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## RESEARCH ARTICLE

# Impact of differences in nutritional quality of wingless and winged aphids on parasitoid fitness

Jennifer A.-L. M. Pirotte<sup>1,\*</sup>, Ange Lorenzi<sup>2</sup>, Vincent Foray<sup>3</sup> and Thierry Hance<sup>1</sup>

## ABSTRACT

Winged aphids are described as hosts of lesser quality for parasitoids because a part of their resources is used to produce wings and associated muscles during their development. Host lipid content is particularly important for parasitoid larvae as they lack lipogenesis and therefore rely entirely on the host for this resource. The goal of this study was to determine to what extent winged and wingless aphids differ from a nutritional point of view and whether these differences impact parasitoid fitness, notably the lipid content. We analysed the energetic budget (proteins, lipids and carbohydrates) of aphids of different ages (third instars, fourth instars and adults) according to the morph (winged or wingless). We also compared fitness indicators for parasitoids emerging from winged and wingless aphids (third and fourth instars). We found that in third instars, parasitoids are able to inhibit wing development whereas this is not the case in fourth instars. Both winged instars allow the production of heavier and fattier parasitoids. The presence of wings in aphids seems to have little effect on the fitness of emerging parasitoids and did not modify female choice for oviposition. Finally, we demonstrate that *Aphidius colemani*, used as a biological control agent, is able to parasitize wingless as well as winged *Myzus persicae*, at least in the juvenile stages. If the parasitism occurs in third instars, the parasitoid will prevent the aphid from flying, which could in turn reduce virus transmission.

**KEY WORDS:** Aphid–parasitoid, Aphid polyphenism, Energetic reserves, Fitness, Host quality, Wing development

## INTRODUCTION

Flight has long been recognized as an expensive activity in terms of energy, usually to the detriment of reproduction (Zera and Denno, 1997). Costs include the energy used during the flight but also to produce the wings and the wing musculature during development (Roff and Fairbairn, 1991). Insects use several flight fuels and their pattern of utilization depends on the insect order but also varies within an insect order. For prolonged flights, lipid is the main flight fuel in Orthoptera (Clark et al., 2013; Zera and Larsen, 2001) and Lepidoptera (Beenackers et al., 1985; Murata and Tojo, 2002) whereas it is mainly sugar and glycogen in Hymenoptera (Amat

et al., 2012; Micheu et al., 2000; Suarez et al., 2005), Diptera (Van Handel, 1984) and Coleoptera (Zebe and Gäde, 1993). Several studies have also demonstrated the use of proline in Hymenoptera (Micheu et al., 2000), Diptera (Scaraffia and Wells, 2003) and Coleoptera (Zebe and Gäde, 1993).

Aphids offer an interesting case study as the same genotype can display a winged and a wingless phenotype depending on environmental conditions (wing polyphenism). Winged and wingless phenotypes differ as they invest either in dispersal or in reproduction, respectively (Braendle et al., 2006; Brisson, 2010). The presence of winged or wingless morphs in the same clone of aphids allows determination of differences in resource allocation according to phenotype and their ecological role. Earlier studies in aphids demonstrated that glycogen is the fuel used at the beginning of flight whereas lipids are used later during prolonged flight (Cockbain, 1961).

The nutritional status of the host may be an important cue for host quality assessment (Li and Mills, 2004) and consequently larger parasitoids emerge from hosts with higher levels of resources (Barrette et al., 2009; Harvey and Vet, 1997; van Emden and Kifle, 2002). However, parasitoid fitness will be a matter not only of resource quantity but also of resource quality (Häckermann et al., 2007) and relies on the ability of parasitoids to exploit the available resources (Sampaio et al., 2008). When an egg is laid in an aphid with wing buds, an inhibition of host wing development by the parasitoid larva is frequently observed (Christiansen-Weniger and Hardie, 1998; Demmon et al., 2004; Kati and Hardie, 2010; Zhang et al., 2009) and can be explained by the need for the parasitoid to redirect resources otherwise not available for its own development (Demmon et al., 2004).

Lipids are important for parasitoids as a maintenance energetic source but also in reproduction for oogenesis (Rivero and Casas, 1999). Lipid levels and availability in hosts during parasitoid larval development are a major constraint as lipogenesis does not occur in adult parasitoid wasps (Giron and Casas, 2003; Strand and Casas, 2008; Visser and Ellers, 2008). Proteins are required for egg production (Rivero and Casas, 1999). Finally, sugar sources in general may also be used in maintenance or in egg production in species that mature eggs after emergence (synovigenic) (Jervis et al., 2008) while glycogen reserves are needed for flight in order to find food, hosts and mates (Amat et al., 2012). Sugar sources such as nectar or honeydew can be found during the adult phase and allow the replenishment of sugar and glycogen reserves (Jervis et al., 2008). However, nectar and honeydew constitute deficient food sources (Jervis et al., 2008) as they contain very small amounts of amino acids (Baker and Baker, 1973; Sabri et al., 2013), proteins (Peumans et al., 1997; Sabri et al., 2013) and lipids (Lee et al., 2004; Strong, 1965).

Our hypothesis was that the winged or wingless forms of the same aphid clone represent hosts of different quality for a female parasitoid. Therefore, we tested the nutritional quality of aphids

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of different ages (third instars, fourth instars and adults) according to the presence or absence of wing buds or full wings. For this, we analysed the aphids' entire energetic budget (proteins, lipids and carbohydrates). We expected wingless individuals to accumulate the resources necessary for reproduction and winged individuals to invest in flight fuels. These differences should appear as soon as the third instar, for which immature winged aphids are recognizable based on wing buds (Ishikawa et al., 2008). Knowledge of the energetic budget through aphid stages will reveal the dynamics of resource accumulation as the host will continue to feed during the parasitoid development (Mackauer, 1986).

Differences in host nutritional status and the ability of the parasitoid to inhibit host wing development may lead to differences in parasitoid fitness. To determine these differences, we compared a series of fitness indicators for parasitoid individuals emerging from winged and wingless aphids (third and fourth instars) in a no-choice and a choice experiment. We predicted that wing inhibition is more important in third instars than in fourth instars (Christiansen-Weniger and Hardie, 1998; Demmon et al., 2004; Kati and Hardie, 2010; Zhang et al., 2009). Consequently, winged or wingless third instars should bring the same fitness as resources required for host wing development will be redirected to parasitoid development (Demmon et al., 2004). However, wing buds might impose a physical constraint on oviposition success by increasing handling time so that winged instars will be less parasitized than wingless instars. In the same way, it might be less advantageous for parasitoids to develop in winged fourth instars if no wing inhibition occurs. Parasitoids emerging from winged fourth instars should therefore show a lower fitness. If this is the case, parasitoids should preferentially lay eggs in wingless fourth instars in a choice experiment.

For this study, we focused on the aphid *Myzus persicae* (Sulzer 1776) (Hemiptera, Aphididae) and one of its parasitoids, *Aphidius colemani* Viereck (Hymenoptera, Braconidae, Aphidiinae). *Myzus persicae* is the aphid species capable of feeding on the largest host plant number and of transmitting the highest number of plant viruses (Essig, 1948; Mackauer and Way, 1976; van Emden et al., 1969). *Aphidius colemani* is widely used for the biological control of *M. persicae*, notably in greenhouses (Fernández and Nentwig, 1997; Takada, 1998), but also parasitizes this aphid species in the field (Starý, 2002).

## MATERIALS AND METHODS

### Insect rearing

*Myzus persicae* was provided by Viridaxis SA (Charleroi, Belgium) from an aphid clone collected in the Czech Republic. Aphid rearing was done on artificial diet to avoid any plant effect (Cambier et al., 2001; Colinet et al., 2005). This artificial diet was also supplied by Viridaxis SA and kept in the dark at 21±2°C, 40–50% relative humidity (RH). In order to obtain third and fourth instars exhibiting wing buds and winged adults, high densities (15 individuals cm<sup>-2</sup>) were applied during development of the mothers and their offspring till second instar. Winged and wingless individuals were both obtained in these crowded conditions unlike in other studies (Shi et al., 2010; Xu et al., 2011).

*Aphidius colemani* individuals were obtained after contact between one parasitoid female and 50 *M. persicae* aged 3–4 days for 24 h. Parasitized aphids were then kept on artificial diet in the dark at 22±1°C, 50–60% RH until mummification. Ten mummies were placed in Petri dishes to allow mating after emergence over a period of 24 h and then used for the experiments.

## Experimental design

### Aphid energy budget

We compared the four main energy components (glycogen, free sugars, lipids and proteins) of third and fourth instars and adults with or without wing buds/wings. Individuals of a given stage are recognizable based on the number and size of antennal segments, and the cauda shape (Sorin, 1958; Sylvester, 1954). All individuals were freeze dried within 3 h of moulting (Shi et al., 2010) at –20°C in 0.5 ml Eppendorf vials. Because of the small size of *M. persicae*, particularly for third instars, 15 individuals per biological replicate were pooled ( $n=13–14$ ) and weighed to the nearest 0.01 mg (Mettler-electrobalance Me22, Mettler-Toledo, Zaventem, Belgium). In total, 210 aphids of each morph and each stage were used for the analysis except for winged adults, for which the number was 195 aphids.

We used biochemical analyses modified from van Handel's methods to quantify the four energy compartments (Foray et al., 2012). Briefly, samples were crushed with 180 µl of buffer solution (prepared with phosphate-buffered saline, pH 7.4; P3813 and DL-dithiothreitol 1 mmol l<sup>-1</sup>; 646563, Sigma-Aldrich, Overijse, Belgium) with a stainless-steel bead (412-3131, VWR, Oud-Heverlee, Belgium) at 25 Hz for 60 s. After centrifugation, 10 µl of the supernatant was transferred to a 96-well quartz microplate (730-009-QG, Hellma Analytics, Müllheim, Germany) with 250 µl of Bradford reagent (B6916, Sigma-Aldrich), and with bovine serum albumin (SB8667, Sigma-Aldrich) as a standard. Afterwards, a chloroform/methanol (1:2 v/v) solution was added to extract total lipids and carbohydrates (free sugars and glycogen), which were further assayed with vanillin (V1104, Sigma-Aldrich) and anthrone (319899, Sigma-Aldrich) reagents, respectively. Lipid and free-sugar content was based on 400 µl and 300 µl of supernatant using triolein (92860, Sigma-Aldrich) and D-glucose (G-7528, Sigma-Aldrich) as standards, respectively.

### Consequences of parasitoid development in winged or wingless aphids

Two different types of experiment were performed in order to study the fitness consequences for parasitoid larvae of developing in aphids with wing buds. In both experiment types, two different aphid stages (third and fourth instars) were used, each with two phenotypes [winged (WD) and wingless (WL)].

First, we carried out a no-choice experiment. Between 4 and 25 aphids of the same morph and stage (third or fourth instar, WD or WL) were placed in a Petri dish and parasitism by a 1–2 day old mated and naive *A. colemani* female was allowed over a period of 2 h. A preliminary analysis showed that the aphid density had an impact on fresh mass, dry mass and lipid content of the parasitoids. Indeed, there was an interaction between aphid morph and aphid density. The morph effect was more visible at low densities. As this interaction does not change our conclusions, in order to have sufficient data, we kept all the replicates regardless of the aphid density (Table S1). The effect of morph and stage on parasitism rate and emergence rate was only analysed in low densities (4–10 aphids). We performed 24 replicates for the winged third instars, 20 replicates for the wingless third instars, 21 replicates for the winged fourth instars and 18 replicates for the wingless fourth instars. After parasitism, aphids were put back into small Petri dishes (55 mm diameter) with artificial diet until mummification and parasitoid adult emergence in the dark at 22±1°C. The artificial diet was changed thrice a week to avoid diet depletion and microorganism contamination. Twenty-four hours after adult emergence, sex was identified, and we measured the fresh mass, dry mass and lipid content of emerging parasitoids.

Second, in a choice experiment, five aphids of the winged and five of the wingless morphs of the same stage (10 aphids in total, third and fourth instars were used separately) were placed in the Petri dish and left to be parasitized by a 1–2 day old naive female parasitoid over a period of 4 h. We performed 10 replicates in total for each stage. After emergence, sex was identified and parasitoids were freeze dried ( $-20^{\circ}\text{C}$ ) and placed in 2 ml Eppendorf tubes. We measured mummy width, tibia size, fresh mass, wing area, wing load (fresh mass/wing area), the number of eggs at emergence for parasitoids emerging from third instars and the reproductive effort (see below).

Lipid quantification from emerging parasitoids was performed as described in Colinet et al. (2006). Parasitoid individuals were first weighed (Mettler-electrobalance Me22; sensitivity: 0001 mg, Mettler-Toledo) to evaluate fresh mass. Individuals were then dried over a period of 3 days at  $60^{\circ}\text{C}$  to obtain dry mass. Dried individuals were placed in Eppendorf tubes with 1 ml of chloroform/methanol (2:1 v/v) solution to extract lipids. The tubes were placed on an agitating table for 2 weeks. After these 2 weeks, the solution was withdrawn, and the samples were dried again at  $60^{\circ}\text{C}$  for 12 h. The lipid content corresponds to the difference between the dry mass before and after extraction.

Photos of the parasitoid left hind tibia, left forewing and mummies were taken with a Sony SSC-DC 198P camera mounted on a binocular microscope ( $20\times$  magnification, Leica MZ6, Leica Microsystems, Diegem, Belgium). The length of the tibia, the wing area and the mummy width were then measured with ImageJ software (Abramoff et al., 2004). The number of eggs at emergence was counted in females under a Nikon SMZ800 binocular microscope ( $63\times$  magnification, Nikon, Brussels, Belgium). The abdomen was separated from the rest of the body on a microscope slide and then opened with entomological pins in a drop of Hanks' balanced salts solution (H6136, Sigma-Aldrich). Ovarioles were then opened and stained with Lactophenol Blue (Sampaio et al., 2008) to count mature eggs (Barrette et al., 2009). The eggs were kept on a sealed slide and photographs of mature eggs were taken. With ImageJ, the volume of 20 eggs per female was estimated as described in Le Lann et al. (2012). The mean egg volume multiplied by the number of eggs provided our estimate of the reproductive effort. Pictures were taken with a Leica DMC4500 camera mounted on a Leica DMLB microscope ( $400\times$  magnification, Leica Microsystems).

The parasitism rate of the parental generation was also evaluated as the number of mummies divided by the number of aphids available for parasitism and the emergence rate as the number of emerging parasitoids divided by the number of mummies. Values were calculated for each female and a mean for all females in a given condition was obtained. Replicates with no mummy formed were not considered.

### Statistical analysis

Generalized linear models were used to assess the impact of aphid stage, aphid morph and parasitoid sex. In the case of aphid fresh mass and parasitoid fresh mass in the no-choice experiment, a log transformation of the values was used. A square root transformation for sugar and protein content in the aphid energy budget and for lipid content of the parasitoids was also used to obtain a normal distribution.

The egg load was analysed by means of a quasi-Poisson error distribution.

As energetic content correlates with fresh mass, it was analysed with fresh mass as the first explanatory variable (Foray et al., 2012,

2014). In the choice experiment, fitness parameters that correlate with mummy width were analysed with mummy width as the first explanatory variable (Demmon et al., 2004).

The parasitoid sex ratio was analysed with a binomial test to see whether it was different from 0.50. The impact of stage and morph on sex ratio, emergence rate and parasitism rate was analysed with a generalized linear model with a binomial error distribution.

The interaction between stage and morph when non-significant was suppressed from models. Contrasts between stage, morph and sex were obtained with the 'lsmeans' function (lsmeans R package, Tukey adjustment). All data are presented as means $\pm$ s.d. Graphs and analyses were performed using the R statistical package, version 3.4.3 (<http://www.r-project.org/>).

The number of individuals used to estimate the different parameters is listed in Table S2.

## RESULTS

### Aphid energy budget

The crowding stimulus induced a winged offspring level of  $3.19\pm 0.53\%$  of all individuals. No winged offspring were ever produced with this clone at lower densities (Pirotte, 2011). Winged aphids were always heavier than wingless aphids (stage:  $F_{2,79}=1108.6$ ,  $P<0.0001$ ; morph:  $F_{1,79}=395.4$ ,  $P<0.0001$ ) (Table 1).

All energy compartments correlated with fresh mass for the wingless (glycogen,  $F_{1,39}=469.3$ ,  $R^2=0.92$ ,  $P<0.0001$ ; free sugars,  $F_{1,39}=90.76$ ,  $R^2=0.69$ ,  $P<0.0001$ ; lipid,  $F_{1,39}=35.64$ ,  $R^2=0.47$ ,  $P<0.0001$ ; protein,  $F_{1,39}=102.1$ ,  $R^2=0.72$ ,  $P<0.0001$ ) and the winged morph (glycogen,  $F_{1,38}=78.9$ ,  $R^2=0.66$ ,  $P<0.0001$ ; free sugars,  $F_{1,38}=132.9$ ,  $R^2=0.77$ ,  $P<0.0001$ ; lipid,  $F_{1,38}=393.3$ ,  $R^2=0.90$ ,  $P<0.0001$ ; protein,  $F_{1,38}=62.65$ ,  $R^2=0.61$ ,  $P<0.0001$ ) (Fig. S1). Therefore, to account for this size effect, the fresh mass was used in the regression. Residuals from the regression are depicted for each morph/stage combination (Fig. 1). The total amount of a given energy compartment per aphid is shown in Table 1.

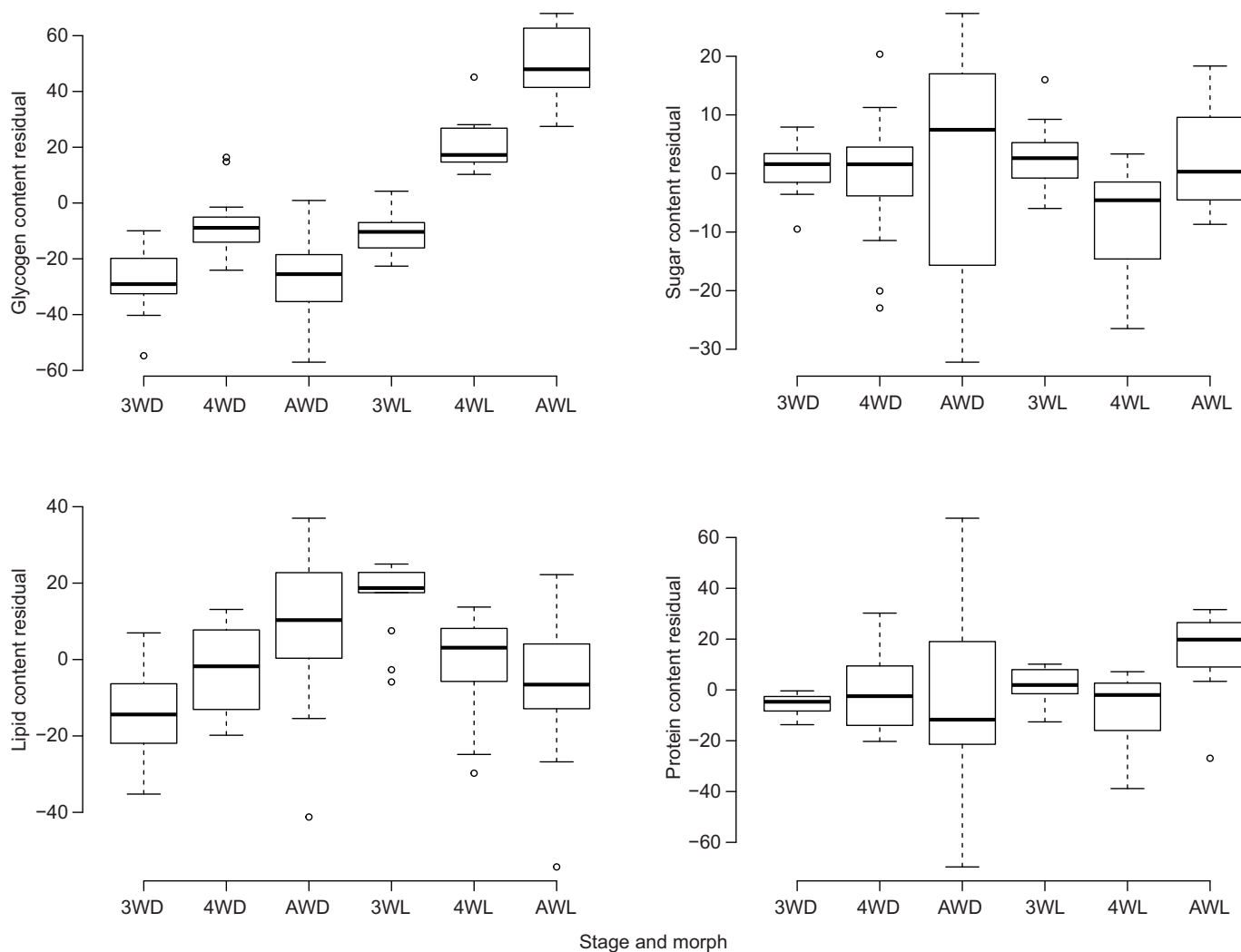
For glycogen content, wingless aphids accumulated more than winged aphids at all stages (Table 1). However, the pattern of accumulation was not the same between morphs. In winged

**Table 1. Summary of the results for aphid fresh mass, glycogen, free sugar, lipid and protein content according to aphid stage and morph**

Parameters	Stage	WD	WL	P-value
Aphid fresh mass ( $\mu\text{g}$ )	Third instars	84 $\pm$ 4	49 $\pm$ 5	<0.0001
	Fourth instars	186 $\pm$ 20	120 $\pm$ 17	<0.0001
	Adults	358 $\pm$ 23	213 $\pm$ 31	<0.0001
Glycogen content ( $\mu\text{g}$ )	Third instars	2.52 $\pm$ 0.74	3.25 $\pm$ 0.46	0.0020
	Fourth instars	5.32 $\pm$ 0.66	6.24 $\pm$ 0.71	0.0004
	Adults	6.39 $\pm$ 0.95	9.41 $\pm$ 0.98	<0.0001
Free sugar content ( $\mu\text{g}$ )	Third instars	1.73 $\pm$ 0.33	1.40 $\pm$ 0.34	0.0482
	Fourth instars	3.00 $\pm$ 0.64	1.63 $\pm$ 0.58	<0.0001
	Adults	5.51 $\pm$ 1.08	3.54 $\pm$ 0.45	0.0015
Lipid content ( $\mu\text{g}$ )	Third instars	1.80 $\pm$ 0.77	3.08 $\pm$ 0.60	0.0002
	Fourth instars	4.82 $\pm$ 0.88	3.47 $\pm$ 0.63	ns
	Adults	9.35 $\pm$ 1.56	5.17 $\pm$ 1.16	0.0035
Protein content ( $\mu\text{g}$ )	Third instars	1.27 $\pm$ 0.29	1.1 $\pm$ 0.37	ns
	Fourth instars	3.42 $\pm$ 0.92	2.07 $\pm$ 0.66	0.0036
	Adults	6.29 $\pm$ 2.58	5.07 $\pm$ 0.93	ns

WD, winged; WL, wingless. Data are means $\pm$ s.d.,  $n=14$  for each group except for winged adults, where  $n=13$ . Each biological replicate corresponds to 15 aphids. Generalized linear model (GLM) with *post hoc* test (Tukey adjustment); ns, no significant difference ( $P>0.05$ ).





**Fig. 1. Boxplots of residuals for glycogen, free sugar, lipid and protein content of aphids (regression with fresh mass) according to stage and morph.** 3, third instar; 4, fourth instar; A, adult; WD, winged; WL, wingless;  $n=14$  for each group except for AWD, where  $n=13$ . Each biological replicate corresponds to 15 aphids. Horizontal bold line: median, box: lower and upper quartiles, dashed lines: smallest and largest non-outlier observations. Generalized linear model (GLM; see Table 1 for details on  $P$ -values).

individuals, the quantity of glycogen reached a maximum value in fourth instars whereas in wingless individuals, the glycogen content increased during development (interaction stage×morph:  $F_{2,76}=15.53$ ,  $P<0.0001$ ) (Fig. 1).

For free sugar content, third wingless instars showed a slightly higher free sugar content than winged ones, but the reverse was the case in fourth instars and adults (interaction stage×morph:  $F_{2,76}=6.19$ ,  $P=0.0032$ ) (Fig. 1, Table 1).

The lipid content was higher in adult winged aphids than in wingless ones, whereas in third instars, the wingless phenotype contained more lipids (Table 1). In general, lipid content increased with age in the winged phenotype and decreased with age in the wingless phenotype (interaction stage×morph:  $F_{2,76}=15.38$ ,  $P<0.0001$ ) (Fig. 1).

The total protein content was on average higher in winged individuals, but it was significant only in fourth instars (Table 1). The accumulation of protein was observed between third and fourth instars for the winged phenotype and between fourth instars and adults for the wingless phenotype (interaction stage×morph:  $F_{2,75}=3.33$ ,  $P=0.041$ ) (Fig. 1).

### Fitness consequences of parasitoid development in winged or wingless aphids

#### No-choice experiment

The parasitism rate was not different according to stage or morph (stage:  $\chi^2_{1,276}=2.98$ ,  $P=0.084$ ; morph:  $\chi^2_{1,276}=3.00$ ,  $P=0.083$ ) (Table 2).

When parasitism occurred for winged third instars, mummies usually presented wing buds (even if they mummified as adults; Fig. 2A) except in 8 cases out of 162 where mummies presented full wings. Individuals that emerged from these mummies were excluded from the analysis. Indeed, this lower number of individuals with wings when parasitism occurred in third instars did not allow us to test the impact of wing inhibition itself on the emergent fitness. When parasitism occurred in winged fourth instar hosts, all mummies presented full wings (Fig. 2B).

The emergence rate was higher in winged fourth instars than in winged third instars (stage:  $\chi^2_{1,164}=3.98$ ,  $P=0.046$ ; morph:  $\chi^2_{1,164}=0.75$ ,  $P=0.39$ ) (Table 2).

Stage or morph did not have any effect on the sex ratio (stage:  $\chi^2_{1,314}=0.019$ ,  $P=0.89$ ; morph:  $\chi^2_{1,314}=0.14$ ,  $P=0.71$ ), which did not

**Table 2. Summary of the main results in the no-choice and choice experiments according to aphid stage and morph**

Experiment	Parameter	Stage	WD	WL	P-value
No-choice	Fresh mass (mg)	Third	0.23±0.043	0.21±0.033	<0.0001
		Fourth	0.19±0.031	0.18±0.017	<0.0001
	Dry mass (mg)	Third	0.094±0.018	0.084±0.014	<0.0001
		Fourth	0.072±0.012	0.066±0.0079	<0.0001
	Lipid content (mg)	Third	0.035±0.0098	0.028±0.0066	0.0001
		Fourth	0.023±0.0057	0.020±0.0046	0.0001
	Parasitism rate	Third	0.73±0.27	0.63±0.29	ns
		Fourth	0.72±0.28	0.51±0.15	ns
	Emergence rate	Third	0.78±0.29	0.81±0.23	ns
		Fourth	0.92±0.14	0.82±0.21	ns
Choice	Fresh mass (mg)	Third	0.24±0.028	0.22±0.024	ns
		Fourth	0.19±0.022	0.19±0.022	ns
	Mummy width (mm)	Third	1.16±0.042	1.12±0.04	0.0087
		Fourth	1.09±0.040	1.08±0.06	0.0087
	Tibia size (mm)	Third	0.58±0.023	0.56±0.020	ns
		Fourth	0.53±0.032	0.52±0.028	ns
	Wing load (mg mm <sup>-2</sup> )	Third	0.41±0.041	0.40±0.042	ns
		Fourth	0.37±0.047	0.38±0.032	ns
	Egg load	Third	201±61	217±40	ns
	Parasitism rate	Third	0.72±0.32	0.74±0.32	ns
		Fourth	0.80±0.094	0.92±0.14	ns
	Emergence rate	Third	0.97±0.083	0.91±0.11	<0.0001
		Fourth	0.92±0.14	0.41±0.25	<0.0001

WD, winged; WL, wingless. Data are means±s.d. Generalized linear model (GLM) with *post hoc* test (Tukey adjustment); ns, no significant difference ( $P>0.05$ ). Binomial error distribution for emergence and parasitism rates.

differ from 0.50 in any of the conditions (sex ratio=0.51,  $P=0.92$  for wingless third instar hosts; sex ratio=0.46,  $P=0.51$  for winged third instar hosts; sex ratio=0.47,  $P=0.86$  for wingless fourth instar hosts; sex ratio=0.51,  $P=1$  for winged fourth instar hosts).

In general, parasitoids emerging from aphids parasitized at the third instars were heavier than those emerging from aphids parasitized at the fourth instars (stage effect on fresh mass:

$F_{1,305}=81.26$ ,  $P<0.0001$ ; dry mass:  $F_{1,304}=139.99$ ,  $P<0.0001$ ) (Fig. 3, Table 2). This was also the case for parasitoids emerging from winged aphids compared with wingless aphids (morph effect on fresh mass:  $F_{1,305}=19.83$ ,  $P<0.0001$ ; dry mass:  $F_{1,304}=11.58$ ,  $P=0.00076$ ) and for female parasitoids compared with males (sex effect on fresh mass:  $F_{1,305}=54.41$ ,  $P<0.0001$ ; dry mass:  $F_{1,304}=30.95$ ,  $P<0.0001$ ) (Fig. 3, Table 2). The dry mass of parasitoids that emerged from fourth instars was not significantly different according to sex. The sex difference was only significant in third instars and females were heavier than males (interaction stage×sex:  $F_{1,304}=5.55$ ,  $P=0.019$ ) (Fig. 3).

The parasitoid lipid content correlated with fresh mass for the wingless ( $F_{1,135}=339$ ,  $R^2=0.71$ ,  $P<0.0001$ ) and the winged morph ( $F_{1,168}=940.9$ ,  $R^2=0.85$ ,  $P<0.0001$ ). Therefore, to account for this size effect, fresh mass was used in the regression. Parasitoids emerging from aphids parasitized as third instars showed a higher lipid content than those emerging from fourth instars (stage:  $F_{1,304}=25.03$ ,  $P<0.0001$ ) as was the case for parasitoids emerging from winged aphids (morph:  $F_{1,304}=23.53$ ,  $P<0.0001$ ). Per unit of fresh mass, males accumulated more lipid than females (sex:  $F_{1,304}=36.62$ ,  $P<0.0001$ ) even if females contained more lipids in total as they were heavier. There was no interaction effect (Fig. 4, Table 2).

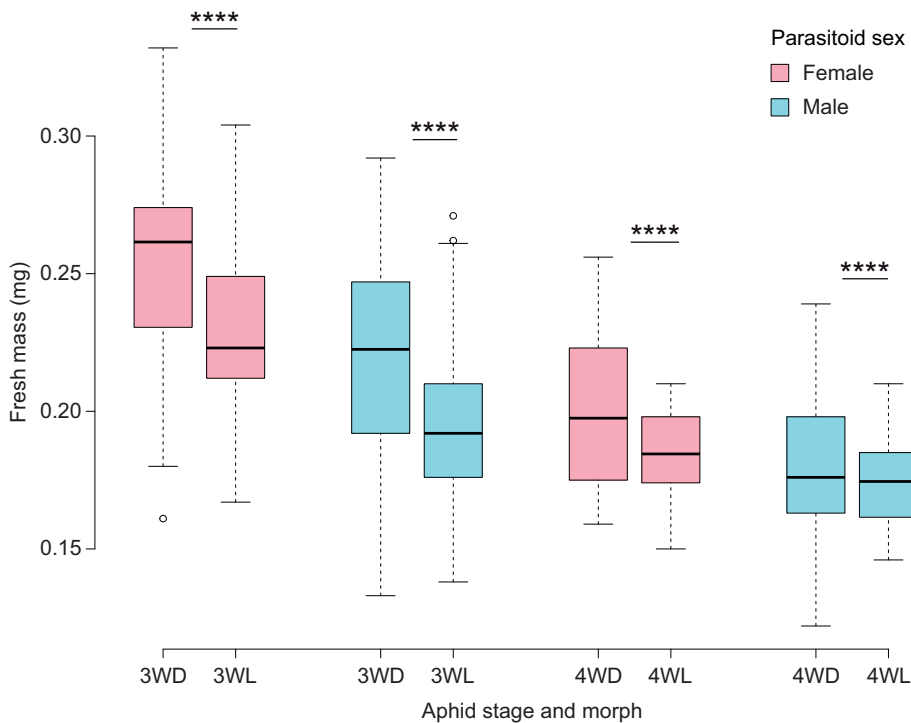
#### Choice experiment

In the choice experiment, the parasitism rate was lower in third instars (stage:  $\chi^2_{1,197}=5.26$ ,  $P=0.022$ ; morph:  $\chi^2_{1,197}=1.55$ ,  $P=0.21$ ) (Table 2).

When parasitism occurred in winged third instars, the mummy usually presented wing buds (even if they mummified as adults) except in 3 cases out of 35 where the mummy presented full wings. Individuals that emerged from these mummies were excluded from the analysis. Again, this low number of individuals with wings when parasitism occurred in third instars did not allow us to test the impact of wing inhibition itself on the emergent fitness. When parasitism occurred in winged fourth instar hosts, all mummies presented full wings.



**Fig. 2. Images of various degrees of wing inhibition when developing winged aphids are parasitized.** (A) Mummy with wing buds (parasitism in third instars). (B) Mummy with full wings (parasitism in fourth instars).



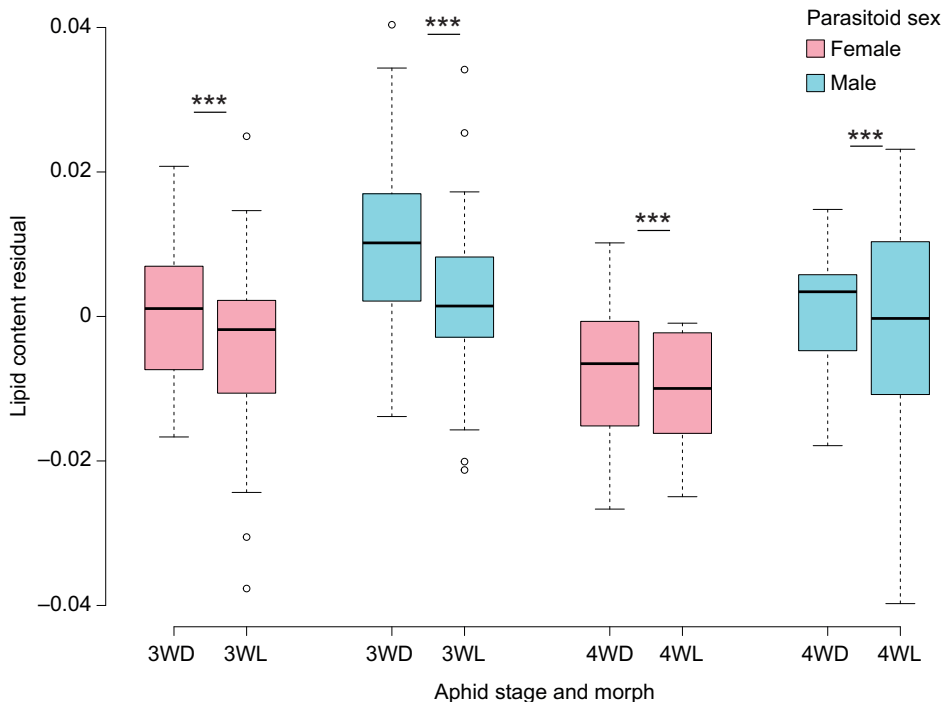
**Fig. 3. Boxplot of fresh mass for male and female parasitoids emerging from two different aphid stages and morphs.** For females,  $n=48$  in 3WD,  $n=55$  in 3WL,  $n=34$  in 4WD and  $n=14$  in 4WL. For males,  $n=56$  in 3WD,  $n=53$  in 3WL,  $n=33$  in 4WD and  $n=16$  in 4WL. GLM with *post hoc* test (Tukey adjustment); \*\*\*\* $P<0.0001$  for differences between winged and wingless individuals at each stage.

The emergence rate was far lower in fourth instars than in third instars and was lower in wingless instars than in winged instars (stage:  $\chi^2_{1,155}=21.21$ ,  $P<0.0001$ ; morph:  $\chi^2_{1,155}=30.43$ ,  $P<0.0001$ ) (Table 2). We dissected mummies from which no adult emerged and in most of them (22 out of 28 mummies of wingless fourth instar hosts) the larva was still present but dead and had not completed metamorphosis. Stage and morph did not have any effect on the sex ratio (stage:  $\chi^2_{1,131}=0.61$ ,  $P=0.44$ ; morph:  $\chi^2_{1,131}=1.67$ ,  $P=0.20$ ), which was female biased in all conditions (sex ratio=0.77,  $P=0.0019$  for wingless third instar hosts; sex ratio=0.74,

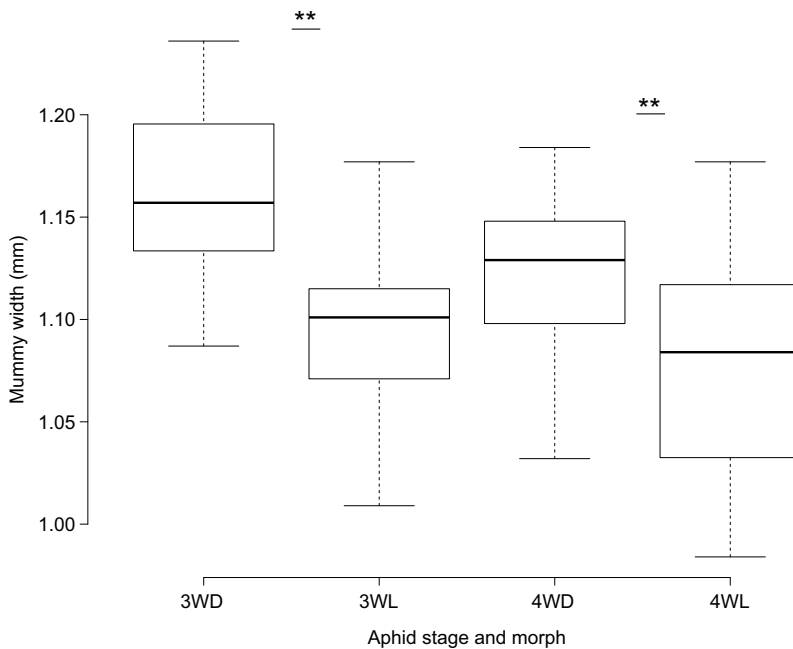
$P=0.0060$  for winged third instar hosts; sex ratio=0.92,  $P<0.0001$  for wingless fourth instar hosts; sex ratio=0.75,  $P=0.0022$  for winged fourth instar hosts).

Very few males were produced. As the emergence rate was very low from the wingless fourth instars, only one male was available for analysis. In consequence, the analysis is only described for females.

Mummies were wider if parasitism occurred in third instars especially if the host was an immature winged aphid (stage:  $F_{1,89}=33.41$ ,  $P<0.0001$ ; morph:  $F_{1,89}=7.21$ ,  $P=0.0087$ ) (Fig. 5, Table 2).



**Fig. 4. Boxplot of residuals for lipid content (regression with fresh mass) for male and female parasitoids emerging from two different aphid stages and morphs.** For females,  $n=48$  in 3WD,  $n=55$  in 3WL,  $n=34$  in 4WD and  $n=14$  in 4WL. For males,  $n=56$  in 3WD,  $n=53$  in 3WL,  $n=33$  in 4WD and  $n=16$  in 4WL. GLM with *post hoc* test (Tukey adjustment); \*\*\* $P\leq 0.001$ .



**Fig. 5. Boxplot of mummy width from parasitism of two different aphid stages and morphs.**  $n=23$  in 3WD,  $n=25$  in 3WL,  $n=29$  in 4WD and  $n=15$  in 4WL. GLM with *post hoc* test (Tukey adjustment); \*\* $P \leq 0.01$ .

Parasitoid tibia size, wing area, wing load and fresh mass were correlated with mummy width for the wingless (tibia size,  $F_{1,45}=31.19$ ,  $R^2=0.39$ ,  $P<0.0001$ ; wing load,  $F_{1,44}=4.25$ ,  $R^2=0.066$ ,  $P=0.045$ ; wing area,  $F_{1,44}=32.55$ ,  $R^2=0.41$ ,  $P<0.0001$ ; fresh mass,  $F_{1,45}=28.82$ ,  $R^2=0.37$ ,  $P<0.0001$ ) and the winged morph (tibia size,  $F_{1,49}=80.18$ ,  $R^2=0.61$ ,  $P<0.0001$ ; wing load,  $F_{1,49}=5.68$ ,  $R^2=0.084$ ,  $P=0.021$ ; wing area,  $F_{1,49}=46.65$ ,  $R^2=0.47$ ,  $P<0.0001$ ; fresh mass,  $F_{1,49}=42.86$ ,  $R^2=0.45$ ,  $P<0.0001$ ). Therefore, to account for this size effect, mummy width was used in the regression. Residuals from the regression are depicted for each morph/stage combination (Fig. S2).

Third instars allowed the production of bigger parasitoids, whatever the fitness parameter considered (tibia size:  $F_{1,88}=28.55$ ,  $P<0.0001$ ; wing load:  $F_{1,87}=5.31$ ,  $P=0.024$ ; fresh mass:  $F_{1,88}=24.05$ ,  $P<0.0001$ ; mean wing area:  $F_{1,87}=16.15$ ,  $P=0.00012$ ). Morph did not have any effect on these fitness parameters (tibia size:  $F_{1,88}=1.22$ ,  $P=0.27$ ; wing load:  $F_{1,87}=0.28$ ,  $P=0.60$ ; fresh mass:  $F_{1,88}=0.18$ ,  $P=0.67$ ; mean wing area:  $F_{1,87}=1.92$ ,  $P=0.17$ ) (Table 2; Fig. S2).

As bigger differences were observed for the third instars for all other fitness parameters, we also focused our attention on parasitoids emerging from this stage as far as the egg load and the reproductive effort are concerned. There was no significant effect of morph on any variable related to reproduction (egg load:  $\chi^2_{1,37}=12.02$ ,  $P=0.34$ ; mean egg volume:  $F_{1,37}=0.56$ ,  $P=0.46$ ; reproductive effort:  $F_{1,37}=0.76$ ,  $P=0.39$ ) (Table 2; Figs S3 and S4).

## DISCUSSION

Immature winged aphids do not represent hosts of equal quality compared with immature wingless aphids and this is the case for both aphid instars. Indeed, both winged instars allow the production of heavier and fatter parasitoids (Table 2).

This can be explained by the greater mass of winged instars compared with wingless instars in our case. This morph should accumulate more resources to cope with the variability of food availability during dispersal and to build up reserves for flight (Zera and Harshman, 2001). Indeed, winged individuals are usually more resistant to starvation (Hazell et al., 2005).

Our results showing that winged aphids are heavier than wingless aphids are in contrast with previous studies on other species like *Sitobion avenae* (Castañeda et al., 2010) or *Aphis gossypii* (Shi et al., 2010). Nonetheless, not all studies demonstrate significant differences between winged and wingless individuals for mass. Some studies have produced these two morphs by different methods, which may modify the final results, as is the case, for example, when the difference in fecundity between morphs is considered (Groeters and Dingle, 1989). Studies usually apply a crowding stimulus to induce the production of winged aphids whereas wingless aphids are obtained in isolation (Shi et al., 2010; Xu et al., 2011). However, when the same rearing method is applied to both forms as in our study, winged and wingless individuals of the same stage have a similar mass in *Acyrtosiphon pisum* (Tsumuki et al., 1990) or winged are heavier in *S. avenae* (Newton and Dixon, 1990). A difference in favour of wingless aphids is only found for adults in these species (Castañeda et al., 2010; Newton and Dixon, 1990; Tsumuki et al., 1990).

Moreover, it seems that winged individuals might be heavier than wingless individuals in some species including *M. persicae* (Dixon and Kindlmann, 1999). The general assumption that migrants should be smaller is, in aphids, particularly true for large species (Dixon and Kindlmann, 1999). In *Megoura crassicauda* or *A. gossypii*, winged individuals have longer tibias than wingless ones, which is the opposite of what is found for *A. pisum* (Ishikawa and Miura, 2007; Wall, 1933). This could be linked to the need of one morph compared with another to disperse by walking (Ishikawa and Miura, 2007).

However, the use of clones reared in the laboratory for several years could lead to a reduced response to wing-inducing cues such as the presence of alarm pheromone (Clegg and Barlow, 1982; Thieme and Dixon, 2015). The conditions of rearing would select for individuals that do not fly or those with a lower flying ability as flyers would escape from the cages (Bush et al., 1976; Dabrowski, 1988; Dutton and Bigler, 1995). This could explain why immature winged individuals are heavier than their wingless counterparts if they behave not as migrants but as non-flyers. Those intermediate forms should resemble more the wingless individuals (Shull, 1940).



As stated in the Results section, this clone never produced winged offspring under lower densities (Pirotte, 2011). Further, with *Capsicum annuum* as a host plant, the production of winged offspring can be very rare or continuous according to the plant variety (J.A.-L.M.P. and T.H., personal observation). We therefore do not believe this clone is not responsive to wing-inducing cues but that density is not the proper cue in this non-gregarious species (Müller et al., 2001; Williams et al., 2000). Moreover, different laboratory-reared clones of *Aphis fabae* have been shown to be perfectly capable of flying even if they do so over a shorter period than field-collected clones (Cockbain, 1961). Finally, development in immature winged aphids also has a positive impact on parasitoid lipid content in the field with a field-collected clone (J.A.-L.M.P., L. Ferrais, A. Goldarazena and T.H., unpublished).

The higher parasitoid fitness when developing in winged instars could also be explained by the differences in nutritional quality of the two types of morphs, as observed here in *M. persicae*. The content of all energy resources increases with fresh mass, as larger individuals will accumulate more resources (Cockbain, 1961; Strohm, 2000), and this is the case for winged aphids except for glycogen. Winged aphids therefore represent an interesting source of lipids, proteins and sugars. Host lipids and proteins are needed for the parasitoid to produce eggs (Rivero and Casas, 1999) and parasitoids lack lipogenesis (Giron and Casas, 2003; Strand and Casas, 2008; Visser and Ellers, 2008). *Aphidius colemani* is a synovigenic species that matures eggs throughout its adult life (as for all Braconidae; Jervis et al., 2001) and is not a host-feeder species at the adult stage (Romeis et al., 2003). This means that all the nutrients required for reproduction are obtained during larval development (Romeis et al., 2003). However, adult parasitoids can replenish their sugar reserves by feeding on nectar (Jervis et al., 2008). Moreover, the honeydew of certain aphid species can increase *A. colemani* longevity (Wäckers et al., 2008).

Furthermore, *A. colemani* is known to inhibit wing development in *A. fabae* (Kati and Hardie, 2010). In our system, wing inhibition occurred in young instars (third) but not in older instars (fourth) (Zhang et al., 2009, 2012). When developing in both winged instars, parasitoids show a higher fresh mass and lipid content than in wingless instars. This would mean that wing inhibition does not imply an arrest of lipid accumulation. The difference in dry mass between male and female individuals is not significant when developing in fourth instars. As *A. colemani* is a koinobiont species, the host will continue to feed and grow. Therefore, third instars have more time to develop and grow than fourth instars, meaning more resources are available, whereas in fourth instars, the parasitoid larva will quickly experience the arrest of the growth of its host, as it will become an adult.

Previous studies brought results that are not consistent with ours. In Mackauer (1986), no difference in male parasitoid dry mass was found when winged and wingless hosts were compared. In this case, second instars were used for parasitism. Demmon et al. (2004) used fourth instars, and in their case, parasitoids emerging from winged hosts were smaller and lighter. Both studies used *A. pisum* as a host, in which wingless individuals are heavier than winged ones. Even if this difference in mass is taken into account for Demmon et al. (2004), the development in immature winged individuals had a negative impact on parasitoid size. In our case, the positive effect of morph on tibia size and mean wing area disappeared if mummy width was considered.

There was no strong pattern concerning parasitism rates and emergence rates and no effect of morph on sex ratio. Wing buds do

not seem to impose a constraint on oviposition success. However, in the choice experiment, the emergence rate was higher in winged instars than in wingless instars. It might be that winged individuals develop a lower physiological defence and that this is a counterpart of flight and associated costs (Parker et al., 2017). Alternatively, wingless instars might be more superparasitized than winged instars as they suffer from a higher mortality rate (Bai and Mackauer, 1992; Keinan et al., 2012). We did not strictly control for superparasitism as in Khatri et al. (2017). A previous study showed that for the density of aphids used and the time spent by the female in the confined area, *A. colemani* can perform superparasitism (Khatri, 2017). Superparasitism is observed when hosts are available in low numbers (Chow and Mackauer, 1991; Jones et al., 2003; McBrien and Mackauer, 1990). It may be adaptive as superparasitized aphids show a higher feeding activity compared with aphids parasitized only once (Cloutier and Mackauer, 1980). However, studies report that there was no effect of superparasitism on emergent dry mass whether females or males were laid by a mated female. Only a higher fitness for males laid by virgin females is reported in superparasitized aphids (Bai and Mackauer, 1992; Mackauer and Chau, 2001). All our females were mated; therefore, we think that even if some superparasitism occurred, it did not affect the fitness of our parasitoids. Moreover, defensive behaviours displayed by attacked aphids may hinder superparasitism (Gardner et al., 1984). The hosts used, third to fourth instars, are highly capable of defending themselves compared with younger instars (Gerling et al., 1990; Hofsvang and Hågvar, 1986; Khatri et al., 2016; Walker and Hoy, 2003; Weisser, 1994; Wu et al., 2011). An observation of the parasitoid behaviour and subsequent aphid dissection might help us to see whether such superparasitism is displayed more often on wingless instars than on winged instars.

The flight fuel in *M. persicae* seems to be lipid as in Orthoptera (Clark et al., 2013; Zera and Larsen, 2001) and Lepidoptera (Beenackers et al., 1985). The storage of lipids for flight will be done at the expense of glycogen, supporting reproduction. However, winged individuals might recover resources from the breakdown of wing musculature after flight (Ishikawa and Miura, 2009). Moreover, lipids are interesting as they offer twice as much energy per unit mass as carbohydrates (Clements, 1992).

We demonstrated that winged individuals might be high-quality hosts. In our study, immature winged aphids are bigger and have a higher content of resources except glycogen. Lipids and proteins can be especially interesting from the parasitoid point of view for building eggs. Parasitoids are bigger and fatter in winged instars than in wingless instars. Wing inhibition does not seem to be playing a role in the higher fitness brought by winged individuals as fitness differences are also seen in fourth instars where no wing inhibition occurs. Our study also highlights the importance of using the same rearing method for each phenotype as natural enemies might be faced to the two types of host in the same colony.

We also showed that *A. colemani*, currently used as a biological control agent, is able to parasitize wingless as well as winged hosts at least in the juvenile stages. This is a matter for consideration as winged individuals are implicated in the secondary transmission of plant virus (Williams et al., 2000) and the presence of the virus can increase the proportion of winged offspring (Blua and Perring, 1992; Gildow, 1980; Ryabov et al., 2009). If the parasitism occurs in third instars, the parasitoid will even prevent the aphid from flying, which could in turn reduce virus transmission. In fourth instars, where wing inhibition is less frequent, the wings are present and, apparently, flight is not prevented at least as long as the parasitoid egg does not hatch (Rauwald and Ives, 2001; Zhang et al., 2009; Zhang et al., 2012).

Parasitoids may even use those fourth instars for their own dispersal (Derocles et al., 2014; Feng et al., 2007; Walton et al., 2011). However, when the parasitoid larva grows bigger, host wing muscles may be consumed so that no more flights will be possible even if the wings are unfolded (Rauwald and Ives, 2001).

In conclusion, we showed that wing polyphenism leads to different energy budgets that meet the needs for flight as demonstrated in other wing-polymorphic insects (Zera and Larsen, 2001). This has implications for the individual's nutritional value as a host, which in turn has consequences for parasitoid fitness. Very few studies have been interested in the impact of wing polymorphism on the third trophic level (Demmon et al., 2004; Mackauer, 1986) and they lead to very different conclusions. A more global picture is needed to fully understand how this can alter or improve the efficiency of biological control agents.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: J.A.-L.M.P., V.F., T.H.; Methodology: J.A.-L.M.P., V.F., T.H.; Formal analysis: J.A.-L.M.P., V.F.; Investigation: J.A.-L.M.P., A.L.; Writing - original draft: J.A.-L.M.P.; Writing - review & editing: V.F., T.H.; Supervision: T.H.; Project administration: T.H.; Funding acquisition: T.H.

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#### Supplementary information

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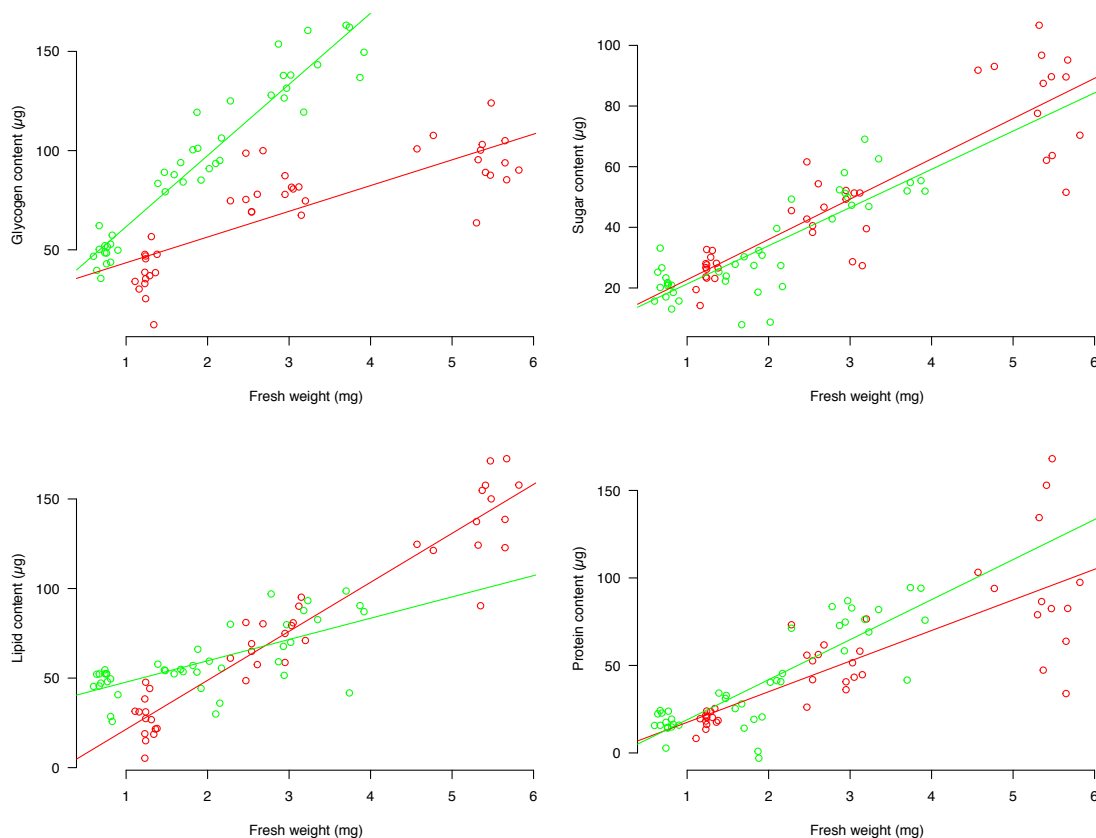
## Supporting information

**Table S1: Density effect on fresh weight, dry weight and lipid content in the no-choice experiment.**

Parameters	Factors of the analysis	p-value
Fresh weight	Sex	<0.0001
	Morph	<0.0001
	Density	ns
	Morph:Density	0.00041
Dry weight	Sex	<0.0001
	Morph	<0.0001
	Density	ns
	Morph:Density	0.00017
Lipid content	Sex	<0.0001
	Morph	<0.0001
	Density	ns
	Morph:Density	0.00016

For females, n=34 in winged morph in high density and n=14 in low density, n=47 in wingless morph in high density and n=8 in low density. For males, n=44, n=12, n=39, n=14 respectively. GLM, ns indicates no significant difference and a p-value above 0.05.

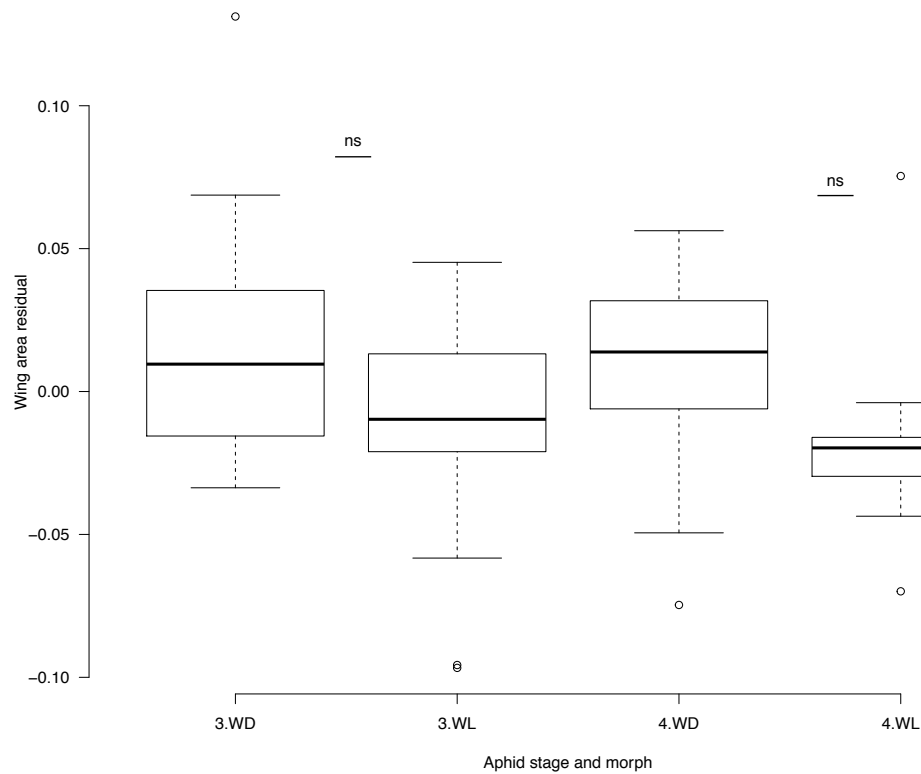
**Figure S1: Linear regressions of energetic compartments (glycogen, sugars, lipids and proteins) ( $\mu\text{g}$ ) along with aphid fresh weight (mg) for wingless (green) and winged (red) aphids.** Each data point corresponds to one biological replicate (15 aphids),  $n=42$  for wingless aphids and  $n=41$  for winged aphids. All energetic compartments correlated with fresh weight for the wingless and the winged morph ( $P<0.0001$ ).



**Table S2: Number of parasitoids used in this study and their distribution among the experimental modalities according to the stage and morph (WD: winged, WL: wingless).**

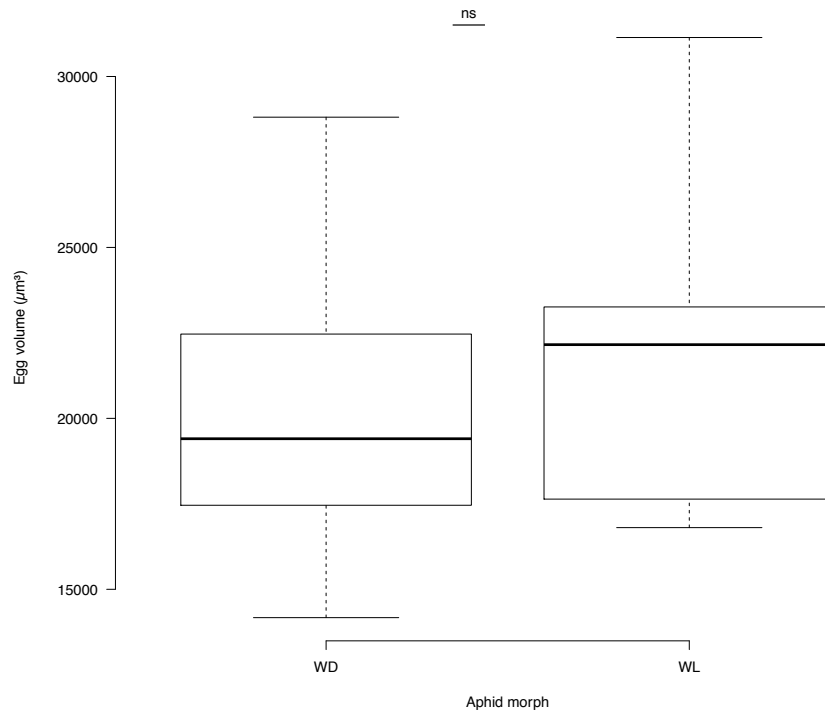
Experiment	Parameter	Aphid morph	Aphid stage	Female	Male
No choice	Fresh weight	WD	Third	48	56
	Dry weight		Fourth	34	33
	Lipid content	WL	Third	55	53
			Fourth	14	16
Choice	Fresh weight	WD	Third	23	-
	Tibia size		Fourth	29	-
	Wing area	WL	Third	25	-
	Wing load		Fourth	15	-
	Egg load	WD	Third	20	-
	Egg volume	WL		19	-

**Fig. S2: Boxplot of residuals for wing area (mm<sup>2</sup>) (regression with mummy size) for female parasitoids emerging from two different aphid stages (3: third, 4: fourth) and aphid morph (WD: winged, WL: wingless). n=23 in 3WD, n=25 in 3WL, n=29 in 4WD and n=15 in 4WL. GLM with post-hoc test (Tukey adjustment).**





**Fig. S3: Boxplot of egg volume ( $\mu\text{m}^3$ ) for female parasitoids emerging from the third aphid stage and from two different aphid morphs (WD: winged, WL: wingless). n=20 in 3WD, n=19 in 3WL. GLM.**



**Fig. S4: Boxplot of reproductive effort for female parasitoids emerging from the third aphid stage and from two different aphid morphs (WD: winged, WL: wingless). n=20 in 3WD, n=19 in 3WL. GLM.**

