

Genetic variation in the behavioural mechanisms involved in the response of the egg parasitoid *Trissolcus brochymenae* to contact chemical cues left by the pest *Murgantia histrionica*

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Abstract. 1. The ability of parasitoid females to perceive chemical traces left by their hosts is of utmost importance in the host location process. The behaviours involved in such ability have thus most likely been promoted by natural selection in the course of the evolutionary time. For this to happen, however, there must be significant genetic variation in natural populations on which natural selection could act.

2. Using the isofemale line method and motion analysis, we detected significant intra-population genetic variation for several walking behaviour traits of the egg parasitoid *Trissolcus brochymenae* (Hymenoptera: Scelionidae) females responding to chemical traces left by its host *Murgantia histrionica* (Heteroptera: Pentatomidae).

3. Besides opening new avenues of research on the reproductive strategies, behaviour, and biological control potential of parasitoid wasps, these results also have implications for understanding their life-history evolution in general.

Key words. Genetic variation, insect egg parasitoids, isofemale lines, response to kairomone, video tracking, walking behaviour.

Introduction

While all insect parasitoid species share a similar life cycle (Clausen, 1940; Jervis & Ferns, 2011), they show a striking degree of diversity in their life histories. At the inter-specific level, detailed comparative studies have provided insights into the selection pressures responsible for trait evolution (Mayhew, 2018). At the intra-specific level, important phenotypic and genotypic variations are also regularly observed (Roush, 1990; Hopper *et al.*, 1993; Wajnberg *et al.*, 2004). Quantifying intra-population genetic variations in parasitoid wasp populations is important since it enables an understanding of how species can evolve in response to environmental selective pressures. This is especially true, for example, when we want to understand what sort of optimised behavioural reproductive

strategies can be adopted by parasitoid females under different environmental conditions (Wajnberg *et al.*, 2008, 2016). Also, provided that the important biological traits for wasp species to be efficient biocontrol agents are known, quantifying genetic variation can aid in developing a selection programme to improve their pest control efficacy (Wajnberg *et al.*, 2004; Lommen *et al.*, 2017).

While searching for hosts to attack, parasitoid females are known to use visual, vibrational, and chemical cues coming from their hosts and/or from the plant in the case of herbivorous hosts (Vinson, 1976). Especially when using chemical cues, parasitoids have to solve a so-called “reliability-detectability problem” (Vet & Dicke, 1992).

The harlequin bug, *Murgantia histrionica* (Hahn) (Hemiptera: Pentatomidae), is an important pest of cole crops in the United States (McPherson, 2018). Adults and nymphs actively feed on aboveground plant tissues, mainly leaves and stems. Feeding injuries can reduce the quality and the yield, and, if damage is severe, plants can be destroyed in a short period of time. One of the methods used to control this pest involves the release of

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biological control agents such as the egg parasitoid *Trissolcus brochymenae* (Ashmead) (Hymenoptera, Scelionidae), which can parasitise up to 45% of *M. histrionica* eggs in a patch (Capinera, 2001).

During the host location process, the parasitoid is known to use chemical footprints, that are cuticular hydrocarbons, left by adult *M. histrionica* on the substrate (kairomones), which represent indirect host-related cues (Conti *et al.*, 2003). Usually, upon recognizing such chemical traces left by their hosts walking on the plant, scelionid females display a drastic change in their walking behaviour associated with an intense antennal drumming behaviour, corresponding to a motivated searching with variation in walking speed and turning frequency (Colazza *et al.*, 2009). Using host chemical cues provided by footprints, parasitoids are able to identify both the instar and the gender of the host that deposited them (Conti *et al.*, 2003; Peri *et al.*, 2016) as well as whether the host female already laid eggs or not (Salerno *et al.*, 2009). *Trissolcus brochymenae* adopts such a motivated host searching pattern so the species appears to be a good model to study the host location process through an accurate analysis of its walking pattern upon searching for hosts.

The ability of *T. brochymenae* females to respond to chemical cues left by their hosts is of utmost importance for them to locate host eggs for their developing offspring. For this to happen, the wasps' traits involved in host location must have been genetically variable enough that natural selection could act (Boulétreau, 1986). Also, since *T. brochymenae* is cosmopolitan and highly generalist (Capinera, 2001; McPherson, 2018), it has most likely to cope with environmental variations coming from a number of potential host species, and the species should thus likely demonstrate important phenotypic variation in their host location behaviours, including in the traits involved in their walking patterns upon discovering chemical traces left by their hosts.

In this work, the intra-population genetic variation in the walking pattern of *T. brochymenae* females upon discovering chemical footprints left by adult *M. histrionica* was quantified using the isofemale lines method (Parsons, 1980; Falconer & Mackay, 1981; Hoffmann & Parsons, 1988; Wajnberg, 2004). Briefly, this method is based on the statistical comparison of different families, each originating from a single mated female taken randomly from the population analysed.

Results demonstrated a significant intra-population genetic variation in the walking behaviours adopted by *T. brochymenae* females upon encountering chemical traces left by their hosts. Such a genetic variation can be the target of selection pressures coming from the environment leading females of this parasitoid species to progressively increase their ability to detect the presence of hosts and to produce progeny.

Materials and methods

Insect rearing

Murgantia histrionica was originally collected from cabbage in Beltsville, USA in 2000. The breeding population was reared inside plastic containers (30 × 19.5 × 12.5 cm) with 5 cm diameter mesh-covered holes for ventilation placed in

climate-controlled rooms (25°C ± 1°C, 60 ± 5% RH, and L:D 16:8). Separate containers were used for nymphs and adults. All stages were fed with cabbage leaves (*Brassica oleracea* var. *capitata*). Inside the cages with adult bugs, paper towels were placed as an oviposition substrate. Egg masses were collected daily and were used to maintain both parasitoid and host breeding populations as well as to carry out experiments. The breeding line of *T. brochymenae* was established from around 80 adults emerged from *M. histrionica* eggs naturally laid on *Cleomella arborea* (Nutt.) Roalson & J.C. Hall in San Diego, California, near Border Field State Park, in 2000. The breeding population was reared on eggs of *M. histrionica* glued to paper strips. Adult wasps were held in 85 ml glass tubes (24 ± 1°C, 55% RH., and L:D 16:8) and provided with honey-water solution (80:20 v/v). Upon emergence, males and females were kept together to allow mating.

Isolation and rearing of isofemale lines

Twenty-one mated *T. brochymenae* females taken at random from the mass-reared population were offered one-day-old *M. histrionica* egg masses (about 10–12 eggs) in 16 ml vials with a drop of honey-water solution to establish 21 isofemale lines. Due to the low number of eggs parasitised per female per day, and in order to produce a sufficient number of daughters in each family, seven days later another host egg mass was offered to each female. An average of 20.19 ± 1.64 (± SE) daughters were produced per family at the F1 generation. These daughters were used in the behavioural test described below, leading to a total of 424 females measured. All newly hatched wasps used for the bioassays were kept together and tested when they were 2–5 days old and naïve to previous contact with host chemical residues. All replicates were randomly distributed over the full experimental period.

Extraction of host insect contact chemicals and setup of arena

Before preparing the extract, newly emerged *M. histrionica* females were individually isolated after mating for two days so that individuals that did not oviposit prior to extraction were of known age. The extract was prepared by submerging three randomly chosen mated females, which had not yet laid eggs, in 1 ml of hexane (Sigma Aldrich ±97% purity) (Colazza *et al.*, 2007; Lo Giudice *et al.*, 2011) in a 15 ml glass vial for 30 min. After removal of the insect bodies, the resulting extract was stored in small vials, closed with Parafilm® and stored at 4°C. The process was repeated several times using new females each time and the different extracts were pooled in order to reduce potential between-female variation in host kairomonal cues.

Behavioural tests were conducted in an open arena consisting of a squared sheet of filter paper (20 × 20 cm; wasp/arena surface ratio: 0.002%). In the middle of each arena, a circular area (6 cm diameter; 7.1% of the total area; wasp/circular area surface ratio: 0.07%) was treated with 100 µl extract. The extract was applied as drops, leaving the surrounding area untreated.

A single female wasp was gently released onto the centre of the treated circular area. Soon after being released, females displayed a characteristic motivated host searching response, *i.e.*, a slower walking behaviour associated to a higher turning tendency with antennae held in contact with the arena surface. Wasps that did not show such response were captured and not tested again. One filter paper was used for sequentially testing two wasps since it has been shown that wasps are not influenced by residues left by previous walking females (Colazza *et al.*, 2007). Only one female was tested if its response lasted more than 10 min.

Setup of the video tracking and the parameters to be tracked/measured

The wasp's movement over the entire arena was recorded by a CCD video camera (Sony SSC M370 CE) fitted with a 12.5–75 mm F⁻¹ 1.8 zoom lens. The analogue signal from the camera was digitised by a video grabber. Digitised data were analysed with Xbug, a video tracking and motion analysis system (Peri *et al.*, 2011). Female behaviours were recorded until they flew away from or walked off the arena. All experiments were conducted between 09.00 h and 13.00 h, in an isolated room at 25 ± 1°C, in an arena illuminated by two 22-W cool white fluorescent tubes (Full spectrum 5900 K, 11 W; Lival, Italy).

After behavioural observations, females were collected and stored in 70% ethanol at 4°C. Soon after, a fine scissor was used to clip off one leg at the hind tibia, which was measured under an optical microscope (Zeiss SteREO Discovery.V12) and using AxioVision SE64 Rel. 4.9.1 software for image acquisition and analysis. The length of the tibia was measured to an accuracy of 0.01 µm. The measurement served as a proxy for adult size (Mills & Kuhlmann, 2000).

Using the video tracking and motion analysis system, walking parameters were defined and used to quantify the characteristic motivated host searching response adopted by the parasitoid females upon encountering chemical traces produced by host females. By definition, these parameters should quantify changes in the females' behaviour by computing differences between their activity inside and outside the treated area. We thus defined the three following parameters: (1) the total residence time in the area treated with chemical extract, (2) the total residence time outside the treated area minus the residence time in the treated area, and (3) the linear walking speed of the females outside the treated area minus the linear walking speed in the treated area. The first parameter describes the overall interest of the females to remain in the treated area. The second parameter gives similar information but takes into account the overall walking activity of the females, estimated by the time she is searching for hosts outside of the treated area, *i.e.*, without chemical cues indicating the host's presence. The last parameter provides an overall information regarding changes in the host searching strategy adopted by the females upon encountering chemical traces left by potential hosts. The stronger the intensity of the motivated host searching response of the females, the higher the values of the total residence in the treated area, and

the difference between the linear walking speed outside and on the treated area (parameters 1 and 3), and the lower the difference between the value of the total residence time outside and on the treated area (parameter 2).

Data handling and statistics

Different experimental protocols can be used to quantify the intra-population genetic variation in quantitative phenotypic traits. We used the so-called family analysis or isofemale lines method (Parsons, 1980; Falconer & Mackay, 1981; Hoffmann & Parsons, 1988), that has been successfully applied to quantify the genetic variation in several behavioural and life-history parameters in parasitic wasps (Wajnberg *et al.*, 1989, 1999, 2004, 2012; Bruins *et al.*, 1994; Pérez-Maluf *et al.*, 1998).

The walking behaviour of parasitoid females is likely linked to their size. Hence, recorded genetic variation in the walking parameters described above might be solely the result of genetic variation in female body size. In order to circumvent this problem, as this was done in Wajnberg *et al.* (2012), we corrected all recorded walking parameters by removing the effect of female size using the linear regression of each measured parameter *vs.* females' tibia length. The correction involved subtracting their predicted values obtained from the linear regressions from the measured walking parameters. After such a correction, there no longer exists, by definition, a relationship between each of the walking traits quantified and female body size. Hence, the walking parameters computed, post-correction, were for females having an average size. Therefore, any observed significant genetic variation in the walking behaviour of the measured females that was evident post-correction could not have been the result of a significant genetic variation in their body size (Wajnberg *et al.*, 2012).

The walking parameters measured, both before and after the correction explained above, were considered to follow normal distributions. Therefore, for each of the walking parameters, the comparison of the averages computed for each family was performed using standard one-way ANOVAs and correlations between parameters were computed using standard Pearson correlation coefficients.

Results

Across all 21 families, females remained an average of 245.58 s (SE ± 8.07) (a bit more than 4 min) looking for hosts on the area treated with extract of host females (Fig. 1a). The difference between female residence time outside the treated area and the residence time in the treated area was on average – 180.90 (± 7.36) sec (Fig. 1b), indicating the females spent actually most of their time on the treated area (almost 75% of their time). Finally, while on the treated area, females reduced their walking linear speed by on average 24.34 (± 0.55) mm s⁻¹. Together, these results indicate that *T. brochymenae* females globally demonstrated a characteristic motivated host searching response upon encountering chemical traces of their hosts.

We found strongly significant variation in all three parameters between the different families (Table 1). Such results strongly

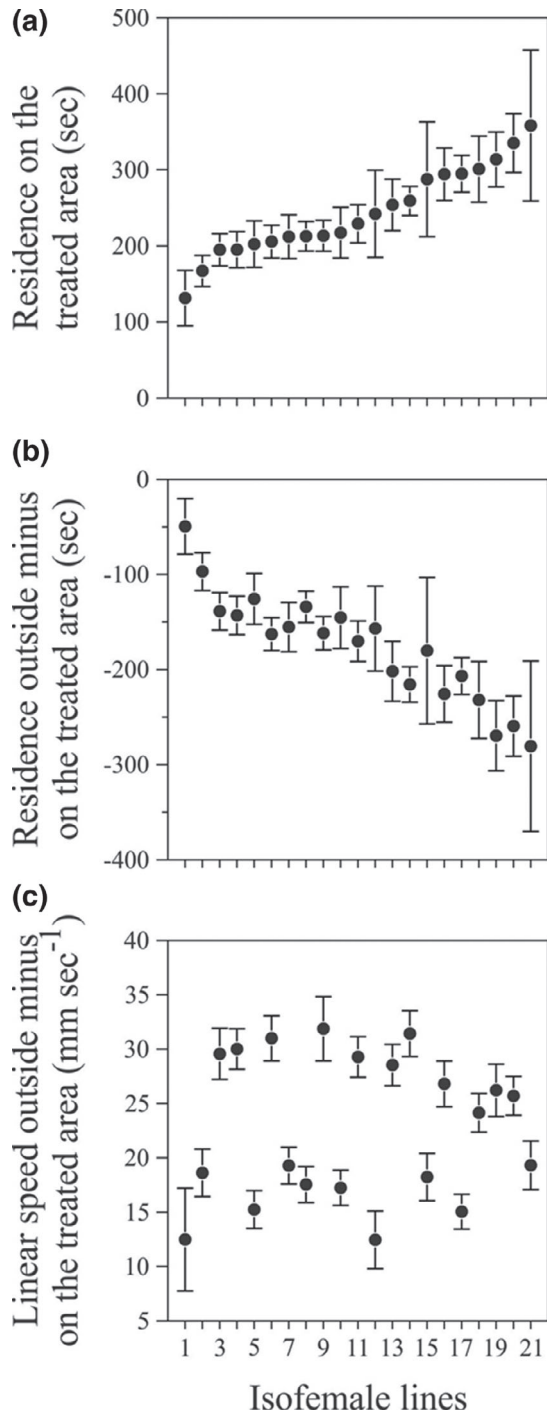


Fig. 1. Average (\pm SE) values of the three parameters used to describe the motivated host searching responses of *Trissolcus brochymenae* belonging to 21 isofemale lines. (a) total residence time in the area treated with chemical extract, (b) residence time outside the treated area minus the resident time in the treated area, (c) linear walking speed of the females outside the treated area minus the linear walking speed in the treated area. On the three graphs, lines are ordered according to increasing average values of the residence time on the treated area. Statistical comparison between the lines in each case are provided table 1.

indicate that, in the *T. brochymenae* population we examined, there is a significant genetic component to the phenotypic variability we observed in the behavioural traits describing the overall motivated host searching response of the wasp females upon encountering chemical traces of their hosts. Within the same parasitoid population, some genotypes are associated with females of some families having average values almost three times higher than others (see Fig. 1).

While the first two parameters appeared to be significantly negatively correlated (Pearson correlation = -0.944 , $P < 0.001$) indicating that the longer the female spent on the treated area, the lower the time they spent outside this area, there was no significant correlation between these two parameters and the third parameter that quantifies the reduction in female walking speed upon entering the treated area (correlation = -0.094 and -0.059 , $P = 0.055$ and $P = 0.221$, respectively). Residence time and walking velocity of the foraging females thus appear to be two independent components of their host searching strategy that are both under a significant genetic variation.

Discussion

In the present work, using a video tracking of the walking behaviour of *T. brochymenae* females in the presence of chemical traces left by *M. histrionica* adult females, we were able to carefully describe how parasitoid females responded to such chemical signals, as this was already demonstrated by Conti *et al.* (2003) and Peri *et al.* (2013). Upon discovering extracts of chemical traces produced by their hosts, females show a characteristic host searching response, with a slower walking behaviour with antennae held in contact with the treated surface.

We observed phenotypic variation among all females tested, and, using the isofemale lines method (Parsons, 1980; Falconer & Mackay, 1981; Hoffmann & Parsons, 1988), we were also able to identify strong variation among isofemale families for all the descriptive parameters recorded on the *T. brochymenae* females. Such inter-family variation suggests that an important part of the phenotypic variation we observed for all the parameters are genetically determined in the *T. brochymenae* population we worked on. This result indicates that, within the population studied, some genotypes result in females remaining longer in the presence of host cues, and/or walking more slowly than others.

The ability of the parasitoid females to respond adequately to the chemical traces could be the result of different mechanisms, including: (1) the number and efficacy of chemoreceptors located on the antennal parts, on the ovipositor, and on the legs of the females (Le Ralec *et al.*, 1996), that they use to recognise chemical compounds in their environment. In particular, the sensilla chaetica present in *T. brochymenae* antennae that allow wasps to perceive the contact semiochemicals associated with residues left by walking host bugs (Iacovone *et al.*, 2016); (2) the intensity of the associated locomotory responses (*e.g.*, walking more or less rapidly upon detecting a chemical cue); and (3) the existence of habituation leading the response to gradually decrease in the course of time (Colazza *et al.*, 1999). The fact that the phenotypic variation observed

Table 1. Results of one-way ANOVAs testing the inter-family variation in the three different walking parameters used to quantify the motivated host searching response adopted by *Trissolcus brochymenae* females upon encountering chemical traces produced by *Murgantia histrionica* host females.

Parameter	Source of variation	df	Mean square	F-value
Residence time in the treated area	Between families	20	53 138.77	2.020**
	Within families	403	26 307.44	
	Total	423	27 576.06	
Residence time outside minus in the treated area	Between families	20	47 895.07	2.206**
	Within families	403	21 708.00	
	Total	423	22 946.16	
Linear speed outside minus in the treated area	Between families	20	774.05	8.136***
	Within families	403	95.14	
	Total	423	127.24	

** $P < 0.01$.*** $P < 0.001$.

appears to be genetically determined could be the consequence of the existence of a genetic variation only in one or in several of these different biological mechanisms, or even in the interaction between them. Irrespective of the biological mechanisms involved, the observed genetic variation is likely characteristic of a polyphagous species like *T. brochymenae*, which is known to attack several host species showing almost certainly strong qualitative and quantitative differences in the chemical traces they deposit of the plant they attack.

The genetic variation observed is most likely the target of natural selective pressures from the environment of the parasitoid females, resulting in increased efficiency in finding hosts. Such genetic variation can also be the starting point for performing breeding selection with the aim to improve the efficacy of *T. brochymenae* females to control *M. histrionica* in biological control programmes (Wajnberg *et al.*, 2004; Lommen *et al.*, 2017). However, in what direction should the traits quantified here be selected to eventually improve the pest control efficacy of the parasitoid females once they are released in a biological control programmes? As a general rule, identifying the biological attributes efficient biocontrol agents should have always been an open question that is still unresolved (Roush, 1990; Wajnberg *et al.*, 2004; Le Hesran *et al.*, 2019). Like many parasitoid species, *T. brochymenae* females are likely time-limited, having potentially a high number of eggs to lay but a limited overall lifetime duration to find all the hosts to lay them. Also, *M. histrionica* females usually lay small egg masses (about 10–12 eggs), dispersed over the plants attacked (McPherson, 2018). Hence, upon encountering chemical traces deposited by their hosts, female parasitoids should likely not spend too much time before discovering a host mass, and should rapidly leave when a host mass has been discovered and exploited, leaving other opportunities to find other hosts later in their life. This would indicate that breeding selection programmes on the behavioural traits quantified in this study should target a lower residence time of the females on the chemical traces they discovered, with a higher walking speed. As far as we know, the work presented in the present paper is the first one quantifying the genetic variation of the response of females of a parasitic wasp to chemical traces left by their hosts. Additional experiments under natural conditions will be needed to verify what would be the best breeding selection strategy to adopt in this case.

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Contribution authors

All authors conceived the ideas and contributed critically to the drafts and gave their approval to the manuscript submitted for publication. MS and MCF collected the data. EW analysed the data and led the writing of the manuscript.

Data availability statement

Data available on request from the authors, i.e., The data that support the findings of this study are available from the corresponding author upon reasonable request.

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