

## **Hole Size Matters: An Indirect Method for Measuring Gall Wasp Community Emergence?**

Ricardo Clark-Tapia<sup>1</sup>, Victor Aguirre-Hidalgo<sup>1</sup>, Juli Pujade-Villar<sup>2</sup>, Cecilia Alfonso-Corrado<sup>1</sup>, and Felipe Tafoya<sup>3,\*</sup>

<sup>1</sup>*Instituto de Estudios Ambientales, Universidad de la Sierra Juárez, Avenida Universidad S/N, Ixtlán de Juárez, Oaxaca 68725, México. E-mail: rclark@unsij.edu.mx (Clark-Tapia), victor@unsij.edu.mx (Aguirre-Hidalgo), liana@unsij.edu.mx (Alfonso-Corrado)*

<sup>2</sup>*Universitat de Barcelona. Departament de Biologia Animal Avda. Diagonal, 645. 08026 Barcelona, Spain. E-mail: jpujade@ub.edu (Pujade-Villar)*

<sup>3</sup>*Tecnológico Nacional de México, Instituto Tecnológico El Llano Aguascalientes, Km. 18 Carretera Aguascalientes -S.L.P., El Llano Aguascalientes, México. C.P. 20330. \*Correspondence: E-mail: ftafoya@yahoo.com (Tafoya)*

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Quantifying the abundance and diversity of small insects, especially those with specific environmental associations or hidden habitats, can be challenging using direct methods. Galls are specialized microhabitats that host a wide range of interactions between species. However, assessing their abundance and associated community diversity takes time and effort. In this study, we propose an indirect approach using the size of gall emergence holes to estimate the abundance of gall wasps (Cynipini) and their associated wasp species. We examined eight different types of gall structures collected in the temperate forest of Mexico. We identified every gall-emerging individual, classifying them as gall inducer, inquiline, or parasitoid. Kruskal-Wallis test, correlation analysis and mixed linear model (LMM) were used to evaluate differences between mesosoma size and gall's hole size for each emerged species in each gall type. Our results showed that mesosoma and hole size significantly differed between Cynipini wasps and their associated wasp species. LMM showed a significant relationship between the mesosoma body and the size diameter of the emergence hole among the analyzed wasp species. Upon validating the method, a low emergence rate and low abundance of the studied Cynipini wasps were observed, attributed to negative interactions and inadequate development of the wasps. We emphasize the potential of gall emergence hole size as an indicator of species abundance and emergence rates within Cynipini gall complexes. Furthermore, strategies are discussed to improve their effectiveness and reliability in future studies to increase our understanding of the ecological dynamics and evolutionary processes of gall-forming wasps.

**Key words:** Cynipini, diameter, mesosoma, negative interactions, wasp

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## BACKGROUND

The estimation of the population size or abundance of insect species, especially those that are difficult to see due to their small size, is a critical ecological indicator (Sileshi 2007; Schowalter 2022). This estimate is essential for understanding the significant fluctuations between different habitats over short periods due to changing environmental conditions or negative interactions with another organisms (e.g., van der Sluijs 2020; Kehoe et al. 2021). Furthermore, understanding insect richness is critical to developing effective management and conservation strategies, especially amid widespread insect declines, in which smaller insects are highly susceptible and vulnerable to environmental changes (Sileshi 2007; Schowalter 2022).

However, quantifying insect abundance and diversity is often a complex and challenging task (Sileshi 2007; Clark-Tapia et al. 2022). Direct methods for estimating insect abundance, such as live insect capture, can be complicated due to difficulties in capturing and handling insects in areas where direct assessment is complex, costly, and unspecific, which may be detrimental to insect populations themselves (Stork 2018) mainly when these populations are fragile or at risk.

Diverse indirect methods have been developed to address this challenge. These methods often entail assessing the marks caused by the insects on their host plants (Dyck et al. 2021). Such approaches include inspecting excreta (Majer 1983; Nichols et al. 2007), plant damage (Schooler and McEvoy 2006) or employing cumulative recording data to obtain valuable measures of range change (Thomas 2005). These methods enable the estimation of insect abundance and diversity without relying on direct observations, which can significantly assist in biodiversity management and species conservation efforts (Nichols et al. 2007; Clark-Tapia et al. 2022). Moreover, indirect methods allow data collection on an extensive time and space scale, which is crucial for understanding biodiversity change trends (Samways et al. 2010 2020).

Nevertheless, it is essential to highlight that these indirect methods are particularly valuable for studying species that are challenging to quantify or have specific survival requirements, such as gall

wasp of the Cynipidae family. In North America, over 80% of gall wasps are from this family, mainly grouped in the tribe Cynipini; all representants of this tribe only utilize the oaks (Fagaceae: *Quercus*) as their primary hosts (Price 2005; Russo 2007), inducing galls or cecidia in response to oviposition (Redfern 2011; Hearn et al. 2019). These structures can serve as new microhabitats, giving rise to the development of ecological niches (Stone and Schönrogge 2003; Gilbert 2009; Pujade-Villar 2013). The recording and observation of gall appearance and structure have proven effective in studying the diversity of Cynipini group and their relationship with host plants (Stone et al. 2002; Ito and Hijii 2004; Lobato-Vila et al. 2022; Martínez-Romero et al. 2022). Gall structures classification is also an effective tool in studies regarding biodiversity patterns and conservation strategies at the ecosystem level (Price et al. 2004; Clark-Tapia et al. 2022; Ward et al. 2022). However, it is essential to note that the appearance of the galls does not provide information about species abundance and diversity. Although only one gall-inducing wasp emerges from each larval chamber of a given type of gall (single or multiple larval chambers), multiple species associations can also emerge. These associations include parasitoid species, primarily from hymenopteran families such as Chalcididae, Braconidae, Ichneumonidae, Eulophidae, Eupelmidae, Eurytomidae, Pteromalidae, Ormyridae, and Torymidae, as well as inquiline species, which are generally other cynipids belonging to the tribe Synergini (Stone et al. 2002; Melika 2006; Russo 2007; Lobato-Vila and Pujade-Villar 2021). In this context, the size of exit holes in galls has been used as indirect evidence of multitrophic interactions (Russo 2007; Stone et al. 2008; Cooper and Rieske 2010).

This study aimed to propose an indirect method to quantify the emergence percentage and abundance of the Cynipini gall wasp and study multitrophic interactions using the size of the insects' emergence hole. To achieve this goal, the methodological proposal was first validated by collecting, monitoring, and measuring emergence holes and interactions in various types of galls. Subsequently, the emergence percentages and abundance of gall wasps were estimated. This will contribute to the general understanding of insect ecology and open new perspectives for research in this field.

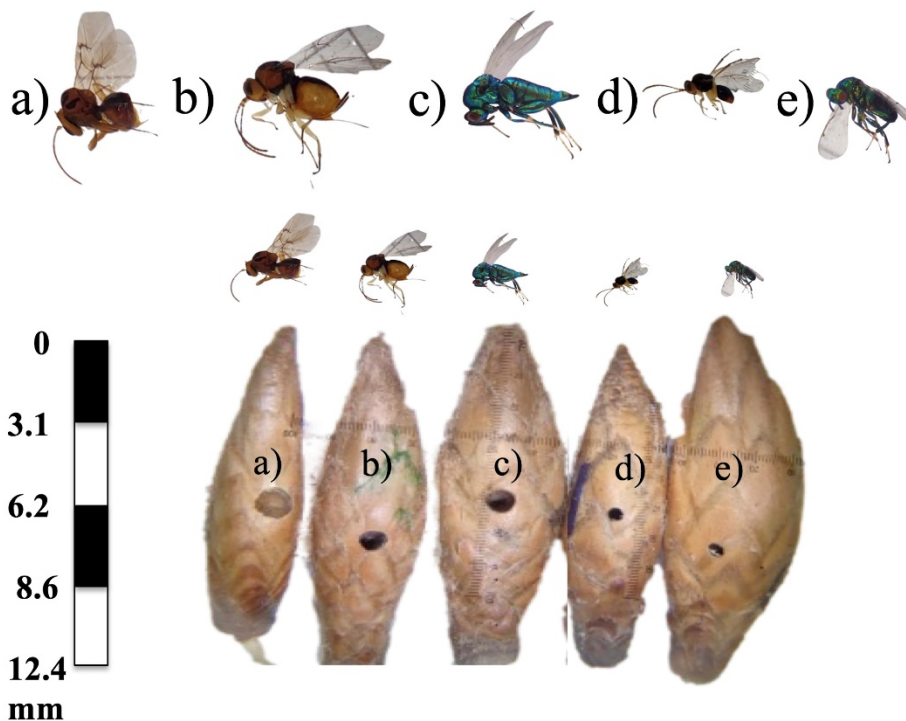
## MATERIAL AND METHODS

### Gall sampling

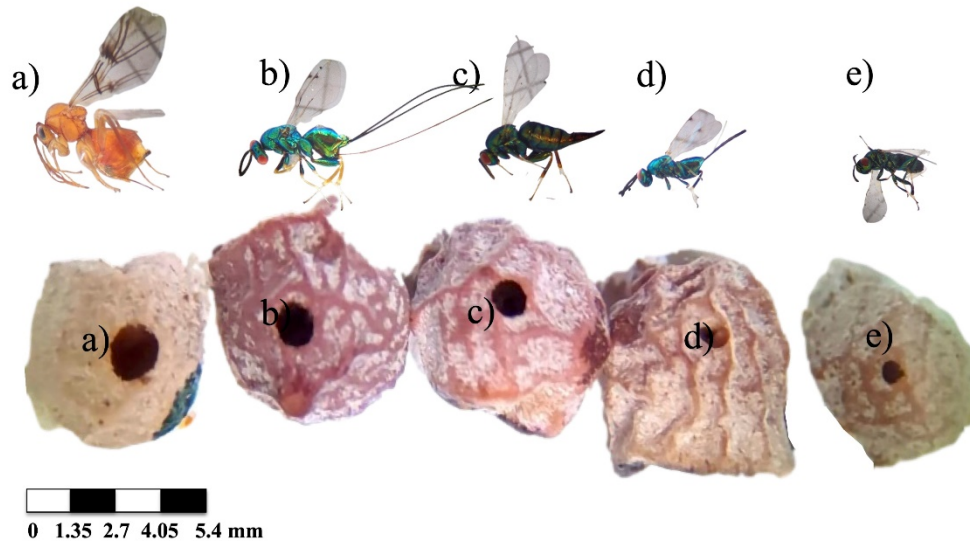
To conduct this study, we collected eight types of Cynipini galls in two temperate forests of Mexico; six came from Sierra Fría, Aguascalientes, and two from Sierra Juárez, Oaxaca, hosted in five

oaks species (see Table 1 for the host species and location of each sampling site). Selected cynipids induce galls, particularly on leaves and stems, exhibiting different shapes and sizes (Figs. 1-8, Table 1). Sampling was carried out in each forest by selecting an area with the presence of the host corresponding to each type of gall. In each sampling area, three transects were established, each with a length of 100 meters and separated by 500 meters. Subsequently, a sample of each gall type was collected from the sampled trees without evidence of emergence or damage. It is important to note that the number of galls collected per tree varied depending on the abundance of each type, and not all trees had galls. Therefore, we aimed to obtain at least 50 samples of each gall type (see Table 1 for inducer and associates gall species, gall size, sample size, and sampling sites).

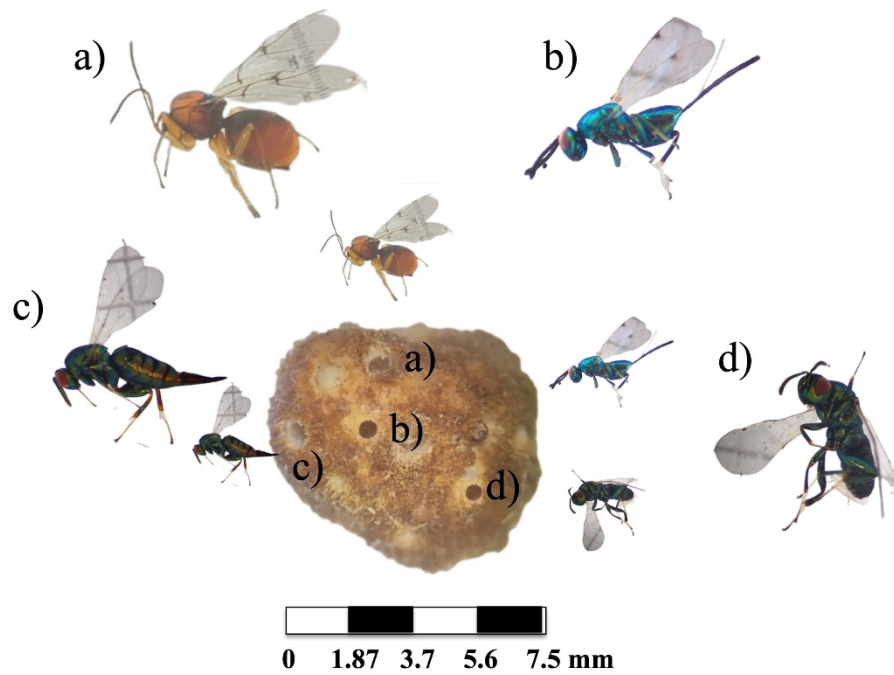
We visited each sampling site during the fieldwork to establish transects for gall collection (see Table 1). In Aguascalientes, the collection period was extended from October 15 to November 10, while in Oaxaca it occurred from November 15 to December 20, 2022, at the beginning of the gall wasp emergence period.



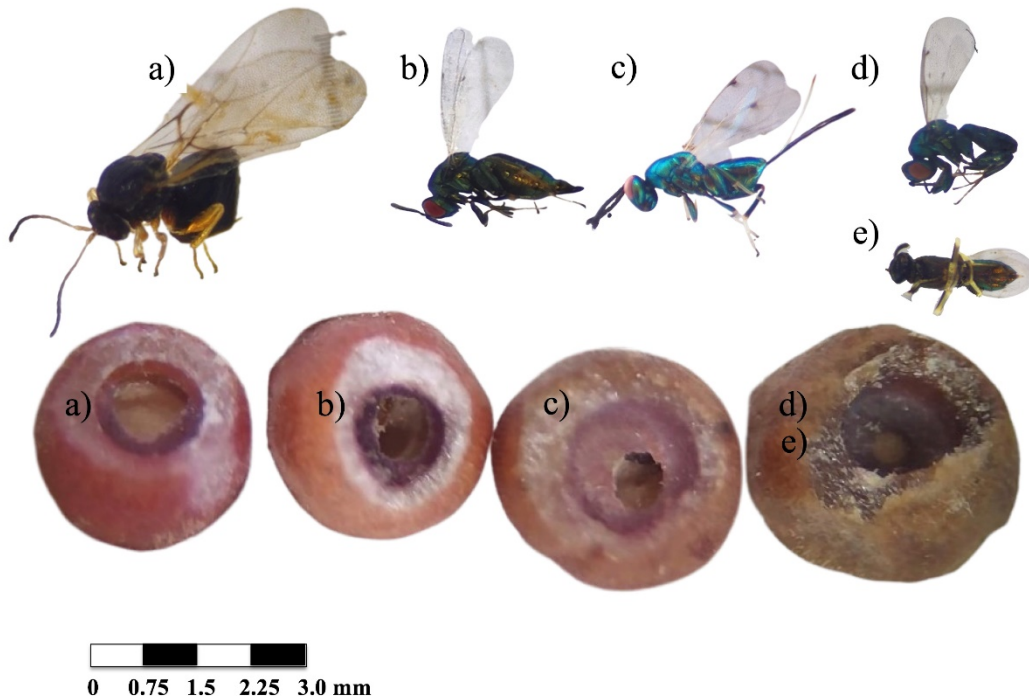
**Fig. 1.** Dimensions in the emergence holes of the gall induced by *Erythres hastata* ♂ (a), and associated genera: *Synergus* sp. ♀ inquiline (b), *Ormyrus* sp. ♀ parasitoid (c), *Synergus* sp. ♂ inquiline (d), and *Ormyrus* sp. ♂ parasitoid (f), at 2:1 scale for enhanced visualization and 1:1 real scale estimated using the average gall size.



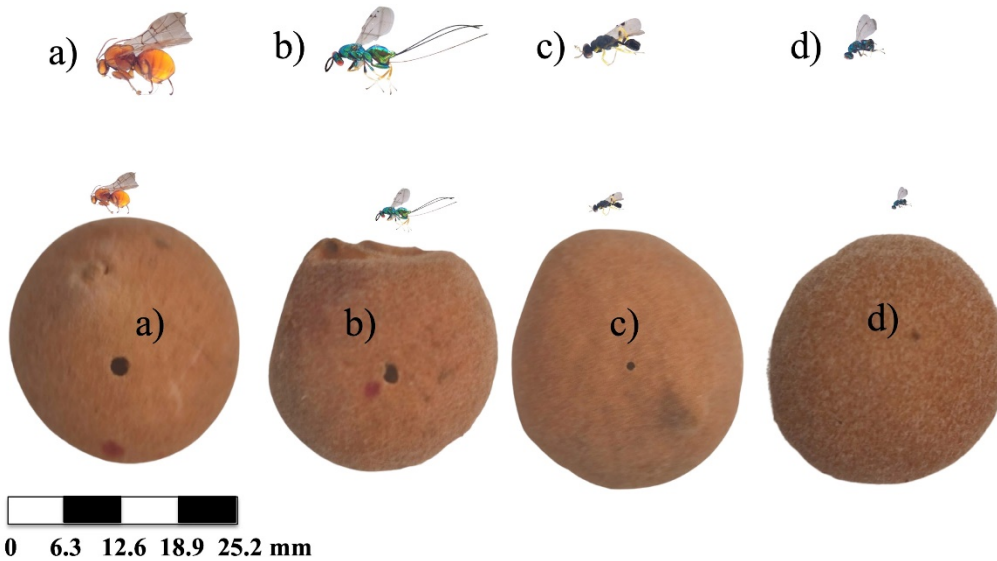
**Fig. 2.** Dimensions in the emergence holes of the gall induced by *Cynips* sp. ♂ (a), and parasitoids associated genera: *Torymus* sp.1 ♀ (b), *Ormyrus* sp. ♀ (c), *Torymus* sp.2 ♀ (d), and *Ormyrus* sp. ♂ (f), at a 1:1 real scale estimated using the average gall size.



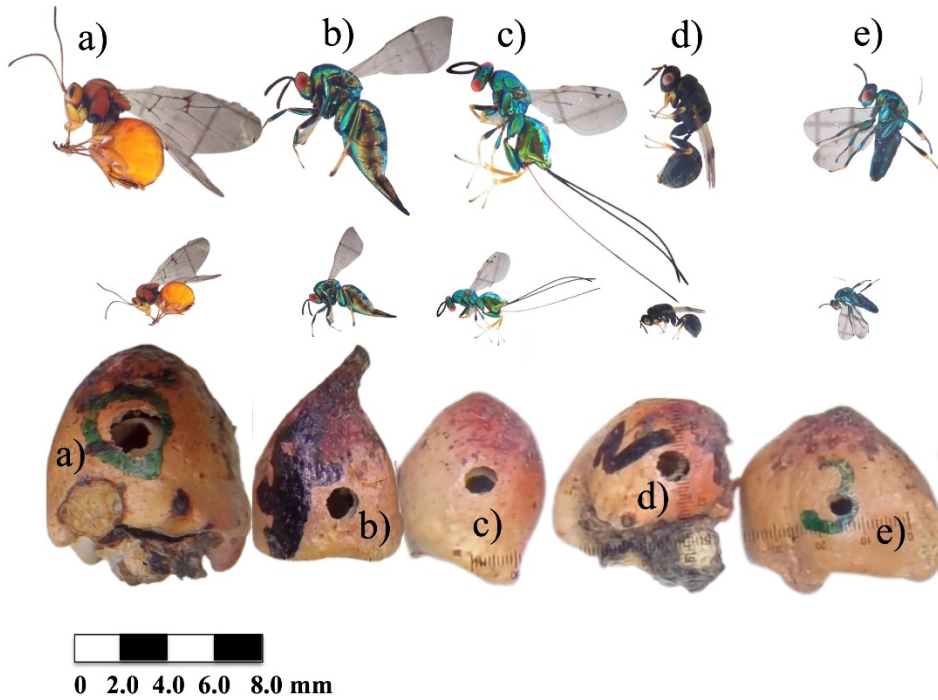
**Fig. 3.** Dimensions in the emergence holes of the gall induced by *Druon* sp. ♂ (a), and parasitoids associated genera: *Torymus* sp.2 ♀ (b), *Ormyrus* sp. ♀ (c), and *Ormyrus* sp. ♂ (d), at 2:1 scale for enhanced visualization and 1:1 real scale estimated using the average gall size.



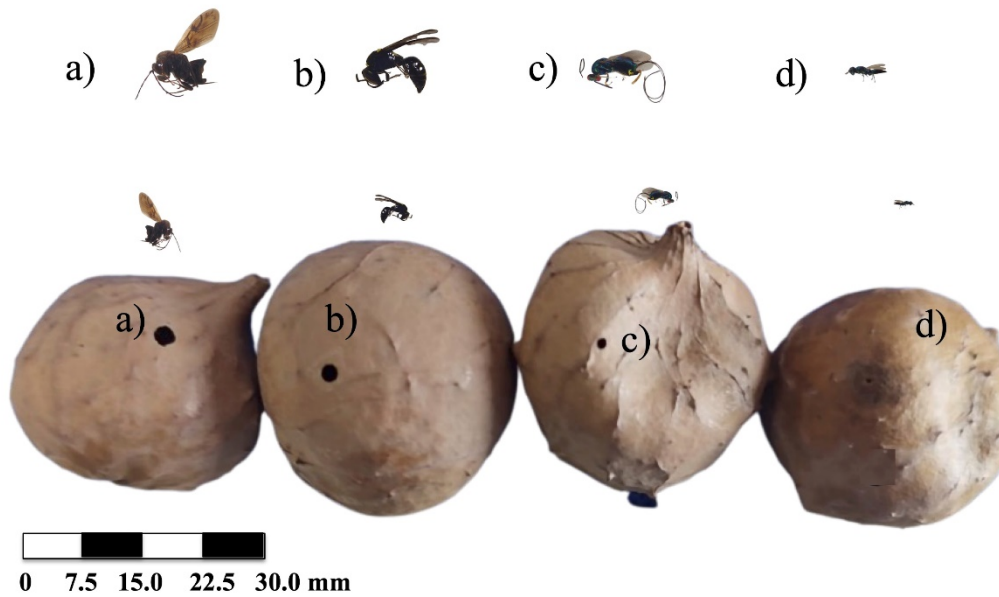
**Fig. 4.** Dimensions in the emergence holes of the gall induced by *Andricus* sp. ♂ (a), and parasitoids associated genera: *Ormyrus* sp. ♀ (b), *Torymus* sp2. ♀ (c), *Ormyrus* sp. ♂ (d), and *Eupelmus* sp. ♀ (e), at a 1:1 real scale estimated using the average gall size.



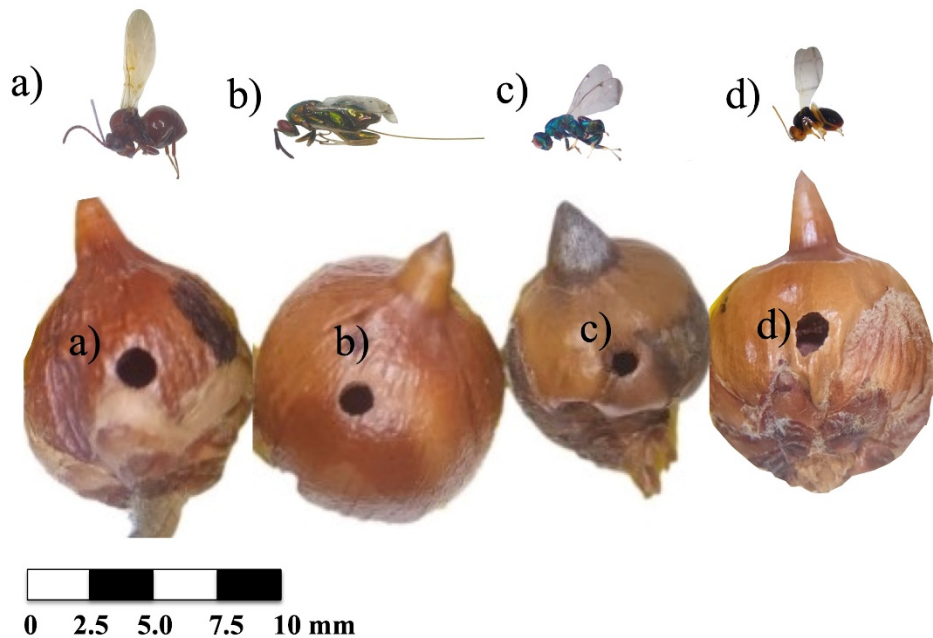
**Fig. 5.** Dimensions in the emergence holes of the gall induced by *Kinseyella quercusobtusata* ♂ (a), and parasitoids associated genera: *Torymus* sp1. ♀ (b), *Sycophila* sp. ♂ (c) and *Torymus* sp1. ♂ (d), at 2:1 scale for enhanced visualization and 1:1 real scale estimated using the average gall size.



**Fig. 6.** Dimensions in the emergence holes of the gall induced by *Disholcaspis* sp. ♂ (a), and parasitoids associated genera: *Ormyrus* sp. ♀ (b), *Torymus* sp. ♀ (c), *Eurytoma* sp. ♂ (d), *Ormyrus* sp. ♂ (e), at 2:1 scale for enhanced visualization and 1:1 real scale estimated using the average gall size.



**Fig. 7.** Dimensions in the emergence holes of the gall induced by *Atrusca* sp. ♂ (a), associated family Vespidae ♀ (b), and parasitoids associated genera: *Torymus* sp3. ♀ (c), and Eulophidae (d), at 2:1 Scale for Enhanced Visualization and 1:1 real scale estimated using the average gall size.



**Fig. 8.** Dimensions in the emergence holes of the gall induced by *Zapatella gabriellae* ♂ (a), and parasitoids associated genera: *Torymus* sp. ♀ (b), *Torymus* sp. ♂ (c) and *Synergus* sp. ♂ (d), at a 1:1 real scale estimated using the average gall size.

**Table 1.** Characteristics of galls induced by Cynipini Tribe and associated species. Abbreviations for genera *E* = *Erytres*, *K* = *Kinseyella* and *Z* = *Zapatella*

Cynipini and associates species	Sexual Generation type	Species code	Gall size (mm, mean ± SD)	Species host and location	Sample size	Sampling sites
<i>E. hastata</i>	Asexual (♂)	Eh	12.3 ± 0.8	<i>Quercus laurina</i> Humb. and Bonpl.	158	Aguascalientes
<i>Synergus</i> sp.	Female (♀)	Sy_f		steams		22°05'56"N
<i>Ormyrus</i> sp.	Female (♀)	Oy_f			102°42'18"W	
<i>Synergus</i> sp.	Male (♂)	Sy_m				
<i>Ormyrus</i> sp.	Male (♂)	Oy_m				
<i>Cynips</i> sp.	Asexual (♂)	Cy	5.4 ± 1.41	<i>Q. resinosa</i> Liemb.	58	Aguascalientes
<i>Torymus</i> sp.	Female (♀)	Tm_f		leaves		22°10'16"N
<i>Ormyrus</i> sp.	Female (♀)	Oy_f			102°31'15"W	
<i>Torymus</i> sp.	Male (♂)	Tm_m				
<i>Ormyrus</i> sp.	Male (♂)	Oy_m				
<i>Druon</i> sp.	Asexual (♂)	Dn	7.3 ± 2.9	<i>Q. chihuahuensis</i> Trel.	56	Aguascalientes
<i>Ormyrus</i> sp.	Female (♀)	Oy_f		leaves		22°09'46"N
<i>Torymus</i> sp.	Tm_f	Tm_m			102°31'28"W	
<i>Ormyrus</i> sp.	Male (♂)	Oy_m				
<i>Andricus</i> sp.	Asexual (♂)	Ad	2.98 ± 0.09	<i>Q. chihuahuensis</i> Trel.	166	Aguascalientes
<i>Ormyrus</i> sp.	Female (♀)	Oy_f		leaves		22°09'46"N
<i>Torymus</i> sp.2	Female (♀)	Tm_f2			102°31'28"W	
<i>Ormyrus</i> sp.	Male (♂)	Oy_m				
<i>Eupelmus</i> sp.	Female (♀)	Eu_f				



<i>K. quercusobtusata</i>	Asexual (♂)	Kq	25.2 ± 4.4	<i>Q. resinosa</i>	112	Aguascalientes
<i>Torymus</i> sp.	Female (♀)	Tm_f		leaves		22°10'16"N
<i>Sycophila</i> sp.	Male (♂)	Sh_m				102°31'15"W
<i>Torymus</i> sp.	Male (♂)	Tm_m				
<i>Disholcaspis</i> sp.	Asexual (♂)	At_1	7.6 ± 1.8	<i>Q. chihuahuensis</i>	123	Aguascalientes
<i>Ormyrus</i> sp.	Female (♀)	Oy_f		stems		22°09'46"N
<i>Torymus</i> sp.	Female (♀)	Tm_f				102°31'28"W
<i>Eurytoma</i> sp.	Male (♂)	Ey_m				
<i>Ormyrus</i> sp.	Male (♂)	Oy_m				
<i>Atrusca</i> sp.	Asexual (♂)	At_2	30 ± 4.1	<i>Q. obtusata</i> Humb. and Bonpl.	50	Oaxaca
<i>Torymus</i> sp.3	Female (♀)	Tm_f				17°18'55"N
Eulophidae sp. Vespidae	Male (♂)	Elp		leaves		96°28'56"W
	Female (♀)	Vp				
<i>Z. gabriellae</i>	Asexual (♂)	Zg	9.6 ± 1.3	<i>Q. crassifolia</i> Humb. and Bonpl.	53	Oaxaca
<i>Torymus</i> sp.	Female (♀)	Tm_f				17°08'22"N
<i>Torymus</i> sp.	Male (♂)	Tm_m		steams		96°36'44"W
<i>Synergus</i> sp.	Male (♂)	Sy_m				

Author of the species of gall wasps: *E. hastata* Kinsey; *K. quercusobtusata* Pujade-Villar and Melika; *Z. gabriellae* Cuesta-Porta and Pujade-Villar.

## Method validation

### Collection, monitoring, and measurement

During sampling, mature galls were collected and identified in the field by their coloration and previous experience. These galls were placed in cloth bags and transported to the laboratory. Once in the laboratory, all galls were visually examined using a stereoscope to ensure no emergencies or damage. The selected galls were placed individually in sheer organza bags, then placed in a box and stored in the laboratory.

Of all the analyzed galls, only those induced by *Druon* sp. showed multiple chambers (Fig. 3). In this gall type, the filaments or hairs covering the structure were removed to obtain accurate measurements of the emergence holes. Since they were placed in the organza bags, the galls were monitored weekly for three months to detect any wasp emergence, except for *Druon* sp. The galls of this species were checked every day because the simultaneous emergence of wasps was recorded. In multiple emergencies, the species was associated based on the size and shape of the hole. In this gall, each emergence hole was marked with an indelible marker. All emerged wasps (Cynipini, inquiline or parasitoids) were stored in Eppendorf tubes with 96% ethyl alcohol, and subsequent measurements were taken of the emergency hole and the mesosoma of the wasp using a VE-910 eyepiece micrometer previously calibrated with acetate sheets with millimeter marks as a reference. This process was

conducted considering that the mesosoma's width in wasps is a reliable indicator of size (*e.g.*, Ohl and Thiele 2007).

## **Emergence Analysis**

For each gall type, we first identified emerging species (cynipids, parasitoids, inquilines) to genus, except for species in Eulophidae and Vespidae. Subsequently, the exit hole's diameter was measured twice, in perpendicular directions, using a previously calibrated micrometer with an ocular. The diameter values obtained from the two directions measurements were averaged to obtain a representative value of the hole emergence diameter. To establish an association between the size of the exit hole and the mesosoma size of emerging wasps, we compared the diameter of the emergence hole with the width of the mesosoma. This comparison was based on the premise that the mesosoma, being more rigid than the metasoma, provides a reliable measure of size. Therefore, if the width of the mesosoma allowed it to pass through the hole, we inferred that the metasoma would too. This assessment was primarily based on the width of the mesosoma, which has previously been established as a dependable indicator of size in wasps (*e.g.*, Ohl and Thiele 2007).

## **Data analysis**

### **Validation method**

Kruskal-Wallis and Mann-Whitney analyzes were used to compare diameter and mesosoma differences between species and to perform species-specific pairwise comparisons. These analyzes were performed in R v.4.10 (R Core Team 2021) using the nparcom package (Konietschke et al. 2015) and visualized in the ggplot2 (Wickham et al. 2016) and GridExtra v.2.3 (Auguie and Antonov 2017) packages. Holm's correction was also applied to the p values obtained to control type I errors. The relationship between diameter and mesosoma was examined, focusing mainly on analyzing the gall-inducing wasp due to the limited number of individuals in other species. This association was evaluated using Pearson correlation and linear regression through the nparcom package (Konietschke et al. 2015) in R. Additionally, a mixed linear model (LMM) analysis was conducted to investigate the relationship between the diameter size of emergence holes (diameter) and the pronotum length (mesosoma) in relation to insect species (species). The diameter and mesosoma were log-transformed to achieve a normal distribution. The model was fitted using the lmer() function from the lme4 package v. 1.1-27 (Bates and Sarkar 2007) in the R program.

The model was specified as follows:

$$\text{Diameter} = \beta_0 + \beta_1 * \text{Meososma} + \beta_2 * \text{Species} + b_i + \varepsilon$$

where:

- Diameter represents the size of the emergence hole diameter.
- Mesosoma represents the length of the mesosoma.
- Species is a categorical variable indicating the insect species.
- $\beta_0$  is the intercept of the model.
- $\beta_1$  and  $\beta_2$  are the coefficients associated with the explanatory variables Mesosoma and Species, respectively.
- $b_i$  is the random effect of each species, considered as a normally distributed random variable with a mean of zero and variance  $\sigma^2_{\text{Species}}$ .
- $E$  represents the error term.

A random effect for the Species variable was included to capture individual differences between species in the size of the emergence hole diameter. This allowed modeling differences between species as random effects, facilitating the assessment of individual differences between species and their impact on the size of the emergence hole diameter.

The restricted maximum likelihood (REML) method was used for parameter estimation and statistical inference. The significance of the model coefficients was evaluated using hypothesis tests, and the p-value associated with each coefficient was calculated.

### **Abundance estimation**

Finally, with all the collected galls, the proposed method was evaluated. To do this, the percentage of emergence for each gall wasp (GC) was estimated, along with two causes of emergence failure: 1) due to the presence of negative interactions (Pa) and 2) inadequate development of the larvae, considered empty galls (Vn). Kruskal-Wallis and Mann-Whitney analyzes were used to evaluate differences among GC, Pa and Vn to each gall type. Also, the abundance of each species was estimated

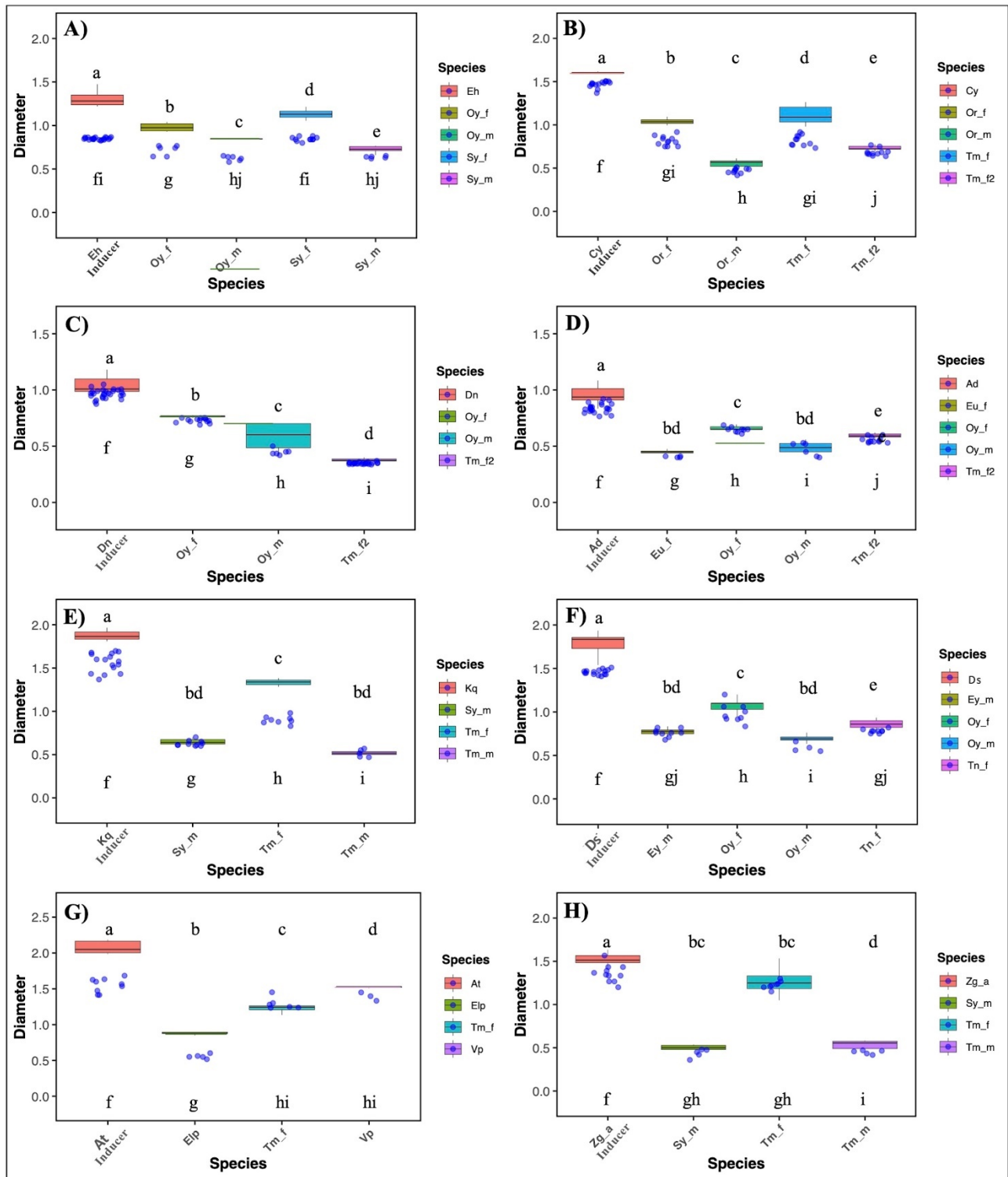
by counting the number of adults that emerged from each species. The tests were executed and visualized in R program.

## RESULTS

### Validation method

In inducer gall wasps, only females were identified (asexual generation), while in associated wasps, individuals of both sexes were observed. Figures 1 to 8 show the gall types induced by each Cynipini wasp and the identified negative interactions, illustrating the size proportions of the species within each gall. It was found that the most frequent negative interactions involved parasitoids of the genera *Torymus* and *Ormyrus*, which were present in 88% and 63% of the analyzed galls species, respectively. Other interactions with wasps, such as *Synergus* (inquilines), *Eurytoma*, *Eupelmus*, and *Sycophila* (parasitoids), as well as Vespidae (predators emerging from the larval chamber), had a frequency of occurrence lower than 25%.

Significant differences were found in the diameter of the emergence hole between Cynipini wasps and associated species (Fig. 9, Table S1). The diameter of the emergence hole of the analyzed galls of inducer wasps was significantly larger than that of all associated species. Furthermore, significant differences were observed between the associated species, especially between the sexes, when present in the samples. Additionally, the mesosoma size of the inducer species was significantly larger ( $H_c = 42.43$ ;  $p < 0.001$ ) than that of the associated wasp species, except for *E. hastata*, which showed no significant differences compared to the females of *Synergus* sp. Furthermore, among the associated gall species, significant differences ( $H_c = 32.15$ ;  $p < 0.01$ ) were observed between females and males in the size of the mesosoma of parasitoid wasps (Fig. 9, Table 1S)



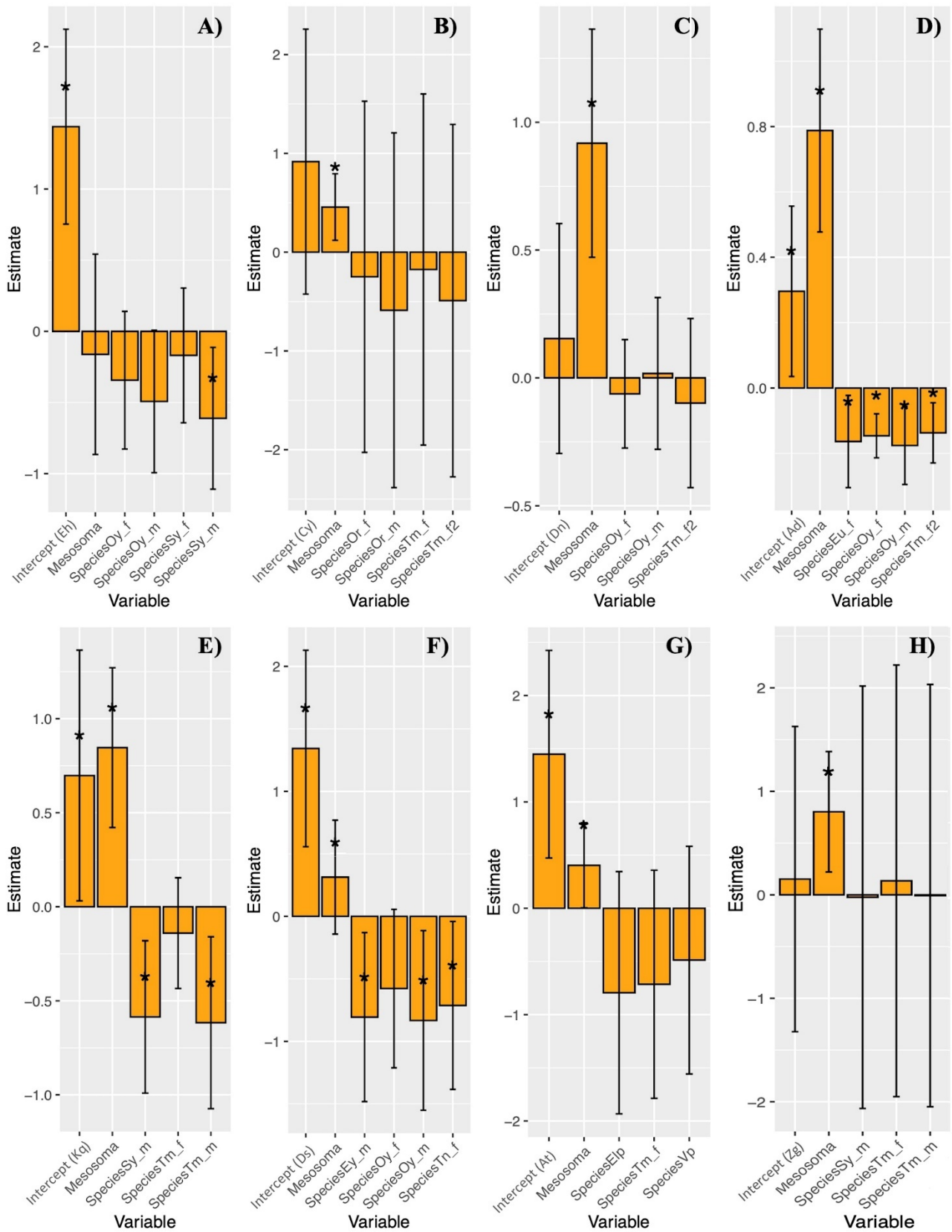
**Fig. 9.** Diameter and mesosome comparisons of each gall wasp inducer and associated species: boxplot representation with standard deviation for diameter and mesosome lengths indicated by blue dots. Different letters indicate significant differences between species mean for diameter (letters a to b, at the top) and mesosome (at the bottom, letters f to j). See Table 1 for species code and Table 1S for results of Kruskal-Wallis tests and Mann-Whitney comparisons with Holm adjustment.

Association analysis revealed a close relationship between mesosoma size and the diameter of the hole in several gall wasps. Specifically, a significant association was found in the species *Druon* sp., *K. quercusobtusata*, *Z. gabriellae*, and *Andricus* sp., although this species had a weak association (Table 2). On the other hand, *E. hastata*, *Cynips* sp., *Disholcaspis* sp., and *Atrusca* sp., their association was not statistically significant. Furthermore, in some cases, a negative association between mesosome size and hole diameter was observed in wasp species (inducers, inquilines, or parasitoids). Smaller holes led to failures in emergence, as the wasps became trapped in the hole or inside the gall.

**Table 2.** Relationship between diameter and mesosoma within each gall wasp: Pearson correlation analysis (r) and linear regression (r<sup>2</sup>). The sampling size (N), F statistic and p-value for each test were included

Species	N	F	r	r <sup>2</sup>	p-value
<i>E. hastata</i>	21	1.71	0.29	n.s.	n.s.
<i>Cynips</i> sp.	14	1.98	0.38	n.s.	n.s.
<i>Druon</i> sp.	27	19.61	0.66	0.44	0.001
<i>Andricus</i> sp.	22	6.43	0.49	0.24	0.02
<i>K. quercusobtusata</i>	16	9.69	0.64	0.41	0.08
<i>Disholcaspis</i> sp.	15	3.7	0.47	n.s.	n.s.
<i>Atrusca</i> sp.	9	0.15	0.14	n.s.	n.s.
<i>Z. gabriellae</i>	11	10.01	0.73	0.53	0.012

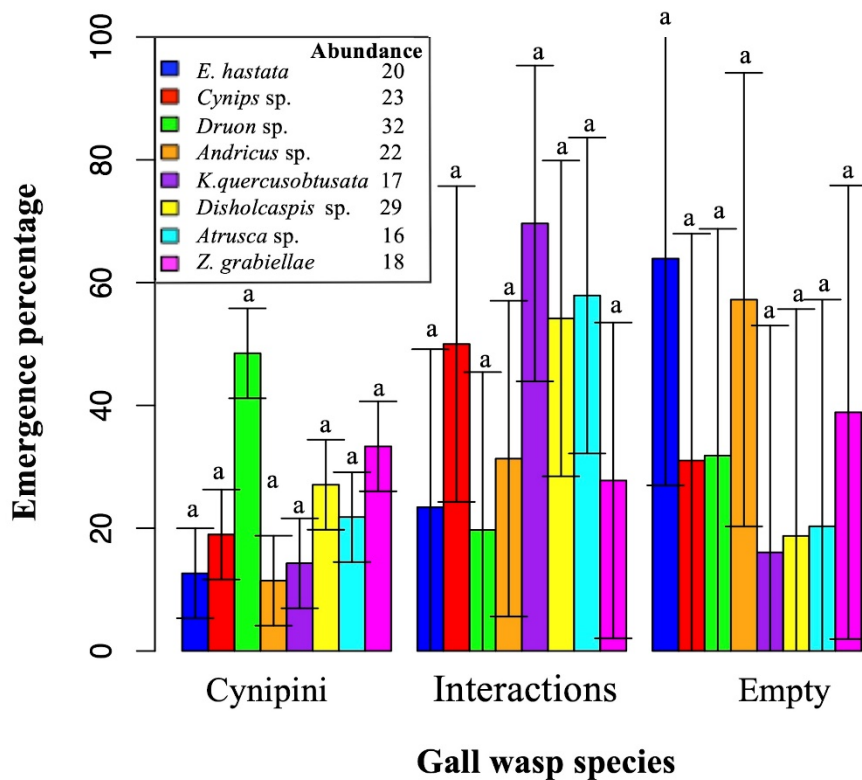
On the other hand, linear mixed models showed a significant relationship between the mesosoma body and the size diameter among the analyzed wasp species. On average, across all species examined, an increase in mesosoma correlated with an increase in hole diameter, with an estimated average coefficient of 0.527 ( $p < 0.05$ ). However, it is worth noting that the magnitude and direction of this association varied among species, with some significant coefficients indicating differences in emergence hole diameter between specific species. Except for *E. hastata*, all models had positive coefficients for the mesosoma, indicating an increase in mesosoma size associated with an increase in diameter (Fig. 10).



**Fig. 10.** Estimation of fixed effects on the size of the Mesosoma of gall wasp and associates' species, in relation to gall emergence hole diameter. Significance levels \* = 0.05 are showed. See table 1 for species code.

### Abundance estimation

According to the proposed method, it was found that most gall wasp species had low emergence percentages. However, two species, *Druon* sp. and *Z. gabriellae* had emergence percentages greater than 30%, with *Druon* sp. having the highest percentage at 48.5%; the remaining Cynipini species showed emergence rates below 30% (Fig. 11). Specifically, in *K. quercusobtusata*, *Disholcaspis* sp., and *Atrusca* sp., their low emergence percentages were attributed to negative interactions (predation), which had percentages of 69.6%, 54.2%, and 55.7%, respectively. On the contrary, the non-appearance in *Andricus* sp. and *E. hastata* was due to inadequate development of the wasp (Fig. 11). No significant differences were observed between the analyzed Cynipini gall wasps, presence of negative interactions or species association, and empty percentages. The estimated abundances of all species were also low, and no significant differences were found between them (Fig. 11).



**Fig. 11.** Percentage of Cynipini wasp emergence, negative interactions, and empty or non-emerged galls. Abundance indicates the estimate of the abundance of each wasp species per hectare. The lines represent standard deviations. Different letters indicate significant differences in emergence between species at a significance level of  $p < 0.05$ .



## DISCUSSION

The study highlights a significant difference in the emergence hole diameter between Cynipini wasps and associated wasp species. In particular, the relationship between the increase in the mesosoma and the size of the hole suggests that the larger the size of the gall wasp, the greater its hole size, an aspect reported for Cynipini wasps (Stone et al. 2002). The proposed indirect method can potentially be a tool to estimate the emergence rate and abundance of gall wasps and associated wasps. However, although the importance of hole size has been demonstrated, obtaining a comprehensive understanding of the biology and ecology of the wasps associated with each gall to improve its effectiveness and reliability is necessary.

For example, initially, the method requires constant monitoring of the emergence time, especially for galls with multiple chambers, which can generate simultaneous emergences in a short time, leading to potential errors in data reading. This aspect has been reported for various galls (e.g., Cuesta-Porta et al. 2022), so it should be considered. Although the exact shape of the hole and the dimensions of the cuts made by the jaws were not evaluated, differences between species were observed. These differences were used to associate species in the case of multiple emergences. Hole shape could be influenced by differences in jaw type between Cynipini wasps and associated species (e.g., Vårdal 2004), and its use in the proposed method could help differentiate and classify multiple emergences or similar hole sizes.

Second, the hole dimensions can vary significantly between sexes of the same species. Although only asexual generations were found and analyzed in the galls of Cynipini, there are differences in the size of the holes between parasitoids of the genus *Torymus* and *Ormyrus*. These species show differences in size between sexes due to sexual dimorphism, which is common in parasitoid wasps (Hurlbutt 1987). Future studies should consider this aspect, especially those that address sexual generations of Cynipini wasps since males can be of similar or smaller size than females (e.g., Melika 2006; Hood et al. 2018; Egan et al. 2012), which makes it necessary to have adequate taxonomic knowledge of gall wasps and associated wasps.

However, one challenge arises from the incomplete taxonomy of the gall wasps (Pujade-Villar et al. 2009; Martínez-Romero et al. 2022) and associated wasp species (Noyes 2003; Cibrián-Tovar 2017), particularly in Mexico. Nevertheless, when viewed the gall as a microecosystem with diverse communities and multi-trophic interactions (Price et al. 2005; Hayward and Stone 2005;

Pujade-Villar et al. 2009; Lobato-Vila et al. 2022), the hole size could function as a reference for studies that evaluate different levels of organization, from population to ecosystem, using only taxonomic identification at higher levels such as genus or family.

For instance, Stone et al. (2002) suggest that larger holes are associated with larger sizes of Cynipini wasps, indicating a possible connection with greater resource availability. By analyzing the difference in hole sizes between species at a population level, we estimated each gall wasp's emergence rate and abundance in a specific area and compared it among Cynipini species. Furthermore, understanding the variation in emergence values among species, some with rates less than 30% and others exceeding 50%, is similar to findings reported by Fernández and Price (1992) and Almeida et al. (2006). This result is significant as the ability to estimate the emergence rate of a particular gall wasp can be crucial for supporting the management and control of species that have become pests (Bewick et al. 1988).

The hole size allowed us to determine the number of galls without adult emergence (empty galls), which ranged between 17% and 64%, a percentage like that reported by Cooper and Rieske (2010). However, to gain a more comprehensive understanding of the interactions present in galls further information is required through studies of associated communities. Notably, the study found no differences in the number of associated wasps and the percentage of negative interaction among the galls analyzed, and this could be due to complex and multiple interactions between the associated wasps. This approach could enhance studies of multitrophic interactions and investigate possible variations between the impacts of apparent competition and interspecific competition, as suggested by Hayward and Stone (2005), Bailey et al. (2009), and Holt and Bosall (2017).

Although this study provides information on only 8 gall morphs, it opens the potential for groundbreaking research on over 200 types found in the temperate forests of Aguascalientes and Oaxaca. These results could lead to significant advancements in our understanding of the impact of annual variation, sites, and climate change on the abundance and distribution of gall wasps and associated wasp species that support conservation efforts.

## CONCLUSIONS

After analyzing and validating the proposed method, we emphasize the potential effectiveness of using the size of gall emergence holes to estimate the abundance of gall wasps (Cynipini) and their associated species. This result significantly expands our understanding of the interactions and dynamics

between inducer wasps and associated species, laying the foundation for future research into non-invasive assessment methods within these ecological communities.

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**Competing interests:** The authors declare that they have no competing interests. Availability of data and materials: All specimens are deposited at the Universidad de Barcelona, Catalonia (J. Pujade-Villar) and Universidad de la Sierra Juárez, Mexico (R. Clark-Tapia).

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**Supplementary materials**

**Table S1.** Statistical differences obtained with the Kruskal-Wallis test (Hc) for diameter and mesosoma among inducer and associated species in each gall, and significance values from Mann-Whitney comparisons (p Un: unadjusted p, p Ad: p adjusted with Holm's method).