



Geometric morphometric study of sexual dimorphism and its associated allometry in wings of *Pelopidas thrax* (Lep.: Hesperiiidae)

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ABSTRACT. *Pelopidas thrax* (Hübner) (Lep.: Hesperiiidae) is a widespread species in southwest Iran. We aimed to use geometric morphometric techniques to investigate the sexual dimorphism and allometric effects associated with the wings of *P. thrax*. Fore- and hind wings of 40 and 39 individuals, respectively, of each sex which were collected from Ahvaz city were digitized and analyzed. Sexual dimorphism was observed in size and shape of fore- and hind wings of *P. thrax* and was graphically illustrated. Multivariate statistics confirmed significant differences in shape of fore- and hind wing between sexes. Centroid size of both wings showed greater values in females than males and visualized by boxplots. Various multivariate regressions of shape coordinates on centroid size were significant and visualized by the thin plate splines. Allometry explained shape variance in each case between 0.8% to 19.6%. The analysis demonstrated different allometric patterns for sexes in both fore- and hind wings. Significant shape differences between wings of males and females were still remained after removing allometric effects. The variation that is not related to size could be attributed to specific behaviors such as flight speed and performance in each sex. However, specific experiments are needed to confirm the association of the wing shape variation expressed in this study with the flight traits.

Key words: wing shape, wing morphology, skipper butterflies, southwest Iran

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Introduction

Lepidoptera (butterflies and moths) are known for their incredible diversity in wing morphology, which has fascinated naturalists for centuries. Wing morphology including characteristics of size, shape, pigmentation, and venation, has played a major role in taxonomic and evolutionary studies of the Lepidoptera (Strauss, 1990; Scoble, 1992). The importance of wing morphology in a wide variety of aspects of an insect's life, such as sexual and territorial display, foraging, defence mechanisms, thermal regulation, and the

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aerodynamics and the energetic costs of flight have studied so far (Hernández-L et al., 2010). Wing morphology, shape in particular, can further be used as an indicator of changing – and often stressful – environmental conditions (Hoffmann et al., 2005). Sexual dimorphism, defined as morphological differentiation of sexually mature males and females in forms of widespread intraspecific variation. Much of this variation is genetically based and likely due to variation in selection, primarily sexual selection, among species/populations (Fairbairn, 1997; Allen et al. 2011). Sexually dimorphic traits can function in reproductive contexts such as fecundity and mate attraction and location (Wiklund, 2003) or have nonreproductive reasons such as mimetic color pattern (Allen et al. 2011). Sexual dimorphism may occur from differences in size, shape or by the dependence of shape in relation to size (i.e., allometry) (Tobler & Nijhout, 2010). Changes in the shape of structures or organs must accompany changes in size to preserve the original function of the structure (Fairbairn, 1997). There is rampant sexual dimorphism within the Lepidoptera. Analyses demonstrate that many lepidopteran species exhibit female-biased sexual size dimorphism (Allen et al., 2011). Female butterflies tend to have patrolling flight in search of suitable foraging and host plants, while males spend most of their adult life locating sexually active females and guarding their territory. For example, the short and broad wing characteristics observed in female *Tongeia fischeri* (Eversmann) would favor slow flight for patrolling (Betts & Wootton, 1988; Jeratthitikul et al., 2014).

In recent two decades, Iranian entomologists were also interested in the study of sexual dimorphism. Zahiri et al. (2006) investigated sexual dimorphism in *Chilo suppressalis* (Walker) (Lep.: Pyralidae) and showed significant wing shape difference in the male and female. Mozaffarian et al. (2007) demonstrated that the wings shape of *Ectomyelois ceratoniae* (Zeller) (Lep.: Pyralidae) differs significantly in males versus females on all tested host-plants. They also found the larger wing size in females than males and indicated that in spite of allometric growth in the examined specimens, significant shape differences are still remaining in constant size. A survey on sexual dimorphism of *Cydia pomonella* (L.) (Lep.: Tortricidae) in the northwest of Iran revealed the centroid size of females was significantly greater than that of males. Allometry investigation showed non-significant association between centroid size and wing shape changes in males and females. Analysis displayed well discrimination between sexes, especially based on the hind wing landmarks. Overall shape deformation indicated wider basal part of the wing in females compared with males especially in the hind wing (Khaghaninia et al., 2008). Alavi (2018) studied sexual dimorphism in the wing shape and size of *Catocala abacta* Staudinger and *Catocala brandti* Hacker & Kautt (Lep.: Erebidae). Results showed that the shape of fore wings was significantly different between males and females of both species.

Pelopidas thrax (Hübner) was identified and recorded by Zergani et al. (2019) as a widespread species in Khuzestan, southwest Iran. In this area, green larvae of this species feed on sugarcane leaves and roll them using white silk. Outside Iran, it is known as the millet skipper and occurs from the eastern Mediterranean islands to North Africa, the Middle East, India and Pakistan (Cock, 2009). There is a prominent sexual dimorphism in the wing pattern of this species. Males of *P. thrax* can be easily distinguished from its females by having androconial patches (oblique grey-white brands) with some small white spots on the median area of fore wing whereas females lacking such brand and having greater white spots in this area. This raised the question of whether there is any dimorphism between males and females in wing venation and morphology, apart from dimorphism in wing pattern. Here, we used a geometric morphometric approach to test our question.

Material and methods

Sampling

Specimens were mainly collected from Shahid Chamran University of Ahvaz campus in November 2017 by a standard insect collecting net (Fig. 1A, B). Microscopic slides from the wings were prepared to study sexual dimorphism in the butterfly *P. thrax*. To avoid asymmetry bias between the two wings, only the right wings of specimens were used in this study (Rohlf & Slice, 1990). After the wings were discoloured in solution of sodium hypochlorite, they were photographed at the same magnification with a Canon SX40 camera mounted on the Olympus SZX12 stereomicroscope. The studied specimens were deposited in the Insect and Mite Collection of Ahvaz, (IMCA), Department of Plant Protection, Shahid Chamran University of Ahvaz.

Data acquisition

Wing size and shape variations were examined and recorded from the fore wings of 40 males and 40 females as well as the hind wings of 39 males and 39 females of the butterfly by the landmark based geometric morphometric method (Bookstein, 1991; Adams et al., 2004). The tps (thin plate spline) series of softwares was used to perform the numerical and geometric analysis of the x,y coordinates of the digitized landmarks (Rohlf, 2015). A total of 18 landmarks on the fore wing and 14 landmarks on the hind wing positioned at vein intersections or terminations (landmarks type I) (Fig. 1C, D) were collected and digitized (Bookstein, 1991).

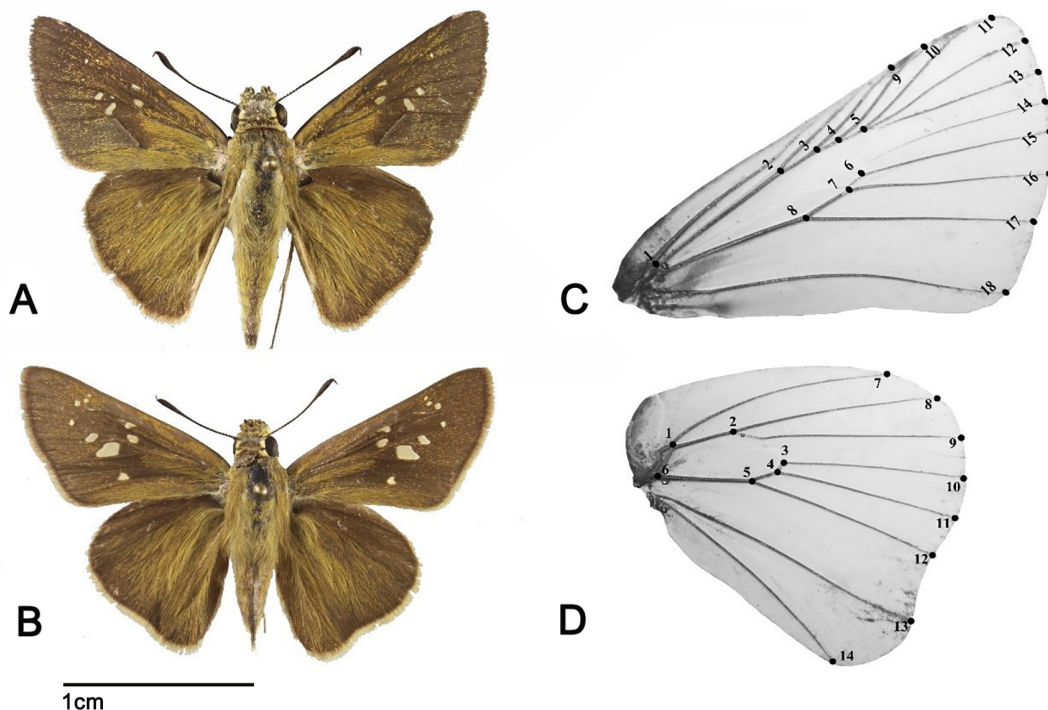


Figure 1. *Pelopidas thrax* (Hübner), wing pattern: **A.** Male, **B.** Female; depicting wing venation and position of landmarks used in this study: **C.** Female fore wing, **D.** Female hind wing.

Morphometric and statistical analyses

Centroid size which is the square root of the sums of squared distances of a set of landmarks from their centroid is a good estimate of overall size and was used to calculate wings size (Zelditch et al., 2004). The differences in centroid size were analyzed by T-test. The normal distribution of the obtained data was confirmed by the Shapiro-Wilkinson and Kolmogorov-Smirnov normality test. Differences and sexual dimorphism were visualized using boxplots. Statistical analyses were performed by SPSS software 24.0 (IBM Corp., 2016). The variation not related to shape (variation by location, scale and orientation) were removed from raw landmarking data by superimposing of images using generalized procrustes analysis (GPA). We performed a relative warp analysis by using the tpsRelw program to obtain the shape variables of the aligned specimens. It makes an interpolation that projects the data in a Euclidean plane (Bookstein, 1991; Rohlf, 2015). To visualize the patterns of shape variations among specimen, graphical depiction of results were presented by using thin plate spline deformation grids.

To test the observed differences in graphical shape variation, we performed a multivariate one-way Procrustes Analyses of Variance (Procrustes ANOVA) which is identical to non-parametric permutational-MANOVA (Anderson, 2001). It was carried out by the R package geomorph v. 3.0.4, using procD.lm (Adams & Otarola-castillo, 2013; Sherratt, 2016). Static allometry was tested by regressing shape coordinates onto centroid size of males and females. Goodall's F-test was calculated for testing the significance of multivariate regression to determine the percentage of allometric shape changes for wings of sexes separated and mixed sexes. It was carried out by parametric test and nonparametric permutation tests, using tpsRegr (Goodall, 1991; Kikukawa & Hikida, 2012; Rohlf, 2016). If the null hypothesis (absence of allometry) was rejected due to the significance of regressions, shape changes of wings would be related to size. To test if patterns of allometric shape variation differ between groups (e.g. sex, interaction effects), a multivariate analysis of covariance (Mancova) should perform, using shape variables as dependent variables, centroid size as covariate and sex as a fixed factor (D.C. Adams & M.L. Zelditch, personal communication). Therefore, a multivariate Procrustes ANOVA which is particularly useful for shape data was carried out as a non-parametric permutational-MANOVA by the R package geomorph v. 3.0.4, using procD.lm (Adams & Otarola-castillo, 2013; Sherratt, 2016; Tamagnini et al., 2018).

Results

Relative warp analysis

*Sexual dimorphism in shape of fore wing of *P. thrax**

Shape variations in fore wing of 40 males and 40 females of *P. thrax* were visualized graphically on relative warp components. Principal component analysis of partial warps scores is known as relative warps analysis (Bookstein, 1991). Among 32 relative warp components, the first relative warp component (RW1) and the second one (RW2) explained 44.17% and 11.09% of landmark shape variation, respectively (Fig. 2). Distribution of female and male *P. thrax* specimens based on the fore wing shape using relative warp components was shown in Fig. 2, with thin plate spline deformation grids for the mean specimens of males and females.

RW1 completely separated male and female specimens so that males were located on the positive direction of RW1, except one which was a bit overlapped with females. Shape deformation grids indicate male fore wings were more elongated than female fore wing's one in apex and termen (landmarks 10, 11, 12, 13, 14 & 15) which makes costa and discal cell close to apex. However, dorsum in male fore wings became broader.

Sexual dimorphism in shape of hind wing of P. thrax

Shape variations in hind wing of 39 males and 39 females of *P. thrax* were visualized graphically on relative warp components. Among 24 relative warp components, the first relative warp component (RW1) explained 65.29% and the second (RW2) 14.87% of the all hind wing shape variability (Fig. 3). Distribution of female and male *P. thrax* specimens based on the hind wing shape and along RW1 and RW2 axes was presented in fig. 3, with thin plate spline deformation grids for the mean specimens of males and females. RW1 distinctly separated male and female specimens with no overlapping, so that males were located on the negative direction of RW1. Shape deformation grids indicate female hind wings were more elongated than male hind wings in apex and termen (landmarks 7, 8, 9, 10 & 11) which makes costa and discal cell more elongated as well.

To test the significance of the shapes changes observed on relative warp components, a multivariate Procrustes ANOVA with 10000 permutation on shape variables of fore- and hind wings was performed (Table 1; Table 2). Sex variation was found to be significant for shape variables of fore- and hind wings indicating differences in at least one dependent variables (shape variables).

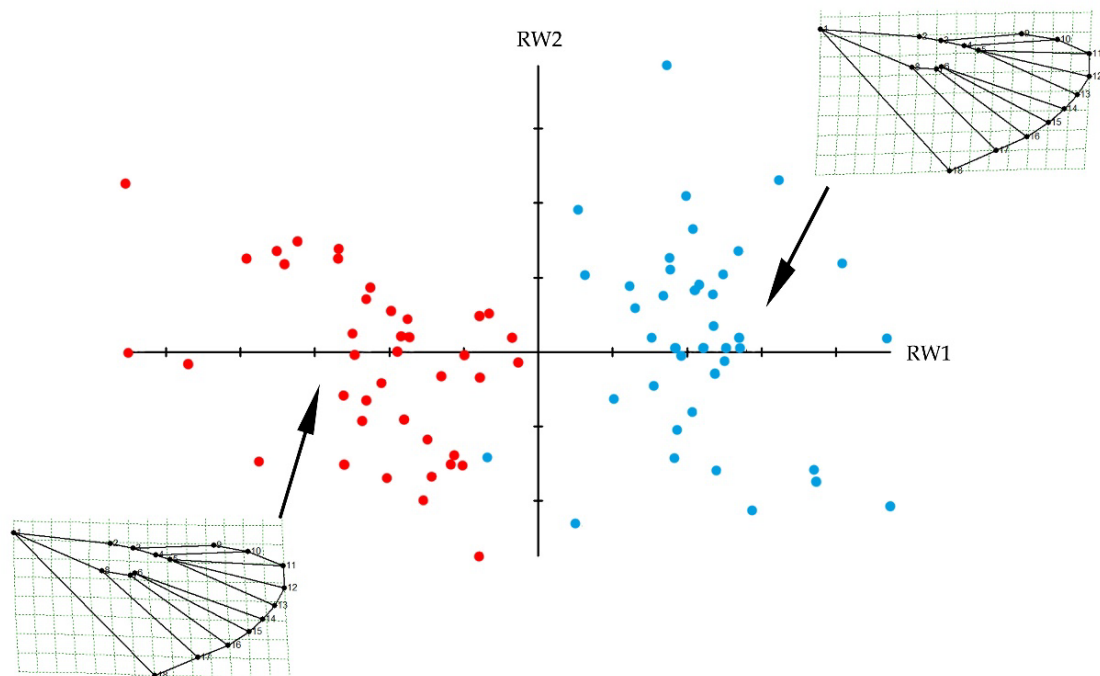


Figure 2. Distribution of male (blue) and female (red) specimens of *Pelopidas thrax* based on fore wing shape changes with thin plate spline deformation grids for the mean specimens of two groups around RW1 axis.

Table 1. Results of multivariate Procrustes ANOVA on fore wing shape variables in *Pelopidas thrax*. Significant difference is marked with an asterisk.

	df	SS	MS	r ²	F	Z	p-value
Sex	1	0.038548	0.038548	0.35057	42.105	6.6945	0.0001*
Error	78	0.071411	0.000916	0.64943			
Total	79	0.109959					

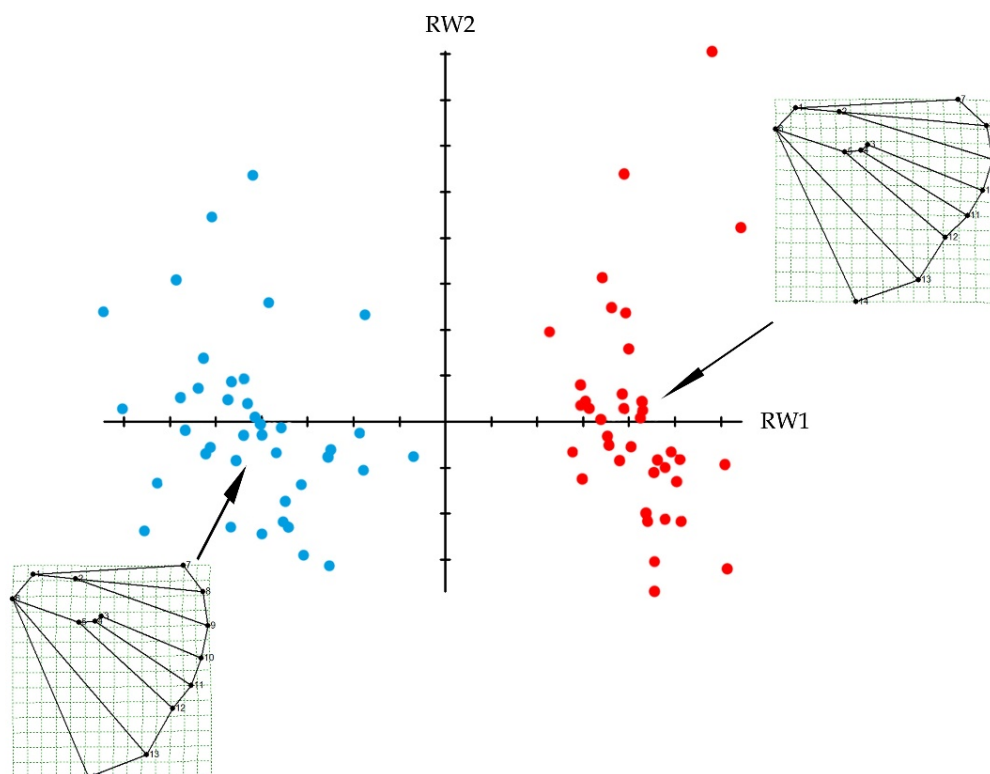


Figure 3. Distribution of male (blue) and female (red) specimens of *Pelopidas thrax* based on hind wing shape changes with thin plate spline deformation grids for the mean specimens of two groups around the RW1 axis.

Table 2. Results of multivariate Procrustes ANOVA on hind wing shape variables in *Pelopidas thrax*. Significant difference is marked with an asterisk.

	df	SS	MS	r ²	F	Z	p-value
Sex	1	0.137638	0.137638	0.6019	114.9	6.2608	0.0001*
Error	76	0.091036	0.001198	0.3981			
Total	77	0.228674					

Sexual dimorphism in the wing centroid size

Wing centroid size of male and female *P. thrax* were analyzed by T-test. Significant size differences were detected between sexes in fore wing ($t=-6.698$, $p<0.001$) and hind wing ($t=-8.535$, $p<0.001$). Boxplots showed greater values in female than in male wings, for the centroid size (Figs. 4; 5).

Static allometry

All multivariate regressions of shape variables on centroid size were significant except one (fore wing, mixed sexes). In fact, shape changes were significantly correlated with changes in size (allometry) and the null hypothesis (isometry) was rejected (Table 3). Allometry explained a notable percentage of shape changes which varied between 0.8% in the fore wing of male and female to 19.6% in the female hind wing. The results of the permutation method in all cases were consistent with the parametric results of Table 3.

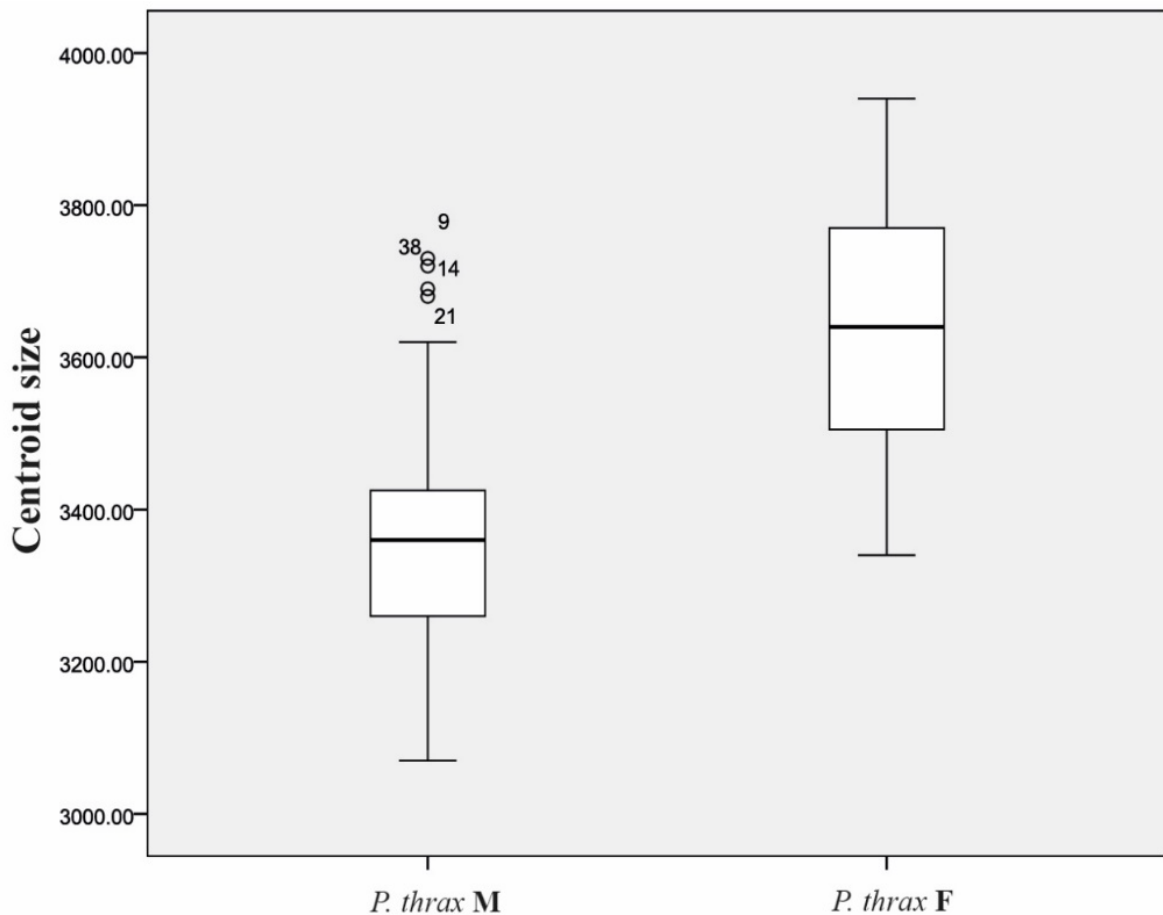


Figure 4. Box-whisker plot of male (M) and female (F) fore wing centroid size of *Pelopidas thrax*. Each box is depicted from first quartile to the third quartile, its horizontal line indicates the median, whiskers extend from minimum to maximum values and circles represent outliers.

Visualizing allometric shape changes

We used the thin plate splines to visualize allometric shape changes in fore- and hind wing of *P. thrax* (Figs. 6; 7). In female fore wing, landmarks of costa and termen were elongated to apex in individuals with larger wings than smaller ones. Male fore wing of larger individuals in post discal area has a small bending to costa and anterior part of discal cell. In the female hind wing of larger individuals, veins and discal cell were elongated to apex. It is almost the same in males. Hind wing in mixed sexes was retracted from termen and inner margin to discal cell in larger individuals than in smaller ones.

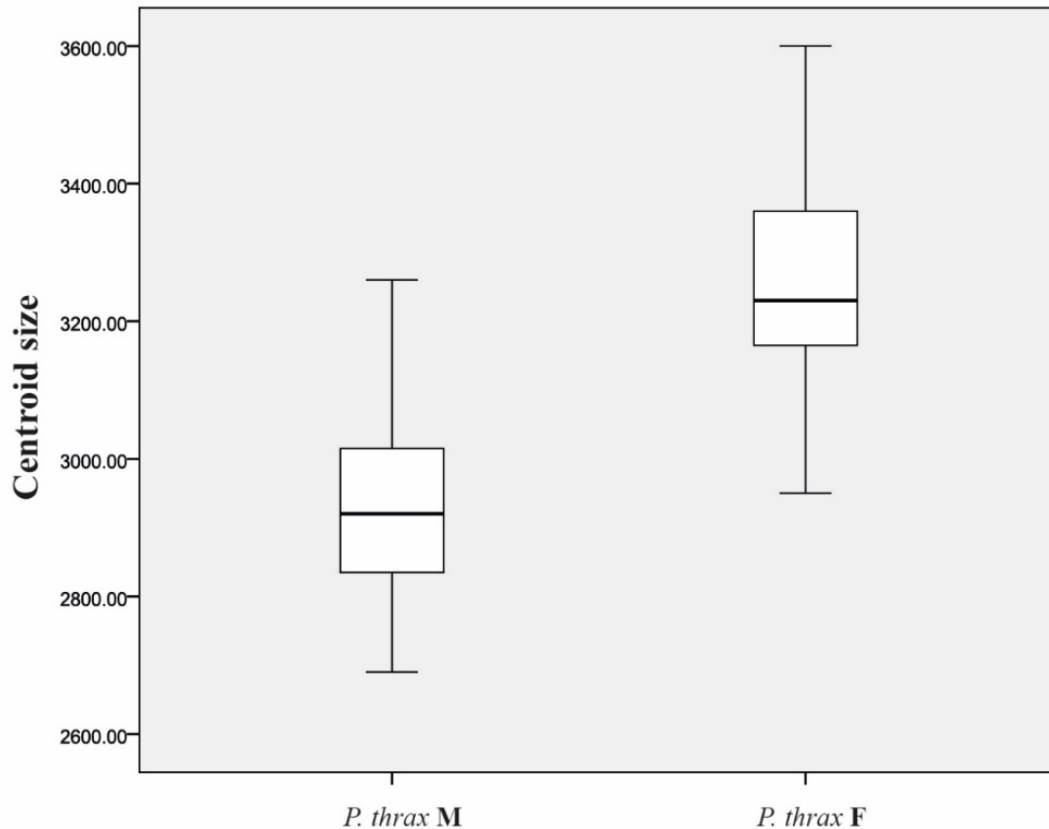


Figure 5. Box-whisker plot of male (M) and female (F) hind wing centroid size of *Pelopidas thrax*. Each box is depicted from first quartile to the third quartile, its horizontal line indicates the median and whiskers extend from minimum to maximum values.

Table 3. Results of multivariate regression of shape variables on centroid size in the fore- and hind wing of *Pelopidas thrax*.

Sex	% Explained	Fore wing			% Explained	Hind wing		
		Goodall's <i>F</i> -test				Goodall's <i>F</i> -test		
		P-value	df	F		P-value	df	F
Female	3.7	0.0481	32	1.46	19.6	0.0001	24	9.05
Male	9.2	0.0001	32	3.86	6.9	0.0001	24	2.74
Mixed	0.8	0.9164	32	0.67	2.9	0.0003	24	2.33

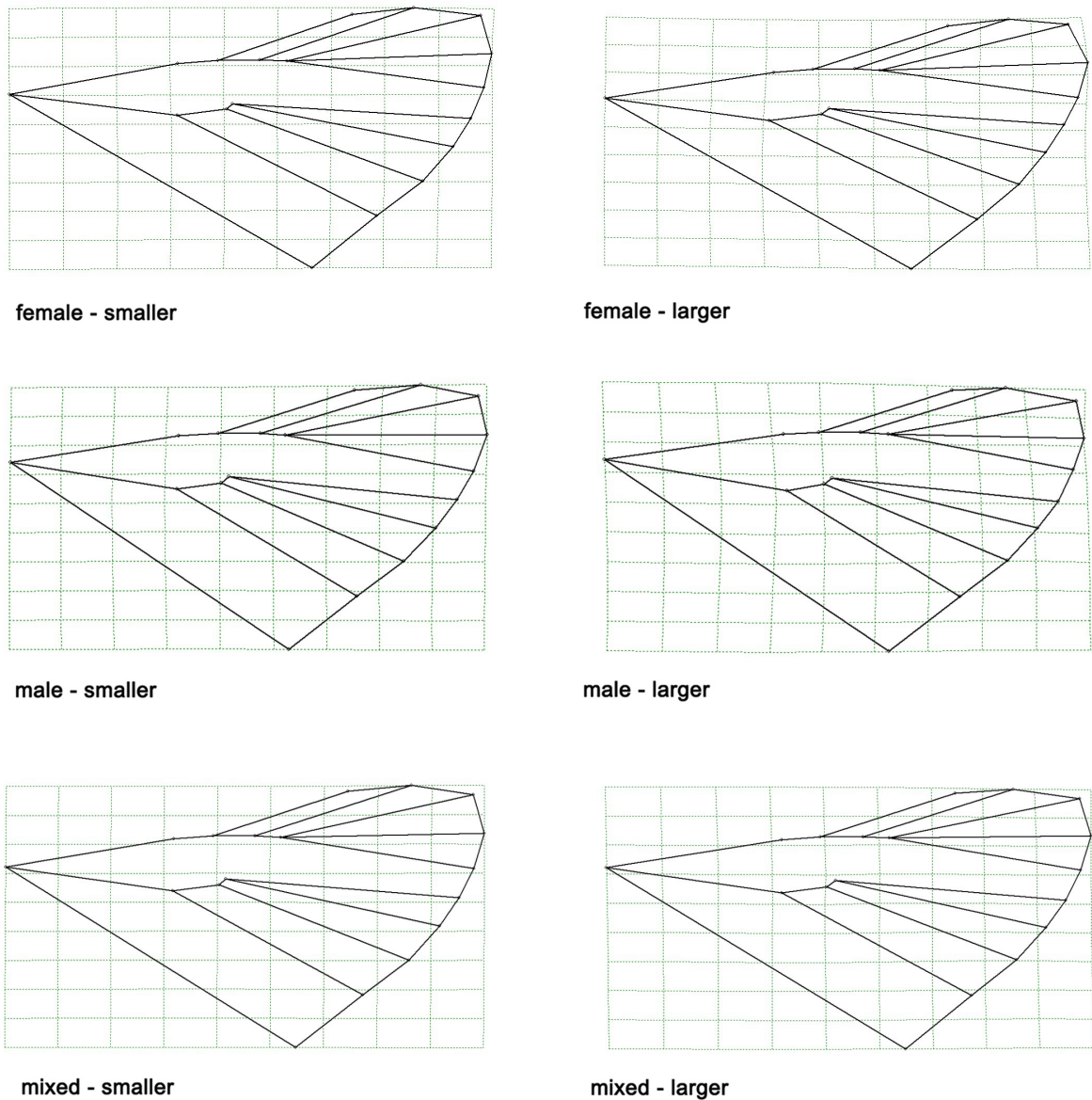
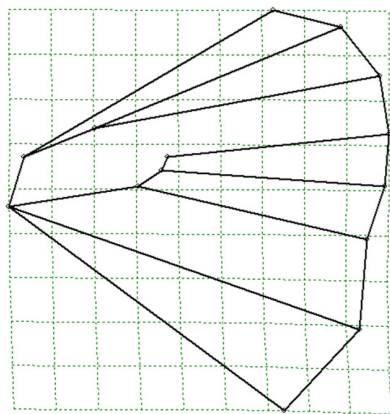


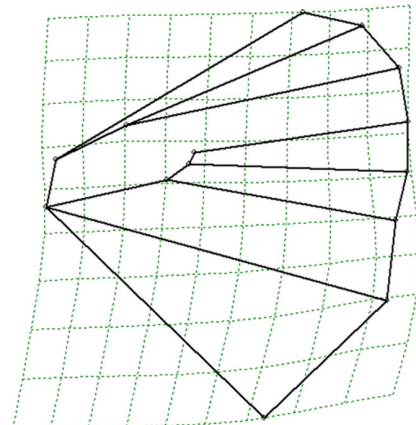
Figure 6. Allometric shape changes from smaller to larger centroid size in fore wing of *Pelopidas thrax* (non-significant in mixed sexes).

Allometric pattern in male and female

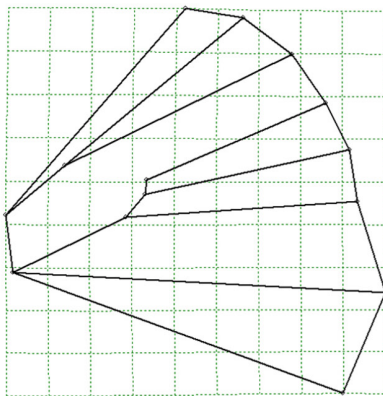
A two-way Procrustes ANOVA with 10000 permutations on fore wing data confirmed the differences in allometric trajectories between sexes (Table 4). A significant interaction between centroid size and sex was also detected ($p = 0.0482$). This analysis on hind wing data confirmed the differences in allometric trajectories between sexes as well (Table 5). A significant interaction between centroid size and sex was also found ($p = 0.0071$). Therefore, the allometric pattern differs between male and female wings. Significant shape differences between wings of males and females were still remained after removing allometric effects.



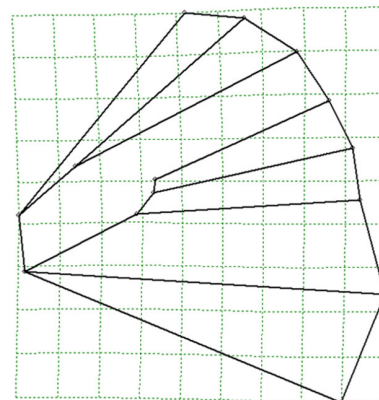
female - smaller



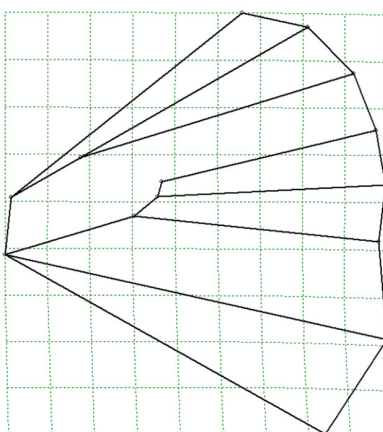
female - larger



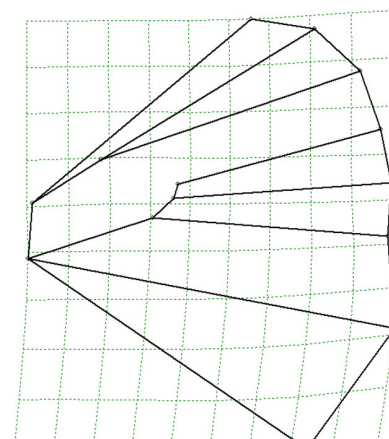
male - smaller



male - larger



mixed - smaller



mixed - larger

Figure 7. Allometric shape changes from smaller to larger centroid size in hind wing of *Pelopidas thrax*.

Table 4. Results of two-way Procrustes ANOVA to test the impact of centroid size and sex (and their interaction) on fore wing shape in *Pelopidas thrax*. Significance is marked with an asterisk.

	Df	SS	MS	r ²	F	Z	p-value
size	1	0.012788	0.0127885	0.11630	13.9621	4.7219	0.0001*
sex	1	0.026460	0.0264601	0.24064	28.8883	6.8060	0.0001*
size: sex	1	0.001099	0.0010986	0.00999	1.1994	1.6224	0.0482*
Residuals	76	0.069612	0.0009159	0.63307			
Total	79	0.109959					

Table 5. Results of two-way Procrustes ANOVA to test the impact of centroid size and sex (and their interaction) on hind wing shape in *Pelopidas thrax*. Significance is marked with an asterisk.

	Df	SS	MS	r ²	F	Z	p-value
size	1	0.077829	0.077829	0.34035	65.326	5.4539	0.0001*
sex	1	0.061095	0.061095	0.26717	51.280	6.7509	0.0001*
size: sex	1	0.001588	0.001588	0.00694	1.333	2.4887	0.0071*
Residuals	74	0.088163	0.001191	0.38554			
Total	77	0.228674					

Discussion

In sexual dimorphic species, separating genders has been misleading and caused taxonomic confusion. In some cases, one dimorphic species has been described as two different species (Laguerre, 2016; Przybyłowicz & Tarcz, 2015). Most species of Hesperidae, especially for the sexually dimorphic species, were described based on male individuals and females of many species have not yet been identified. Identification of female specimens is rather difficult especially when similar species are often found to be sympatric (Xue et al., 2016; Zhai et al., 2017). We were also confused early in this research before detecting that males and females belong to one species, *P. thrax*, because of prominent sexual dimorphism in the wing patterns. Therefore the sexual association of insect species including dimorphic lepidopterans could be a necessary work for research in taxonomy, phylogeny and biology.

Sexual dimorphism in Lepidoptera wings shape has already been shown in various studies (e.g. Mozaffarian et al., 2007; Benitez et al., 2011; Camargo et al., 2015; Shi et al., 2015; Benitez & Vargas, 2017; Moraes et al., 2017; Moreno & Vilorio, 2020). Sexual size dimorphism in Lepidoptera wings has also been documented in several kinds of researchs (e.g. Zahiri et al., 2006, Mozaffarian et al., 2007; Khaghaninia et al., 2008; Jeratthitikul et al., 2014; Camargo et al., 2015; Shi et al., 2015; Moreno & Vilorio, 2020). The present study was one of the rare research projects dealing with sexual dimorphism in the Hesperidae family, using the geometric morphometric method. Our results indicate that in *P. thrax* fore- and hind wings of females were significantly larger than those of males. The intersex shape changes in wing venation and morphology were found to be significant in fore- and hind wings, apart from dimorphic wing pattern. The findings confirm female-biased sexual size dimorphism (females being larger than males), which is a frequent trend in insects and very common in

Lepidoptera (Stillwell et al., 2010; Iglesias et al., 2012; Jeratthitikul et al., 2014). Alavi (2018) performed a geometric morphometric study on sexual dimorphism in the wings of *Catocala abacta* and *C. brandti* and found that the wing size in both fore- and hind wings of *C. abacta* females was larger than of the males, whereas in *C. brandti* wing size of males was larger than of females. She suggested that dietary restrictions or different development period length in males versus females during the larval stages may have resulted in sexual dimorphism in favor of males in *C. brandti*.

Size is an important indicator of insect fitness. In most of dioecious animals, males and females have different body size, and this phenomenon is called sexual dimorphism in size. In fact, the body size of males and females is determined by forces that affect survival and reproduction. Larger individuals have longer life duration and more reproductive success than smaller ones. Larger males are more successful in competing for mating with females and larger females produce more eggs. Although environmental factors such as food availability are limiting factors, genetic differences between individuals also play a role in growth and development (Arak, 1988; Stillwell et al., 2010; Beukeboom, 2018). Wing shape and size are important factors in reproductive-related dimorphism and can reduce mating costs and increase insect performance in flight-based mating tactics (Wiklund, 2003). Larger size of females in *P. thrax* indicates they have more growth rate than males in larval period to obtain more ability for egg reproduction in maturity phase. Wing sexual dimorphism in Lepidoptera seems to be arisen largely by the natural and sexual selection that affects males and females differently (Allen et al., 2011). Vargas et al. (2010) suggested that the wing sizes of insects are related to the growth of the larvae. The larger size of the female is a natural selection to allocate resources in the larval period for future reproduction, but it increases the risk of predation. Males also incur costs if they increase in size, but in contrast, they gain less than females when they mature (Allen et al., 2011).

The fore wings of males are triangular on their extremities and have straighter edges. This would reduce its friction with the air (Lockwood et al., 1998; Camargo et al., 2015) and may result in faster flight and less maneuverability than females which yield in better fitness in finding females. The fore wings of the females are rounded at the extremities and have a larger surface that provides them more maneuverability and slower flight and patrolling over host plants to choose oviposition site (Camargo et al., 2015). Triangular wingtip (apex) in insects, birds and bats demonstrate an evolutionary consequence that increases energy efficiency during the migration (Lockwood et al., 1998). It has showed, for example, in fore wings of Sphingidae species by geometric morphometrics (Camargo et al., 2015). Wing shape changes in males and females that were presented graphically in the current study exhibited that the fore- and hind wings were congruent to above described features for males and females, confirming results of Camargo et al. (2015) and other similar reserches. Maybe the sex-specific flight behaviors contribute to the evolution of sexual dimorphism in *P. thrax* wings.

The variation that is not related to size could be described by specific behaviours in each sex. Just as females fly and search for a host plant to lay eggs, males must always be in search of mates. These activities require different selection of specific traits pertain to flight speed and performance, such as wing size and shape, to reduce associated costs (Lockwood et al., 1998; Camargo et al., 2015). Allometry explained less than 20% of wing shape changes and significant shape differences between wings of sexes that were not related to size also detected in our findings and could be connected to those behaviors. On the other hand,

allometry was important in intraspecific variation of wing shape in Sphingidae, represented up to 60% of variation (Camargo et al., 2015). In *E. ceratoniae* (Pyrilidae) there were still significant differences between shapes of wings when size was held constant (Mozaffarian et al., 2007) and in *C. pomonella* (Tortricidae) no allometry was detected at all (Khaghaninia et al., 2008). Such differences between Lepidoptera species/families may also arise from behavioral traits.

Generally, the observed morphological change on the veins between males and females can affect issues such as dispersion, migration, mating time, territory selection, host plant finding and nutrition (Benson et al., 1989; Dudley, 2000; Camargo et al., 2015; Benitez et al., 2011). Thus, selection would optimize flight-related traits by affecting wing shape as stated by DeVries et al. (2010). However, specific experiments are needed to confirm the association of the wing shape variation expressed in this study with the flight performance.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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مطالعه ریخت‌شناسی هندسی دوشکلی جنسی و روابط آلومتری در بالهای *Pelopidas thrax* (Lep.: Hesperiiidae)

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چکیده: گونه *Pelopidas thrax* (Hübner) (Lep.: Hesperiiidae) در جنوب غربی ایران پراکنش گسترده‌ای دارد. هدف ما کاربرد تکنیک‌های ریخت‌شناسی هندسی در بررسی دوشکلی جنسی و اثرات آلومتری در بال‌های *P. thrax* بود. بال‌های جلو و عقب به ترتیب ۴۰ و ۳۹ فرد از هر جنس نر و ماده که از اهواز جمع‌آوری شده بود لندمارک‌گذاری و تحلیل شد. دوشکلی جنسی در اندازه و شکل بال‌های جلو و عقب *P. thrax* مشاهده و بطور گرافیکی نمایش داده شد. تحلیل چندمتغیره وجود تفاوت‌های معنی‌دار در شکل بال جلو و بال عقب بین دو جنس را تایید کرد. اندازه متوسط هر دو بال نشان داد که اندازه ماده‌ها از نرها بزرگتر است و توسط باکس پلات نمایش داده شد. رگرسیون‌های چند متغیره متغیرهای شکلی روی اندازه متوسط معنی‌دار بودند و به‌وسیله thin plate splines نمایش داده شدند. آلومتری، تنوع شکل در هر مورد را بین ۰/۸ تا ۱۹/۶ درصد بیان نمود. تحلیل‌ها الگوهای متفاوتی از آلومتری را در بال‌های جلو و عقب در دو جنس نشان دادند. تفاوت‌های معنی‌دار در شکل بال‌ها بین نر و ماده بعد از حذف اثرات آلومتری همچنان باقی ماند. تنوعی که با اندازه مرتبط نباشد می‌تواند به رفتارهای خاصی مانند سرعت و عملکرد پرواز در هر جنس نسبت داده شود. با این حال تأیید ارتباط بین تنوع شکل بال مشاهده شده در این پژوهش با خصوصیات پروازی نیازمند انجام آزمایشات ویژه است.

واژگان کلیدی: شکل بال، مرفولوژی بال، پروانه‌های جهنده، جنوب‌غرب ایران