


# Compensatory mechanisms during development contribute to overcome intraspecific competition in a dipteran solitary ectoparasitoid with larval active host seeking behaviour

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**Abstract.** 1. Superparasitism has been rendered many times as detrimental for fitness, although there are examples in which the competing winning larvae compensate for its development. Here, we studied if, in the host-parasitoid system *Cyclocephala signaticollis* (Coleoptera: Scarabaeidae) – *Mallophora ruficauda* (Diptera: Asilidae), larvae have compensatory mechanisms during development to overcome competition.

2. Through a combination of behavioural and artificial parasitism protocols, we investigated if host instar or time between parasitism events influences the probability of a larva winning the competition. In addition, we studied the effect that superparasitism has in terms of some fitness traits like sex ratio, size, and shape.

3. Our results showed that *M. ruficauda* has compensatory mechanisms like differential larval survival according to host availability. In addition, we found that as the time between parasitism events increased, the second larva lowers its chance to win the competition in favour of the first arriving larva. Larvae under competition develop at a similar rate compared to larvae that had not experienced competition for hosts. Finally, our results show that wing shape is different between groups, and this could influence flight and courtship.

4. Our study suggests that superparasitism in *M. ruficauda* is an advantageous strategy under some circumstances although it might influence adult performance with flight related behaviours.

**Key words.** Compensatory mechanisms, competition definition, diptera, ectoparasitoid, geometric morphometry, superparasitism.

## Introduction

Competition is an interaction between individuals when a shared requirement for a resource leads to a reduction in the survival, growth, and/or reproduction of at least some of the competing individuals concerned (Begon *et al.*, 2006). When speaking of parasitoids, two main categories of competition

are defined: extrinsic (when free-living adults searching for hosts are involved) and intrinsic (when immature parasitoids developing on or inside the host compete) (Harvey *et al.*, 2013).

The intrinsic competition is known as superparasitism if parasitism is performed by parasitoids of the same species or multi-parasitism when a host is parasitised by parasitoids of different species (Godfray, 1994; Moraes & Mescher, 2005; Cusumano *et al.*, 2012; Harvey *et al.*, 2013; Poelman *et al.*, 2014). Superparasitism has been extensively studied and the influence of several factors on fitness related traits like the number of emerging parasitoids, the developmental time, or even the size of the progeny are well established for parasitoids where females search for hosts (van Alphen & Visser, 1990; Potting

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*et al.*, 1997). However, for parasitoids with free host seeking larvae, it has long been thought that superparasitism might not be avoided due to the lack of host discrimination mechanisms (Feener Jr & Brown, 1997).

There are studies showing different strategies used by females when faced with the decision of accepting hosts already parasitised for laying eggs (Wajnberg *et al.*, 2008). Theoretical models show that patch residence time can increase or decrease in response to ovipositions, that is incremental or decremental rules (Wajnberg *et al.*, 2008). Other studies show that clutch size can be modified given the amount of competitors in the foraging area and even if encountered hosts should be used for host-feeding or oviposition as a response to the host quality (van Alphen & Visser, 1990; Visser *et al.*, 1990; Visser *et al.*, 1992; Heimpel & Collier, 1996; van Alphen *et al.*, 2003; Wajnberg *et al.*, 2008; Yang *et al.*, 2012). In turn, studies dealing with the effects of superparasitism on the offspring are usually expressed in terms of survival, developmental time, final size reached, or some other characteristic related to the adult performance of the developing larvae (Bai & Mackauer, 1992; Tunca *et al.*, 2016; Devescovi *et al.*, 2017).

The outcome of competition is influenced by several factors such as the developmental stage of the host, the developmental rate of the parasitoid, the number of eggs on a host, the order in which ovipositions occur, or even by the time interval between parasitism events (van Strien-van Liempt, 1982; Tillman & Powell, 1992; Moraes *et al.*, 1999). Superparasitism can cause smaller body sizes, longer developmental times, and changes in adult sex ratio (Godfray, 1994). Although the negative effects have been many times reported, there are recent examples where superparasitism does not seem to have negative effects on parasitoids (Chen *et al.*, 2020). The fact that superparasitism does not have important fitness related costs could be explained by compensation responses involved during larval development (Metcalf & Monaghan, 2001; Dorn & Beckage, 2007; Nestel *et al.*, 2016). Physiological and metabolic responses refer to compensation responses as diapause, nutrient regulation, or reabsorption of internal tissues (Nestel *et al.*, 2016). On the other hand, behavioural compensation responses include ovicide, increases in feeding through higher loads of ingestion or faster feeding (Mayhew, 1997; Nestel *et al.*, 2016). Other responses include faster development of the most impaired larvae to compensate with the competitor that had more time to develop (Nestel *et al.*, 2016). All these responses evolved as compensating factors for nutritional deficiencies that can have a profound impact on the next stages of the individual. There are cases of differences in body size, dispersal capabilities, male competitiveness for females, male performance, and between other mechanisms (Nestel *et al.*, 2016).

Given that parasitoids acquire all resources for development from a single host, a direct relationship exists between host quality and parasitoid fitness (Lewis *et al.*, 2002). Host quality may be modified by competing individuals giving place to different strategies and mechanisms that define the outcome of competition (Harvey *et al.*, 2013). For instance, endoparasitoids possess several adaptations for development in the host hemocoel as a thin cuticle suited for respiration by passive diffusion and uptake of nutrients (Lewis *et al.*, 2002). But also, they have developed

structures like heavily sclerotised heads, enlarged mandibles, or caudal structures that can be used in combat against competing for parasitoid larvae (Lewis *et al.*, 2002; Harvey *et al.*, 2013). On the contrary, ectoparasitoids usually lack specialised adaptations for attaching to hosts or fighting with other larvae (Lewis *et al.*, 2002). These feeding habits of parasitoid larvae are usually associated with whether the parasitoid attaches externally or inside its host. While ectoparasitoids are almost exclusively idiobionts, that is they kill or paralyse their hosts at the time of parasitism, endoparasitoids are primarily koinobionts where the host continues feeding, growing, and can defend themselves after parasitism has occurred (Harvey *et al.*, 2013). This distinction is important since it is often argued that resources obtained by idiobionts are static and their quality is positively correlated with host size. On the other hand, koinobionts attack resources that are highly dynamic and their quality is harder to define (Lewis *et al.*, 2002; Harvey *et al.*, 2013).

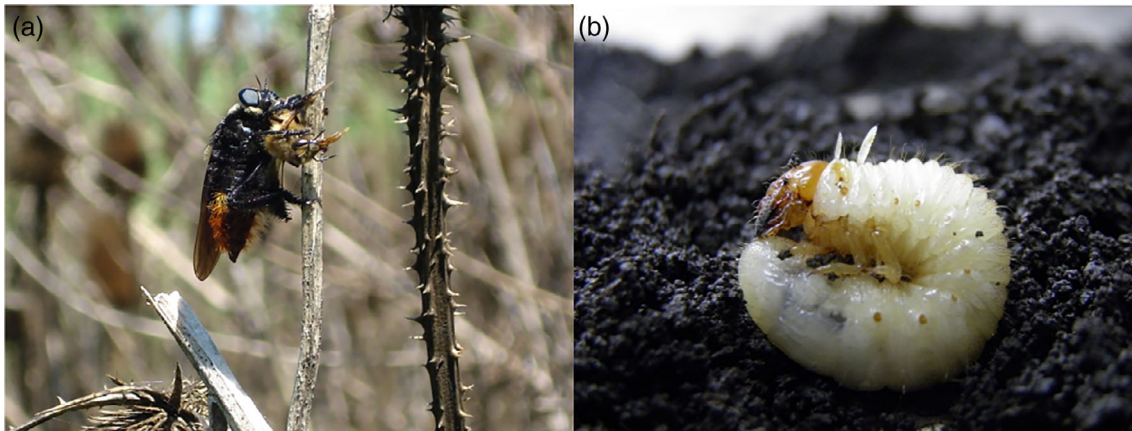
The vast majority of parasitoids belong to the Hymenoptera although dipteran parasitoids are important in many communities (Godfray, 1994). Most dipteran parasitoids are endoparasitoids, except for bombyliids and some species of asilids, which are mostly ectoparasitoids (Feener Jr & Brown, 1997; Smith *et al.*, 2007; Burington *et al.*, 2020; Ferguson *et al.*, 2020). Although dipteran parasitoids do not paralyse or arrest a host's development, there is much variation in the amount of host development after oviposition (Feener Jr & Brown, 1997). In general, larvae of dipteran parasitoids counter the host's immune response by maintaining contact with the outside air through a hole in the integument or by remaining in specific protected tissues like nerve ganglia, muscles, or glands (Feener Jr & Brown, 1997). This is true for dipteran endoparasitoids, but there is almost no information on how dipteran ectoparasitoids resolve competition, and which are the compensating responses they have.

In this work, we addressed, which factors influence intrinsic competition in the solitary koinobiont dipteran ectoparasitoid *Mallophora ruficauda* Wiedemann (Diptera: Asilidae). In particular, we tested whether the first larva that parasitises the host has more probability of outcompeting its competitor and if it depends on host instar or the time between parasitism events. In addition, we studied if this species has developmental compensatory responses to reduce the negative effect of superparasitism, and we analysed the cost of superparasitism on fitness related traits on pupae or adult parasitoids like body weight, form, or sex ratio.

## Materials and methods

### *Biology of Mallophora ruficauda*

In this work, we used the species *M. ruficauda* Wiedemann (Diptera: Asilidae), which is a common robber fly found in the open grasslands of the Pampas region of South America (Corley & Rabinovich, 1997). Adults are known to prey on many flying insects but most frequently feed on *Apis mellifera* L. (Hymenoptera: Apidae) (Fig. 1). *Mallophora ruficauda* has an annual life cycle with oviposition occurring from December to March. Oviposition takes place as clusters of several eggs on



**Fig. 1.** (a) Adult female of *Mallophora ruficauda* feeding on *Apis mellifera*. (b) Third instar larvae of *Cyclocephala signaticollis* superparasitised by larvae of *M. ruficauda*.

high substrates like fences, standing dry dead plants but also on living plants. After birth, larvae are wind dispersed and bury themselves in the soil, resulting in the coexistence of larvae from many different egg-clusters (Castelo & Corley, 2004; Castelo *et al.*, 2006). This means of dispersal is proven to maximise single parasitism for such small, very tiny larvae ( $1.35 \pm 0.11$  mm of length and  $0.32 \pm 0.27$  mm wide, Castelo *et al.*, 2006).

Larvae of *M. ruficauda* are solitary koinobiont ectoparasitoids of masked chafer larvae, *Cyclocephala signaticollis* Burmeister (Coleoptera: Scarabaeidae) (Fig. 1, Castelo & Corley, 2010). Hosts, however, are not available until mid-February. Hence, early born larvae must await for the host to become available (Crespo & Castelo, 2010). After burying in the soil, parasitoid larvae moult to the second instar in absence of the host 7 days since birth on average (Crespo & Castelo, 2010). This step is of extreme importance since only second instar larvae are capable of orienting to the host (Crespo & Castelo, 2008). Once hosts become available, larvae begin an active search following chemical cues arising from the host hindgut (Crespo & Castelo, 2008; Groba & Castelo, 2012). After parasitism occurs, *M. ruficauda* larvae remain externally attached to their host as the second instar during the cold seasons (autumn and winter). At the beginning of spring, host metabolism speeds up and it is then that the parasitoid speeds up its development and kills the host very fast (Copello, 1922; Crespo & Castelo, 2010). Although *M. ruficauda* is a solitary species, superparasitism of two or three larvae per host is frequent in the field (Crespo & Castelo, 2009).

Previous studies have shown that *M. ruficauda* larvae have the ability to discriminate different characteristics of the host like species, instar, between parasitised, and unparasitised hosts and it has been determined that larvae are repelled from stressed hosts (Crespo & Castelo, 2009; Crespo *et al.*, 2015). Although the third instar is the preferred instar, the presence of conspecific larvae (competitors) in the ground modulates this selectivity inducing orientation to second instar and parasitised hosts in a dense-dependent way (Castelo & Capurro, 2000; Castelo & Corley, 2010; Crespo *et al.*, 2015; Martínez *et al.*, 2017).

#### Collection of insects

Larvae of *M. ruficauda* were obtained from egg-clusters collected on high plants in grasslands in Moreno ( $34^{\circ}46'S$ ,  $58^{\circ}93'W$ ) and Mercedes ( $34^{\circ}37'S$ ,  $59^{\circ}27'W$ ), localities associated with apiaries in Buenos Aires province, Argentina, between the months of January and March from 2010 to 2019. The egg-clusters were stored in Falcon type tubes and were observed daily to register hatching under laboratory conditions ( $25 \pm 1^{\circ}C$ ,  $50 \pm 3\%$  RH, and LD 12:12 h photoperiod). When the eggs hatched, the larvae were stored in groups in glass jars with commercial gardening soil as substrate (Humidity: 35–40%; Organic Matter: 15–20%, pH: 7.0; Carbon/Nitrogen: 7.6; and conductivity:  $1.3 \mu S cm^{-1}$ ). For this, larvae were dropped from the top of the glass and allowed to bury themselves. All larvae bury in few minutes or otherwise they could die by desiccation. Each glass contained 50 larvae at a density of 1 larva  $ml^{-1}$  of soil. Only one larva per egg-cluster was used in a glass. In addition, larvae that were born on the same day were used thus assuring that no relatedness between two larvae exists. Glass jars were stored in complete darkness at  $25 \pm 1^{\circ}C$  until used in the experiments. Except for studying preparasitism survival, larvae used were all born late in the season. *Cyclocephala signaticollis* hosts were also collected underground in the same grasslands as the parasitoid larvae in Moreno, and Mercedes, localities of *M. ruficauda* from Buenos Aires province, between the months of March and August from 2010 to 2019. Hosts were maintained individually at  $25 \pm 1^{\circ}C$  in black tubes filled with commercial gardening soil as substrate and were fed weekly with fresh pieces of carrots until used in the experiments.

#### Factors influencing intraspecific competition

*Effect of host instar.* To study if host instar influences the probability of the parasitoid larva to win the competition, we artificially superparasitised hosts of different instars with second instar *M. ruficauda* larvae. The artificial superparasitism procedure consists of offering either a second or a third instar host to a second instar parasitoid larva (first larva). After

confirmation of positive parasitism, we placed another second instar parasitoid larva (second larva) on the same host and checked after 48 h for superparasitism to occur. In case that during this procedure a larvae did not attach to a host, the host was removed from the experiment. Different treatments differed in the host instar at the moment of parasitism of the first and second larva. Briefly, we designed three treatments where: (a) first and second parasitoid larvae were offered a second instar host ( $N = 17$ ); (b) first parasitoid larva was offered a second instar host, and, after host moulting, the second larva was exposed to it as third instar host ( $N = 44$ ); and (c) first and second parasitoid larvae parasitised a third instar host ( $N = 24$ ). The host with both parasitoid larvae was placed in a 30 ml tube filled with commercial gardening soil and fed twice a week with fresh pieces of carrot until the definition of competition under controlled laboratory conditions ( $25 \pm 1$  °C,  $50 \pm 3\%$  RH and LD 12:12 photoperiod).

In order to identify, in the previous experiment, which larva had attached first to the host and which 1 s, we developed a non-invasive marking code. This method consisted of cutting a tiny part of a particular caudal seta of the parasitoid larva. In this species, larvae have four pairs of locomotory setae in the terminal segment of the abdomen. So, by cutting either the outer left or right one, we can clearly identify both larvae. Prior to using this method, we evaluated the survival of larvae after cutting a seta compared to non-treated larvae. We cut the left or right caudal setae of 50 s instar 20 days-old larvae (25 larvae per side), and another group of 50 larvae remained intact as a control. To cut the seta, the larva was placed on a microscope slide over a cooling gel pack that lowered the corporal temperature of the larva, thus immobilising it. After the seta was cut with a scalpel, larvae were placed individually in an Eppendorf type tube containing a piece of filter paper moistened with mineral water as a substrate. Larvae for the control group were only exposed to the cooling gel pack and then placed back in the tubes. Tubes were stored in darkness at  $25 \pm 1$  °C and  $50 \pm 3\%$  RH. After 1 month, the number of surviving larvae in each group was registered.

*Effect of time between parasitism events.* The rationale behind this experiment was to study if a larva that arrived earlier to the host could outcompete a second larva. To achieve this, two parasitoid larvae (with different time between parasitism events) marked as mentioned previously with a caudal seta cut, were placed on the thorax of a healthy third instar host aided with a paintbrush. The time between parasitism events was 2 ( $N = 20$ ), 7 ( $N = 63$ ), or 30 days ( $N = 21$ ). Three days after each exposure of the parasitoid larva to its host, we checked whether the larva had attached to the host integument. In case no parasitism had occurred that host was removed from the study. During the experiment, hosts were evaluated weekly and their status (healthy H, parasitised P, or superparasitised SP) registered. At the beginning of the experiment, every host was SP. If both parasitoid larvae died during the experiment, the host changed its status from SP to H and that observation was taken out from the analysis. If the host changed its status from SP to P, the experiment ended and the remaining larva was declared

the winner. When a larva was found dead attached to the integument or was not found during three consecutive weeks, it was considered the loser. Finally, the host's developmental state (third larval stage or prepupa) at the time competition was resolved was registered. Hosts with parasitoid larvae were placed in a 30 ml tube filled with commercial gardening soil and fed twice a week with fresh pieces of carrot in darkness and under controlled laboratory conditions ( $25 \pm 1$  °C,  $50 \pm 3\%$  RH). It should be noted that since not every host survived until the end of the experiment due to attacks of fungus or other entomopathogens, number of replicates varied between treatments.

#### *Preparasitism survival as compensatory response*

In this section, we analysed if larvae have any compensatory responses. So, we first tested if early born larvae have any increased survival that allows them to better await the appearance of the host. For this, we used 500 larvae born early in January and 500 larvae born in February collected from egg-clusters in the field and placed them without hosts on individual vials with a piece of moistened filter paper in darkness and under controlled laboratory conditions ( $25 \pm 1$  °C,  $50 \pm 3\%$  RH). Survival of free-living larvae was registered daily until death.

In a different experiment, we registered the developmental time of individuals that experienced superparasitism and compared it to the developmental time of larvae developed on single parasitised hosts. For this, we artificially parasitised and superparasitised 36 and 28 third instar hosts, respectively, with second instar parasitoid larvae and followed them until pupation registering the number of days from parasitism until pupation. Hosts with parasitoid larvae were placed in 30 ml tube filled with commercial gardening soil and fed twice a week with fresh pieces of carrot under controlled laboratory conditions ( $25 \pm 1$  °C,  $50 \pm 3\%$  RH).

#### *Costs of superparasitism*

*Effect of superparasitism on fitness-related traits.* In this section, we analysed the cost of superparasitism on some fitness-related traits. To this goal, we raised either superparasitised hosts ( $N = 48$ ) or hosts with only one parasitoid larva ( $N = 327$ ). The host with parasitoid larvae was kept in 30 ml tube filled with commercial gardening soil and fed twice a week with fresh pieces of carrot in darkness and under controlled laboratory conditions ( $25 \pm 1$  °C,  $50 \pm 3\%$  RH). After reaching pupation, pupae were weighed and its sex registered. We then analysed if the sex ratio and weight of pupae differed between superparasitised or single parasitised hosts. Both traits, sex ratio, and size are useful fitness related traits since they can have and influence on the amount of females in the population (sex ratio) and the size of those individuals (Roitberg *et al.*, 2001; Boivin, 2010). Bigger individuals may outperform others through more egg production or higher survival (Bezemer *et al.*, 2005).

*Effect of superparasitism on adult parasitoid wing size and shape.* We analysed the effect of superparasitism on adult

parasitoid form through wing size and shape by landmark-based geometric morphometrics (Theska *et al.*, 2020). To this, we captured wing images with an Olympus SP-800UZ digital camera attached to an Olympus SZ51 binocular microscope. We took images of 176 individuals raised under no competition and 76 from individuals that suffered superparasitism of two larvae per host (individuals came from Sections 2.3.2–2.5.1). After obtaining the images, the General Procrustes Analysis was used to further analyse them (Gower, 1975).

All experiments conformed to the legal requirements of Argentina and to accepted international ethical standards, including those relating to conservation and animal welfare.

### Statistical analysis

*Effect of larva marking method on survival.* In order to validate our marking code, we compared through a two proportions test if there were differences between the proportion of larvae that survived after a month since the procedure was done in marked and non-marked larvae.

*Effect of host instar.* To model the probability of winning the competition as a function of the host instar, a Binomial GLMM with a logit link function was used. The logit link function ensures fitted values between 0 and 1, and the Binomial distribution is typically used for probability data. Fixed covariates were host instar when the first larva parasitised (categorical with two levels, second and third instar) and host instar when the second larva parasitised (categorical with two levels, second and third instar). To incorporate the dependency among observations of the same year, we used year as random intercept. Every time the first larva won the competition was counted as a success (noted with a 1) and whenever the second larva won the competition a failure was registered (noted as 0).

*Effect of time between parasitism events.* To model the probability of winning the competition as a function of the time between parasitism events, a Binomial GLMM with a logit link function was used. Fixed covariables were time between parasitism events (categorical with three levels, 2, 7, or 30 days intervals) and if the host was in prepupa stage when competition resolved (categorical with two levels, host in prepupa stage or not). To incorporate the dependency among observations of the same year, we used year as random intercept. Every time the second larva won, the competition was counted as a success (noted with a 1) and whenever the first larva won, the competition a failure was registered (noted as 0).

To select the final model, we performed a model selection procedure in which we evaluated the significance of the covariables with the function `DROP1`, eliminating them if the difference in Akaike Information Criterion after refitting a model without including the variable was less than 2 (Sakamoto *et al.*, 1986). In that case, the model was refit without including that covariable and further elimination of covariables were evaluated. This procedure continued until only covariables that were important to keep in the model were found or no other covariables could be excluded.

*Preparasitism survival as compensatory response.* To compare survival of first and second instar larvae born, we performed a Cox proportional-hazard model, including moment of birth (early in the season or not), instar [first (I) or second (II)], and the interaction between them as fixed explanatory variables. The Cox proportional-hazard model allowed us to obtain the hazard ratio between groups. In the case of first instar, larvae could either moult to the second instar or die. In turn, the only possible outcome for second instar larvae is death since no moulting to the third instar could occur without parasiting a host. Regarding the difference in developmental time, a *t*-test was performed between time until pupation between single parasitised hosts and superparasitised hosts (R Core Team, 2020).

### Costs of superparasitism.

*Effect of superparasitism on fitness related traits.* To model the probability that a pupa was female or male as a function of the number of larvae in the host, a Binomial GLMM with a logit link function was used. Fixed covariates were number of larvae in the host (categorical with two levels, hosts with only one parasitoid larva or hosts with two larvae). Success (being a female) was noted with a 1 and 0 if the pupa was a male. Finally, in order to model the weight of pupa as a function of the number of larvae during development, a GLM with Gaussian link function was used. Whenever the assumptions were not met, variance modelling was performed with a `VARIDENT` structure. In both models, we incorporated the dependency among observations of the same year, using year as random intercept.

### Effect of superparasitism on adult parasitoid wing size and shape.

After obtaining the images, we placed 27 landmarks on each right wing using the `STEREOMORPH R` package (Fig. 3a; Olsen & Westneat, 2015). We used centroid size as a proxy for wing size, calculated as the square root of the sum of the squared distances between each landmark and the centroid of each wing. After obtaining the landmarks for each wing, data were modified according to the General Procrustes Analysis. This analysis extracts shape information from landmark configurations and removes variation not related to shape like differences in rotation, orientation, or size. The Procrustes coordinates are projected into a linear tangent space through a principal components analysis (PCA). In order to compare differences in wing size between single parasitised and superparasitised groups, we performed a linear model of the centroid size. For comparing wing shape between groups, we performed multivariate statistical analyses. We did a Procrustes `MANOVA` to identify biologically relevant differences in mean shape since this analysis has no assumption of multivariate normality. Finally, in order to test if differences between groups were because of allometry, we did a PCA on predicted values obtained from a multivariate regression of shape on size. Then, the first PC of this PCA is plotted against the logarithm of the centroid size of each individual and compared the trend lines.

All the analyses were done using the `R v3.6.3` 'Holding the Windsock' software (R Core Team, 2020). The

**Table 1.** Summary of the results of the model for the effect of host instar '*Cyclocephala signaticollis*' on the probability of *Mallophora ruficauda* larva to win the competition in superparasitism experiments.

Host instar when first larva attaches/second larva attaches	Probability	Standard error	d.f.	Lower confidence interval	Upper confidence interval
2nd/2nd	0.647	0.1159	81	0.400	0.834
2nd/3rd	0.455	0.0751	81	0.313	0.604
3rd/3rd	0.542	0.1017	81	0.343	0.728

Contrast	Odds ratio	Standard error	d.f.	Lower confidence interval	Upper confidence interval
2nd–2nd/2nd–3rd	2.200	1.300	81	0.537	9.02
2nd–2nd/3rd–3rd	1.551	1.012	81	0.327	7.36
2nd–3rd/3rd–3rd	0.705	0.359	81	0.209	2.38

The first part shows the predicted probabilities for the first larva in the different treatments tested. The inferior part shows the odds ratio between the groups. When performing contrasts between groups, it should be noted that if the confidence interval includes 1, there is no significant difference between them.

package GLMMTMB and NLME were used to fit the models (Brooks *et al.*, 2017; Pinheiro *et al.*, 2021). For testing model assumptions, we used the package DHARMA (Hartig & Lohse, 2021). Graphs were done using the package GGLOT2 (Wickham, 2016). Survival analysis and the Cox-Hazard proportional-hazard model were done with functions from the package survival (Therneau *et al.*, 2021). Geometric morphometric analyses were performed using several functions from the packages MORPHO, GEOMORPH v.3.3.2 (Schlager, 2017; Adams *et al.*, 2021).

## Results

### Validation of marking code of parasitoid larvae

First of all, we found that our marking code for identification of individual larvae did not affect the survival of larvae after a month. We observed that 90% of intact larvae and 92% of larvae with setae cut were alive, rendering no statistical difference between both groups ( $Z = 0.349$ ,  $P = 0.726$ ). Hence, this technique could be successfully implemented in the experiments that required the identification of individuals assuming insignificant cost on survival.

### Factors influencing intraspecific competition

*Effect of host instar.* We found that the host instar had no influence on the probability of winning the competition (Appendix S1). If both larvae attached to a second instar host, the first larva had a probability of winning the competition of 0.647 (Table 1). This probability was lower (0.455) but not significantly different if the second larva attached to the host after it moulted to the third instar (Table 1). Finally, the probability for the first larva to win was 0.542 when both were larvae attached to a third instar host (Table 1). When comparing groups, no difference was found between them (Table 1).

*Effect of time between parasitism events.* Our results show that the time interval between parasitism events has an influence

**Table 2.** Summary of the results of the model selection procedure to study the effect of time between parasitism events in the system *Cyclocephala signaticollis* – *Mallophora ruficauda*.

Model	AIC	LRT	<i>P</i> value
Result~treatment * prepupa + (1 year)	132.00		
Result~treatment + prepupa + (1 year)	131.05	3.052	0.2174
Result~treatment + (1 year)	129.07	0.025	0.8738
Result~ prepupa + (1 year)	136.35	9.303	<0.001

Result denotes if first or second larva attaching to the host wins the competition. Treatment includes time between parasitism events (2, 7, or 30 days). Prepupa indicates if the host was in prepupa stage when parasitoid competition was resolved. AIC shows the difference in the Akaike Information Criterion used to drop or keep a model. LRT is the likelihood ratio test that is performed by the DROP1 function used to perform model selection. Finally, the *P* value indicates if the value of the LRT should be considered significant.

on the probability of the second larva to win the competition. After the model selection procedure, we ended with a model that included time interval between parasitism events as the only important variable (Table 2 and Appendix S1). In particular, we found that the predicted probability of the second larva to win the competition is 0.920 with a 2-day interval between parasitism events (Table 3). When the time between parasitism events was 7 days, the probability of the second larva winning decreased to 0.718 although not statistically different from the 2-day treatment. Finally, when the time between parasitism events was 30 days, a lower probability (0.361) was found (Table 3). We also found that the probability for the second larva to win the competition is significantly less in the 30-day group compared to the 2-day group, although no difference was found for the 7-day group (Table 3).

### Compensatory responses

*Preparasitism survival.* We found that early born free living larvae have higher survival than larvae born later in the

**Table 3.** Summary of the results of the model for the effect of time between parasitism events of the host '*Cyclocephala signaticollis*' on the probability of *Mallophora ruficauda* larva to win the competition in superparasitism experiments.

Treatment (days)	Probability	Standard error	d.f.	Lower confidence interval	Upper confidence interval
2	0.920	0.097	100	0.462	0.993
7	0.718	0.183	100	0.298	0.939
30	0.361	0.231	100	0.072	0.804

Contrast	Odds ratio	Standard error	d.f.	Lower confidence interval	Upper confidence interval
2/7	4.49	5.55	100	0.238	84.8
2/30	20.28	25.59	100	1.008	408.1
7/30	4.51	4.91	100	0.338	60.2

The first part shows the predicted probabilities for the second larva in the different treatments tested. The inferior part shows the odds ratio between the groups. When performing contrasts between groups, it should be noted that if the confidence interval includes 1, there is no significant difference between them.

**Table 4.** Summary of the results of the Cox proportional-hazard model to test the preparasitism survival in larvae of *M. ruficauda* born at different moments of the season.

Moment-instar	Probability	Standard error	d.f.	Lower confidence interval	Upper confidence interval
Early – I	0.822	0.0608	Inf	0.711	0.950
Middle – I	1.000	0.0000	Inf	1.000	1.000
Early – II	0.256	0.0203	Inf	0.219	0.299
Middle – II	0.405	0.0319	Inf	0.347	0.473

Contrast	Odds ratio	Standard error	d.f.	Lower confidence interval	Upper confidence interval
Instar = I Middle/early	1.22	0.090	Inf	1.05	1.41
Instar = II Middle/early	1.58	0.123	Inf	1.36	1.84

The first part shows the predicted probabilities for the different groups. The inferior part shows the odds ratio between the groups. When performing contrasts between groups, it should be noted that if the confidence interval includes 1, there is no significant difference between them. Degrees of freedom are infinite because they are asymptotic results and indicates that estimates are tested against the standard normal distribution –  $z$  tests.

oviposition season. Regarding the moment of the season larvae are born, we found that first instar larvae born in the middle-end of the season have 0.22 times more chance to die than first instar larvae born early in the season (Table 4). This difference is even higher for free living second instar larvae. Larvae that were born during the middle-end of the season had 0.58 times greater chances of dying than early born larvae (Table 4).

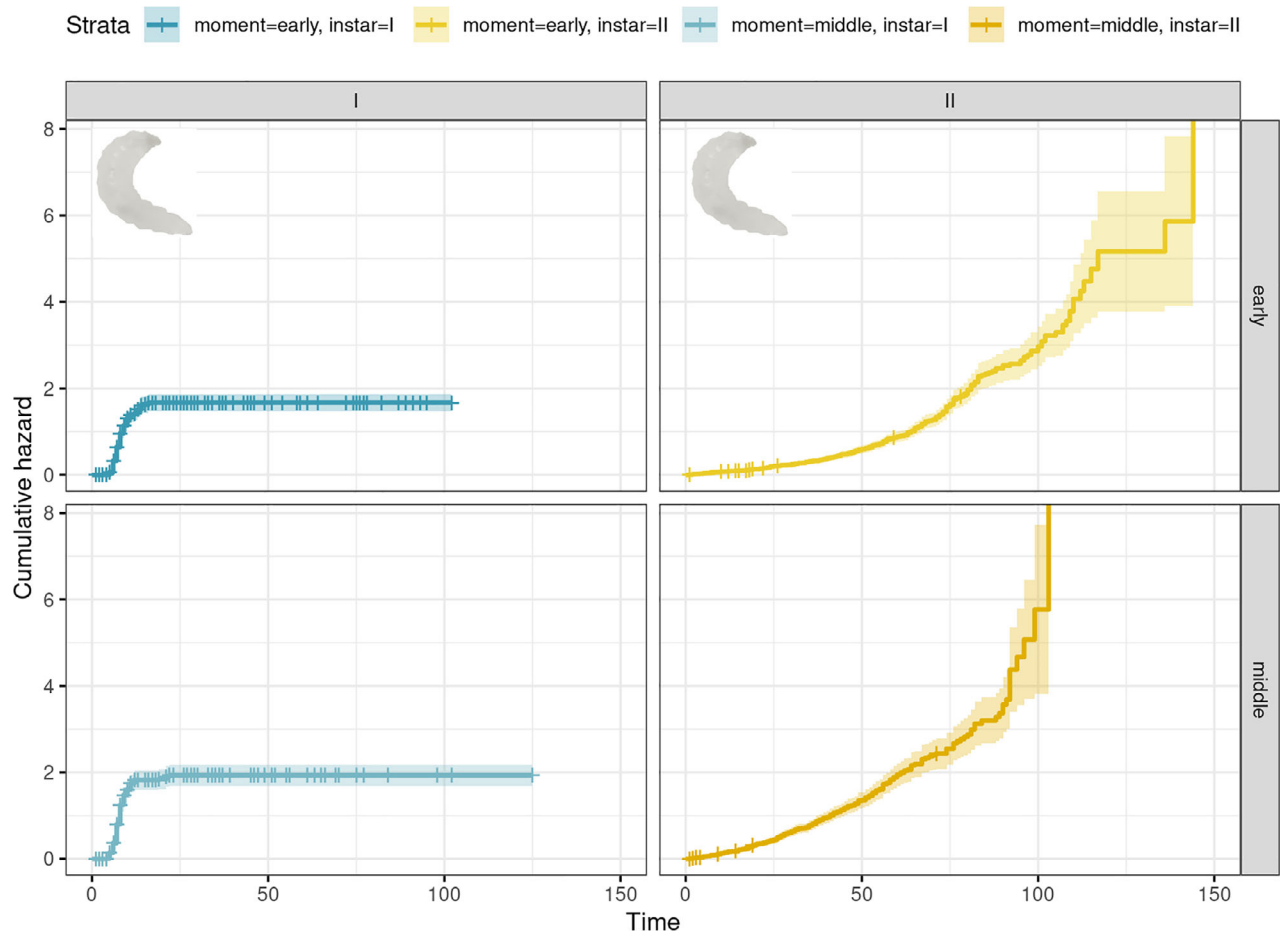
The cumulative hazard in time for each group is shown in Fig. 2. For first instar larvae, the hazard accumulates fast during the first days from birth due to moulting to the second instar. In the right panel, the hazard for second instar larvae shows a clear steeper increase for middle born than early born larvae.

Finally, regarding developmental time, no difference was found between larvae developed from single and superparasitised hosts (mean  $\pm$  SE: single parasitised = 73.78 days  $\pm$  7.04; superparasitised = 87.39 days  $\pm$  8.59;  $t = -1.225$ , d.f. = 55.98,  $P = 0.226$ ).

### Cost of superparasitism

*Effect of superparasitism on fitness related traits.* Regarding the sex ratio of the pupa, we found that the probability of obtaining females was similar under single or superparasitism (0.514 vs. 0.457 and Table 5 for model results). A similar result was found in the case of the pupa's weight. When we analysed the effect of superparasitism on the weight achieved by the pupa, we found no significant effect of the host parasitism status ( $F_{1,365} = 1.234$ ,  $P = 0.2674$ ). Furthermore, the mean weight was of 240 versus 259 mg between single and superparasitised groups.

*Effect of superparasitism on adult parasitoid wing size and shape.* Regarding wing size, we found no significant difference between both groups ( $F_{1,250} = 2.57$ ,  $P = 0.1105$ ). As for the wing shape analysis, differences between adult parasitoids raised from single parasitised and superparasitised groups were found. The PCA analyses showed that the first 16 principal



**Fig. 2.** Cumulative survival hazard along days for the first and second instar larvae at different moments of reproductive season. First instar larvae could either moult or die, while second instar larvae can only die as second instar.

**Table 5.** Summary of the results of the model for the sex ratio in superparasitism by *Mallophora ruficauda*.

Treatment	Probability	Standard error	d.f.	Lower confidence interval	Upper confidence interval
Single parasitism	0.514	0.0277	368	0.459	0.568
Superparasitism	0.457	0.0734	368	0.319	0.601
Contrast	Odds ratio	Standard error	d.f.	Lower confidence interval	Upper confidence interval
1/2	1.26	0.396	368	0.676	2.34

The first part shows the predicted probabilities for the winning larva of being a female. The inferior part shows the odds ratio between the groups. When performing contrasts between groups, it should be noted that if the confidence interval includes 1, there is no significant difference between them.

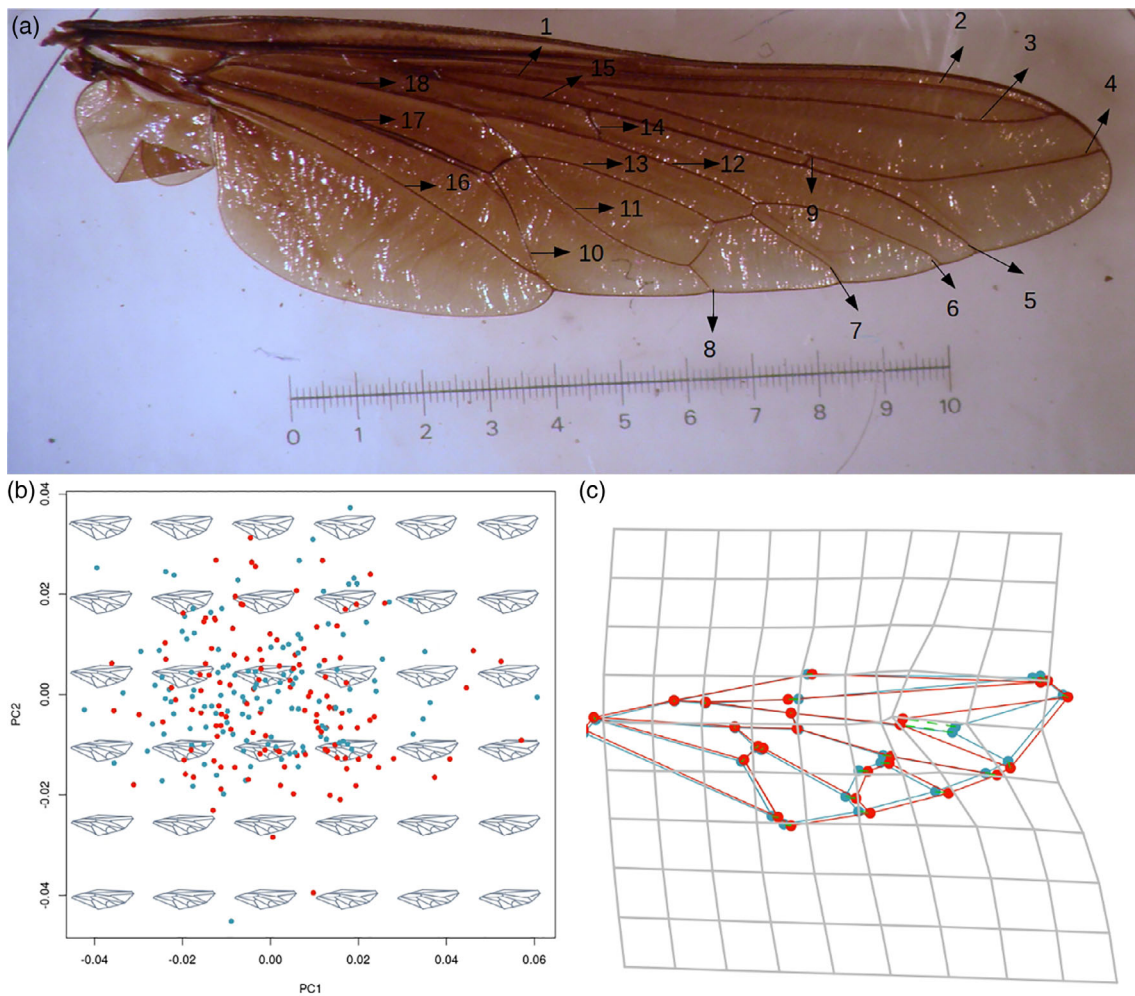
components (PC) were the most informative accounting for ~92.61% of the total variation. The PCA plot of the first two PCs shows that the distribution of specimens in the tangent space is somewhat superimposed (Fig. 3a).

The procrustes analysis of variance showed a significant effect of the parasitism condition (single or superparasitised) on adult parasitoid wing shape ( $F_{1,250} = 3.154$ ,  $P < 0.003$ ). Detailed analysis of wing shape variation showed that differences between

groups are mainly due to changes in the position of a crossvein between R4 and R5 (Fig. 3b). In particular, individuals raised under superparasitism show a proximal relative displacement of this crossvein compared to individuals raised alone in the host (Fig. 3b). Finally, the wing margin of the superparasitised group shows an expansion seemingly rendering a wider wing (Fig. 3b).

The analysis of allometry of wing shape variation showed that there are significant shape changes as size increases although





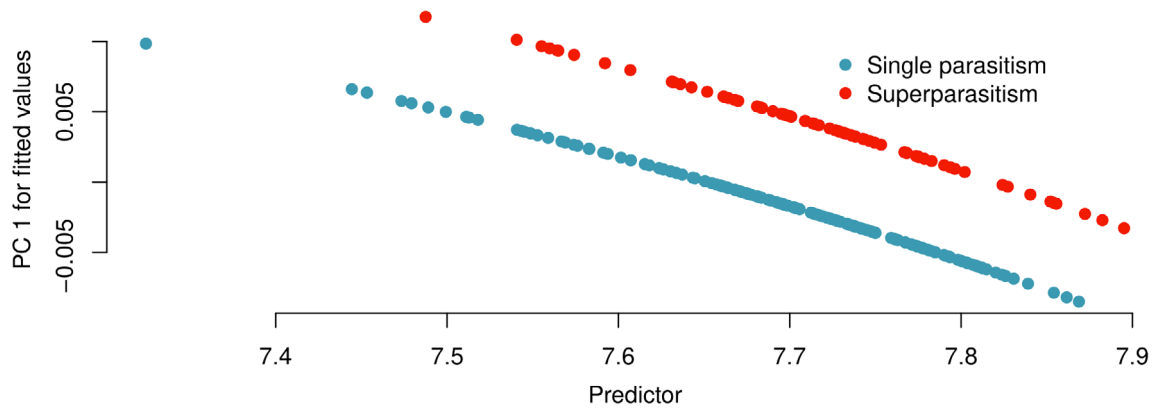
**Fig. 3.** (a) Photograph of *Mallophora ruficauda* wing. Vein references: 1. radial sector; 2. anterior branch of radius; 3. second and third posterior branch of radius; 4. fourth posterior branch of radius; 5. fifth posterior branch of radius; 6. first posterior branch of media; 7. second posterior branch of media; 8. third posterior branch of media; 9. R4 with recurrent vein arising its junction with R5; 10. second anterior branch of cubitus; 11. first anterior branch of cubitus; 12. and 13. Veins forming the discal cell; 14. radial-medial crossvein; 15. fourth and fifth posterior branch of radius; 16. 1st branch of anal vein; 17. cubitus; 18. media. (b) Principal components analysis plot of the first two principal component's for the analysis of effects of superparasitism on adult parasitoid wing size and shape. Light blue points represent individuals from the single parasitised group. Red points represent individuals from the superparasitised group. (c) Deformation grid showing the relative displacement of landmarks between single parasitised group (light blue) and the superparasitised group (red).

both groups have the same allometric pattern (Size effect  $F_{1,249} = 3.316$ ,  $P < 0.005$ ; Parasitism condition:  $F_{1,249} = 3.275$ ,  $P < 0.001$ ). However, at any given size, there exists variation between the single parasitised and the superparasitised groups, meaning that they differ in shape (Fig. 4).

## Discussion

In this work, we studied several aspects of factors influencing the probability of winning the competition for the larva arriving first or second to the host in a solitary dipteran koinobiont ectoparasitoid. We found that the time between parasitism

events but not the host instar could be involved in the competition for the host in the *C. signaticollis* – *M. ruficauda* host-parasitoid system. Both aspects are rendered as important factors in other parasitoids influencing the probability of winning the competition of the first or second arriving larva (van Alphen & Visser, 1990; Visser *et al.*, 1992; Godfray, 1994). For *M. ruficauda* these factors should be of extreme importance because of their particular life cycle. *Mallophora ruficauda* starts oviposition somewhere in mid-December when the host (second/third instar) is still unavailable in the field and continues until mid-March (Austral summer). Adult hosts make their appearance in December where they start laying their eggs underground. It is only in February that the availability of



**Fig. 4.** Allometric trajectory of the adult parasitoid wing shape for single parasitised (light blue) and superparasitised (red) groups showing the effects of superparasitism in flight traits.

potential usable hosts, that is second instar host, starts increasing. This characteristic of the system implies that early born parasitoid larvae are already in the soil waiting for their hosts to become available. The first hosts available are second instar hosts that, although not optimal, are likewise parasitised when conspecific larvae are abundant in the soil also searching for hosts (Crespo *et al.*, 2015; Martínez *et al.*, 2017). However, in February, the field scenario is somewhat different. Larvae born in February have second and third instar hosts already available, albeit some of them already parasitised. Our experiments showed that host instar does not influence the probability of a parasitoid larva winning competition. However, we found that as the time between parasitism events increased, the first larva lowered its chance to win the competition in favour of the second arriving larva.

With the information obtained here, we have evidence indicating that, in *M. ruficauda*, the time between parasitism events influence the probability of winning the competition for an individual larva meaning that the order of arrival does influence the result of competition. These results are coherent with recent results that show that larvae of *M. ruficauda* do not select hosts directly based on their nutritional quality but seem to favour the assurance of a resource unit (Zermoglio *et al.* unpublished data). It has been shown for other parasitoids that the first arriving larva does not always win the competition (Godfray, 1994; Yamada & Miyamoto, 1998; Yamada & Sugaura, 2003; Harvey *et al.*, 2013; Bili *et al.*, 2016). In *Haplogonatopus atratus* Esaki and Hashimoto (Hymenoptera: Dryinidae), the order of arrival does not always favours the first arriving larva. It has been seen that, when the interval between the first and second ovipositions was 1 h, the second larva had higher survival rates than the first one. However, if the interval was increased, the first larva increased its survival rate (Yamada & Miyamoto, 1998). In this parasitoid, the mechanism by which one larva is eliminated is through infanticide by the female although it is still not clear why the second larva has higher survival rates on some occasions (Yamada & Sugaura, 2003). Our results show that the second larva increases its chances of winning the competition when the time interval increased up to 30 days. A possible explanation for this is that the first larva must fight the host immune

system while the second larva could take advantage of this and win the competition. Another possibility is that larvae have different competitive abilities if they are born early or later in the season. However, in our experiments, larvae were all born at the same time. What is clear is that competition between larvae occurs by other modalities than fighting unlike what happens in hymenopterans because *M. ruficauda* larvae stay attached with their head capsule buried in the integument of the host. An interesting behaviour that has been recorded in this species is cannibalism (J. Crespo, unpublished data). Whenever a larva has a low life expectancy and has consumed almost all the teneral reserves, it is more prone to show cannibalism and, in this way, eliminates a competitor. However, in our experiments, larvae were the same age so this extreme behaviour was unlikely to occur.

Despite the existence of mechanisms to eliminate the competitor larva, compensatory responses could also help in diminishing the negative effects of superparasitism. Compensation mechanisms have been shown to aid in many species where individuals with impaired nutrition can still equal individuals with good nutrition reaching the adult stage with similar body sizes (Metcalf & Monaghan, 2001). In general, physiological compensation mechanisms such as entering diapause or increasing developmental rates are common examples that can be paid with smaller adult size, fecundity, or shorter life expectancy (Metcalf & Monaghan, 2001; Nestel *et al.*, 2016).

In the case of our experiments, we found that larvae born early in the season, where there are no hosts available for parasitism, have higher survival than larvae born in February. This result shows that free living early born larvae await for the host to appear in the environment, thus increasing the probability of parasitism of a healthy host. Arriving early to a healthy host could be an adaptive strategy for attaching alone to a host and start feeding. Assuring parasitism in this species has been shown to be a decision of paramount importance since they only change host in case it dies (M. Castelo, unpublished data). In case a second larva arrives at the host, the first larvae would still have a high probability of winning the competition although less than developing alone on a host. As shown in the paper by Crespo and Castelo (2010) a parasitoid larvae developing on a third instar *C. signaticollis* has at least 0.7 probability of

reaching the adult stage thus rendering competition an important factor. In turn, larvae born in February showed lower survival as free-living larva. The reduced survival could be indicative of a higher proneness to accept superparasitism.

We also indirectly tested whether *M. ruficauda* has additional compensation mechanisms for superparasitism and if it has a cost later in life. In this regard, we found no difference in the pupa's weight, sex ratio, or developmental time between parasitoids of single and superparasitism groups. These results might be indicating that *M. ruficauda* can compensate for the costs of superparasitism once attached to a host during the larval stage in a competition scenario if, by competing, larvae would be deriving resources that could otherwise be used for development. Since both groups seem to attain similar weight, it could be argued that compensation would let larvae reach a similar physiological state. However, body condition indices could not be the only estimate of fitness since it has been argued that they do not always reflect lipid reserves (Wilder *et al.*, 2016).

Another dimension in which superparasitism could be influencing is adult development. To tackle this, we analysed differences in adult parasitoids' wing size and shape between larvae raised with no competition or superparasitism conditions. Wings are frequently used as an adult developmental proxy since they are easy structures to study (Dellicour *et al.*, 2017). In addition, wing size and shape are involved in flight performance, foraging activity, and even there are some reported cases that these traits can influence sexual selection and reproduction (Wootton, 1992; Outomuro & Johansson, 2011; Dellicour *et al.*, 2017). We found similar wing sizes but there were differences between groups in wing shape. The main differences were found to be a relative displacement of a crossvein between R4 and R5 and an apparent increase in wing area. Differences in wing shape could influence wing deformation, flexibility, and flight control (Wootton, 2020). *Mallophora ruficauda* captures its prey on flight and they show a huge capability of flight dispersal. Particularly relevant, differences in flight could have an influence on sexual selection since males exhibit a courtship behaviour in which flight performance is central (Video S1). Finally, regarding wing shape differences, we found an allometric effect indicating that the difference between groups is independent of particular wing size. This result is relevant because it shows that superparasitism might impair development and adult performance with negative consequences on fitness.

The cost of superparasitism might even be evident with a reduced adult survival or lower fecundity, two aspects not studied here. In this species, it has proven to be impossible the adult maintenance and oviposition in captivity, rendering it almost impossible to test these aspects.

In conclusion, we have shown that although a solitary species, superparasitism in *M. ruficauda* might be an advantageous strategy for larvae with many competitors searching for hosts since it can increase the probability of winning a competition from 0 to 1 in case the second larva wins the competition. At a population level, this strategy implies that any larva in presence of conspecific larvae will increase its survival chances from certain death (should the larva keep waiting for a healthy host and never obtain it) to win competition half the times. Although no important costs seem to act against superparasitism after

winning the competition, we were able to show some subtle effects in adult parasitoids that could reduce sexual performance and flight efficiency in prey capture and dispersal. However, our results of superparasitism on adult development could be underestimated and the effect of the time between parasitism on development time or adult morphological traits should be studied more thoroughly. This study brings together information on the effect of superparasitism in a solitary ectoparasitoid with host-seeking larvae, filling a gap in our knowledge of the cost of superparasitism in insect parasitoids.

## Acknowledgements

We would like to thank many beekeepers that allowed us to collect the samples. We are grateful to Gustavo Agustín Martínez, María Eugenia Barrantes, and Agustín Sebastián Atala for their contributions in some of the experiments. A special recognition to Pablo Milla Carmona for his assistance and help with the geometry morphometrics analyses. This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (grant number PICT BID No2614); Consejo Nacional de Investigaciones Científicas y Técnicas (grant number PIP 0368); and Universidad de Buenos Aires (grant numbers UBACyT 0059BA, 0019BA). We would like to thank the three anonymous reviewers that greatly improved the manuscript with their suggestions. Authors declare no conflict of interest.

## Author contributions

JEC and MKC both contributed equally in the design, collection, data collection, and paper writing. JEC performed the data analysis.

## Data availability statement

Data openly available in a public repository that issues datasets with DOIs

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Appendix S1:** Supporting information

**Video S1:** Supporting information

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Accepted 18 October 2021

Associate Editor: Saskya van Nouhuys