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Article

Watershed-Mediated Ecomorphological Variation: A Case Study with the Twin-Striped Clubtail Dragonfly (*Hylogomphus geminatus*)

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Simple Summary: Changes in the size or shape of aquatic or amphibious organisms can signal environmental alterations. The Twin-striped Clubtail is a dragonfly in the southeastern US with known body size variation. Our objective was to characterize the significance and extent of size differences in the species and determine whether those differences were associated with watershed across the species' limited range. In this species, total body length varied by watershed. Significant size differences were noted in many watershed comparisons, most notably in adjacent watersheds on either side of the Apalachicola River, FL, which differed in land-cover types; smaller individuals were associated with more disturbed forms of land cover. Our results suggest there are watershed-level differences in body size across the range of the species that should be examined more closely with respect to different potential environmental stressors, such as poor water quality from changing land cover associated with agriculture and urbanization.

Abstract: Anthropogenic land-cover change is modifying ecosystems at an accelerating rate. Changes to ecomorphologically variable taxa within those ecosystems serve as early-warning signs that resources on which humans and other animals depend are being altered. One known ecomorphologically variable taxon is *Hylogomphus geminatus*, a species of dragonfly in the southeastern United States that shows pronounced variation in total body length across its limited geographic range. We measured total length of live as well as preserved museum specimens of *H. geminatus* and the sympatric species *Progomphus obscurus* (as a means for comparison). Both species showed significant size differences by HUC-8 watersheds in which they occur. *H. geminatus* showed additional significant differences on either side of the Apalachicola River, Florida, for all comparisons by sex. In overlapping watersheds, the species tended to show the same trends in size relative to their respective averages. Smaller body size was associated with more urban and agricultural land cover. These findings indicate that ecomorphological variation is tied to the watershed scale and point to significant variations on either side of the Apalachicola River. More focused research and continuous stream monitoring should be implemented to understand the extent that phenotypic plasticity plays in this variation.

Keywords: *Hylogomphus geminatus*; ecomorphology; odonata; watershed; landcover

1. Introduction

Because species' physical traits are shaped by environmental conditions, trait variation can inform extinction risk [1,2]. Thus, examining patterns of ecomorphological variation may improve our ability to predict how species may respond to environmental changes, such as those stemming from anthropogenic land-cover changes. Land-cover changes have altered water quality and availability in many parts of the world [3], which may be reflected in development and subsequent body morphology of some animals [4]. Identifying environmental factors influencing ecomorphologically variable taxa is thus crucial in understanding effects of anthropogenic activities and landscape disturbance on aquatic resources.

Ectotherms such as aquatic insects are especially sensitive bioindicators that respond to anthropogenic impacts on both abiotic and biotic environmental variables [5,6]; members of the insect order Odonata (dragonflies and damselflies) have been noted as particularly useful gauges of environmental conditions [7]. The presence, abundance, and morphology of these amphibious predators in an ecosystem can be used to signal terrestrial and aquatic environmental aberrations [8,9]. With relatively short generation times, their morphological traits (e.g., body size) may signal recent environmental changes [10], but environmental factors that affect ecomorphology in odonates in the field are poorly understood. Many abiotic variables known to influence insect morphology include aquatic factors, like depth, conductivity, pH, temperature, turbidity, and dissolved oxygen (DO); some biotic variables, like prey availability, also have relationships with size [11]. As odonates spend two-thirds of their lives growing in water, these aquatic variables should have a larger impact on their morphology than would terrestrial variables because odonates do not continue growing once they have emerged from the water as winged adults. Once an adult, dispersal allows gene flow across populations, so consistent ecomorphological patterns in populations indicate the presence of environmental drivers of variation.

Land-cover changes are occurring at an accelerating rate in many parts of the world. Deforestation and waterway modifications change aquatic habitats and induce stressors like increasing pollutants, temperatures, or stream velocity, or they may reduce habitat entirely, leaving large swathes of unsuitable land cover that act as barriers to even vagile dragonfly species. In the United States, the state of Florida has experienced relatively recent and rapid land-cover changes resulting from increases in the extent of urbanization and agriculture since the early 1900s [12,13]. Florida increased in population by 2.95% per year between 1960 and 1997 [14], associated with a 60% increase in agricultural lands between 1936 and 1995 and a 632% increase in urbanized lands during the same time period [12]. These landscape changes are associated with environmental consequences and modifications of hydrology that could be responsible for variations in odonate morphology through deforestation, streambank destabilization, and stream channelization. Thinner canopies via deforestation can lead to more sunlight permeation in streams, which is associated with warming water. Warmer water temperatures correlate with decreased body size in odonates [15,16]. There also may be indirect effects on odonates, such as impacts on attack rate and food handling time [17–19], which are crucial functional responses for an organism's metabolic rate and, therefore, its developmental rate. Furthermore, channelization of naturally dendritic streams causes a reduction in spatial heterogeneity and increases in stream depth and velocity, which not only affects odonates, but also the prey on which they depend.

Warming waters also have a reduced ability to hold DO, a stressor that has been shown to reduce overall odonate diversity in streams [20] and could trigger changes in ecomorphologically variable taxa. Streambank destabilization can exacerbate erosion, causing increases in turbidity and sedimentation which can reduce an organism's ability to successfully find food, limit light permeation necessary for aquatic primary producers, and clog the delicate gills of DO-breathing organisms. Agricultural and urban runoff pollute streams with heavy metals, salts, and other chemicals that change stream pH, total dissolved solids, and specific conductance, reducing water quality for aquatic macroinvertebrates. Water pollution is rarely limited to a single stretch of stream at the source of the disturbance, but generally travels much further downstream, affecting the large majority of any watershed within which the disturbance occurs. First-order headwater streams in particular supply roughly 55% of the mean-annual water volume received by higher-order navigable rivers [21], so even small-scale, point-source pollution in low-order streams has the potential to affect a large extent of the landscape and therefore impact a great deal more aquatic taxa than just those immediately around the disturbance.

One known ecomorphologically variable odonate is *Hylogomphus geminatus* [22], the Twin-striped Clubtail (Gomphidae), a species that primarily inhabits relatively small, sandy-bottomed streams across the panhandle of Florida and surrounding areas along the Gulf Coast of the United States. In Carle's original description of *H. geminatus* (Carle, 1979), he noted size differences in adults from populations on either side of the Apalachicola River, Florida, but did not suggest any mechanisms for these differences [22]. Subsequent unpublished observations have noted these size differences in *H. geminatus* as well. [23]. Although the river spans several hundred feet across much of its width [24], that distance should not act as a barrier to gene flow for vagile organisms such as odonates.

Some intraspecific body size variation in odonates is the result of nymphal development time, with longer time spent as a nymph generally producing larger adults; the duration of nymphal development time, in turn, is driven by environmental conditions [25–28]. It is well-documented in adult odonates that body size is positively associated with thermoregulatory ability, competitive ability, and fecundity [29–31]. Thus, the implications of differences in body size in *H. geminatus* across populations that are being driven by environmental conditions include potential mate limitation (if different populations are emerging at different times because of different nymphal development times) and access to resources. Anecdotally, it has been observed that smaller individuals west of the Apalachicola River tended to emerge earlier in the year than the larger individuals east of the river [23]. The major river drainage within the core range of *H. geminatus* is the Apalachicola River, a crucial freshwater resource for the surrounding biotic community, with a drainage that includes four major rivers spanning three U.S. states and an area of almost 51,800 sq km [32].

Although some dragonflies are capable of crossing oceans during annual migratory events, these species tend to occupy lentic habitats whereas many members of the gomphid family occupy lotic habitats and are not known to travel far from the water sources from which they emerge [33]; *H. geminatus* is no exception. Therefore, it is hypothesized that adult body size is likely to be a function of the environmental conditions in the watershed within which they occurred when captured for measurements. It is unknown if these differences in body size are present in the nymphs of the species as well, but the adult size discrepancies may be indicative of potential stressors in nymphal habitat, like warming water temperatures or direct modifications to hydromorphology potentially due to changes in land use and land cover.

H. geminatus is listed by the International Union for Conservation of Nature (IUCN) as globally vulnerable, vulnerable in Florida, critically imperiled in Mississippi, and imperiled in Alabama and Georgia [34], so variations in body size may be signaling stressors in its aquatic habitat that, if left unnoticed, could lead to its extirpation from some of these areas. Although vulnerable, populations are currently stable and the species' biggest threats are habitat loss due to land-cover changes, like development and forestry [34].

Those threats occur across broad spatial and temporal scales. Characterizing effects at a watershed scale based on the morphology of individuals within that watershed is a way to gauge watershed changes across broad temporal and spatial gradients. Many species of odonates can be hard to find due to land access issues, abbreviated flight seasons, limited geographic range, or downright scarcity. *H. geminatus* is an uncommonly encountered species with an abbreviated flight season and limited geographic range; there are not enough records of the species to justify examination of body length within fine-scale watersheds, so medium-scale 8-digit Hydrologic Unit Code (HUC-8) "subbasins" [35] were chosen as the area to which populations' total body lengths would be examined. Patterns within and amongst subbasins where *H. geminatus* occurs can be analyzed for significance, which can inform ecomorphological variation as a function of subbasin occurrence. As these subbasins around the core range of *H. geminatus* continue to transform due to anthropogenic activities, this uncommon species could see retractions in its distribution and eventual extirpation from areas where its optimal habitat no longer exists. Understanding the causes of variation in its ecomorphology now may allow us to prevent future losses of this species and many others that depend on crucial freshwater systems.

2. Materials and Methods

2.1. Measurement of Museum Specimens

A total of 154 adult *H. geminatus* specimens preserved at the International Odonata Research Institute (IORI) within the Florida State Collection of Arthropods in Gainesville were measured by J.S.G. with digital calipers. (The IORI is one of the largest repositories of odonate specimens in North America.) Total length (from head to tip of cerci), hindwing length, forewing length, and

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metathoracic femur length were measured to the nearest hundredth of a millimeter when possible. The left wings and femora were measured; right side measurements were made if necessary due to damage to left wings/femora. Total length of 159 Common Sanddragon (*Progomphus obscurus*) adults was also measured as a means for comparison to determine whether any patterns by watershed were consistent between species rather than a species-specific idiosyncrasy; presence of consistent patterns between species would indicate external (landscape) drivers at work. This confamilial species was chosen because it occurs across a broad geographic range that encompasses *H. geminatus'*, and it occurs in the same sandy streams as *H. geminatus*, often collected alongside it. *P. obscurus* specimens from IORI were only selected from collection locations within and adjacent to the geographic range of *H. geminatus*. All specimens were primarily kept in plastic envelopes with wings folded together above the bodies so that only the lateral aspect was available for measurements (Figure 1).

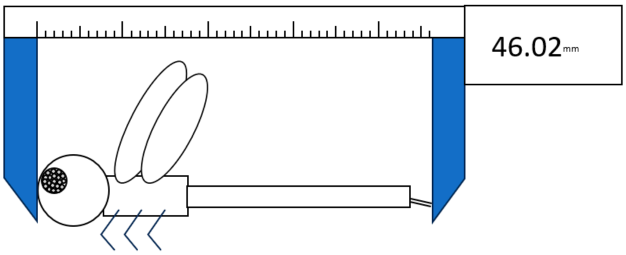


Figure 1. The general approach to measuring total length of odonate specimens (from head to tip of cerci) housed in plastic envelopes in IORI.

H. geminatus specimens whose bodies were broken into three or more pieces did not have their total length recorded (*n*=19). One specimen from Tennessee was not used in analyses due to questionable identification.

2.2. Selection of Field Locations and Live Specimen Measurements

We used museum specimen locations as well as expert opinion and citizen science databases (iNaturalist, OdonataCentral, GBIF) to identify specific field localities to capture *H. geminatus* adults. A novel method for measuring live adult dragonflies was implemented during this study. Adults were captured with an aerial insect net and placed into a clear plastic page protector where they "perched" motionless with wings spread, allowing easy measurements to be taken with digital calipers from the dorsal aspect (Figure 2).

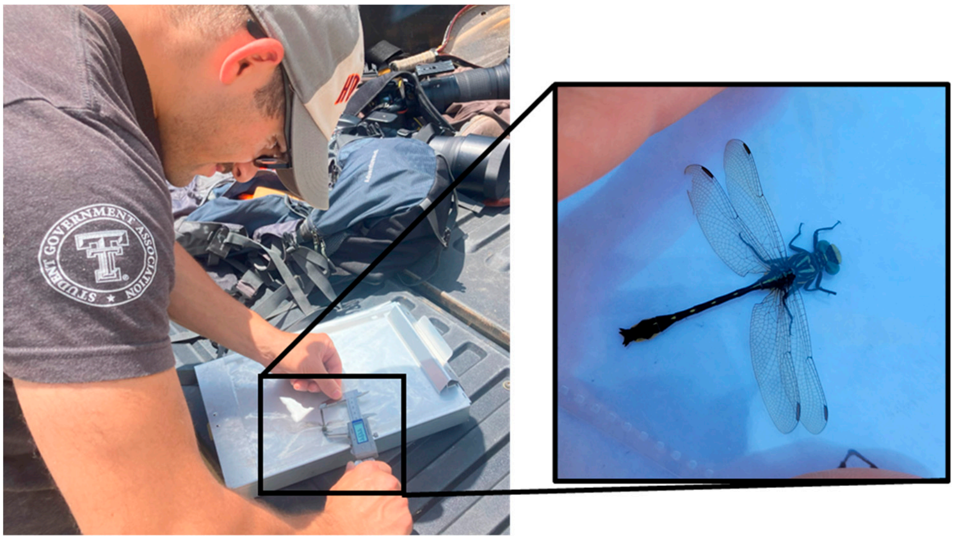


Figure 2. The general approach to measuring total length of live odonate specimens by temporarily placing them in a clear page protector and measuring head to tip of cerci across the dorsal aspect.

Total body length and the left fore- and hindwings were measured on live *H. geminatus* specimens in this way. After the individuals were sexed, their wings were marked with unique identifiers with a Sharpie marker to avoid recapture and they were photographed and released (n=27). Metathoracic femur lengths were not measured due to the likelihood of inflated error associated with such small measurements on live specimens. Three of the hand-caught individuals were not used in analyses due to fears that their teneral measurements may not be indicative of their sexually mature dimensions.

2.3. Watersheds

Body sizes in *H. geminatus* and *P. obscurus* were examined by watershed to determine (1) whether there were differences by watershed and (2) whether both species exhibited similar patterns by watershed. There are six sizes of watershed, given by hydrologic unit codes (HUCs). These range in size and specificity from large-area "regions" (HUC-2) down to small-area "subwatersheds" (HUC-12) [35]. Although characterizing watershed effects at the finest possible resolution would be desirable in many scenarios, the need to balance statistical power (higher sample size) with watershed scale in this case necessitates the use of a "medium-scale" watershed boundary, HUC-8. The HUC-8 "subbasin" scale allows enough specificity to characterize effects at a relatively fine scale but still allows a high enough sample size within each subbasin for statistical power.

2.4. Statistical Analyses

Length data on either side of the Apalachicola River were analyzed for significance for both species in the RStudio version 2023.06.1 (R Core Team, 2023) base package *stats* using a non-parametric Wilcoxon rank-sum test. Eight-digit Hydrologic Unit Code (HUC-8) watersheds were downloaded to ArcGIS Pro (Esri, Redlands, California, USA) from the USGS National Hydrography Dataset [36]. Watershed boundaries and locations of measured adults were combined using a spatial join where they overlapped. Location data were then overlaid onto a map of HUC-8 watersheds and the watershed boundaries were extracted for use in further analyses for each species (Table 1, Figure A1).

Table 1. HUC-8 watersheds where *H. geminatus* and *P. obscurus* occurred. *P. obscurus* specimens were collected from a wider geographic range than *H. geminatus*, hence the additional number of codes.

Hylogomphus geminatus	*Progomphus obscurus*
3130011	3150204
3140105	3140104
3140104	3140102
3130012	3140301
3140103	3140103
3130005	3140203
3140304	3140201
3140305	3140101
3140102	3130012
3170007	3130011
3140101	3120003
3140203	3130003
3160205	3110103
3120003	3110206
	3080102
	3080103
	3070204

Land-cover data were downloaded from Florida Fish and Wildlife's and Florida Natural Areas Inventory's Florida Cooperative Landcover Map [37] and land-cover types were classified into one of six mutually exclusive categories (Upland/Sandhill, Perennial Wetland, Seasonal Wetland, Estuarine Wetland, Urban/Developed, and Rural/Agriculture). Land-cover data were clipped to subbasin polygons in ArcGIS Pro for analyses on either side of the Apalachicola River. One subbasin (3130011) was split by the river and its land cover east of the river was merged with the eastern subbasin (3120003) whereas its land cover west of the river was merged with the western subbasin (3130012).

Significance of adult total lengths as a function of watershed occurrence was then assessed using a Kruskal-Wallis test and boxplots were made using the R package *ggplot2* [38] to show total adult lengths in each watershed (Figures 5a and 6a) as well as male and female lengths in each watershed (Figures 5b and 6b). From there, we used a pairwise Wilcoxon rank sum test with a BH adjustment [39] to establish significant differences in total lengths amongst pairwise comparisons (Tables A1 and A2). To understand if all *Hylogomphus* species show high levels of morphological variation, we compared the published minimum and maximum total lengths of all 6 members of the genus [40] (all occur in eastern North America) as well as the minimum and maximum lengths of individuals from our own measurements. The same was done for *P. obscurus*.

3. Results

3.1. Patterns in Body Size

Hylogomphus geminatus adult lengths were originally grouped into two categories: those east of the Apalachicola River (n = 22) and those west of it (n = 144). Significant size differences were found (Wilcoxon rank-sum test: W = 2793, p = 4.317e-09). *Progomphus obscurus* adults also showed significant differences when eastern specimens (n = 91) were compared to western (n = 68) (Wilcoxon rank-sum test: W = 4246, p = 3.05e-05) (Figure 3). Eastern *H. geminatus* adults averaged 7.99% larger than their western counterparts; eastern *P. obscurus* averaged 2.67% larger than theirs.

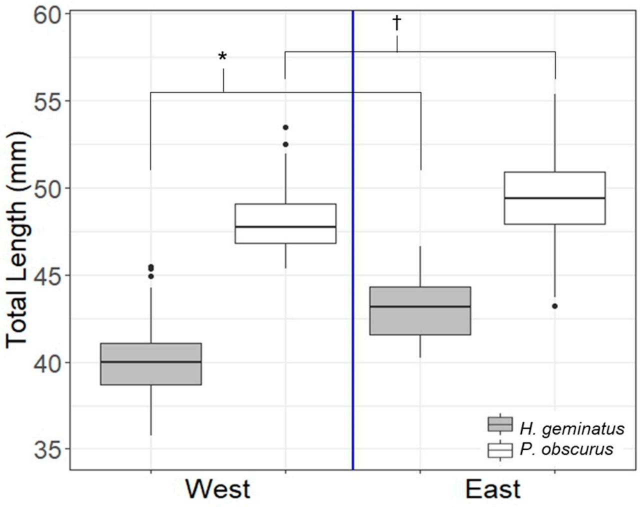


Figure 3. *H. geminatus* individuals averaged significantly larger east of the Apalachicola River (represented by the vertical blue line) than west (W = 2793, p = 4.317e-09). *P. obscurus* individuals also showed the same significant trend (W = 4246, p = 3.05e-05). Significance between *H. geminatus* comparisons is denoted with an asterisk and that of *P. obscurus* is denoted by †.

When adult *H. geminatus* lengths on either side of the Apalachicola River were parsed on the basis of sex, they were significant for all comparisons: west males (n = 93) against west females (n = 51) (W = 1480, p = 9.887e-05), east males (n = 16) against east females (n = 6) (W = 15, p = 0.006674), west males against east males (W = 135.5, p = 9.648e-08), and west females against east females (W = 21, p = 0.0003139). *P. obscurus* showed no significance for west males (n = 54) against west females (n = 14)

(W = 379, p = 0.497), east males (n = 67) against east females (n = 24) (W = 880.5, p = 0.2468), and west females against east females (W = 130, p = 0.1301), but did show significance for west males against east males (W = 1054, p = 4.176e-05) (Figure 4).

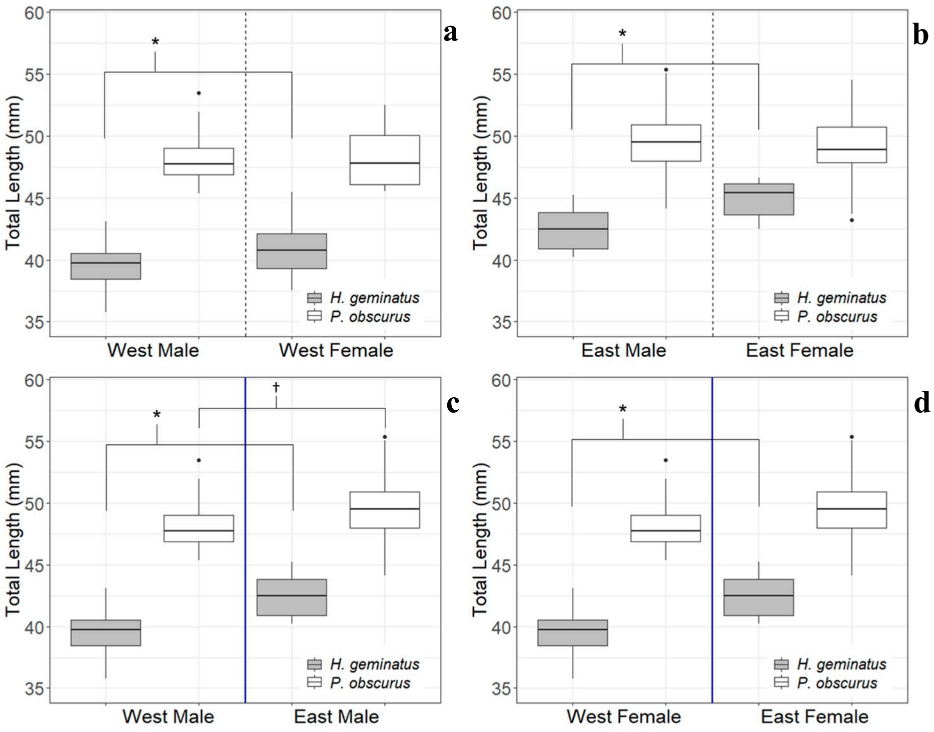
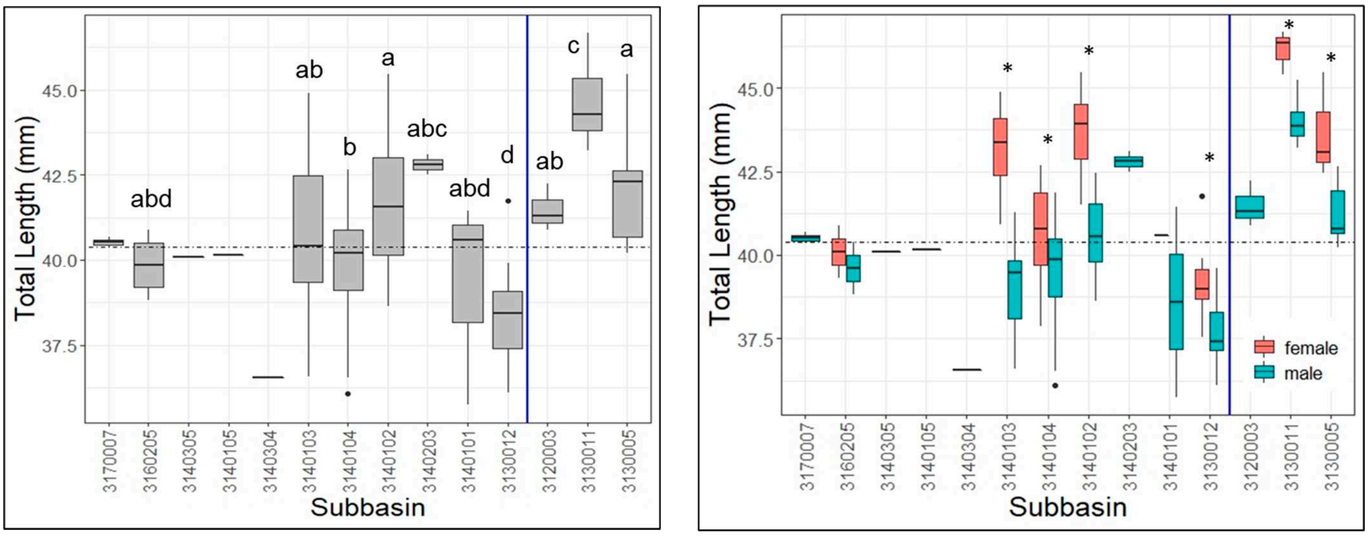


Figure 4. All comparisons between *H. geminatus* individuals of either sex on either side of the Apalachicola River showed significant differences whereas *P. obscurus* only showed significant differences in length between east and west males. Significance between *H. geminatus* comparisons is denoted with an asterisk and that of *P. obscurus* is denoted by †. Note that the vertical blue line separating western and eastern data in Figure 4c,d represents the Apalachicola River. (**a**) Western female *H. geminatus* were significantly larger than western males (W = 1480, p = 9.887e-05) but there was no significant difference for *P. obscurus* (W = 379, p = 0.497); (**b**) eastern female *H. geminatus* were significantly larger than eastern males (W = 15, p = 0.006674) but there was no significant difference for *P. obscurus* (W = 880.5, p = 0.2468); (**c**) eastern male *H. geminatus* and *P. obscurus* were significantly larger than their western conspecifics (W = 135.5, p = 9.648e-08) and (W = 1054, p = 4.176e-05), respectively; (**d**) eastern female *H. geminatus* were significantly larger than western females (W = 21, p = 0.0003139) but there was no significant difference for *P. obscurus* (W = 130, p = 0.1301).

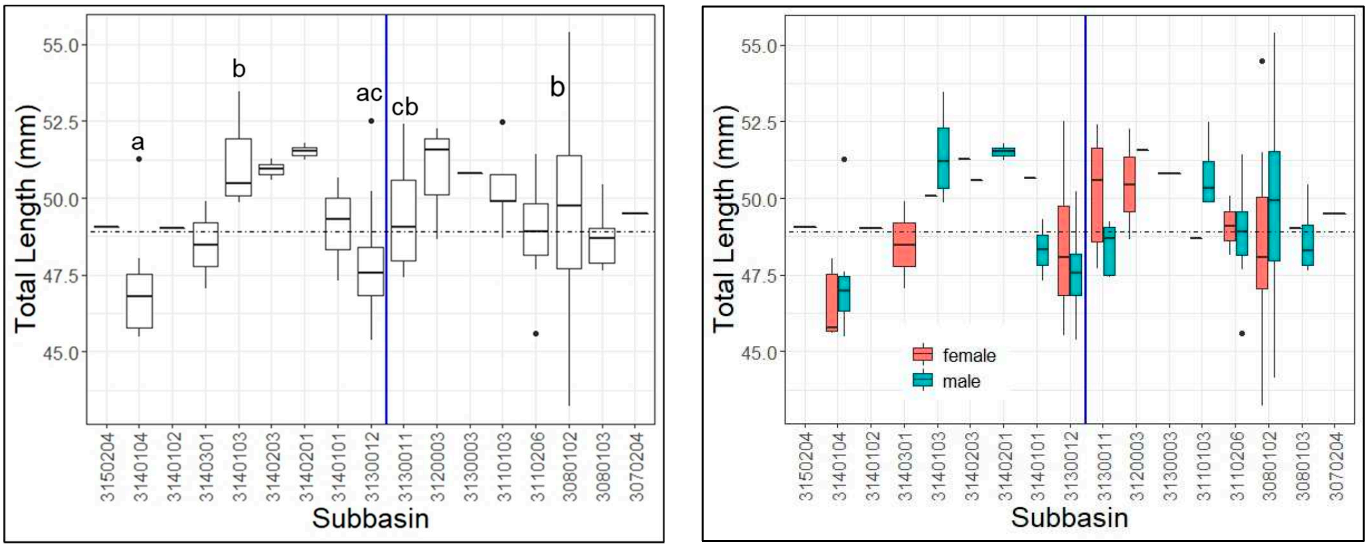
3.2. Lengths by Watershed

After total lengths for both species were assessed on either side of the Apalachicola River, we then plotted average total lengths for each species within each subbasin of occurrence and did the same broken down by sex (Figures 5 and 6). The average length of *H. geminatus* females was significantly larger than that of males in 6 of the 7 subbasins with enough individuals of each sex to run analyses. The average length of *P. obscurus* males and females did not significantly differ in any of the 5 subbasins with enough individuals of both sexes to run analyses, and females did not always average larger in each subbasin as they did in *H. geminatus*. *H. geminatus* showed significance of total length as a function of subbasin occurrence (Kruskal-Wallis: X^2 = 77.796, df = 13, p = 2.861e-11); there were significant differences in total lengths of 16 of the possible 91 pairwise subbasin comparisons (17.58%) (Table A1). *P. obscurus* also exhibited significant differences in total length as a function of subbasin occurrence (Kruskal-Wallis: X^2 = 47.898, df = 16, p = 4.928e-05) but only showed significant differences in total lengths of 5 of the possible 136 pairwise subbasin comparisons (3.68%) (Table A2).



(**a**) (**b**)

Figure 5. *H. geminatus* total lengths within each subbasin (HUC-8 codes from Table 1). Note that the vertical blue lines separating subbasins in Figure 5a,b represent the Apalachicola River and its placement relative to associated subbasins. The horizontal dashed line represents the average total length of all *H. geminatus* specimens measured. (**a**) *H. geminatus* varied in length significantly in 16 pairwise subbasin comparisons (Table A1). This is especially noticeable in subbasins directly on either side of the river. (**b**) *H. geminatus* total lengths by subbasin broken down by sex. Females averaged larger than males for all subbasins. Significant differences between male and female sizes are seen in 6 of the 7 subbasins with enough specimens of each sex to assess significance. Subbasins containing significant size differences between males and females were denoted with an asterisk.



(**a**) (**b**)

Figure 6. *P. obscurus* total lengths within each subbasin (HUC-8 codes from Table 1). Note that the vertical blue lines separating subbasins in Figure 6a,b represent the Apalachicola River and its placement relative to associated subbasins. The horizontal dashed line represents the average total length of all *P. obscurus* specimens measured. (**a**) Analyses of total length in each subbasin revealed significant size differences in 5 pairwise comparisons (Table A2). (**b**) *P. obscurus* total lengths by subbasin broken down by sex. Females were not always larger than males and no significant differences between male and female sizes were found in any subbasin with enough of each sex to assess significance.

There were 8 overlapping subbasins that contained specimens of both species (Figure 7). Both species tended to show the same trends in length relative to their respective averages in 6 of the 8 overlapping subbasins. This was most notable around the Apalachicola River.

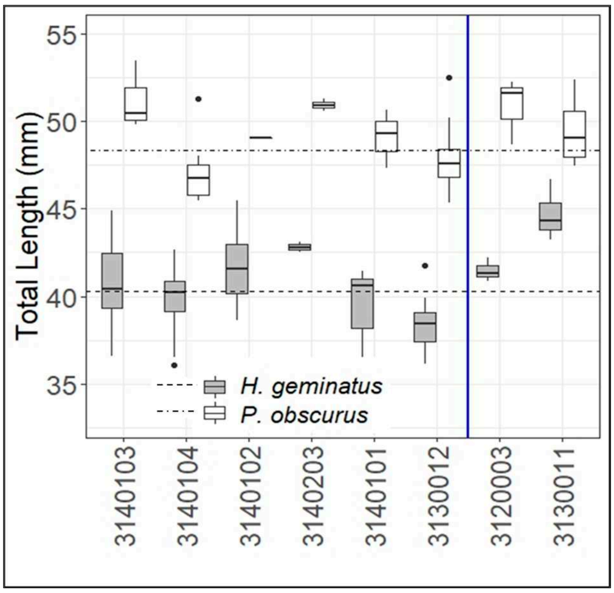


Figure 7. Average lengths of *H. geminatus* and *P. obscurus* in 8 overlapping subbasins (HUC-8 codes from Table 1). The vertical blue line separating subbasins represents the Apalachicola River and its placement relative to associated subbasins. The dashed horizontal lines represent the overall average lengths of the respective species. Notably, the subbasin immediately west of the river (3130012) contained smaller individuals of both species and the one immediately east of the river (3120003) contained larger individuals of both species.

3.3. Comparisons of Body Size with Published Values

A comparison of published total lengths of the 6 species of *Hylogomphus* showed that *H. geminatus* is known to have the most variation in total body length (20.5% difference) followed closely by *H. parvidens* (18%) from which *H. geminatus* was split during its original species description. Three other members of the genus showed variation in body length by 5.7% or less. Our own measurements of *H. geminatus* specimens increases the published range of variation from 20.5% to 30.5%. Published values for *P. obscurus* show total body length of individuals varies by 12.8% but our own measurements increase that to 28% (Table A3).

3.4. Land-cover Analysis

The land cover of the three subbasins surrounding the Apalachicola River (3130012, 3120003, 3130011) was analyzed on the basis of the side of the river on which the land occurs (Table 2). The subbasins directly to the west of the river were characterized by high Rural/Agricultural influences that represented more than half of the land area. Additionally, Urban/Developed was 0.3% more common (as a function of total land area) in the west than the east. The east side of the river had proportionately more wetlands and Upland/Sandhill land cover.

Table 2. Florida land-cover data for three watersheds surrounding the Apalachicola River. Both sides had relatively high Rural/Agricultural inputs, but the west side of the river had notably more and was also characterized by less Upland/Sandhill habitat and overall wetlands.

Land-cover Type	West of River	East of River
Upland/Sandhill	10.9%	21.7%

Perennial Wetland	20.8%	25.6%
Seasonal Wetland	8.5%	12.7%
Estuarine Wetland	0.3%	1.3%
Urban/Developed	5.5%	5.2%
Rural/Agriculture	54.1%	33.5%

4. Discussion

4.1. Patterns in Body Size

These findings support the size discrepancies noted in the original species description of *H. geminatus* and show that both *H. geminatus* and *P. obscurus* are significantly different sizes on either side of the Apalachicola River, although the size discrepancy of the former species is much greater than that of the latter. Body size in odonates is positively associated with breeding rate and fitness [29,41], so adults in subbasins containing larger than average individuals may not have much reason to move to other subbasins. Dispersal from breeding sites seems to be undertaken by smaller, less dominant individuals when larger, more dominant individuals are also present [41], but it is also impacted by the age and sex of individuals [42]. It stands to reason that in areas where there are mostly larger (dominant) individuals (east of the river), there would be fewer smaller (subordinate) individuals dispersing to new areas, which might account for the small geographic area *H. geminatus* occupies east of the river. There is a wider range of sizes in subbasins west of the river, so smaller individuals likely disperse to new areas more frequently, colonizing new subbasins and increasing their geographic range.

It is possible that whatever stressors are acting on *H. geminatus* and *P. obscurus* are affecting them to different extents, and while the Apalachicola River does not pose a significant barrier to gene flow for either species, they may simply have no reason to fly across if they have success breeding and laying eggs around the streams from which they emerged. If smaller individuals west of the river are able to cross it, they may not have success breeding in the presence of larger, more dominant individuals in the east, so the population in the east would still contain mostly larger individuals' genes. This is a pattern that has been noted in the literature since the late 1970s [22]. The size differentiation could have arisen by phenotypic plasticity, but if individuals are not crossing the river and sharing genes, this could lead to genotypic differences. DNA would need to be collected to assess the impact genotypic differences may have on size. Continuous stream monitoring with data loggers is another option to collect data with which we can form models to predict size, but it is still difficult to assign an individual dragonfly to the stream where it was caught, as it may have developed as a nymph in an adjacent watershed.

H. geminatus females were significantly larger than their male counterparts, but that was rarely the case with *P. obscurus*. Although *P. obscurus* did show significant differences on either side of the river when sexes were grouped together, the only significant difference when lengths were broken out by sex was between east males and west males. This could suggest that eastern males have taken advantage of their larger size as a byproduct of phenotypic plasticity and have excluded smaller males from the breeding pool. Overall, however, *P. obscurus*' smaller effect size on either side of the river suggests that *P. obscurus* morphology may not be as closely tied to watersheds as is *H. geminatus*' morphology.

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4.2. Lengths by Watershed

This supposition is further supported when examining the significance of size amongst pairwise subbasin comparisons (Tables A1 and A2). *P. obscurus* rarely showed significant differences in size amongst subbasins and did not show any significant differences between the two specific subbasins on either side of the river. *H. geminatus* had a much higher number of significant comparisons and specifically showed a large difference on either side of the river. *H. geminatus* also showed significant differences in several adjacent subbasins, which supports the cause of morphological differences being from phenotypic plasticity rather than genotype, as adjacent watersheds not separated by a river should still see some amount of gene flow. When sizes of both species were analyzed in overlapping subbasins with respect to each species' average length (Figure 7), trends within each subbasin relative to the average lengths were roughly the same for both species. Despite this, *H. geminatus* showed more significant differences across those subbasins than did *P. obscurus*.

4.3. Comparison of Body Size with Published Values

A summary of published lengths of the species within *Hylogomphus* shows it is not a highly morphologically variable genus, but the species from which *H. geminatus* was split does exhibit similar morphological variation based on published values (Table A3). Our measurements also showed higher variability in total lengths for both *H. geminatus* and *P. obscurus* than published values showed. Intraspecific size variability of odonates is not a new concept [43,44] but has typically been attributed to differences in climate and water temperature based on latitude. Climate and temperature should not have an effect on *H. geminatus*, whose geographic range is only across a small area on a mostly longitudinal gradient. Primary productivity has been suggested as a mechanism predicting odonate body size [45] and should be investigated as a factor in the streams inhabited by *H. geminatus*.

4.4. Land-cover Effects on Length

Since the original species description of H. geminatus, the populations directly west of the Apalachicola River have consisted of smaller individuals [22]; this is especially concerning given that more than half of the area within the subbasins to the immediate west of the river was comprised of agricultural land. Not only does agricultural development reduce the number of viable streams in which H. geminatus can live through direct waterway modification, it also reduces the amount of upland/sandhill habitat that deposits the sediments into the streams on which H. geminatus is so dependent. Hylogomphus geminatus is only found in streams with primarily sandy substrate and as that substrate disappears due to agricultural development, more silt may be deposited into the streams instead, creating a possible stressor that is negatively impacting the species. The smaller individuals in these areas could be signaling decreased fitness due to environmental stressors [29,41] that may eventually lead to extiraption of this and similar species. Agricultural practices are commonly associated with high levels of nitrogen (N) and phosphorus (P) runoff [46] as well as various herbicides and pesticides [47]. This is especially concerning in waterways because aquatic organisms can bioaccumulate these chemicals and may be more susceptible to them than terrestrial organisms [47], and while collateral damage to non-target organisms in agricultural settings may be limited, once chemicals enter nearby streams, side effects can be drastic. Chemicals are carried further away by streams, often ending up as far as the ocean, and many more non-target organisms fall victim to them [48].

By contrast, H. geminatus east of the Apalachicola River are known to be larger than their western conspecifics. Rural/Agricultural land cover east of the river is still relatively high (proportionately just over a third of total land area) but much more of the land area is still covered by native wetlands and adjacent upland/sandhill habitat that deposits the vital substrate to which H. geminatus is so closely tied. Hylogomphus geminatus total length is significant as a function of the watershed in which they occur, so it stands to reason that land use inputs in those watersheds are creating or have historically created environmental stressors that are impacting the fitness of the species. These trends should be verified with more focused land-cover analyses, especially with respect to the temporal scale, and an increase in N, P, and pesticide monitoring in agricultural-adjacent waterways.

Understanding the real-time changes of biotic and abiotic variables within the specific waterways in which *H. geminatus* exists would require continuous stream monitoring with permanent data loggers. Significant size differences amongst adjacent watersheds serve as an early warning sign that some aquatic aberrations are inducing effects in the aquatic community; those effects may directly impact *H. geminatus*' size or indirectly by decreasing available prey. Continuous stream monitoring can submit real-time data, but dragonflies can live in streams for up to three years as aquatic nymphs. Their morphology and/or presence may thus be indicative of environmental aberrations across a large temporal scale. These effects suggest not only a threat to resources on which we depend, but also on the existence of rare and endemic species as well as those of economic importance within the core range of *H. geminatus*. Future research should include DNA sampling within different watersheds, but especially on either side of the Apalachicola River, in order to rule out genotypic differences. Data loggers should be implemented in stretches of streams where *H. geminatus* is known to occur to monitor stream variables and build more mechanistic predictive models. Quantitative aquatic macroinvertebrate surveys should be used to assess the density and diversity of prey species available to *H. geminatus* nymphs. Satellite imagery should be used to assess the effects of terrestrial changes (like land clearing, urbanization, and agriculture) that might impact adjacent waterways. Finally, quantitative morphological analyses should be used to assess differences among the *H. geminatus* core population, disjunct populations, and *H. parvidens* (e.g., patterns and colors on individuals can be quantified and the morphology of mating appendages can be measured in detail for comparisons). Analyses like these would allow us to assess the scale of morphologic differences. In general, more data are needed to assess what the significance of these size differences means, and more specimens from areas east of the river where *H. geminatus* seems to be less common should be measured and analyzed.

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Data Availability Statement: Data available in a publicly accessible repository. The data presented in this study are openly available in GitHub at https://github.com/Joseph73192/H_geminatus_adults.

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Appendix A

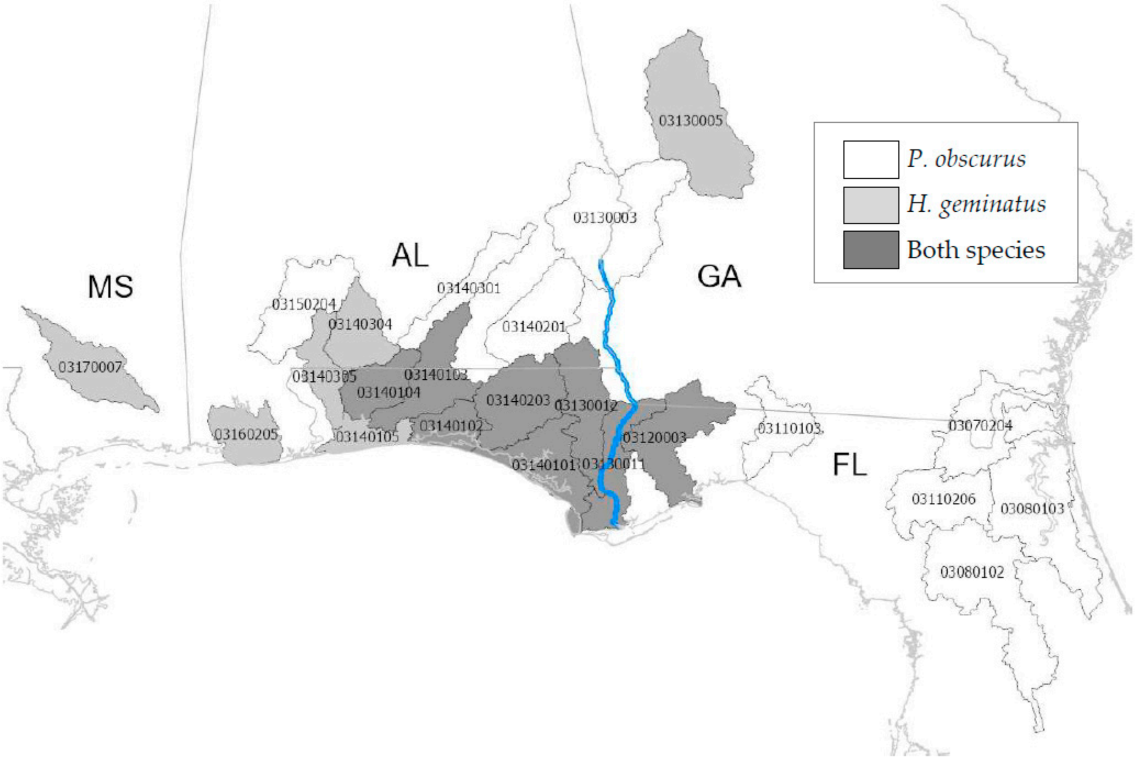

Figure A1. Labeled HUC-8 subbasin boundaries across the states of Mississippi, Alabama, Georgia, and Florida used in analyses. Note the Apalachicola River, represented by the blue line, that extends down from the AL-GA border through the FL panhandle and into the Gulf of Mexico.

Table A1. Pairwise comparisons of significant differences in total lengths of *H. geminatus* amongst subbasins. The 16 significant comparisons are highlighted and bolded.

	3120003	**3130005**	**3130011**	**3130012**	**3140101**	**3140102**	**3140103**	**3140104**	**3140105**	**3140203**	**3140304**	**3140305**	**3160205**
3130005	1.0000												
3130011	**0.0398**	**0.0132**											
3130012	**0.0133**	**0.0000**	**0.0000**										
3140101	0.6838	0.4270	**0.0398**	0.7566									
3140102	1.0000	0.8655	**0.0044**	**0.0000**	0.4361								
3140103	0.8554	0.5490	**0.0119**	**0.0302**	0.8873	0.5431							
3140104	0.1640	**0.0132**	**0.0000**	**0.0000**	1.0000	**0.0063**	0.6786						
3140105	0.7712	0.4333	0.4333	0.4044	1.0000	0.8655	1.0000	1.0000					
3140203	0.4333	0.6319	0.1313	**0.0301**	0.4333	0.6594	0.6618	0.1136	0.8667				
3140304	0.7712	0.4333	0.4333	0.4333	1.0000	0.3165	0.4333	0.3816	1.0000	0.8667			
3140305	0.7712	0.4333	0.4333	0.4044	1.0000	0.8655	1.0000	1.0000	1.0000	0.8667	1.0000		
3160205	0.2823	0.1992	**0.0152**	0.1136	1.0000	0.3357	0.8182	1.0000	1.0000	0.4044	0.6838	1.0000	
3170007	0.4333	0.4912	0.1313	0.0921	1.0000	0.6838	1.0000	0.7712	0.8667	0.6319	0.8667	0.8667	1.0000

Table A2. Pairwise comparisons of significant differences in total lengths of *P. obscurus* amongst subbasins. The 5 significant comparisons are highlighted and bolded.

	3070204	**3080102**	**3080103**	**3110103**	**3110206**	**3120003**	**3130003**	**3130011**	**3130012**	**3140101**	**3140102**	**3140103**	**3140104**	**3140201**	**3140203**	**3140301**
3080102	1.0000															
3080103	0.9749	0.8889														
3110103	0.9749	0.9094	0.4626													
3110206	0.9749	0.8065	1.0000	0.4119												
3120003	1.0000	0.7953	0.7727	1.0000	0.4979											
3130003	1.0000	0.9749	0.7953	0.9749	0.7110	1.0000										
3130011	1.0000	1.0000	0.9749	0.6757	1.0000	0.8210	0.9749									
3130012	0.6628	**0.0352**	0.3971	0.0600	0.0878	0.2108	0.5216	0.0870								
3140101	1.0000	1.0000	1.0000	0.8210	1.0000	0.8369	0.9749	1.0000	0.6456							
3140102	1.0000	1.0000	1.0000	0.9749	1.0000	1.0000	1.0000	1.0000	0.6757	1.0000						
3140103	0.7953	0.6456	0.2540	0.9749	0.0878	1.0000	1.0000	0.3198	**0.0363**	0.7727	0.7953					
3140104	0.7953	**0.0363**	0.1026	0.0870	0.0515	0.0878	0.7953	**0.0363**	0.2590	0.5019	0.7953	**0.0444**				
3140201	0.9749	0.6757	0.4626	0.7953	0.3198	1.0000	0.9749	0.7473	0.2309	0.6800	0.9749	1.0000	0.2590			
3140203	0.9749	0.8065	0.4626	0.9749	0.4119	1.0000	1.0000	0.8454	0.2309	0.8369	0.9749	1.0000	0.2590	0.9749		
3140301	1.0000	0.9749	1.0000	0.7953	1.0000	0.8369	0.9749	0.9749	0.9749	1.0000	1.0000	0.6757	0.8356	0.7953	0.7953	
3150204	1.0000	1.0000	0.9749	0.9749	1.0000	1.0000	1.0000	1.0000	0.6757	1.0000	1.0000	0.7953	0.7953	0.9749	0.9749	1.0000

Table A3. Differences in total length from published values of all species of *Hylogomphus* and *P. obscurus* as well as differences in measured values of *H. geminatus* and *P. obscurus*.

Species	Minimum Length (mm)	Maximum Length	Difference (mm)	Difference (%)
H. viridifrons	45	46	1	2.22
H. abbreviatus	34	35	1	2.94
H. apomyius	35	37	2	5.71
H. adelphus	39	45	6	15.38
H. parvidens	39	46	7	17.95
H. geminatus	39	47	8	20.51
Measured	**35.76**	**46.66**	**10.9**	**30.48**
P. obscurus	47	53	6	12.77
Measured	**43.24**	**55.38**	**12.14**	**28.08**

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