

Evolution

How relevant is the relationship between ostiole size and wasp head shape in the *Ficus*-Agaonidae mutualistic interaction?

¿Qué tan relevante es la relación entre el tamaño del ostiolo y la forma de la cabeza de las avispas en la interacción mutualista Ficus-Agaonidae?

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Abstract

The mutualism between *Ficus* species (Moraceae) and their pollinating wasps (Agaonidae) is a widely recognized coevolutionary model. In *Ficus* species from the Paleotropic, it has been determined that the ostiole acts as a morphological filter that affects the head dimensions of pollinating female wasps. Here, for the first time, the allometric relationship between ostiole size (diameter and length) and the shape of the head (length/width) of pollinating wasps is quantitatively explored in 6 Neotropical *Ficus* species (3 sect. *Americanae* and 3 sect. *Pharmacosycea*). In the case of sect. *Americanae*, wasp head shape was significantly correlated only with ostiole length, while in sect. *Pharmacosycea* both ostiole variables were correlated with head shape. The ordination analysis (NMDS) clearly reflected associations of these traits in species for both sections. The results support what has been interpreted in previous studies as reciprocal evolution between the analyzed traits, which contribute, along with other morphological and ecological traits, to the specificity between *Ficus* species and their pollinating wasps.

Keywords: Mutualism; Ostiole; *Pegoscapus*; *Tetrapus*

Resumen

El mutualismo entre *Ficus* (Moraceae) y sus avispas polinizadoras (Agaonidae) es un modelo coevolutivo ampliamente reconocido. En especies de *Ficus* del Paleotrópico, se ha determinado que el ostiolo actúa como un filtro morfológico que afecta las dimensiones de la cabeza de las hembras polinizadoras. Por primera vez se explora,

cuantitativamente, la relación alométrica entre el tamaño del ostiolo (diámetro y largo) y la forma de la cabeza (largo/ancho) de las avispas polinizadoras en 6 especies de *Ficus* neotropicales (3 sect. *Americanae* y 3 sect. *Pharmacosycea*). En el caso de sect. *Americanae*, la forma de la cabeza de las avispas se correlacionó significativa y únicamente con el largo del ostiolo, mientras que en la sect. *Pharmacosycea*, ambas variables del ostiolo se correlacionaron con la forma de la cabeza. El análisis de ordenación (NMDS) reflejó claramente asociaciones de estos atributos por especie para ambas secciones. Los resultados apoyan lo expuesto en estudios previos de una evolución recíproca entre los atributos analizados, lo que contribuye, en conjunto con otros atributos morfológicos y ecológicos, a la especificidad entre *Ficus* y sus avispas polinizadoras.

Palabras clave: Mutualismo; Ostiolo; *Pegoscapus*; *Tetrapus*

Introduction

The interaction between species of the genus *Ficus* L. (Moraceae) and wasps of the family Agaonidae Walker (Hymenoptera) is a classic example of a mutualistic interaction, in which the phenomena of co-diversification, co-speciation, and co-evolution are well documented (Borges, 2021; Bronstein, 1988; Jusselin et al., 2003; Rønsted et al., 2005; Wang et al., 2019; Wiebes, 1979) and is estimated to date to ca. 74.9 Ma (60.0-101.9 Ma; Cruaud et al., 2012; Zhang et al., 2019). For many years it was assumed that 1 *Ficus* species was pollinated exclusively by 1 wasp species (e.g., Ramírez, 1970; Wiebes, 1966, 1979), but a growing amount of evidence contradicts this assumption (Cook & Rasplus, 2003; Cook & Segar, 2010; Herre et al., 2008; Machado et al., 2005; Molbo et al., 2003; Oldenbeuving et al., 2023; Peng et al., 2008; Su et al., 2008; Weiblen, 2002).

Regardless of the degree of specificity, this ancestral and close mutualistic relationship involves complex morphological modifications (Clement et al., 2020; Galil & Eisikowich, 1968). The flowers of *Ficus* species are found inside an urn-shaped inflorescence (syconium). The development of the syconium traditionally has been divided into 5 phases (Galil & Eisikowich, 1968; Ibarra-Manríquez et al., 2012; Verkerke, 1989): *A*) floral or female, *B*) receptive or female, *C*) interfloral, *D*) male, and *E*) post floral. Pollinators can only access the flowers during phase B, through the ostiolo, which is an apical opening in the syconium made up of numerous overlapping bracts (Castro-Cárdenas et al., 2022; Machado et al., 2013; van Noort & Compton, 1996; Verkerke, 1989). The ostiolo plays a fundamental role in the specificity of pollinating wasps among the different sections of the genus *Ficus*, since it acts as a physical barrier, blocking the entry of non-pollinating wasps and/or regulating the size of the pollinating wasps that are able to reach the syconial cavity (Chen et al., 2001; Janzen, 1979; Jusselin et al., 2003; Liu et al., 2011; Ramírez, 1974; Verkerke, 1989).

One of the morphological modifications recorded for Agaonidae wasps was suggested by Ramírez (1974), who proposed that the shape of the head of female wasps presents various modifications that facilitate their passage through the ostiolo, indicating that heads that are subquadrangular or subhemispherically flattened (usually as long as broad across the eyes) have been recorded in *Ficus* species in different groups (e.g., subgen. *Ficus* or the sections *Americanae* or *Conosycea*), whose syconia present spirally arranged ostiolar bracts. On the other hand, wasps with thin elongated heads have been recorded in *Ficus* species possessing syconia with linearly arranged ostiolo bracts, which mostly point downwards (e.g., sections *Galoglychia* or *Pharmacosycea*).

van Noort & Compton (1996) evaluated the convergence in head shape between wasps from 2 subfamilies (Agaonidae and Sycoecinae), pollinators and non-pollinators, respectively, of the same *Ficus* species (subg. *Urostigma* sect. *Galoglychia*). These authors suggested that selection would have favored convergence in the shape of the head of both lineages of wasps, due to the pressure derived from achieving a successful entry of pollinating females into the syconium cavity. Furthermore, they emphasized that the length of the ostiolo is the main factor in determining this trait in wasps, since the arrangement of the ostiolo bracts in sect. *Galoglychia* is constant between species (Verkerke, 1989). However, the authors suggested that confirmation of their results required obtaining measurements of the ostiolo during phase B of syconium development since the measurements used in the analysis were obtained from taxonomic literature. This is an important proposal, considering that there are records of *Ficus* species in which the size of the syconium differ among the different stages of development (Delgado-Pérez et al., 2020; Piedra-Malagón et al., 2019). Finally, Jusselin et al. (2003) reconstructed the evolution of the shape of the ostiolo (spiral or linear) and the shape of the head of pollinating wasps (short or elongate) using molecular markers only (ITS and ETS) and concluded that both traits are correlated throughout the genus *Ficus*.

The genus *Ficus* is represented in the Neotropics by the subgenera *Pharmacosycea* (Miq.) Miq. and *Spherosuke* Raf. (Berg, 1989; Pederneiras et al., 2015), whose sections are endemic to the American continent (*Pharmacosycea* (Miq.) Griseb. and *Americanae* (Miq.) Corner, respectively). The diversity of section *Pharmacosycea* is estimated to be 20 species, which are exclusively pollinated by wasps of the genus *Tetrapus* Mayr 1885, whose heads are longer than wide. The sect. *Americanae* includes a larger number of species (ca. 120) whose pollinators belong to the genus *Pegoscapus* Cameron 1906, which possess subquadrangular or subhemispherical heads (Berg, 1989; Joussetin et al., 2003; Ramírez, 1974).

The main objective of the present study was to determine the relationship between the size of the ostiole in species belonging to the sections of *Ficus* and the shape of the heads of pollinating wasps, a relationship that has not been previously analyzed quantitatively. The inclusion of *Ficus* species belonging to the 2 Neotropical sections is relevant, since it will allow a further evaluation between fig and fig wasp traits from a phylogenetic context (Joussetin et al., 2003).

Materials and methods

Fieldwork was carried out in the NW portion of the Los Tuxtlas region in the state of Veracruz, Mexico (Fig. 1). This region forms part of the Upper Tertiary and Middle Quaternary volcanic complex and is composed of basaltic andesite and basalt rock (Verma et al., 1993) and is bordered by Holocene lava flows (Nelson & Gonzalez-Caver, 1992). The study area reaches elevations between 100 and 600 m. The predominant climate is warm and humid, with rain all year round (A), an average annual temperature between 24.1 and 25 °C, and an average annual precipitation of 1,939 to 3,928 mm (Fig. 2; SMN, 2010). Rainfall is more frequent and abundant during the summer, whereas a drier season occurs between March to May, the latter being the driest month (SMN, 2010; Soto, 2004). The species richness of vascular plants documented for the Los Tuxtlas region is 2,548 species (2,391 native; 307 are endemic to Mexico), distributed in 208 families and 1,018 genera (Villaseñor et al., 2018). The families with the highest number of species are Fabaceae (212), Orchidaceae (171), Asteraceae (150), Rubiaceae (143), and Poaceae (82). Villaseñor et al. (2018) point out that the predominant biome in the region is the humid tropical forest. The location of the individuals of *Ficus* sampled in the present study are indicated in Figure 1.

The collection and measurement of syconia and pollinating wasps. Three species were chosen from each section of *Ficus*, which represented the size range

of the syconia described for both sections in Mexico (Ibarra-Manríquez et al., 2012): *F. apollinaris* Dugand, *F. insipida* Willd., and *F. yoponensis* Desv. from sect. *Pharmacosycea*, and *F. colubrinae* Standl., *F. isophlebia* Standl., and *F. obtusifolia* Kunth from sect. *Americanae*. Detailed descriptions and illustrations of the species can be consulted in Ibarra-Manríquez et al. (2012), Cornejo-Tenorio et al. (2019), and Hernández-Esquivel et al. (2020).

The material collected included 5 individuals per *Ficus* species with syconia in the development phases B and D (Galil & Eisikowitch, 1968; Ibarra-Manríquez et al., 2012). The collected syconia for phase B allowed us to measure the size of the ostiole at the time when the pollinating wasps enter the syconium, whereas for the syconium in phase D (male phase), we were able to capture the emerging pollinating wasps. In phase B syconia, the ostioles were dissected and fixed and stored in 70% ethanol. Phase D syconia were placed in airtight bags until the pollinating wasps emerged and then each wasp was collected with a small brush, fixed, and stored in 70% ethanol. Wasps were identified to genus level using identification keys (Bouček, 1993; Rasplus & Soldati, 2006) and classified into morphospecies (Fig. 3). We measured the ostioles for a total of 150 syconia and 170 individuals of pollinating wasps.

The size of the ostiole was recorded using 3 measurements: 2 diameters of the area that is covered by the superficial ostiolar bracts (Fig. 4A, B), and the length, which indicates the distance between the internal and superficial ostiolar bracts (Fig. 4C, D). The head of pollinating wasp individuals were dissected and measured under a Zeiss stereoscopic microscope model Axio Zoom V16. We recorded the length (the distance between the posterior margin of the head and the apex of the jaw) and the width at maximum eye level (Fig. 4E, F). Both measurements allowed us to determine the shape of the wasp's head (average head length to head width ratio), as described by van Noort & Compton (1996).

Data analysis. We calculated the Pearson correlation coefficients between diameter 1, diameter 2, and length of the ostiole. We also applied generalized linear models and carried out multiple comparisons using the 'multcomp' package to assess differences in ostiole diameter and Agaonidae wasps' length/width ratio among species of each section. We performed a Nonmetric Multidimensional Scaling (NMDS) analysis to examine the multivariate relationship of the ostiole proportions (diameters and length) and head size (length and width) among species of each section of *Ficus*. We applied the decostand function of 'Vegan' to obtain a standardized matrix constructed with values to reduce the extremes values effect (Oksanen et al., 2019). We implemented NMDS with Bray-Curtis's

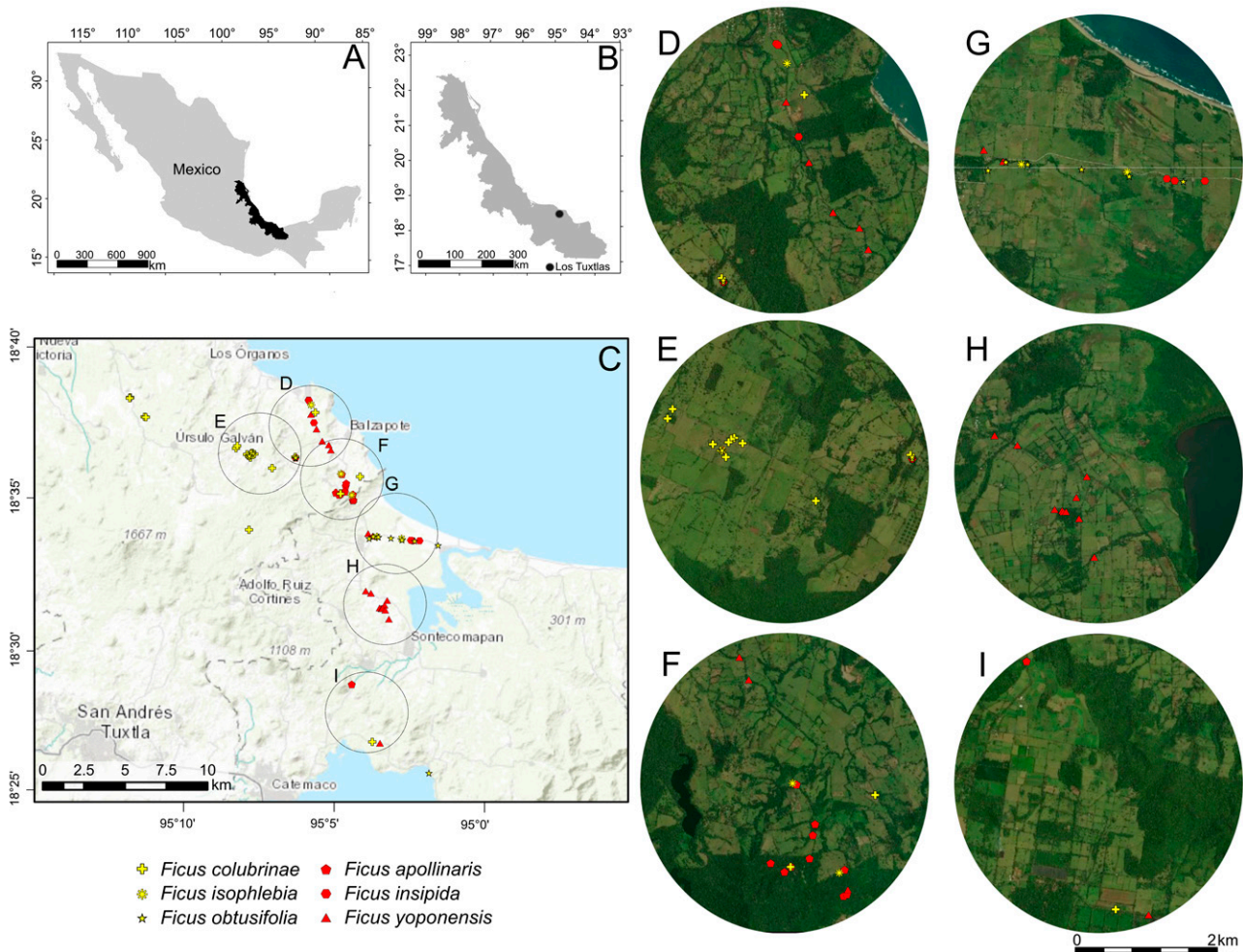


Figure 1. Study site localization and sampling area. A) Veracruz state (black area) in Mexico; B) location of the Los Tuxtlas Region (black dot) within Veracruz, Mexico; C) syconium and wasps' collection points are shown in the Los Tuxtlas Region and a zoom of the vegetation cover is shown in areas for each species included in the study (D - I). Points were placed on base maps (OpenStreetMap and World Imagery) of ArcGIS® software.

dissimilarity. Finally, we added convex hulls with the ordihull function of 'vegan' to highlight point clusters based on species. Since NMDS uses rank order information, this technique provides a highly flexible quantitative method that allows distribution-free inferences and, because it has a high flexibility in the number of observations, it is particularly useful with unbalanced experimental designs to explore the proximities and resemblances in the structure of the data among groups (Tong, 1992). Therefore, these analyses allowed us to identify the structure and association degree of ostiole-wasp heads among species of each section. All the analyses were conducted using R software.

Results

We found a positive correlation between the 2 diameters of the ostiole, both for sect. *Americanae* (0.99, $p < 0.000$) and for sect. *Pharmacosyceae* (0.96, $p < 0.001$). In the latter section, a high positive correlation was also obtained between the length of the ostiole and both diameters (0.93, $p < 0.000$), a relationship that was lower for sect. *Americanae* (diameter 1, 0.37 and diameter 2, 0.39, $p < 0.01$). Figure 5 shows that the diameter of the ostiole differs significantly between the 3 species included in each section (*Americanae*, $F_{2,73} = 2156922$, $p < 0.000$; *Pharmacosyceae*, $F_{2,74} = 3.81$, $p < 0.000$). The same result was found for the species from

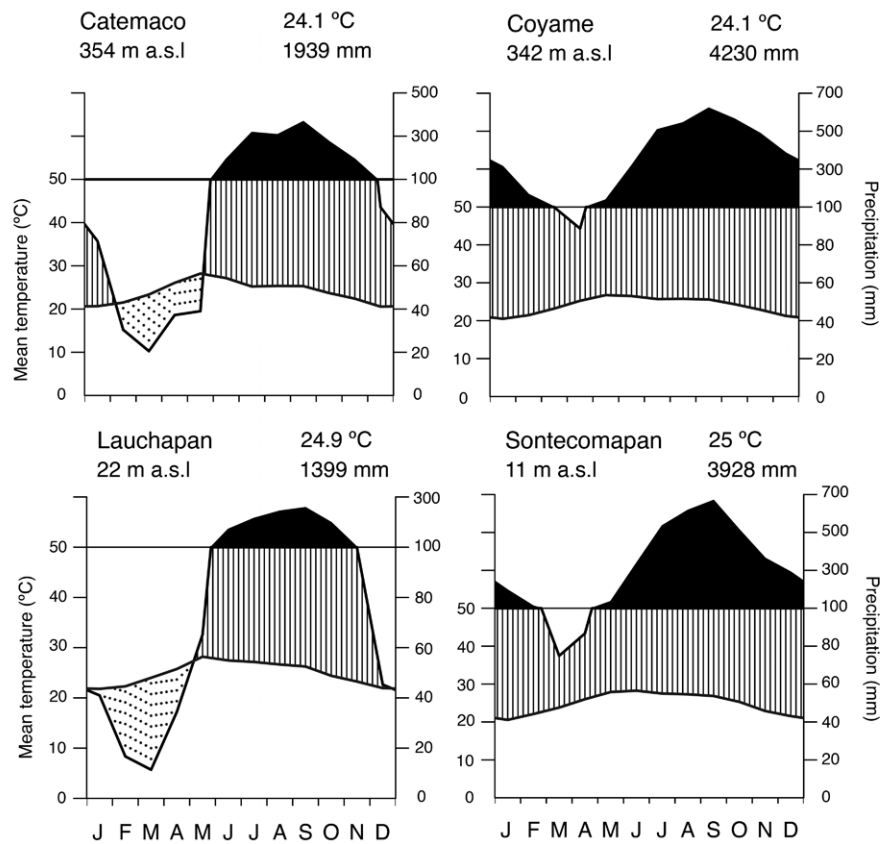


Figure 2. Ombrothermal diagrams of meteorological stations in the Los Tuxtlas Region, Veracruz. The data correspond to 30 years of records from the SMN (2010).

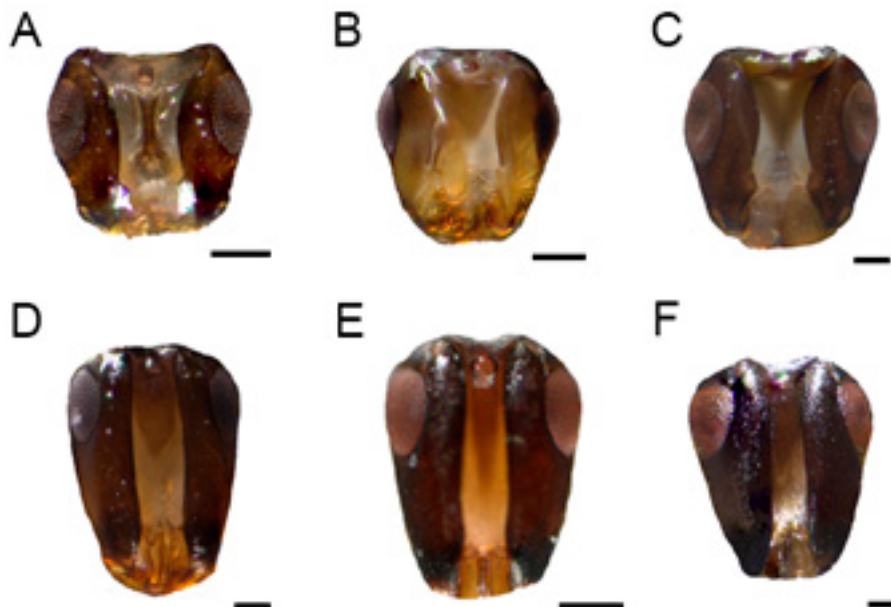


Figure 3. Heads of pollinating wasps. A) *Pegoscapus* sp. 1 (*Ficus colubrinae*); B) *Pegoscapus* sp. 2 (*F. isophlebia*); C) *Pegoscapus* sp. 3 (*F. obtusifolia*); D) *Tetrapus* sp. 1 (*F. apollinaris*); E) *Tetrapus* sp. 2 (*F. insipida*); F) *Tetrapus* sp. 3 (*F. yoponensis*). Scale bar: 200 μ m.

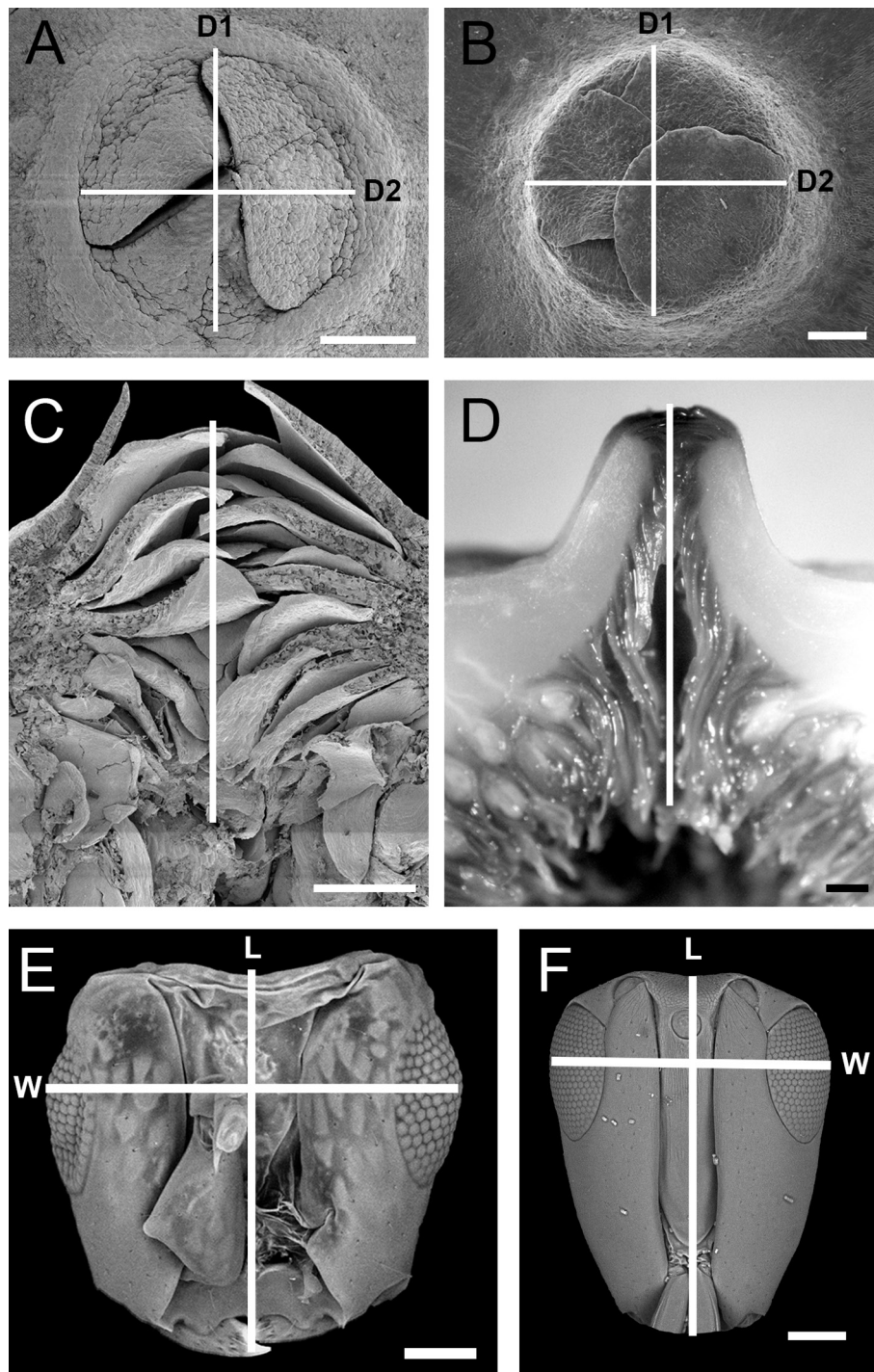


Figure 4. Variables considered in the study. A-B) Frontal view of the ostiole; D1, diameter 1 and D2, diameter 2; C-D) longitudinal section of the ostiole; E-F) frontal view of the head of pollinating wasps; L, length of the head (from the protuberances of the clypeal margin to the mandible) and W, width of the head (Interocular distance). A, C) *Ficus colubrinae*; B) *F. yoponensis*; D) *F. insipida*; E) *Pegoscapus* sp. 1; F) *Tetrapus* sp. 1. Scale bars: a, c, d = 500 μ m, b = 250 μ m, e = 50 μ m, f = 100 μ m.

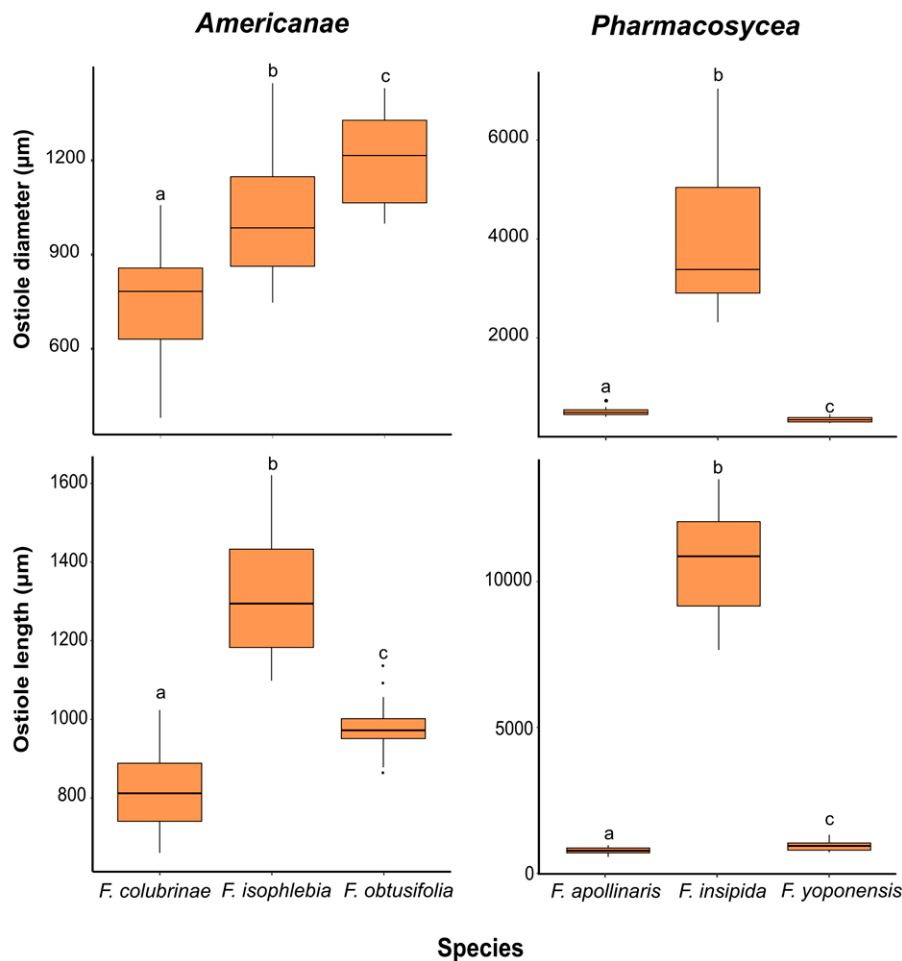


Figure 5. Diameter of the ostiole in the *Americanae* and *Pharmacosycea* sections. Syconia by species (25). Different letters indicate significant differences at a $p < 0.001$ level.

both sections when considering the length of the ostiole (*Americanae*, $F_{2, 71} = 0.74$, $p < 0.000$; *Pharmacosycea*, $F_{2, 74} = 1.88$, $p < 0.000$).

A positive correlation was recorded between the length and width of the head of the pollinating wasps in both genera; however, the relationship was greater in *Pegoscapus* than in *Tetrapus* ($\rho = 0.85$ and 0.75 , respectively). The wasps in this latter genus have elongated heads and differ from those recorded in the *Pegoscapus* species, which are subquadrangular (Fig. 6). For the case of sect. *Americanae*, the head proportion of *F. isophlebia* wasps differed significantly from *F. colubrinae* and *F. obtusifolia* ($F_{2, 37} = 0.079$, $p < 0.000$), while for sect. *Pharmacosycea*, differences were also found between the 3 species analyzed ($F_{2, 74} = 0.291$, $p < 0.000$) (Fig. 6).

In the correlation analysis between the size of the ostiole (diameter 1 and length) and the shape of the wasp's head, sect. *Americanae* only showed significance for ostiole length (0.6 , $p < 0.000$), while for *Pharmacosycea* both ostiole variables are correlated, although with low values (diameter, 0.35 , $p < 0.01$; length - 0.25 , $p < 0.05$). The ordination analysis (NMDS) reflects associations by species (Fig. 7). For sect. *Americanae* (stress = 0.01), the species with smaller ostioles (*F. colubrinae* and *F. isophlebia*) are found at 1 end of the ordination, with a greater width in the dispersion of the data for *F. isophlebia*. Likewise, in sect. *Pharmacosycea* (stress = 0.02), the species with the smallest ostiole size (*F. apollinaris* and *F. yoponensis*) are located at 1 end of the ordination, distant from *F. insipida*, which shows greater variation in the traits analyzed.

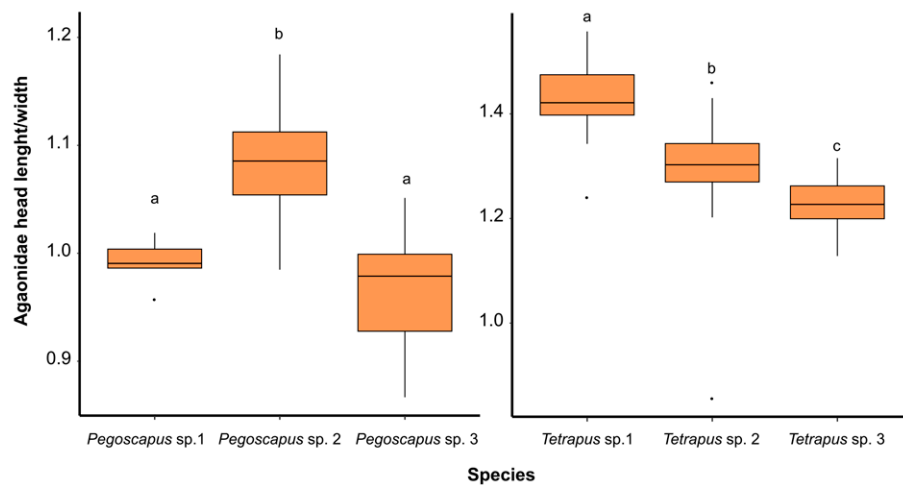


Figure 6. Head length/width of wasps by section. Distinct letters indicate significant differences at $p < 0.001$ level.

Discussion

The present study is the first to quantitatively analyze the relationship between the dimensions of the ostiole and the shape of the head of pollinating wasps for the Neotropical sections of the genus *Ficus* and it contributes to support the reciprocal evolution in their mutualism. Here, it is important to point out that our results were obtained during phase B of syconia development, which was not the case in the study by van Noort & Compton (1996), who used measurements of syconia size from the literature. In addition to the above, Castro-Cárdenas et al. (2022) hypothesized that the ostiole could play a selective filter role that only allows the entry of a particular pollinating wasp morphospecies into the syconium, as has been suggested in previous studies (Dunn, 2020; Herre, 1989; Jandér et al., 2016; Liu et al., 2011). The differences found for the diameter and length of the ostioles among the species studied in both sections (Fig. 5) support this hypothesis.

As regards to the shape of the heads in the Agaonidae wasps proposed by Ramírez (1974), for the genera recorded in America, that information coincides with our results, since the species belonging to the genus *Pegoscapus* presented subquadrangular heads, while those of *Tetrapus* were clearly more elongated than wide (Figs. 3, 6). With respect to the 2 categories in wasp head shape (short heads, with a ratio ≥ 1 ; elongate, ratio ≤ 1), proposed by Jousselin et al. (2003), the ranges of variation found in the species evaluated (*Americanae*, 0.9-1.2; *Pharmacosycea*, 0.9-1.6), do not coincide exactly, although short and elongate are equivalent terms to those proposed by Ramírez (1974).

The close relationship between the metrics of the ostiole and the head of the pollinating wasps in the American sections (Fig. 7) coincides with previous studies of a reciprocal evolution between both mutualistic partners (Cook & Segar, 2010; Galil, 1977; Ramírez, 1974; Weiblen, 2002). Furthermore, interspecific variation in head shape suggests a closer modification, at the species level, in response to ostiole morphology, as has been argued in various previous studies (Cook & Rasplus, 2003; Cook & Segar, 2010; Jousselin et al., 2003; Liu et al., 2011; Ramírez, 1974; Rasplus et al., 2020; van Noort & Compton, 1996). Similar patterns have been reported for other genera of the family Agaonidae, such as *Alfonsiella* Waterston, *Allotriozoon* Grandi, *Courtella* Kieffer, *Elisabethiella* Grandi, and *Pleistodontes* Saunders (Dunn et al., 2008; van Noort & Compton, 1996).

The close correlation recorded between the shape of the head of the pollinating wasps and the size of the ostiole coincides with that proposed by Cruaud et al. (2012) and van Noort & Compton (1996), who indicate that these traits are part of a complex mechanism (the lock-and-key shapes of fig ostioles and wasp heads), that determines the specificity between *Ficus* species and their pollinating wasps. As previously mentioned, this mechanism may explain the role of the ostiole, not only as a physical filter for pollinators but also as a chemical filter, since it is associated with the emission of volatiles for the attraction of legitimate pollinating wasps (Castro-Cárdenas et al., 2022; Okamoto & Su, 2021; Wang et al., 2013). This mechanism is considerably important since it has been shown that closely related species can emit different volatile compounds to attract their pollinating

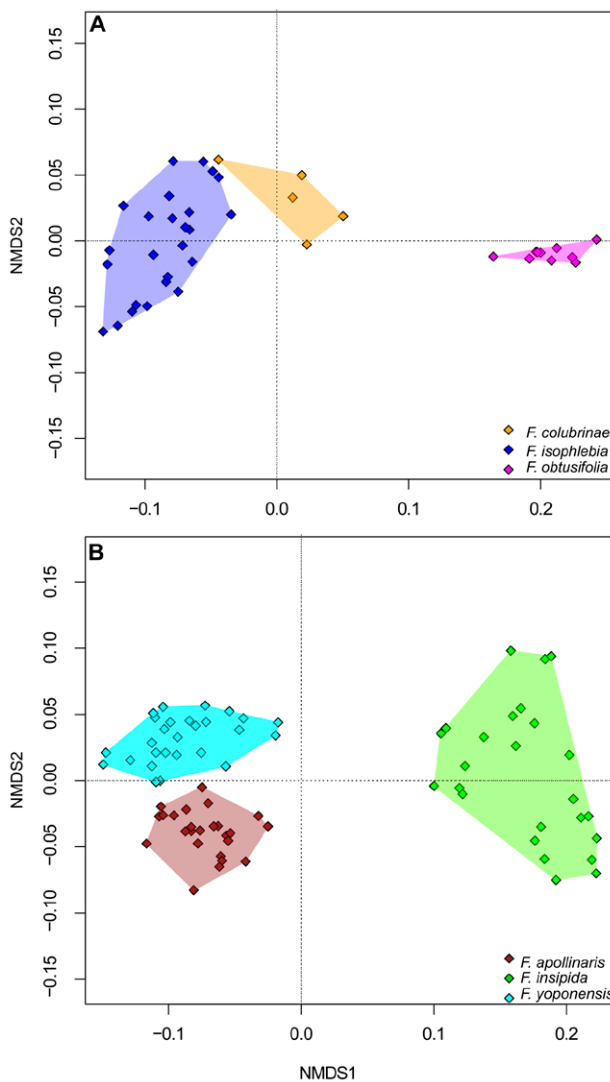


Figure 7. Grouping based on the characteristics of the diameters and length of the ostiole and the shape of the head of the wasps in the species of the *Ficus* sections included in the study.

wasps (Okamoto & Su, 2021; Oldenbeuving et al., 2023; Souto-Vilarós et al., 2018).

A critical aspect for the advancement in the understanding of the connection between the head shape of pollinating wasps and the dimensions of the ostiole is to carry out studies with a greater geographic scope, since the taxonomy of *Ficus* species in the Neotropical sections is not completely resolved (Berg, 1989; Ibarra-Manríquez et al., 2012). Global studies are required to resolve the taxonomic complexes that have been detected

(Berg, 2007; Hernández-Esquivel et al., 2020; Pederneiras et al., 2023). These studies need to be performed together with a precise identification of the pollinating wasps, since there is evidence of *Ficus* species with more than 1 wasp pollinator (Jackson et al., 2008; Machado et al., 2005; Molbo et al., 2003; Su et al., 2008), some of which can be cryptic (Haine et al., 2006; Molbo et al., 2003). Future studies where these elements can be integrated are thus required to validate and estimate the possible contribution of each trait in this intriguing mutualism.

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