2009, 2010 and 2011; and FOB 2009, 2014. Ellipses represent 50% frequency.

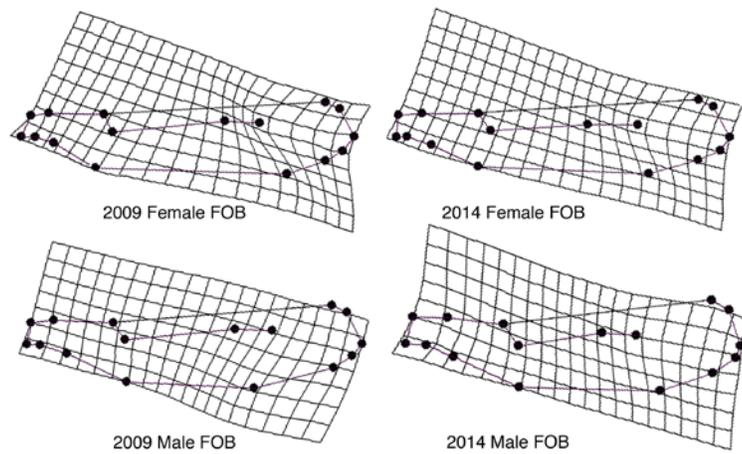


Figure 2.9. Variation of wing shapes between females and males collected at FOB 2009 and 2014. The deformation grids indicate the mean shape of each group in relation to the overall mean of the groups being compared. Minimal increases in factor level were occasionally used to highlight differences.

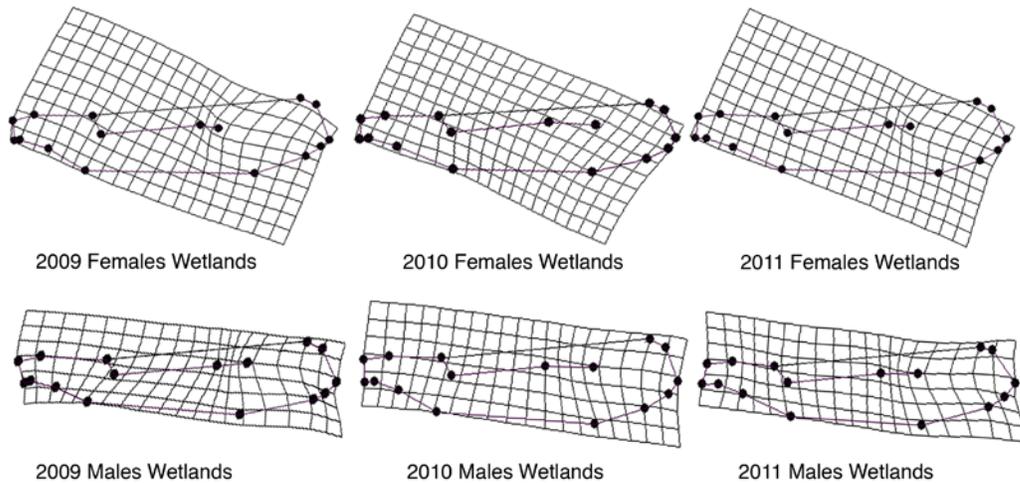


Figure 2.10. Variation of wing shapes between females and males collected at Wetlands 2009, 2010, and 2011. The deformation grids indicate the mean shape of each group in relation to the overall mean of the groups being compared. Minimal increases in factor level were occasionally used to highlight differences.

Centroid size

Annual differences in wing centroid sizes for females and for males were examined for collections from four years (2009, 2010, 2011, 2014). Significant size differences were found between male populations collected at FOB and between those collected at the Wetlands. Wing sizes for the female comparison from FOB were significantly different, as were two of the three female comparisons made at the Wetlands. ANOVA indicated that wing centroid sizes of females from FOB 2009 (centroid \bar{x} = 869.66) were significantly smaller than those for females from FOB 2014 (centroid \bar{x} = 904.32) ($F = 12.61$, $p < 0.001$). Wing centroid sizes for males from FOB 2009 (centroid \bar{x} = 802.88) were significantly smaller than those of males from FOB 2014 (centroid \bar{x} = 868.51) ($F = 38.70$, $p < 0.001$).

ANOVA indicated significant differences among years for centroid sizes of females from Wetlands 2009, 2010 and 2011 ($F = 77.42$, $p < 0.001$). Pair-wise comparisons indicated significant differences in centroid sizes for females from 2009 versus 2010 (Tukey's $Q = 14.35$, $p < 0.001$) and from 2010 versus 2011 (Tukey's $Q = 12.03$, $p < 0.001$). However, centroid sizes for females from 2009 versus 2011 were not significantly different (Tukey's $Q = 2.32$, $p = 0.228$). For these female populations, wing sizes were largest in 2010 (centroid \bar{x} = 951.97), compared to 2011 (centroid \bar{x} = 880.53) and 2009 (centroid \bar{x} = 866.74) (Fig. 2.11).

ANOVA indicated significant differences among years for centroid sizes for males from Wetlands 2009, 2010 and 2011 ($F = 105.20$, $p < 0.001$). Pair-wise comparisons also indicated significant differences for males from 2009 versus 2010

(Tukey's $Q = 10.87$, $p < 0.001$), 2009 versus 2011 (Tukey's $Q = 11.21$, $p < 0.001$), and 2010 versus 2011 (Tukey's $Q = 22.08$, $p < 0.001$). For these male populations wing sizes were largest in 2010 (centroid $\bar{x} = 855.88$) compared to wing sizes in 2009 (centroid $\bar{x} = 818.10$) and in 2011 (centroid $\bar{x} = 779.12$) (Fig. 2.11).

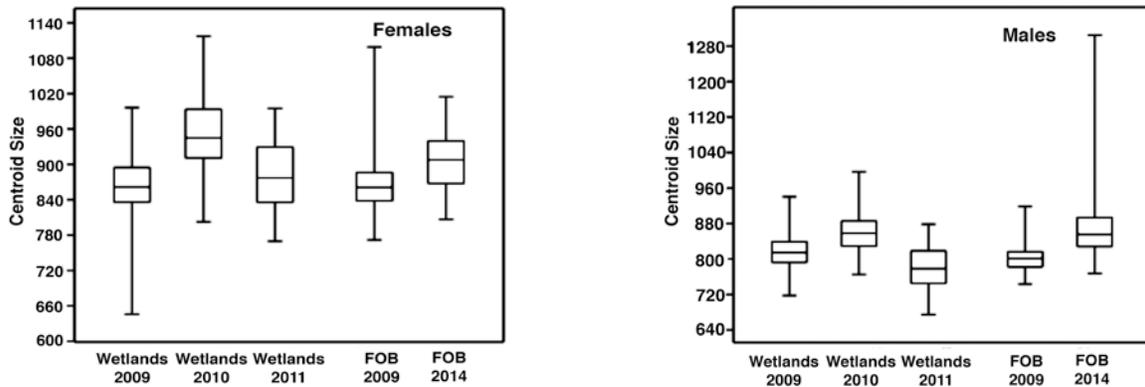


Figure 2.11. Mean centroid sizes of females and males collected at Wetlands 2009, 2010, and 2011 and at FOB 2009, 2014. Note difference in y-axes.

Discussion

Environmental conditions affect morphology of organisms (Outomuro & Johansson, 2011), as genetically similar organisms developing in different conditions may display quite different characteristics (Stearns, 1989). The morphology of insect wings can largely determine the energetic costs and maneuverability of flight (Betts & Wootton, 1988). This study examined the effects of environmental conditions on wing shape and wing size in the damselfly *A. sedula*. We examined these morphologies of males and females emerging early versus late in the flight season, between damselfly populations from different locations in the same year, and among damselflies emerging from the same location but in different years. Wing shape and wing size varied

significantly in most comparisons, suggesting that environmental factors consistently influence wing shape and body size. The frequency of wing shape and wing size differences supports the concept that whole body morphology is a thorough integration of all components of size and shape.

Comparisons of early versus late emerging damselflies showed consistent differences for both wing shape and wing size for all comparisons. Wing shape and wing size, as components of flight morphology, have shown seasonal variation in other studies (Pétavy et al., 1997; Norberg & Leimar, 2002; Debat et al., 2003; Bots et al., 2009; Klingenberg, 2013). In our study, examination of deformation grids revealed that both male and female populations collected at the Wetlands in 2009 and at FOB in 2014 had wider wings late in the season, which would aid maneuverability (Betts & Wootton, 1988). Internal landmark (6) was also frequently variable, and likely contributes to wing stability (Bots et al., 2009).

Wing centroid sizes, and therefore body sizes, were larger for adults collected in early flight season for all years, locations, and both sexes. Other studies have noted similar results for wing size variation (Norberg & Leimar, 2002; Bots et al., 2009). Insects emerging early in the flight season and therefore developing in colder temperatures are frequently larger than those emerging late in the season after developing in warmer temperatures (Norberg & Leimar, 2002; Bots et al., 2009).

Unexpectedly, wing shape for females and for males was not sensitive to environmental differences between the Wetlands and the riverine FOB. Wing sizes also were not significantly different between female populations. However, wing size was larger for males developing at the Wetlands. Interestingly, the body sizes of the most

common species of the damselfly community at the Wetlands are smaller (e.g. *Enallagma spp.*, *Ischnura spp.*) than those dominant species at the FOB community (*Hetaerina spp.*, *Argia spp.*). This difference could be influenced by interspecific interactions. Life histories differ for the territorial *Argia sedula*, which results in different flight patterns for males and females. The observed differences in wing sizes between males but not females might be explained by male territorial behavior. Water discharge and velocity vary greatly between these two locations, with greater discharge and velocity at the riverine FOB. These differences may have contributed to significant wing size variation in males at the contrasting environments. As expected, wing shape and wing size of males collected from Nolan Creek and Harris Creek also did not differ significantly for these similar stream habitats.

Annual differences in wing shape occurred for most populations compared between two years. Norberg & Leimar, (2002) found that annual variations in environmental influences on insect morphology were considerable. Damselflies were collected for three years at the Wetlands (2009-2011) and for two years at FOB (2009, 2014). All male and female populations from both locations showed considerable annual variation, especially in landmark (12) on the lower edge of the wing affecting wing width. Variation in all populations was also notable for internal landmark (6) which likely influences wing stability. In males and females, wing sizes differed significantly among Wetland collections from all three years with largest wings in 2010. At FOB, wing sizes of both males and females varied significantly with larger wings occurring in 2014. Water levels at the Wetlands varied considerably over these three years with the draining of the Wetlands in July 2010 and drought conditions in 2011. Water regime of

the area they inhabit is an environmental factor of primary importance. Their abundance can depend directly on water level and decrease as this level drops (Popova, Haritonov, & Erdakov, 2016).

Wing shape evolution and adaptation involves environmental pressures that optimize efficiency (Outomuro et al., 2013). Our research indicates that significant morphological variation resides in the sensitivity of wing shape development to annual and seasonal variation. Habitat influences on wing shape and body size were not as pronounced as were annual and seasonal differences. The concept that insects embody a rigid, purely deterministic shape should not be assumed. Significant plasticity expressed during development occurs in the damselfly genome. Further research should document the precise relationships between habitat parameters and measurable morphological features.

CHAPTER THREE

Seasonal and Annual Variations on Wing Shape and Wing Size of Eight Damselfly Species (Odonata: Zygoptera)

Introduction

Flight is a key adaptation in winged animals. Wing shape is essential to flight performance, especially maneuverability, agility, lift and thrust production (Bots et al. 2012). The evolution of wing shape, although constrained by aerodynamic limitations, is influenced by selection operating on organismal performance including migration, dispersal, foraging, predator avoidance, specific gender strategies as well as sexual selection (Breuker et al. 2007; Bots et al. 2009; Breuker et al. 2010; Outomuro et al. 2016; Dellicour et al. 2017). Variations in body shape have important fitness consequences, so we can expect environmental selection to act on wing phenotype (Sadeghi et al. 2009).

Wing shape evolves in response to multiple selective pressures optimizing performance under a set of costs and benefits (Outomuro & Johansson 2011; Outomuro et al. 2013). Environmental pressures (e. g. habitat, weather, seasons) especially during larval development are often experienced by organisms over time. These changes may occur either suddenly (e.g. flood), progressively (e.g. climate change), or predictably (e.g. seasonal variation). This temporal environmental heterogeneity likely necessitates flexibility during development in order to produce the appropriate phenotype (Gibbs et al. 2010). Development that flexibly and adaptively responds to environmental variability has been labeled developmental plasticity and the resulting phenotypes display

phenotypic plasticity (Breuker et al. 2010). For example, wing sizes and shapes may vary across years or across a single season (Nylin & Gotthard 1998).

Body size in Odonata shows a strong seasonal pattern (Corbet 1999). Seasonal variation in flight morphology can be expected since insect body size typically declines during the season in response to development time constraints and variations in environmental conditions (e.g. temperature)(Rundle et al. 2007). The adult phenotype of the butterfly *Pararge aegeria* changed in response to local temporal fluctuations in annual temperature and rainfall from 1989 to 1999 (Gibbs et al. 2010). Similarly, seasonal patterns were found for *P. aegeria* following three developmental pathways (pupal winter diapause, larval winter diapause and no winter diapause). These developmental pathways produced different flight morphologies (Van Dyck & Wiklund 2002). *Drosophila simulans*, when exposed to increased temperatures, decreased in size and differed in wing shape (Debat et al. 2003), while wing shape of the damselfly *Calopteryx splendens* varied from early to late season (Gallesi et al. 2016).

Geometric morphometrics is a powerful tool to evaluate correlations between shape and environmental variables. Insect wings are an excellent study system because they are essentially two-dimensional and the homologous wing veins provide well-defined morphological landmarks (Klingenberg & McIntyre 1998). Geometric morphometrics describes shape by examining information from multiple homologous landmarks across the wing to detect more subtle variation than that provided by conventional linear measurements (Bots et al. 2012). Geometric morphometrics can detect subtle shape changes resulting from environmental stresses (e.g. pesticides) (Hoffman et al. 2002; Hoffman et al. 2005), from different locations (Rundle et al. 2007;

Outomuro & Johansson 2011; McTavish et al. 2012), and among subspecies (Sadeghi et al. 2009; Yee et al. 2009; Lee & Lin 2012).

In this study we explored the extent of plasticity over seasons and among years by using landmark-based geometric morphometrics to quantify and analyze wing shape in eight species of damselflies, including *Hetaerina americana* (Fabricius, 1798), *Argia moesta* (Hagen, 1861), *Argia nahuana* (Calvert, 1902), *Argia sedula* (Hagen, 1861), *Enallagma basidens* (Calvert, 1902), *Enallagma civile* (Hagen, 1861), *Ischnura hastata* (Say, 1839) and *Ischnura posita* (Hagen, 1861). Wing shape, a multivariate trait, can be examined using Procrustes techniques in geometric morphometrics to remove variation in orientation, position, and scale while preserving shape (Hoffman et al. 2002; Bots et al. 2012; Ren et al. 2017). Correction for the effects of allometry is accomplished using residuals from a regression of shape on centroid size (Klingenberg 2010). We searched for the effects of seasonality and annual variation on wing shape and wing size of adults from eight damselfly species. Specifically, our comparisons searched for differences in wing shape and wing size for 1) adults emerging early in the flight season versus those emerging late in the flight season, and 2) adults emerging in different years from the same habitats. Our results describe the extent of developmental plasticity across seasons and among years.

Materials and Methods

Field Collections and Morphometric Measurements

This study included damselflies from eight, broadly distributed species of four genera in two families. Calopterygidae was represented by *Hetaerina americana* (Fabricius, 1798). Specimens of Coenagrionidae included *Argia moesta* (Hagen, 1861),

Argia nahuana (Calvert, 1902), *Argia sedula* (Hagen, 1861), *Enallagma basidens* (Calvert, 1902), *Enallagma civile* (Hagen, 1861), *Ischnura hastata* (Say, 1839) and *Ischnura posita* (Hagen, 1861). Habitats for these eight species can be broadly divided into streams and ponds. Stream and river species include *H. americana*, *A. moesta*, *A. nahuana*, and *A. sedula*, while ponds and ditches are preferred by *E. basidens*, *E. civile*, *I. hastata* and *I. posita*. The geographical ranges for *H. americana*, *A. sedula* and *E. civile* are the largest and extend east and west across the United States from southern Canada into Mexico. *A. moesta*, *E. basidens*, *I. posita* and *I. hastata* have more eastern ranges and extend from Ontario to Florida, east to South Dakota, and south to Mexico, including southern California, Arizona and New Mexico. *A. nahuana* ranges more westerly and extends from Oregon south to California and east to Texas, Oklahoma and Mexico (Westfall & May 2006).

To examine variations among wing shapes and wing sizes of adults developing and emerging from varied aquatic environments, collections were made from two sites in Falls and McLennan counties, Texas, USA. Falls on the Brazos (FOB), a site on the Brazos River (Falls County, 31.25 N, 96.92 W), has a rocky bottom and is approximately 150 m wide. Shoreline depth is 0.5 m, and velocity is 5 m sec⁻¹. The Lake Waco Wetlands (McLennan County, 31.61 N, 97.30 W) covers 75 hectares with a residence time of eight days. Water flows slowly through the Wetlands from the North Bosque River into Lake Waco.

To examine variations in wing shape and wing sizes, collections were made of adults emerging early versus late in their flight season. Early flight season emergers (April-June) included adults whose larvae developed primarily over winter. Late flight

season (August-October) included adults whose larvae developed primarily at spring and summer temperatures. Males of *A. nahuana* and *E. civile* were collected in 2009 at the Wetlands. *H. americana* males were collected in 2009, 2013, and 2014 at FOB. Males and females of *A. moesta* were collected in 2017 at FOB.

To examine annual differences among wing shapes and wing sizes, adults were collected from the same environment but during different years. From FOB, males and females of *H. americana* were collected in 2009, 2013, and 2014, and males of *A. sedula* were collected in 2009, 2014 and 2017. At the Wetlands, males and females of *E. basidens* were collected in 2009, 2010, and 2011, and males of *I. hastata* and *I. posita* were collected in 2009 and 2010.

Damselflies were collected with sweep nets, transported to the laboratory, killed using ethyl acetate, and weighed. Wings were spread and scanned at 800 dpi on a flatbed scanner. Damaged wings were not included in the analyses. Homologous landmarks, sixteen for Coenagrionidae and thirteen for Calopterygidae, were designated where veins reached the wing edge, or at vein intersections in the central section of the wing (Figure 1). Scans of right forewings were digitized with tps.Dig2 software (Rohlf 2015a) using these reliable and consistent landmarks to capture wing shape (Outomuro et al. 2013; McTavish et al. 2012). Wing size was quantified as centroid size, which is the square root of the sum of the squared distances between each landmark and the specimen centroid (Outomuro et al. 2012; Gallesi et al. 2016). Wing size commonly and reliably estimates body size (e.g. abdomen length) (Sadeghi et al. 2009; Outomuro & Johansson 2011; Yazdi 2014). To evaluate measurement error, repeat measurements were taken for

a subset of wings, and a Procrustes ANOVA was performed using MorphoJ software (Klingenberg 2011). Measurement error was not significant.

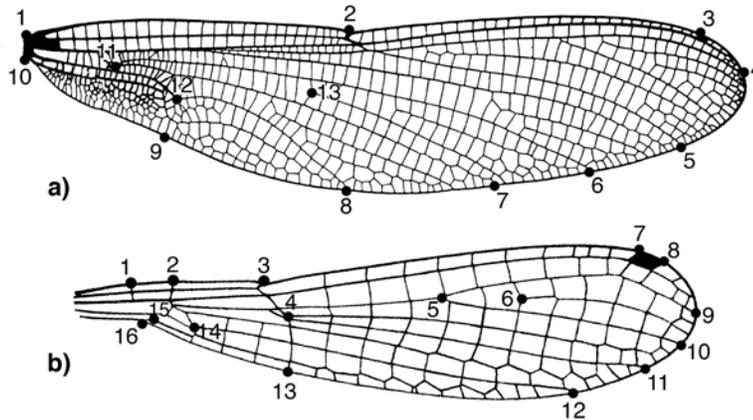


Figure 3.1. Right forewing of a) *H. americana*, Calopterygidae and b) Coenagrionidae with landmark positions defining wing shape. All landmarks were consistent for both sexes. Modified from Westfall & May 2006.

Data Analysis

Geometric morphometrics quantifies wing shape based on landmark coordinates (Outomuro et al. 2013). Procrustes superimposition rotates, translates, and scales landmark configurations into alignment via generalized least squares (Klingenberg & McIntyre 1998; Bots et al. 2012). This superimposition eliminates variation in scale, position, and orientation, but preserves shape (Outomuro & Johansson 2011). Multivariate linear regression of the aligned Procrustes coordinates on centroid size was performed using MorphoJ (Klingenberg 2011) to remove the allometric component of wing shape (Lorenz et al. 2017). Residuals from these regressions were used in subsequent analyses as size-corrected shape variables (Klingenberg & McIntyre 1998; Outomuro et al. 2013; Gyulavari et al. 2017). Analyses were performed only on right forewings.

Given the unknown distribution of Procrustes coordinate data; we used Permutation Multivariate Analysis of Variance (PERMANOVA) with 10,000 random samples on size-corrected shape variables to assess F-ratio significance for variance differences (Hoffmann et al. 2002; Slice 2007). P-values were calculated from Wilks' lambda statistics. PERMANOVA analyses were conducted using PAST software (Hammer et al. 2007) to assess whether differences in wing shape were associated with either seasonality, or annual variation (McTavish et al. 2012).

Principal component calculations reduce data by summarizing multidimensional variation effectively in fewer dimensions (Klingenberg & McIntyre 1998). For meaningful analyses, the first few principal components (PCs) should account for most of the total variation contained in a dataset. PCA of the size-corrected shape variables was conducted with MorphoJ (Klingenberg 2011), which identified landmarks with the highest loading coefficients as those most likely responsible for shape differences (Klingenberg & McIntyre 1998). To visualize wing shape variations between groups, tps.Splint software (Rolff 2004) created deformation grids comparing the mean shape of each group with the mean for the merged groups (Outomuro & Johansson 2011).

Mean centroid size of wings was calculated for each population to assess variation in overall wing size, and therefore overall body size. Correlation between wing centroid size and other body size measurements is high (Outomuro & Johansson 2011; Yazdi 2014). Centroid sizes were calculated using MorphoJ (Klingenberg 2011). One-way ANOVA conducted with PAST tested significance of centroid size differences among populations, and was followed by Tukey's post hoc test to identify pairwise differences

(Hammer et al. 2007). Homogeneity of variance and normality were tested by Levene's test and Shapiro-Wilk test, respectively (Sadeghi & Dumont 2014).

Results

Variation between Early and Late Flight Seasons

Wing shape. Wings collected from damselflies early in the flight season varied significantly in shape from those collected late in the season for all comparisons. Males were collected from four species (*A. nahuana*, *A. moesta*, *E. civile*, *H. americana*) and females from one species (*A. moesta*) during four years (2009, 2013, 2014, 2017). PERMANOVA tests on size-corrected shape variables indicated significant shape differences between wings developing early in the flight season compared to those developing later in the flight season for all species collected for both sexes (Table 3.1).

Table 3.1. Results of PERMANOVA on shape components (regression residuals) for damselflies emerging early in the flight season versus those emerging late in the flight season. See Table 3.2 for sample sizes.

SPECIES	SEX	YEAR	LOCATION	EARLY vs LATE SHAPE DIFFERENCE
<i>H. americana</i>	males	2009	FOB	***F = 7.318, p < 0.0001
<i>H. americana</i>	males	2013	FOB	***F = 17.97, p < 0.0001
<i>H. americana</i>	males	2014	FOB	**F = 4.505, p = 0.0013
<i>A. moesta</i>	males	2017	FOB	***F = 10.17, p = 0.0001
<i>A. moesta</i>	females	2017	FOB	***F = 6.614, p = 0.0001
<i>A. nahuana</i>	males	2009	Wetlands	***F = 5.857, p = 0.0001
<i>E. civile</i>	males	2009	Wetlands	**F = 2.934, p = 0.0078

Note: Significance -*<0.05, -**<0.01, -***<0.001.

PCA performed on size-corrected shape variables visualized the shape variations between early versus late flight season groups. The first two principal components

accounted for a large percentage of the variation between groups. In *A. nahuana* males, PC1 (27.1%) and PC2 (20.3%) indicated greatest variation in landmarks 6,12 and 12,13 respectively, resulting in wings of late emerging damselflies being wider near the nodus. Males of *E. civile* followed a similar pattern of wing shape variation with PC1 (27%) and PC2 (21%) showing greatest position changes in landmarks 6 and 12. Landmark 6, an internal landmark, likely affects wing stability and landmark 12 affects wing width. Wings of late emerging damselflies were slightly wider. Wings of males and females of *A. moesta* exhibited similar patterns of variation between early and late emergers. In both males (31.7%) and females (31.9%), PC1 was associated with variation in landmarks 6, 5 and 12. PC2 for male (23.1%) and female (25.9%) comparisons indicated variation in landmarks 12, 6, and 13. In both sexes, wings of damselflies emerging early were wider and longer than those emerging late in the season. Deformation grids visualized the mean wing shapes of each group (Figure 3.2).

H. americana males were compared over three years. In each year, wing shapes were significantly different between early and late seasons (Table 1). In 2009, PC1 (28%) showed greatest variation in the position of landmarks 13, 7, 8, 3, 4. PC2 (25.5%) showed greatest variation in landmarks 11, 12, 13, and indicated more rounded wing tip curvature by adults emerging early in the season. In 2013, late season wings were wider than early season wings due to position changes in landmarks 13, 8, 9 (PC1, 36.6%) and landmarks 6, 7, 8 (PC2, 24%). In contrast, wings of damselflies collected early in 2014 were wider than those collected later in the season, as shown by variation in landmarks 13 (PC1, 38,4%) and 8 (PC2, 21.2%). Deformation grids visualized the mean wing shapes of each group (Figure 3.3).

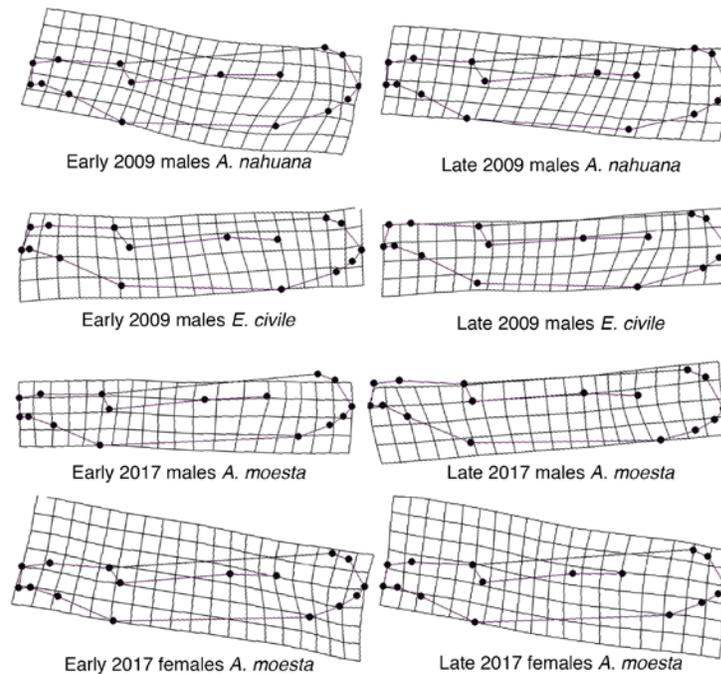


Figure 3.2. Variation of wing shape between damselflies collected early versus those collected late in the flight seasons of Wetlands 2009 and FOB 2017. The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

Wing size. Wing sizes, measured as centroid size, for early versus late flight seasons were compared. One-way ANOVA indicated significant variation between wing size of damselflies of all species and both sexes emerging early versus late in the flight season. In all comparisons, except *E. civile* males, wings of damselflies emerging early were larger than wings of damselflies emerging later in the flight season (Table 3.2) (Figures 3.4 and 3.5).

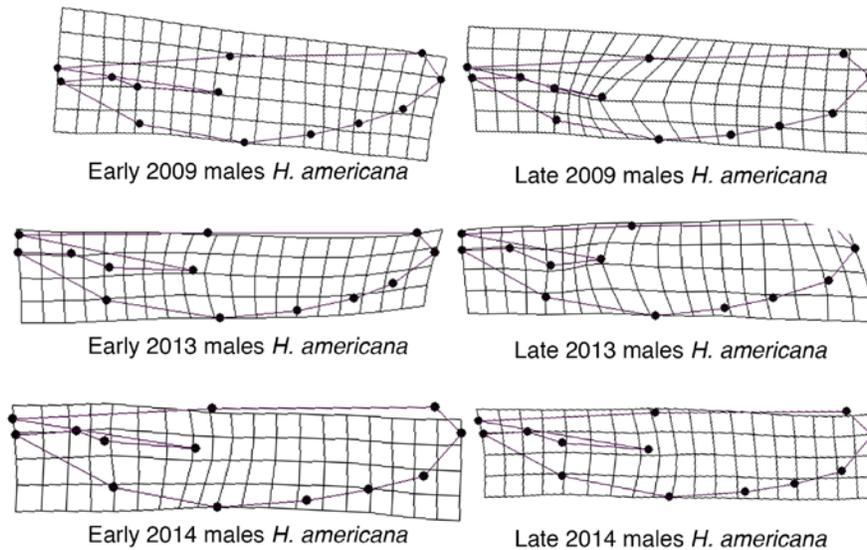


Figure 3.3. Variation of wing shape between *H. americana* males collected early versus those collected late in the flight seasons of FOB 2009, 2013, 2014. The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

Table 3.2. Results of ANOVA on centroid size for damselflies emerging early in the flight season versus those emerging late in the flight season. Values for centroid sizes from t tests.

SPECIES	SEX	YR	SITE	\bar{x} CENTROID	\bar{x} CENTROID	EARLY vs LATE CENTROID DIFFERENCE
				SIZE	SIZE	
				EARLY (n)	LATE (n)	
<i>H. americana</i>	males	2009	FOB	1222 (46)	1135 (44)	***F = 132.4 p < 0.001
<i>H. americana</i>	males	2013	FOB	1214 (63)	1135 (49)	***F = 165.0 p < 0.001
<i>H. americana</i>	males	2014	FOB	1211 (28)	1143 (32)	***F = 46.75 p < 0.001
<i>A. moesta</i>	males	2017	FOB	1217 (77)	1112 (84)	***F = 244.4 p < 0.001
<i>A. moesta</i>	females	2017	FOB	1304 (76)	1192 (68)	***F = 153.7 p < 0.001
<i>A. nahuana</i>	males	2009	Wetlands	837 (43)	769 (59)	***F = 13.81 p < 0.001
<i>E. civile</i>	males	2009	Wetlands	835 (46)	901 (36)	***F = 58.21 p < 0.001

Note: Significance -*<0.05, -**<0.01, -***<0.001.

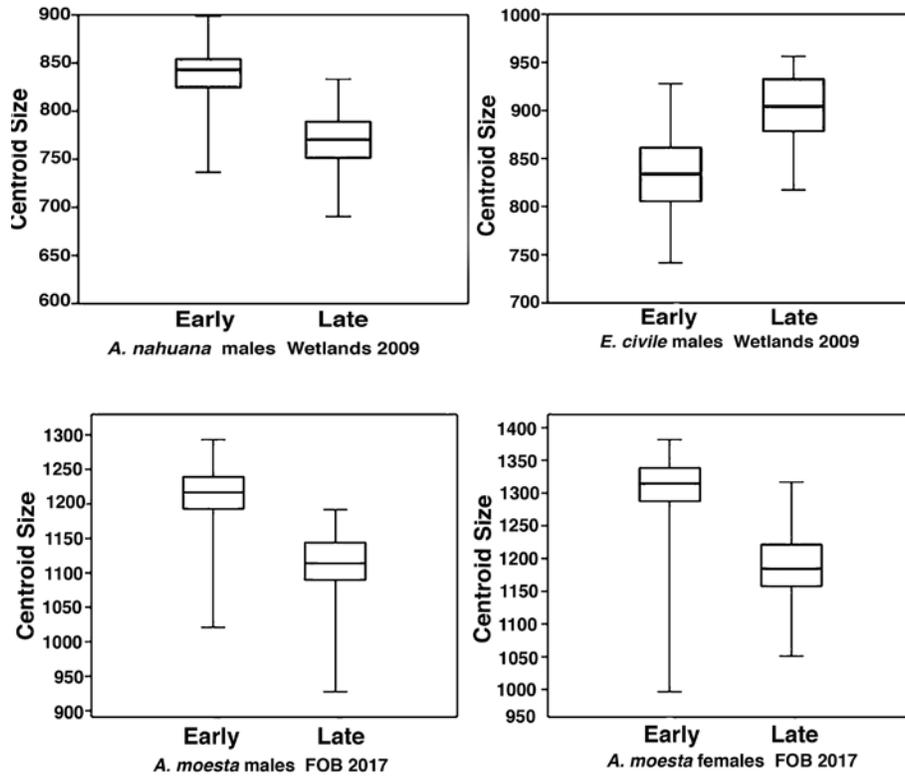


Figure 3.4. Mean centroid sizes of damselflies collected early and those collected late in the flight seasons of Wetlands 2009 and FOB 2017. Note differences in y-axis scales.

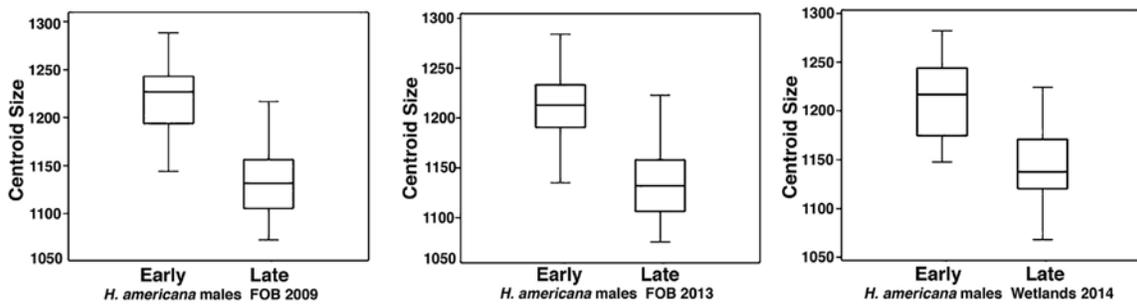


Figure 3.5. Mean centroid sizes of *H. americana* collected early and those collected late in the flight seasons of FOB 2009, 2013, 2014.

Annual Variation

Wing Shape

Consistent annual variation in wing shape was not found when comparing damselflies emerging over different years (Table 3). Males of six species (*E. basidens*, *I. hastata*, *I. posita*, *A. moesta*, *A. sedula*, and *H. americana*) and females of three species (*E. basidens*, *I. hastata*, and *H. americana*) were collected during the flight season in 2009, 2010, 2011, 2013, 2014 and 2017. PERMANOVA run on size-corrected shape variables tested for shape differences for wings of adults developing in different years. Males of *H. americana* collected in 2009, 2013 and 2014 showed significant variations in wing shape in all pairwise comparisons. Significant shape differences occurred in males of *A. sedula* collected in 2009, 2014 and 2017 (Table 3.3), although differences were found in only two of the three pairwise comparisons (2009 versus 2014; 2014 versus 2017). Wing shape did not differ significantly between years for males of *E. basidens*, *I. hastata*, *I. posita*, and *A. moesta*. Likewise, wing shape did not vary significantly between years for females of *E. basidens*, *I. hastata*, and *H. americana*.

To visualize shape variation between groups, PCA was performed on size-corrected shape variables for those groups shown to have significant differences between collection years. Most variation between males of *H. americana* collected in 2009, 2013, and 2014 was accounted for by PC1 (29.3%) at landmark 13 and by PC2 (23.8%) at landmarks 7, 8, 6, 3 and 4. Variation in landmark 13, an internal landmark, likely affects wing stability. Landmarks 6, 7 and 8 define the lower edge of the wing while landmarks 3 and 4 define the wing tip. Comparison of wings collected in 2009 with those collected in 2013 and in 2014 indicated the wings in 2009 were more slender than those from 2013

and from 2014. Damselfly wings collected in 2014 were more slender than those collected in 2013. In *A. sedula* males, most variation was accounted for by PC1 (29.2%) at landmarks 6 and 12 and by PC2 (23.5%) at landmarks 7, 8, 9, 10 and 11.

Table 3.3. Results of PERMANOVA on shape components (regression residuals) for damselflies emerging in different years from the same location. See Table 3.4 for sample sizes.

SPECIES	SEX	SITE	ANNUAL COMPARISONS	YEAR vs YEAR
				SHAPE DIFFERENCE
<i>H. americana</i>	females	FOB	2009 vs 2014	F = 0.913 p = 0.4533
<i>H. americana</i>	males	FOB	2009 vs 2013 vs 2014	***F = 22.23 p = 0.0001
			2009 vs 2013	***Tukey's p = 0.0003
			2009 vs 2014	**Tukey's p = 0.0012
			2013 vs 2014	***Tukey's p = 0.0003
<i>A. moesta</i>	males	FOB	2009 vs 2017	F = 1.655 p = 0.1289
<i>A. sedula</i>	males	FOB	2009 vs 2014 vs 2017	***F = 4.364 p = 0.0001
			2009 vs 2014	***Tukey's p = 0.0009
			2014 vs 2017	**Tukey's p = 0.0024
			2009 vs 2017	Tukey's p = 0.5289
<i>E. basidens</i>	females	Wetlands	2009 vs 2010 vs 2011	F = 1.569 p = 0.0948
<i>E. basidens</i>	males	Wetlands	2009 vs 2010 vs 2011	F = 1.456 p = 0.1565
<i>I. hastata</i>	females	Wetlands	2009 vs 2010	F = 1.408 p = 0.1835
<i>I. hastata</i>	males	Wetlands	2009 vs 2010	F = 1.486 p = 0.1649
<i>I. posita</i>	males	Wetlands	2009 vs 2010	F = 1.057 p = 0.3637

Note: Significance -*<0.05, -**<0.01, -***<0.001.

Landmark 6 is an internal landmark and variation at this location affects wing stability.

Damselfly wings collected in 2009 and in 2017 were longer than those collected in 2014,

and wings collected in 2009 were not significantly different from those collected in 2017.

Deformation grids visualize the mean wing shapes of each group (Figure 3.6).

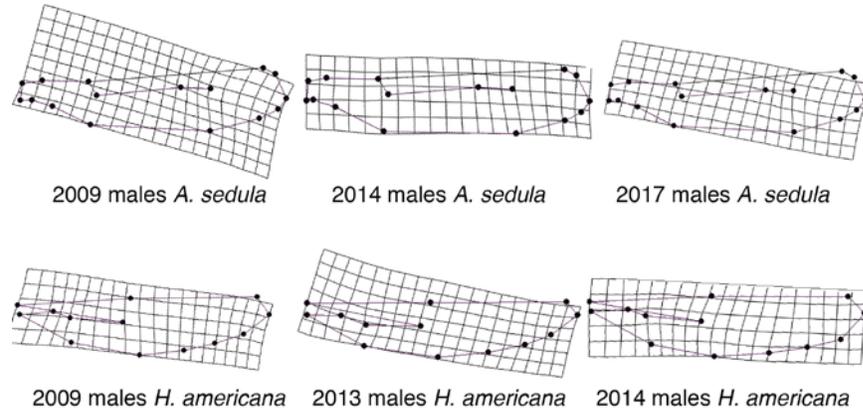


Figure 3.6. Variation of wing shape between *A. sedula* males collected at FOB 2009, 2014, 2017; and *H. americana* males collected at FOB 2009, 2013, 2014. The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

Wing Size

In annual comparisons, wing size (centroid size) was more consistently variable than wing shape. ANOVA showed a significant difference in wing size in ten of the seventeen comparisons between years (Table 3.4). Mean centroid sizes were compared by t-tests. Results for *H. americana* males showed wings collected at FOB in 2009 and in 2013 were larger than in 2014, and wing sizes were not significantly different between 2009 and 2013. *H. americana* females did not show a significant difference in wing size between collections from 2009 and from 2014. Wing sizes for both *E. basidens* males and females followed a similar pattern. For both sexes, wings collected in 2009 and 2010 were significantly larger than those collected in 2011, while wings collected in 2009 did

Table 3.4. Results of ANOVA on centroid size for damselflies emerging in different years from the same location. Values for centroid sizes from t tests.

SPECIES	SEX	SITE	ANNUAL COMPARISONS (n)	\bar{x} CENTROID SIZE	YEAR vs YEAR
					SIZE DIFFERENCE
<i>H. americana</i>	females	FOB	2009 (34) vs 2014 (33)	1177 vs 1199	F = 3.921 p = 0.0519
<i>H. americana</i>	males	FOB	2009 (90) vs 2013 (154) vs 2014 (111)		***F = 5.581 p = 0.0041
			2009 vs 2013	1179 vs 1177	Tukey's p = 0.9660
			2009 vs 2014	1179 vs 1157	**Tukey's p = 0.0071
			2013 vs 2014	1177 vs 1157	*Tukey's p = 0.0154
<i>A. moesta</i>	males	FOB	2009 (31) 2017 (161)	1131 vs 1162	F = 3.609 p = 0.0589
<i>A. sedula</i>	males	FOB	2009 (58) 2014 (178) 2017 (51)		***F = 28.86 p < 0.0001
			2009 vs 2014	804 vs 869	***Tukey's p < 0.0001
			2014 vs 2017	869 vs 815	***Tukey's p < 0.0001
			2009 vs 2017	804 vs 815	Tukey's p = 0.0584
<i>E. basidens</i>	females	Wetlands	2009 (29) vs 2010 (31) vs 2011 (31)		***F = 8.354 p = 0.0004
			2009 vs 2010	583 vs 573	Tukey's p = 0.5800
			2009 vs 2011	583 vs 543	***Tukey's p = 0.0006
			2010 vs 2011	573 vs 543	*Tukey's p = 0.0131
<i>E. basidens</i>	males	Wetlands	2009 (41) vs 2010 (35) vs 2011 (91)		***F = 17.24 p = 0.0001
			2009 vs 2010	519 vs 528	Tukey's p = 0.3635
			2009 vs 2011	519 vs 496	***Tukey's p = 0.0006
			2010 vs 2011	528 vs 496	***Tukey's p = 0.0001
<i>I. hastata</i>	females	Wetlands	2009 (27) vs 2010 (55)	565 vs 545	F = 3.237 p = 0.0757
<i>I. hastata</i>	males	Wetlands	2009 (38) vs 2010 (41)	428 vs 408	***F = 12.55 p = 0.0006
<i>I. posita</i>	males	Wetlands	2009 (29) vs 2010(48)	479 vs 454	***F = 15.13 p = 0.0002

Note: Significance -*<0.05, -**<0.01, -***<0.001.

not differ significantly in size from those collected in 2010. However, for males of *I. hastata* and *I. posita*, wing sizes were larger in 2009 than in 2010. *E. basidens*, *I. hastata*, and *I. posita* were collected at the Wetlands and wing sizes of all three species were

largest in 2009. For *A. sedula* males collected at FOB, wings were largest in 2014 compared to wings collected in 2009 or 2017, and wing sizes did not differ significantly between 2009 and 2017 (Figure 3.7). There were no significant differences annually in collections of *I. hastata* females and of *A. moesta* males (Table 3.4).

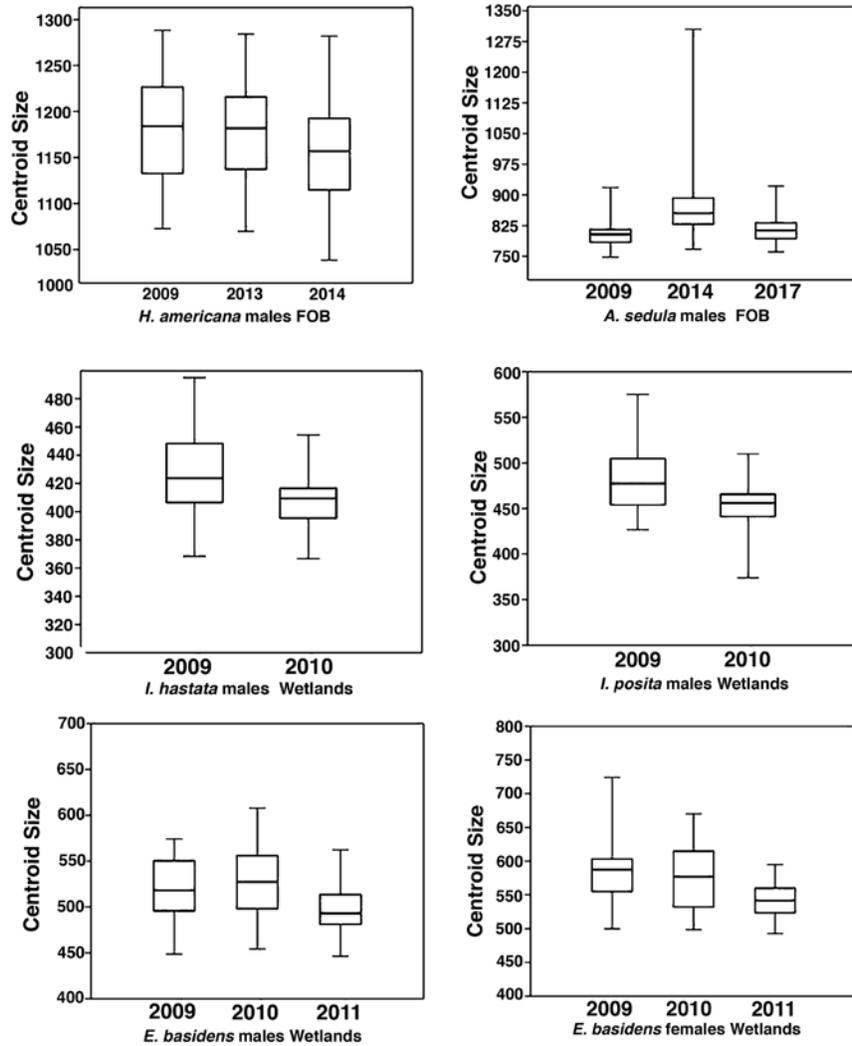


Figure 3.7. Mean centroid sizes of females and males collected at Wetlands 2009, 2010, 2011; and males collected at FOB 2009, 2013, 2014, 2017. Note differences in y-axis scales.

Discussion

Habitat conditions and genetic makeup affect and constrain morphological differences among organisms (Outomuro & Johansson 2011). Individuals of the same species having developed under different conditions often display quite different characteristics (Stearns 1989). Characteristics such as insect wing morphology greatly influence the energetic costs and maneuverability of flight (Betts & Wootton 1988). This study examined the effects of habitat conditions on wing shape and wing size in eight species of damselflies. We examined the wing morphologies of males and females emerging early versus late in the flight season, and adults emerged from the same location but in different years. Wing shape and wing size varied significantly in all early versus late comparisons, of both sexes in all eight species. Results yielded fewer significant differences in annual comparisons, with significant variations in wing size among years found in five species and variations in wing shape found in only two species.

Comparisons of early versus late emerging damselflies showed differences for both wing shape and wing size in comparisons of all four species collected and analyzed. Other studies have shown similar seasonal variation in wing shape and wing size, as components of flight morphology (Pétavy et al. 1997; Norberg & Leimar 2002; Debat et al. 2003; Bots et al. 2009; Klingenberg 2013). In our study, examination of deformation grids revealed no consistent pattern of wing shape variation between damselflies emerging early compared to those emerging late in the flight season. *A. nahuana* and *E. civile* males had wider wings late in the flight season, while the wings of *A. moesta* males and females were wider early in the season. Broader wings enhance maneuverability and agility, which could decrease predation risk (Betts & Wootton 1988; Outomuro &

Johansson 2015). *H. americana* males were collected for early versus late emergence in three seasons and displayed contrasting results for each season. Wings collected in 2009 had a more rounded wing tip early in the season, while wings collected in 2013 were wider late in the season in contrast to wings collected in 2014 which were wider early in the season. Relative positions of internal landmarks for all species were also frequently variable, and likely contribute to wing stability (Bots et al. 2009, Talucdher & Shivakumar 2013).

Wing centroid sizes, and therefore body sizes, were larger for adults collected early in the flight season for all species and both sexes except *E. civile* males who were larger late in the season. Other studies have noted similar variation for wing size (Norberg & Leimar 2002; Bots et al. 2009). Insects emerging early in the flight season and therefore having developed in colder temperatures are frequently larger than those emerging late in the season after developing in warmer temperatures (Norberg & Leimar 2002; Bots et al. 2009).

Annual differences in wing shape occurred in only two of the six species and only in males compared between two years. Norberg & Leimar (2002) found that annual variations in environmental influences on insect morphology were considerable. *H. americana* males collected for three years at FOB (2009, 2013, 2014) showed significant wing shape differences between all annual comparisons. Wings collected in 2009 were the most slender, wings collected in 2013 were the most wide, and wings from 2014 intermediate between 2009 and 2013. Slender wings, adapted to reduce flight energy and to enhance lift production, may optimize flight performance as ambient temperatures may affect lift efficiency (Bots et al. 2009; Gallesi et al. 2016). Broader wings enhance agility

and maneuverability and aid predator avoidance (Svensson & Friberg 2007; Outomuro & Johansson 2015; Gallesi et al. 2016). Broader wings for males may enhance mating success for territorial damselflies such as *H. americana* that depend on large pigmented wing spots for sexual displays (Bots et al. 2012; Outomuro et al. 2013; Outomuro et al. 2014). An internal landmark (13) affecting stability also varied significantly. *Argia sedula* males were collected at FOB in 2009, 2014, and 2017. Wings collected in 2009 and in 2017 were longer than wings collected in 2014, with no significant difference between wing shapes of collections from 2009 and 2017. Variation was also notable for internal landmark (6) which likely influences wing stability.

Wing centroid sizes, and therefore body sizes differed significantly in many but not all of the annual comparisons made from males and females of the six species collected. *H. americana* males collected at FOB in 2014 were smaller than those collected in 2009 and 2014, while *A. sedula* males from FOB were larger in 2014 than those collected in 2009 and 2017. *H. americana* are more territorial and larger (36-51 mm) than *A. sedula* (29-34 mm) which could influence their relative size differences in 2009 (Abbott 2005). Males and females of *E. basidens*, collected at the Wetlands over three years (2009, 2010, 2011), indicated that damselflies were smallest in 2011 and similar in size between 2009 and 2010. Males of *I. hastata* and *I. posita* were significantly larger in 2009 than in 2010. Water levels at the Wetlands varied considerably over these three years with the draining of the Wetlands in July 2010 and drought conditions in 2011. Water regime of the area they inhabit is an environmental factor of primary importance. Their abundance depends directly on water level and decreases as this level drops (Popova et al. 2016).

This study contributes to the growing awareness that selective pressures produce considerable temporal variation among years (Gyulavari et al. 2015) and between seasons within a year (Van Dyck & Wiklund 2002; Debat et al. 2003; Gibbs et al. 2010; Gallesi et al. 2016). Phenotypic plasticity can promote wing shape evolution involving environmental pressures that optimize efficiency (Outomuro et al. 2013, Gallesi et al. 2016). Our research indicates that significant morphological variation and plasticity resides in the sensitivity of wing shape development to seasonal variation and in a lesser extent to annual variation. Studies among species show that both ecological and behavioral factors can affect flight morphology (Norberg & Leimar 2002). The frequency of wing shape and wing size differences supports the concept that flight morphology is under strong selection for optimal fitness.

CHAPTER FOUR

Effects of Mating Status and Sex on Wing Morphology of Ten Damselfly Species (Odonata: Zygoptera)

Introduction

For flying organisms, morphology closely relates to their ecology and behavior (Wickman 1992; Petavy et al. 1997; Bots et al. 2012). Wing shape is expected to adapt to maximize performance and is influenced by costs and benefits related to habitat and social systems (Wickman 1992; Van Dyck & Wiklund 2002; Outomuro & Johansson 2012). Differences in wing morphology may result from adaptive evolution driven by natural selection. Natural selection may adapt individuals and optimize their biological processes such as dispersal, migration, predator escape, foraging, and mating strategies (Bots et al. 2009; Outomuro & Johansson 2012; Outomuro et al. 2016; Gyulavari et al. 2017). In territorial damselfly species, speed and maneuverability may be favored at the expense of energetically efficient flight in mating systems (Wickman 1992). Few studies have focused on the effects of mating status on wing morphology.

Wing shape is linked to fitness through its effect on flight performance and energetics. The relationship between the ecology and flight performance of a species becomes more complex when natural and sexual selection processes work simultaneously on wing shape (Outomuro & Johansson 2012). Flight ability combines agility, maneuverability, and endurance (Swillen 2009). Sexual selection could align with natural selection on wing morphology if for example females prefer males with superior flight performance that also enhances survival. Sexual selection could oppose natural selection

in species where for example wing morphology that maximizes territorial defense and displays is energetically costly (Outomuro et al. 2016). Selection can act on all traits involved in reproduction, including sexual signals, preferences in mate choice (Cigognini 2014) as well as physiological and morphological traits such as wing shape (Swillen 2009).

Sexual selection can enhance dimorphism of wing shape as it affects mating behavior. *Calopteryx* spp. show strong sexual dimorphism related to differences in life histories of males and females. (Anderson 1994; Van Dyck & Wiklund 2002; Bots et al. 2009; Breuker et al. 2010; Outomuro & Johansson 2012). In male butterflies, sexual selection should affect male wing design because most of their time is allocated to mate location (Wickman 1992). Males of some territorial species exhibit different mating strategies, which select different wing morphologies (Wickman 1992; Outomuro & Johansson 2012). Perching males defend a territory by rapidly flying from their perch to intercept passing males and then return to their perch. For perching males, selection should favor wings designed for high acceleration ability and speed. Males of territorial species unable to obtain a territory and males of non-territorial species are patrollers. They must fly to seek out mates and engage in scramble competition to successfully mate. For scrambling males, selection should favor wing shapes efficient for flight endurance (Wickman 1992). In general, long and narrow wings enhance lift and reduce flight costs, whereas short and broad wings tend to increase maneuverability at the cost of increased energy (Betts & Wootton 1988; Outomuro & Johansson 2015).

Damselfly body size is a major phenotypic trait under selection from both male-male competition and female choice (Steele et al. 2011). Flight energetics are also

strongly affected by body size. In territorial mating systems selection favors larger males (Anderson 1994). Smaller flying males tend to be more agile and maneuverable, which may increase mate search time and confer an adaptive advantage in scrambling mating systems (De Block & Stoks 2007). Also, adult body size may vary according to the ecological environments in which immatures develop.

Changes in a population's range of phenotypes can be adaptive or non-adaptive. In damselflies, female mate choice is partly determined by the fit between male (cerci) and female reproductive structures (mesostigmal plates) which both scale to body size. The distribution of female sizes at any given time largely shapes the mating fitness experienced by males (McPeck et al. 2008; Steele et al. 2011). Temporal variation in phenotypic distributions of both male and female body size may affect the temporal dynamics of selection on male body size (Blanckenhorn et al. 1999; Steele et al. 2011; Gyulavari et al. 2017).

In this study we investigated aspects of sexual selection on wing shape and body size by comparing mated versus unmated adults and comparing male versus female adult damselflies. Geometric morphometrics was used to quantify and analyze wing shape as well as provide centroid sizes used as proxy for body size. Wing shape, a multivariate trait, was examined using Procrustes techniques in geometric morphometrics, to remove variation in orientation, position, and scale while preserving shape (Hoffman et al. 2002; Bots et al. 2012; Ren et al. 2017). Correction for allometry effects was accomplished using residuals from a regression of shape on centroid size (Klingenberg 2010). Three damselfly species, including *Argia moesta* (Hagen, 1861), *Argia sedula* (Hagen, 1861), and *Enallagma basidens* (Calvert, 1902), were field collected for the mated versus

unmated comparisons, a well-established method to study aspects of sexual selection (Blanckenhorn et al. 2003; Blanckenhorn et al. 2004; Gosden & Svensson 2008; Therry et al. 2014). These comparisons often correlate to mating success and are often used to investigate sexual selection in damselflies (Gosden & Svensson 2008; Steele et al. 2011).

For male versus female comparisons, populations of ten damselfly species including *Argia moesta* (Hagen, 1861), *Argia nahuana* (Calvert, 1902), *Argia sedula* (Hagen, 1861), *Argia translata* (Hagen in Selys, 1865), *Enallagma basidens* (Calvert, 1902), *Enallagma civile* (Hagen, 1861), *Hetaerina americana* (Fabricius, 1798), *Ischnura hastata* (Say, 1839), *Ischnura posita* (Hagen, 1861), and *Ischnura ramburii* (Selys, 1842) were sampled. Both territorial and scrambling male mating strategies are represented by these species. Selection should favor wing morphologies and body sizes most advantageous for each mating strategy. We expected wing morphology and body size to vary not only between sexes and life history traits and associated behaviors, but also within a sex (Shuster & Wade 2003; Breuker et al. 2007). Specifically we tested: (1) whether mated and unmated adults differed in wing morphology and body size, and (2) whether males and females differed in wing morphology and body size.

Materials and Methods

Field Collections and Morphometric Measurements

This study included damselflies from ten broadly distributed species of four genera in two families. Calopterygidae was represented by *Hetaerina americana* (Fabricius, 1798). Populations of Coenagrionidae included *Argia moesta* (Hagen, 1861), *Argia nahuana* (Calvert, 1902), *Argia sedula* (Hagen, 1861), *Argia translata* (Hagen in Selys, 1865), *Enallagma basidens* (Calvert, 1902), *Enallagma civile* (Hagen, 1861),

Ischnura hastata (Say, 1839), *Ischnura posita* (Hagen, 1861), and *Ischnura ramburii* (Selys, 1842). Habitats for these ten species can be broadly divided into streams and ponds. Stream and river species included *H. americana*, *A. moesta*, *A. nahuana*, *A. sedula*, and *A. translata*, while ponds and ditches are preferred by *E. basidens*, *E. civile*, *I. hastata*, *I. posita*, and *I. ramburii*. Geographical ranges for *H. americana*, *A. sedula*, *E. civile*, and *I. ramburii* are the largest and extend east and west across the United States from southern Canada into Mexico. *A. moesta*, *A. translata*, *E. basidens*, *I. posita*, and *I. hastata* have more eastern ranges and extend from Ontario to Florida, east to South Dakota, and south to Mexico, including southern California, Arizona and New Mexico. *A. nahuana* ranges more westerly and extends from Oregon south to California and east to Texas, Oklahoma and Mexico (Westfall & May 2006).

To examine variations among wing shapes and wing sizes of mated versus unmated adults, and between adult males and females, collections were made in 2009, 2010, 2011, 2014, 2015, and 2017 from three sites in Falls, McLennan, and Bell counties, Texas, USA. Falls on the Brazos (FOB), a site on the Brazos River (Falls County, 31.25 N, 96.92 W), has a rocky bottom and is approximately 150 m wide. Littoral depth is 0.5 m, and velocity is 5 m sec⁻¹. Lake Waco Wetlands (McLennan County, 31.61 N, 97.30 W) covers 75 hectares with an approximate residence time of eight days. Water flows slowly through the Wetlands from the North Bosque River into Lake Waco. Nolan Creek (Bell County, 31.04 N, 97.44 W) is approximately 25 m wide, shoreline depth 0.3 m, and velocity 2 m sec⁻¹.

To examine differences between wing shapes and wing sizes of mated versus unmated adults, nine collections were made from three species at three locations over

four years. From FOB, *A. sedula* males were collected in 2009, *A. sedula* males and females in 2014 and *A. sedula* males, *A. moesta* males and females in 2017. From the Wetlands, *E. basidens* females were collected in 2009 and 2010, and *A. sedula* females in 2010. We considered males in tandem or wheel position to be mated whereas males without females were considered unmated. This study does not measure lifetime mating success because males captured singly may have mated previously.

To examine variations in wing shapes and wing sizes for male versus female adults, collections were made in 2009, 2010, 2011, 2014, 2015, and 2017 at FOB, the Wetlands, and Nolan Creek. Collections were made at the Wetlands in 2009 of *A. nahuana*, *A. sedula*, *E. basidens*, *E. civile*, *I. hastata*, *I. posita*, and *I. ramburii*. At the Wetlands in 2010, collections were made of *A. sedula*, *E. basidens*, and *I. hastata*, and in 2011 of *A. sedula* and *E. basidens*. At FOB, collections were made in 2009 and 2014 of *A. sedula* and *H. americana* and in 2017 of *A. moesta*. At Nolan Creek in 2015, *A. moesta*, *A. translata*, and *H. americana* were collected.

Damselfly adults collected with sweep nets were transported to the laboratory, killed with ethyl acetate, and weighed. Wings were spread and scanned at 800 dpi on a flatbed scanner. Damaged wings were eliminated from the analyses. Homologous landmarks, thirteen for Calopterygidae and sixteen for Coenagrionidae, were designated where veins reached the wing edge, or at vein intersections in the interior of the wing (Figure 4.1). Scans of right forewings were digitized with tps.Dig2 software (Rohlf 2015) using these consistent landmarks to capture wing shape (Outomuro et al. 2013; McTavish et al. 2012). Wing size was quantified as centroid size, which is the square root of the sum of the squared distances between each landmark and the specimen centroid

(Outomuro et al. 2012; Gallesi et al. 2016). Wing size commonly and reliably estimates body size (e.g. abdomen length) (Sadeghi et al. 2009; Outomuro & Johansson 2011; Yazdi 2014). To evaluate measurement error, repeated measurements were taken for a subset of wings, and Procrustes ANOVA was performed using MorphoJ software (Klingenberg 2011). Measurement error was not significant.

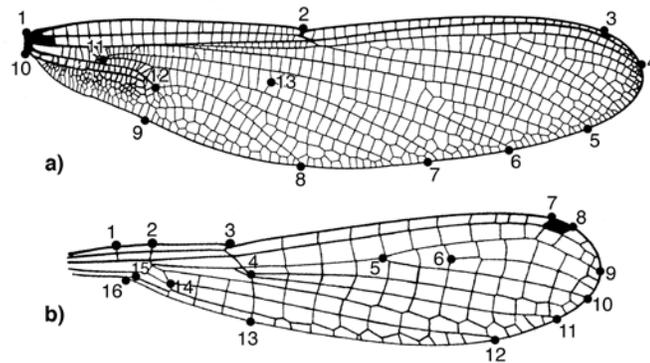


Figure 4.1. Right forewing of a) *H. americana*, Calopterygidae and b) Coenagrionidae with landmark positions defining wing shape. All landmarks were consistent for both sexes. Modified from Westfall & May 2006.

Data Analysis

Geometric morphometrics quantifies wing shape based on landmark coordinates (Outomuro et al. 2013). Procrustes superimposition rotates, translates, and scales landmark configurations into alignment via generalized least squares (Klingenberg & McIntyre 1998; Bots et al. 2012). This superimposition eliminates variation in scale, position, and orientation, but preserves shape (Outomuro & Johansson 2011). Multivariate linear regression of the aligned Procrustes coordinates on centroid size was performed using MorphoJ (Klingenberg 2011) to remove the allometric component of wing shape (Lorenz et al. 2017). Residuals from these regressions were used in

subsequent analyses as size-corrected shape variables (Klingenberg & McIntyre 1998; Outomuro et al. 2013; Gyulavari et al. 2017). Analyses were performed only on right forewings.

Given the unknown distribution of Procrustes coordinate data, we used Permutation Multivariate Analysis of Variance (PERMANOVA) with 10,000 random samples on size-corrected shape variables to assess F-ratio significance for variance differences (Hoffmann et al. 2002; Slice 2007). P-values were calculated from Wilks' lambda statistics. PERMANOVA analyses were conducted using PAST software (Hammer et al. 2007) to assess whether differences in wing shape were associated with either mated versus unmated, and male versus female variation (McTavish et al. 2012).

Principal component calculations reduce data by summarizing multidimensional variation effectively in fewer dimensions (Klingenberg & McIntyre 1998). For meaningful analyses, the first few principal components (PCs) should account for most of the total variation contained in a dataset. PCA of the size-corrected shape variables was conducted with MorphoJ (Klingenberg 2011), which identified landmarks with the highest loading coefficients as those most likely responsible for shape differences (Klingenberg & McIntyre 1998). To visualize wing shape variations between groups, tps.Splin software (Rohlf 2004) created deformation grids comparing the mean shape of each group with the mean for the merged groups (Outomuro & Johansson 2011; Klingenberg 2013).

Mean centroid size of wings was calculated for each population to assess variation in overall wing size, and therefore overall body size. Correlation between wing centroid size and other body size measurements is high (Outomuro & Johansson 2011; Yazdi

2014). Centroid sizes were calculated using MorphoJ (Klingenberg 2011). One-way ANOVA conducted with PAST tested significance of centroid size differences among populations, and was followed by Tukey's post hoc test to identify pairwise differences (Hammer et al. 2007). Homogeneity of variance and normality were tested by Levene's test and Shapiro-Wilk test, respectively (Sadeghi & Dumont 2014).

Results

Variation between Mating and Not Mating Damselflies

Wing shape. The shape of wings collected from mated damselflies varied significantly from wings collected from unmated damselflies in only one of nine populations compared. Males were collected from two species (*A. moesta*, *A. sedula*) and females from three species (*A. moesta*, *A. sedula*, *E. basidens*) during four years (2009, 2010, 2014, 2017). PERMANOVA tests on size-corrected shape variables indicated significant shape differences only between wings of mated female *E. basidens* compared to unmated females (Table 4.1).

Table 4.1. Results of PERMANOVA on shape components (regression residuals) for mated compared to unmated damselflies. See Table 4.2 for sample sizes.

SPECIES	SEX	YEAR	LOCATION	MATED vs UNMATED SHAPE DIFFERENCE
<i>A. moesta</i>	males	2017	FOB	F = 0.453, p = 0.8654
<i>A. moesta</i>	females	2017	FOB	F = 1.911, p = 0.0845
<i>A. sedula</i>	males	2009	FOB	F = 0.1977, p = 0.9905
<i>A. sedula</i>	males	2014	FOB	F = 0.6188, p = 0.7041
<i>A. sedula</i>	males	2017	FOB	F = 1.04, p = 0.3831
<i>A. sedula</i>	females	2010	Wetlands	F = 0.3825, p = 0.9147
<i>A. sedula</i>	females	2014	FOB	F = 0.3319, p = 0.9495
<i>E. basidens</i>	females	2009	Wetlands	F = 0.89, p = 0.4753
<i>E. basidens</i>	females	2010	Wetlands	*F = 2.413, p = 0.0269

Note: Significance -*<0.05, -**<0.01, -***<0.001.

PCA of size-corrected shape variables quantified patterns of shape variation between groups. The first two principal components accounted for most of the variation between groups. For *E. basidens* females collected at the Wetlands (2010), loading coefficients for PC1 (37.4%) and PC2 (25.8%) indicated variation at landmarks 5 and 12, and 13, 7, 8, 9, and 10 respectively, indicating that wings of mated damselflies were wider and shorter than wings of unmated damselflies. Variations in an internal landmark (5) influence wing stability. Deformation grids visualized the mean wing shapes of each group (Figure 4.2). No significant differences occurred between wing shapes of mated and unmated *A. moesta* males or mated versus unmated females collected at FOB (2017). *A. sedula* males collected at FOB (2009 and 2017), males of *A. sedula* and females collected at FOB (2014), females of *A. sedula* collected at the Wetlands (2010), and females *E. basidens* collected at the Wetlands (2009) (Table 4.1).

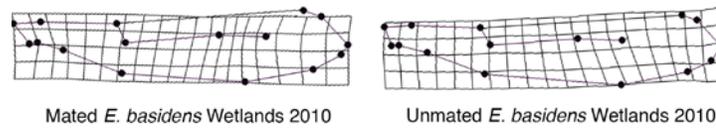


Figure 4.2. Variation of wing shape between *E. basidens* females collected mated versus those collected unmated in the flight season of Wetlands (2010). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

Wing Size

Wing sizes, measured as centroid size, were also compared. One-way ANOVA indicated significant variation between wing sizes of mated and unmated damselflies in only three of nine comparisons. ANOVA indicated that wings from mated males of *A. sedula* were significantly smaller than those from unmated males collected from FOB (2014). ANOVA indicated that mated females of *A. sedula* collected at the Wetlands (2010) were significantly larger than unmated females (Figure 4.3). Similarly, ANOVA indicated that wings of mated females of *E. basidens* collected at the Wetlands (2010) were significantly larger than those of unmated females of *E. basidens* (Figure 4.4) (Table 4.2).

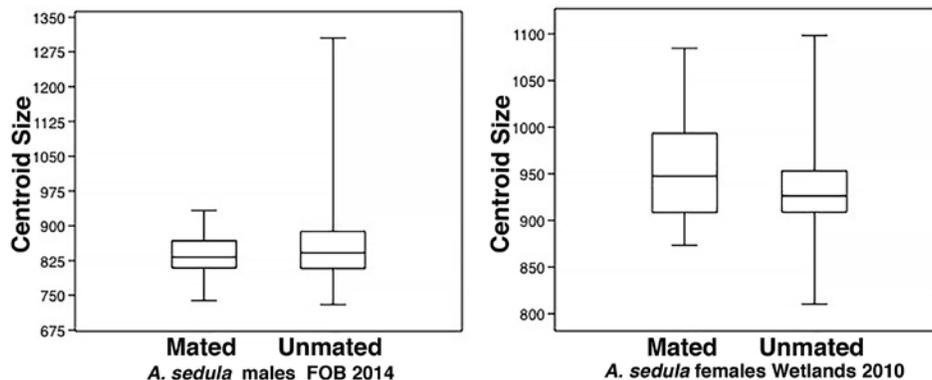


Figure 4.3. Mean centroid sizes of *A. sedula* males collected mated and those collected unmated in the flight season of 2014 (FOB); and *A. sedula* females collected mated and those collected unmated in the flight season of 2010 (Wetlands). Note differences in y-axis scales.

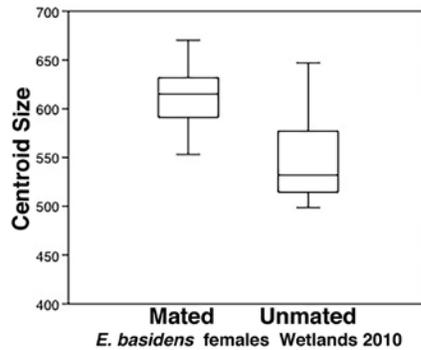


Figure 4.4. Mean centroid sizes of *E. basidens* females collected mated and those collected unmated in the flight season of Wetlands (2010).

Table 4.2. Results of ANOVA on centroid size for mated compared to unmated damselflies. Values for centroid sizes from t tests.

SPECIES	SEX	YEAR	SITE	\bar{x} CENTROID	\bar{x} CENTROID	MATED vs UNMATED
				SIZE	SIZE	
				MATED (n)	UNMATED (n)	DIFFERENCE
<i>A. moesta</i>	males	2017	FOB	1159 (99)	1169 (82)	F = 1.18 p = 0.2788
<i>A. moesta</i>	females	2017	FOB	1239 (91)	1262 (73)	F = 3.738 p = 0.0549
<i>A. sedula</i>	males	2009	FOB	800 (27)	805 (32)	F = 0.3212 p = 0.5731
<i>A. sedula</i>	males	2014	FOB	834 (91)	858 (160)	*F = 6.134 p = 0.0139
<i>A. sedula</i>	males	2017	FOB	816 (24)	813 (27)	F = 0.1207 p = 0.7298
<i>A. sedula</i>	females	2010	Wetlands	958 (28)	932 (66)	*F = 4.922 p = 0.0289
<i>A. sedula</i>	females	2014	FOB	890 (99)	893 (16)	F = 0.0681 p = 0.7945
<i>E. basidens</i>	females	2009	Wetlands	586 (15)	581 (15)	F = 0.1068 p = 0.7463
<i>E. basidens</i>	females	2010	Wetlands	608 (13)	545 (15)	***F = 18.89 p = 0.0002

Note: Significance -*<0.05, -**<0.01, -***<0.001.

Variation between Male and Female Damselflies

Wing Shape

Differences in wing shape occurred between male and female damselflies in multiple species. Males and females of nine species (*A. moesta*, *A. nahuana*, *A. sedula*, *E. basidens*, *E. civile*, *H. americana*, *I. hastata*, *I. posita*, and *I. ramburii*) were collected during the flight seasons of 2009, 2010, 2011, 2014, 2015, and 2017. PERMANOVA on

size-corrected shape variables tested for significant shape differences between wings of males and females. Significant variations in wing shape occurred for males versus females in 19 of 20 pairwise comparisons. Significant differences in wing shape were found in both populations of *A. moesta*, the single population of *A. translata*, the single population of *A. nahuana*, all five populations of *A. sedula*, two of three populations of *E. basidens*, the single population of *E. civile*, all three populations of *H. americana*, both populations of *I. hastata*, the single population of *I. posita*, and the single population of *I. ramburii*. Wing shape did not differ significantly between males and females in the population of *E. basidens* collected at the Wetlands (2009) (Table 4.3).

PCA of size-corrected shape variables visualized wing shape variation between males and females. The first two principal components accounted for most of the variation. For *A. moesta* collected at Nolan Creek (2015), loading coefficients of PC1 (32.4%) and PC2 (21.4%) indicated that most variation occurred at landmarks 6 and 13, and landmarks 12, 7, 8, 9, and 10 respectively. For *A. moesta* collected at FOB (2017), coefficients of PC1 (30.2%) and PC2 (23.6%) indicated that most variation occurred at landmarks 6 and 12, and 7, 8, 9, and 10 respectively. For *A. nahuana* collected at the Wetlands (2009), coefficients of PC1 (31.4%) and PC2 (27.2%) accounted for most variation at landmarks 6 and 12, and 5, 7, 8, 9, and 10 respectively. Most variation for *A. translata* collected at Nolan Creek (2015) was accounted for by loading coefficients of PC1 (30.4%) and PC2 (25.6%) for landmarks 6 and 12, and 5 and 11 respectively.

Table 4.3. Results of PERMANOVA on shape components (regression residuals) for male damselflies compared to female damselflies. See Table 4.4 for sample sizes.

SPECIES	YEAR	LOCATION	MALE vs FEMALE SHAPE DIFFERENCE
<i>A. moesta</i>	2015	Nolan Creek	*F =3.013 p = 0.0106
<i>A. moesta</i>	2017	FOB	***F =5.373 p = 0.0004
<i>A. nahuana</i>	2009	Wetlands	***F = 5.627 p = 0.0002
<i>A. sedula</i>	2009	FOB	***F =5.429 p = 0.0004
<i>A. sedula</i>	2014	FOB	***F =8.415 p = 0.0001
<i>A. sedula</i>	2009	Wetlands	***F =5.131 p = 0.0007
<i>A. sedula</i>	2010	Wetlands	***F =9.658 p = 0.0001
<i>A. sedula</i>	2011	Wetlands	***F = 4.592 p = 0.0008
<i>A. translata</i>	2015	Nolan Creek	*F =2.517 p = 0.0269
<i>E. basidens</i>	2009	Wetlands	F =1.372 p = 0.2099
<i>E. basidens</i>	2010	Wetlands	*F =2.564 p = 0.0278
<i>E. basidens</i>	2011	Wetlands	*F =2.991 p = 0.0161
<i>E. civile</i>	2009	Wetlands	*F =2.913 p = 0.014
<i>H. americana</i>	2009	FOB	***F =14.08 p = 0.0001
<i>H. americana</i>	2014	FOB	***F =8.338 p = 0.0001
<i>H. americana</i>	2015	Nolan Creek	***F =16.37 p = 0.0001
<i>I. hastata</i>	2009	Wetlands	***F =28.05 p = 0.0001
<i>I. hastata</i>	2010	Wetlands	***F =67.14 p = 0.0001
<i>I. posita</i>	2009	Wetlands	***F =3.295 p = 0.0165
<i>I. ramburii</i>	2009	Wetlands	***F =6.837 p = 0.0002

Note: Significance -*<0.05, -**<0.01, -***<0.001.

Variation in internal landmarks 5 and 6 affect wing stability. Landmarks 12 and 13 define the lower edge of the wing while landmarks 7, 8, 9, and 10 define the wing tip.

Comparisons of wings from populations of *A. moesta* indicated that the wings of females

were longer and more slender than those of males (Figure 4.5). For *A. nahuana* and *A. translata*, wings of females were more slender with more rounded wingtips than wings of males (Figure 4.6).

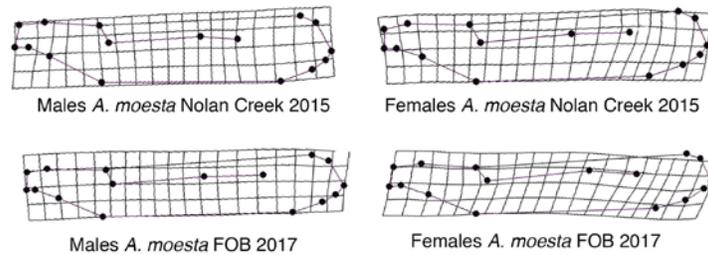


Figure 4.5. Variation of wing shape between *A. moesta* males and females collected in the flight seasons of Nolan Creek (2015) and FOB (2017). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

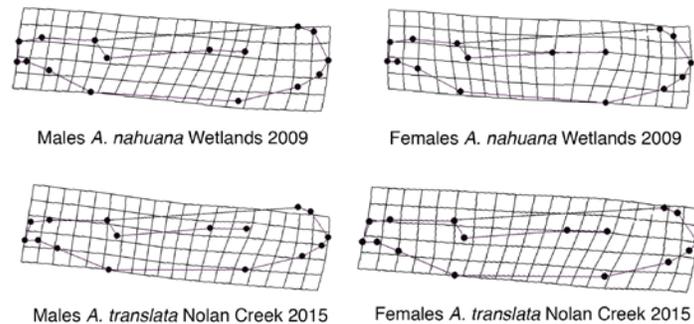


Figure 4.6. Variation of wing shape between *A. nahuana* males and females collected at Wetlands (2009); and *A. translata* males and females collected at Nolan Creek (2015). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

PCA of size-corrected shape variables indicated that most variation between wings of males and females was accounted for by PC1 and PC2. For *A. sedula* collected at FOB (2009), loading coefficients of PC1 (36.2%) and PC2 (18%) indicated that most

variation at landmarks 6, 12, and 5, 7, 8, 9, and 10 respectively. Most variation for *A. sedula* collected at FOB (2014) was accounted for by coefficients of PC1 (29.8%) and PC2 (24%) at landmarks 12 and 6, and 5, 7, 8, 9, and 10 respectively. Most variation for *A. sedula* collected at the Wetlands (2009, 2010, and 2011) was accounted for by coefficients of PC1 (32.8%; 32.9%; 31.5%) at landmarks 12 and 6 and by PC2 (27%; 22.6%; 22.3%) at landmarks 7, 8, 9, and 10. For all *A. sedula* populations sampled, wings of females were more slender and longer than wings of males (Figures 4.7 & 4.8).

PCA of size-corrected shape variables indicated that PC1 and PC2 accounted for most variation in wing shape between males and females. For *E. basidens* collected at the Wetlands (2010 and 2011), coefficients of PC1 (36.8%; 36.1%) and PC2 (22.5%; 25.5%) indicated that most variation occurred at landmarks 12, 7, 8, 9, 10, and 6 respectively. For *E. civile*, coefficients of PC1 (37.8%) and PC2 (22.5%) indicated variation at landmarks 6 and 12, and 7, 8, 9, and 10, respectively.

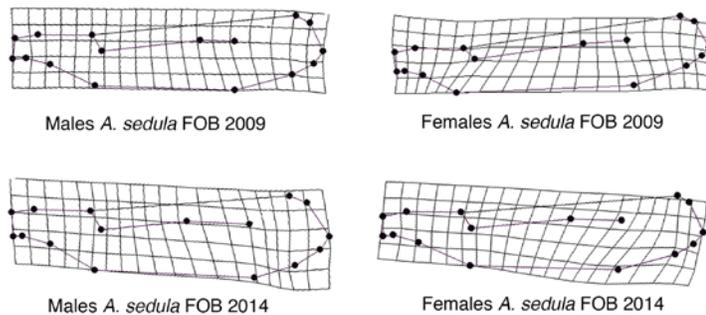


Figure 4.7. Variation of wing shape between *A. sedula* males and females collected at FOB (2009), (2014). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

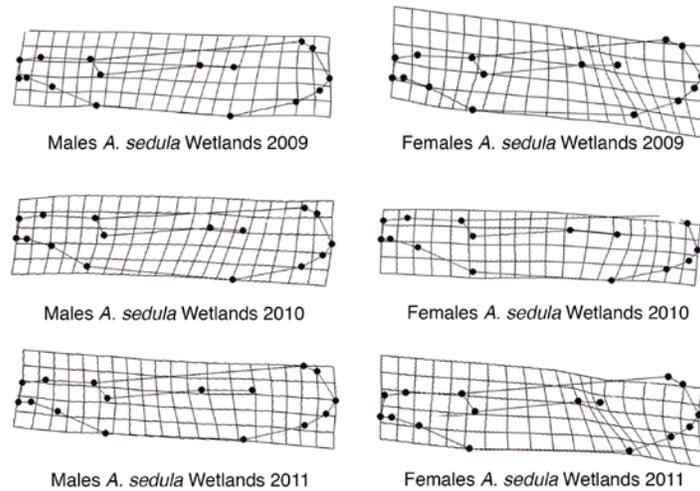


Figure 4.8. Variation of wing shape between *A. sedula* males and females collected at Wetlands (2009), (2010), (2011). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

Wings of females were longer and more slender than for males of *E. basidens* (2010, 2011) and for *E. civile* (2009) collected at the Wetlands. Wing shapes were not significantly different between males and females of *E. basidens* collected at the Wetlands in 2009 (Figure 4.9).

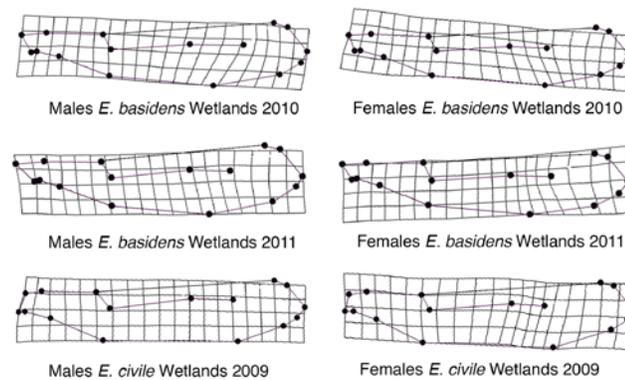


Figure 4.9. Variation of wing shape between *E. basidens* males and females collected at Wetlands (2010), (2011); and *E. civile* males and females collected at Wetlands (2009). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

PCA of size-corrected shape variables visualized significant wing shape variation between males and females in four populations from three species of genus *Ischnura*. Most variation in wing shape between males and females of *I. hastata* collected at the Wetlands (2009 and 2010) was accounted for by loading coefficients of PC1 (38.3%; 52.1%) and PC2 (23.5%; 16.1%) for landmarks 6 and 12, and 7, 8, 9, and 10, respectively. In contrast to other species, wings of female *I. hastata* were wider with more rounded wingtips than wings of males (Figure 4.10). For *I. posita* and *I. ramburii* collected at the Wetlands in 2009, loading coefficients of PC1 (46.4%; 33.4%) and PC2 (19.4%; 16.8%) indicated the most variation for landmarks 6,12 and 7, 8, 9, and 10 respectively, indicating that females had more slender wings and more rounded wingtips (Figure 4.11).

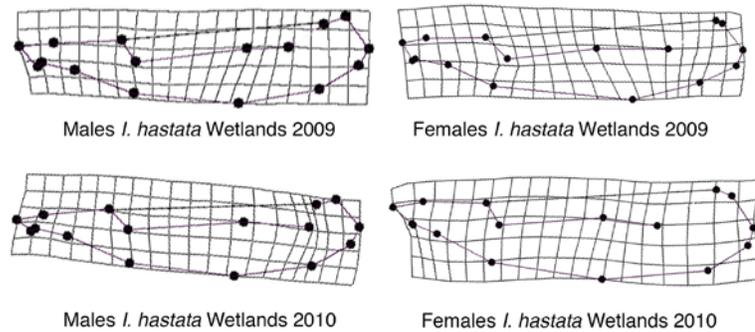


Figure 4.10. Variation of wing shape between *I. hastata* males and females collected at Wetlands (2009), (2010). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

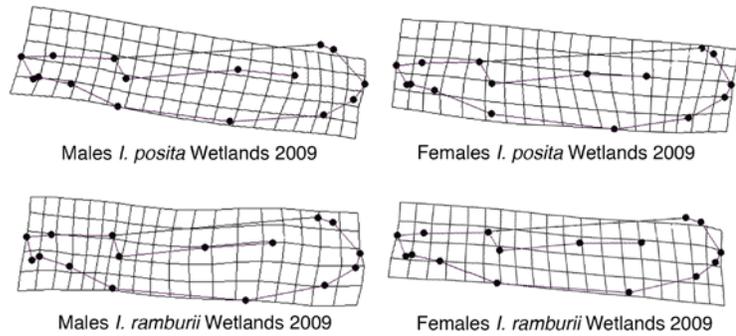


Figure 4.11. Variation of wing shape between *I. posita* males and females collected at Wetlands (2009); and *I. ramburii* males and females collected at Wetlands (2009). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

PCA of size-corrected shape variables indicated that most variation between wing shapes of male and female *H. americana* collected at FOB (2009 and 2014) and at Nolan Creek (2015) was accounted for by coefficients of PC1 (36.3%; 30.9%; 38.7%) at

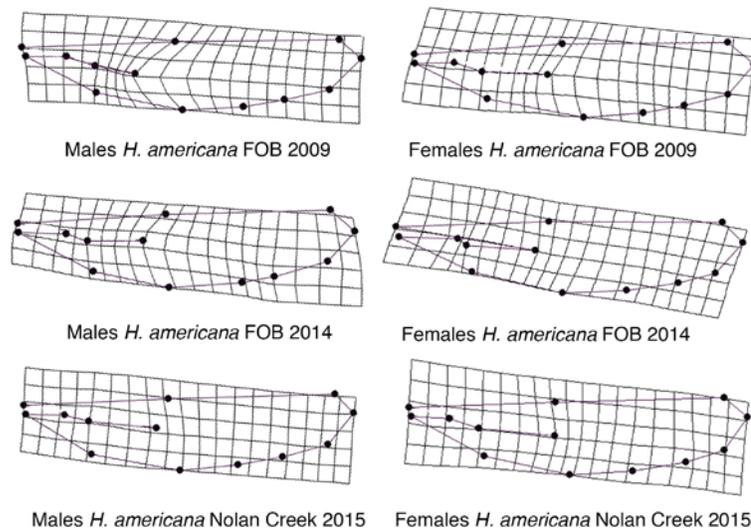


Figure 4.12. Variation of wing shape between *H. americana* males and females collected at FOB (2009), (2014) and at Nolan Creek (2015). The deformation grids indicate the mean shape of each group in relation to the overall mean of the combined groups. Minimal increases in factor level were uniformly used to highlight differences.

landmark 13 and PC2 (25.2%; 23.2%; 20.4%) for landmarks 7, 8, and 9. Landmark 13, an internal landmark, contributes to wing stability and landmarks 7, 8, and 9 define the lower edge of the wing. Female wings, were wider, thinner at the base, and shorter at the tip than were male wings (Figure 4.12).

Wing Size

For male versus female comparisons of wing size (centroid size), of the twenty populations compared ANOVA found a significant difference in wing size for nineteen of the male versus female comparisons. Wings from females were significantly larger than wings from males in all comparisons except *E. civile* (Table 4.4) (Figures 4.13-4.16).

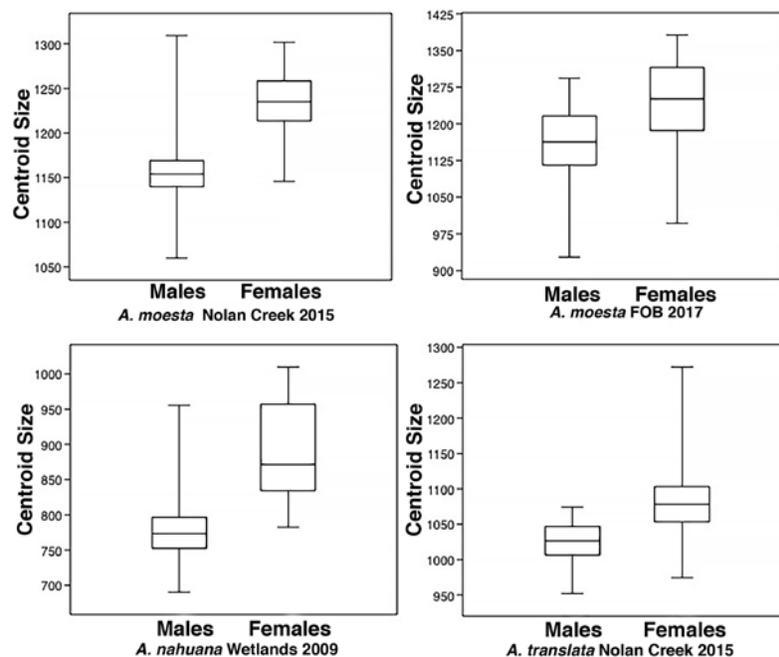


Figure 4.13. Mean centroid sizes of *A. moesta* males and females collected at FOB (2017) and Nolan Creek (2015); *A. nahuana* males and females collected at Wetlands (2009); and *A. translata* males and females collected at Nolan Creek (2015). Note differences in y-axis scales.

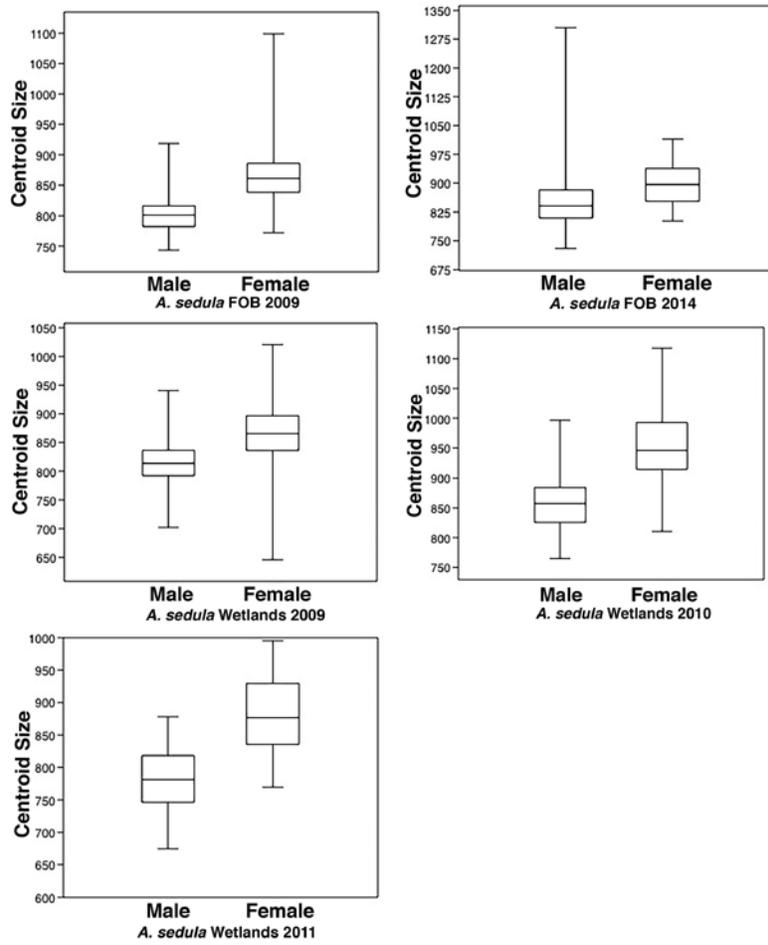


Figure 4.14. Mean centroid sizes of *A. sedula* males and females collected at FOB (2009) and (2014); and at Wetlands (2009), (2010), (2011). Note differences in y-axis scales.

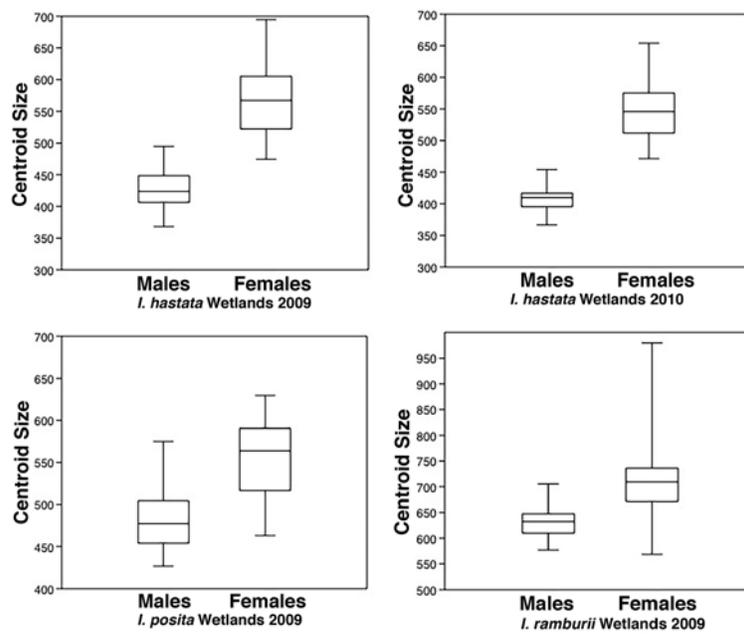


Figure 4.15. Mean centroid sizes of *I. hastata* males and males collected at Wetlands (2009), (2010); *I. posita* males and females collected at Wetlands (2009); and *I. ramburii* males and females collected at Wetlands (2009). Note differences in y-axis scales.

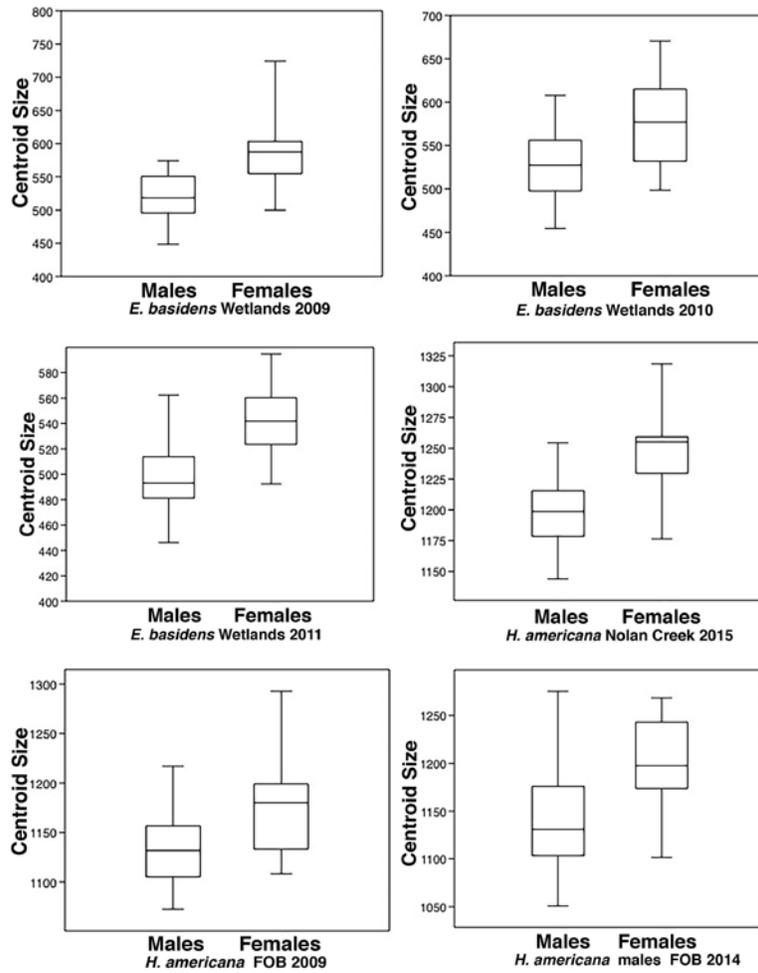


Figure 4.16. Mean centroid sizes of *E. basidens* males and females collected at Wetlands (2009), (2010), (2011); and *H. americana* males and females collected at Nolan Creek (2015) and FOB (2009), (2014). Note differences in y-axis scales.

Table 4.4. Results of ANOVA on centroid size for male damselflies compared to female damselflies. Values for centroid sizes from t tests.

SPECIES	YEAR	SITE	SEX COMPARISONS (n)	\bar{x} CENTROID SIZE	MALE vs FEMALE
					SIZE DIFFERENCE
<i>A. moesta</i>	2015	Nolan	Males (37) vs Females (38)	1157 vs 1234	***F = 76.49 p < 0.0001
<i>A. moesta</i>	2017	FOB	Males (182) vs Females (164)	1164 vs 1249	***F = 127 p < 0.0001
<i>A. nahuana</i>	2009	Wetlands	Males (122) vs Females (42)	776 vs 892	***F = 193.5 p < 0.0001
<i>A. sedula</i>	2009	FOB	Males (59) vs Females (40)	803 vs 870	***F = 54.82 p < 0.0001
<i>A. sedula</i>	2014	FOB	Males (270) vs Females (130)	852 vs 896	***F = 37.73 p < 0.0001
<i>A. sedula</i>	2009	Wetlands	Males (257) vs Females (131)	817 vs 867	***F = 105.9 p < 0.0001
<i>A. sedula</i>	2010	Wetlands	Males (130) vs Females (119)	855 vs 954	***F = 257.49 p = 0.0001
<i>A. sedula</i>	2011	Wetlands	Males (114) vs Females (56)	780 vs 881	***F = 162.7 p < 0.0001
<i>A. translata</i>	2015	Nolan	Males (41) vs Females (31)	1023 vs 1082	***F = 36.96 p < 0.0001
<i>E. basidens</i>	2009	Wetlands	Males (41) vs Females (30)	520 vs 584	***F = 51.86 p < 0.0001
<i>E. basidens</i>	2010	Wetlands	Males (35) vs Females (29)	528 vs 573	***F = 16.43 p = 0.0001
<i>E. basidens</i>	2011	Wetlands	Males (91) vs Females (31)	497 vs 543	***F = 80.16 p < 0.0001
<i>E. civile</i>	2009	Wetlands	Males (46) vs Females (31)	836 vs 848	F = 0.803 p = 0.3731
<i>H. americana</i>	2009	FOB	Males (44) vs Females (34)	1135 vs 1177	***F = 19.19 p < 0.0001
<i>H. americana</i>	2014	FOB	Males (48) vs Females (33)	1142 vs 1199	***F = 27.61 p < 0.0001
<i>H. americana</i>	2015	Nolan	Males (38) vs Females (34)	1198 vs 1251	***F = 49.84 p < 0.0001
<i>I. hastata</i>	2009	Wetlands	Males (38) vs Females (27)	428 vs 565	***F = 164.1 p < 0.0001
<i>I. hastata</i>	2010	Wetlands	Males (41) vs Females (55)	408 vs 546	***F = 413.6 p < 0.0001
<i>I. posita</i>	2009	Wetlands	Males (29) vs Females (33)	480 vs 557	***F = 62.21 p < 0.0001
<i>I. ramburii</i>	2009	Wetlands	Males (59) vs Females (65)	632 vs 708	***F = 77.16 p < 0.0001

Note: Significance -* < 0.05, -** < 0.01, -*** < 0.001.

Discussion

In damselflies, flight is critical for foraging, avoiding predators, and mating success. Therefore wing morphology, influenced by ecology and behavior, is under

selection for optimal survival and reproductive success (Wickman 1992; Petavy et al. 1997; Breuker et al. 2007; Bots et al. 2012; Outomuro et al. 2016). This study examined variations in wing shape and body size between mated and unmated damselflies as well as differences between males and females. In our study of mated versus unmated damselflies, we examined nine populations for three species, including *A. moesta*, *A. sedula*, and *E. basidens*. We found significant differences in wing shape only for the *E. basidens* females and significant body size differences for *E. basidens* females, the *A. sedula* females, and one population of *A. sedula* males. When examining variations in wing shape and body size between males and females, we consistently found differences between males and females for the twenty populations in ten species studied. In nineteen of twenty comparisons females were larger than males.

Our study did not find widespread differences in wing shape between mated and unmated damselflies for males or for females. Nine comparisons of mated versus unmated adults included both sexes of *A. moesta*, both sexes of *A. sedula*, and females of *E. basidens*. These collections were from FOB and the Wetlands during the flight seasons of four years. The only significant difference in wing shape occurred in the female population of *E. basidens* collected at the Wetlands (2010). In that population, wings of mated females were wider and shorter than those of the unmated females with most variation occurring at landmark 12 (lower edge) and 5 (internal). This shape suggests that selection favors maneuverability in females at a cost of increased energy demand (Betts & Wootton 1988; Outomuro et al. 2016). Other studies have not detected significant differences between mated and unmated damselflies (Bots et al. 2012; McTavish et al. 2012). Natural selection for survival as well as sexual selection may work in either the

same direction or in opposing directions. Selective forces working in concert could magnify adaptive evolution, whereas these selective forces working in opposition could maintain variation (Steele et al. 2011).

Body size, correlated to centroid size, significantly differed in only three of the nine mated versus unmated comparisons. Size differences between mated and unmated adults occurred in one of three male populations of *A. sedula*, in one of two female populations of *A. sedula*, and in one of two female populations of *E. basidens*. In the male population of *A. sedula*, the unmated males were larger, while mated females were larger for *A. sedula* and *E. basidens* populations. Body size may be an important component of successful male mating systems. Larger males more successfully defend sites than smaller males in territorial systems (Wickman 1992; Norberg & Leimar 2002). Smaller males can mate by patrolling for females away from the territorial sites (Gosden & Svensson 2008; Gyulavari et al. 2014; Therry et al. 2014). Size differences between mated and unmated damselflies are also influenced by temporal variations within seasons and among years (Gosden & Svensson 2008; Steele et al. 2011; Gyulavari et al. 2017).

As expected, significant wing shape differences were consistently found between males and females across twenty populations of ten species. Male and female damselflies have different life histories, including differences in mating behavior. Males defend territorial sites or patrol for females, while females forage and enter territories to mate and oviposit (Van Dyck & Wiklund 2002; Bots et al. 2009 99). *E. basidens* males and females collected at the Wetlands in 2009 did not show a significant difference in wing shape. Significant differences occurred between the *E. basidens* populations sampled in 2010 and 2011, with females having longer, more slender wings that aid in flight

endurance and agility. This difference in shape occurred in all species except *I. hastata* whose wings of females were wider. The most variable landmarks were 12 (lower edge of wing) and 5, 6 (internal landmarks).

Comparisons between male and female body sizes, as measured by centroid size, mirrored the consistent results of wing shape comparisons. In populations compared, females were significantly larger than males in all comparisons except for *E. civile*. Similar results in other studies are consistent with life history differences between the sexes (Breuker et al. 2007; Bots et al. 2009; McTavish et al. 2012). In species with two female morphs, the density of the female morphs influences male body size (Gosden & Svensson 2008). Variation in adult size is influenced by environmental conditions and food availability (Wickman 1992; Outomuro & Johansson 2012).

This study contributes to our understanding of sexual selection on flight morphology of winged animals. Wing design is closely related to ecology and behaviors, such as mating (Wickman 1992; Swillen et al. 2009; Bots et al. 2012). Sexual selection should strongly affect wing morphology because adult damselflies have a relatively short life span devoted mainly to reproduction (Corbet 1999; Vilela et al. 2017). Selection acts on reproductive structures or behavior, such as mate choice and male mating systems; so one can expect variations in flight morphology associated with these differences (Anderson 1994; Shuster & Wade 2003; Bots et al. 2009; Cigognini et al. 2014). Our research found scant evidence of variation in wing shape and body size between mated and unmated damselflies representing both sexes of three species, in two locations, over four years. Studies finding significant variations often compared mated and unmated males of one species (Steele et al. 2011; Gyulavari et al. 2014; Therry et al. 2014; Tuzun

et al. 2017). In contrast to wing shape, we found widespread differences in body size, as measured by centroid size, between smaller males and larger females. These results along with similar results in other studies are most likely consistent with life history differences between the sexes, ecology, and selection forces (Van Dyck & Wiklund 2002; Gosden & Svensson 2008; Bots et al. 2009; McTavish et al. 2012). This study supports the concept that sexual selection affects important variables associated with flight performance and mating success.

CHAPTER FIVE

Summary and Conclusions

Summary

Chapters Two and Three present results from geometric morphometric studies examining environmental effects on wing morphologies of damselflies. Chapter Two results strongly suggest that temporal environmental factors (i.e. early versus late emergence) affect wing morphology in *A. sedula* more consistently than do spatial environmental factors (different habitats). Significant variations in both wing shape and wing size were shown in all four comparisons between *A. sedula* emerging early in the flight season and those emerging late in the flight season. These results were consistent for both sexes, in two years, and at two locations. These results are supported by previous studies (Bots et al. 2009; Gallesi et al. 2016). Chapter Two also report significant variations in wing shape and wing size between years. Taken together, these results suggest that temporal (i.e. seasonal) environmental factors consistently affect wing morphology in both sexes of *A. sedula*. In contrast, spatial environmental factors (i.e. different habitats) showed little affect on wing shape and wing size.

Chapter Three used findings from Chapter Two to further examine temporal environmental effects on wing morphology to include five damselfly species in addition to *A. sedula*. Significant differences in wing shape and wing size for early versus late seasonal emergers for seven populations (six male and one female) of five species suggest that this seasonal effect on wing morphology is widespread among damselfly species. Although *A. sedula* exhibited significant variations in wing morphology in seven

of eight comparisons between years, this pattern was not seen for the additional six species. Few comparisons between years indicated significant variations in wing shape (three of seventeen comparisons) and wing size (six of seventeen comparisons). Wing morphology within damselfly species does not frequently vary between years. Results for the six species included in Chapter Three do not parallel the findings for *A. sedula* in Chapter Two for annual variations in wing morphology. Within a habitat, annual environmental differences are not significant enough to affect wing morphology within a species.

Chapter Four examined wing morphologies varying between mated and unmated damselflies and between male and female damselflies. These results are discussed in the context of behavioral differences of mated versus unmated adults of both genders. Significant variations in wing shapes were rarely found between mated and unmated damselflies. Some studies have reported significant differences between mated and unmated males (De Block & Stoks 2007; Swillen et al. 2009), while other studies have not found significant differences (Steele et al. 2011). Most studies examined multiple traits (e.g. body mass, wing aspect ratio, flight performance) that did not include geometric morphometric analysis of wing shape. These studies typically were conducted on only males of one species. This study is unique in that three species, including males and females, were compared using geometric morphometric analyses of wing shape and wing size. All studies were conducted using field-collected specimens either mating or not mating at the time of collection. Although this is the standard experimental design, it does not capture lifetime mating success. Chapter Four also reported results of comparisons from male and female wings. As reported in other studies, significant

differences in wing shape and wing size consistently occurred between males and females (Outomuro & Johansson 2011; Gallesi et al. 2016). Females are larger and have more slender wings than males. Geometric morphometric analysis accurately detects differences in wing shape between males and females, which suggest that it is a tool that could be more widely used in analyses of mating behaviors.

This dissertation provides an extensive body of research using geometric morphometric techniques to analyze data and elucidate variations in damselfly wing morphology caused by environmental and behavioral effects. Patterns of consistent variation in phenotype suggest evidence of developmental plasticity. This work adds considerably to our knowledge of which environmental factors most influence wing morphology in damselflies, which in turn contributes to their survival and fitness. These patterns of influence could extend beyond damselflies.

Future Directions

Although there have been many studies of environmental effects on organisms, the genetic and developmental basis of plasticity remains unclear, possibly because of the lack of an appropriate tool to relate phenotypic outcomes to developmental mechanisms (Debat et al. 2003). Integrating genomic data with the field ecology knowledge of many odonate species could be a major step forward in understanding eco-evolution (ByBee et al. 2016). Studies measuring gene expression patterns during larval stages should reveal the varied degree of plasticity evidenced by adult stages of odonates.

Further fieldwork exploring sexual selection should incorporate geometric morphometric as an additional technique to analyze differences between mated and unmated damselflies. Most studies of mating status have been done with only males of

only one species. More studies should include multiple species and both sexes. In addition to comparing mated versus unmated males, studies should explore possible differences between males of alternate mating system (e.g. territorial and scrambling systems).

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