PLOS ONE

RESEARCH ARTICLE

Innate Host Habitat Preference in the Parasitoid *Diachasmimorpha longicaudata*: Functional Significance and Modifications through Learning

Diego F. Segura[1,2]*, Ana L. Nussenbaum[1,2], Mariana M. Viscarret[3], Francisco Devescovi[1,2], Guillermo E. Bachmann[1,2], Juan C. Corley[4], Sergio M. Ovruski[5], Jorge L. Cladera[1]

1 Laboratorio de Genética de Insectos de Importancia Económica, IGEAF, CICVyA, INTA, Hurlingham, Argentina, **2** Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina, **3** Insectario de Investigaciones para Lucha Biológica, IMyZA, CICVyA, INTA, Hurlingham, Argentina, **4** Grupo de Ecología de Poblaciones de Insectos. INTA EEA Bariloche, San Carlos de Bariloche, Argentina, **5** PROIMI Biotecnología, CCT Tucumán CONICET, División Control Biológico de Plagas, San Miguel de Tucumán, Argentina

* segura.diego@inta.gob.ar

OPEN ACCESS

Citation: Segura DF, Nussenbaum AL, Viscarret MM, Devescovi F, Bachmann GE, Corley JC, et al. (2016) Innate Host Habitat Preference in the Parasitoid *Diachasmimorpha longicaudata*: Functional Significance and Modifications through Learning. PLoS ONE 11(3): e0152222. doi:10.1371/journal.pone.0152222

Editor: Cesar Rodriguez-Saona, Rutgers University, UNITED STATES

Received: November 18, 2015

Accepted: March 10, 2016

Published: March 23, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The work was supported by the following: Fondo para la Investigación Científica y Tecnológica, PICT 2010-1324 (http://www.agencia.mincyt.gob.ar/frontend/agencia/fondo/foncyt); and CONSEJO NACIONAL DE INVESTIGACIONES CIENTIFICAS Y TECNICAS, PIP 2011–2013 (www.conicet.gov.ar).

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Parasitoids searching for polyphagous herbivores can find their hosts in a variety of habitats. Under this scenario, chemical cues from the host habitat (not related to the host) represent poor indicators of host location. Hence, it is unlikely that naïve females show a strong response to host habitat cues, which would become important only if the parasitoids learn to associate such cues to the host presence. This concept does not consider that habitats can vary in profitability or host nutritional quality, which according to the optimal foraging theory and the preference-performance hypothesis (respectively) could shape the way in which parasitoids make use of chemical cues from the host habitat. We assessed innate preference in the fruit fly parasitoid *Diachasmimorpha longicaudata* among chemical cues from four host habitats (apple, fig, orange and peach) using a Y-tube olfactometer. Contrary to what was predicted, we found a hierarchic pattern of preference. The parasitism rate realized on these fruit species and the weight of the host correlates positively, to some extent, with the preference pattern, whereas preference did not correlate with survival and fecundity of the progeny. As expected for a parasitoid foraging for generalist hosts, habitat preference changed markedly depending on their previous experience and the abundance of hosts. These findings suggest that the pattern of preference for host habitats is attributable to differences in encounter rate and host quality. Host habitat preference seems to be, however, quite plastic and easily modified according to the information obtained during foraging.

Introduction

Host plants are a major source of information for insect parasitoids during host searching [1–3], even when they are not infested [4–6]. The damage caused by the herbivore can induce the plants to release specific compounds (termed herbivore-induced plant volatiles) that increase the attraction of parasitoids [7–9]. Communication between the first and third trophic level is expected to be particularly enhanced when the herbivores are concealed within the host plant, thus reducing to a minimum the available information about their location [10].

The type of chemical cues used by parasitoids during host searching has been related by Vet and Dicke [11] to the range of plant species where the host can be found. When the hosts are generalists, female parasitoids should rely on general cues, common to all possible host plant species, and would follow specific cues only after they acquire experience and associate conspicuous cues (mainly from the plant) to the host presence, through associative learning [12–15]. However, Steidle and van Loon [16] found many examples of parasitoids of generalist hosts that innately respond towards chemical cues from the host habitat. Vet and Dicke's [11] concept does not consider that different host plants species may differ in their profitability (host density and/or encounter rate) as well as in the quality of the hosts they harbour [17–19]. In fact, if host availability differs among host plants, optimal foraging theory [20,21] predicts that females should always prefer the most profitable host plant. Therefore, an innate response towards chemical cues could have evolved from some host-plant systems if the reward in terms of cumulative fitness varies among them [22]. Likewise, if the nutritional quality of the herbivore host varies among plant species, natural selection should favour female parasitoids that are attracted to plants in which the hosts are nutritionally better [23–26], a concept known as the preference–performance hypothesis (PPH) [27,28]. PPH has been widely studied in bi-trophic scenarios: host plant-herbivore (see Gripenberg et al. [28] and references herein) and parasitoid-herbivore host [29–31].

Previous studies have shown that the plant species on which the herbivores develop influences parasitoid's offspring performance [32–35]. Furthermore, there are robust evidences that host plant species affect the second and third trophic levels simultaneously [36–41]. Concurrently, several studies on parasitoids reported a preference for particular host habitats [42–44]. Nonetheless, there is a limited number of studies that simultaneously addressed preference and performance considering the first and third trophic level [45], and are mainly focused on parasitoids associated to Brassicaceae species [45–47] or to Tephritidae fruit fly species [48,49].

Diachasmimorpha longicaudata (Ashmead) is a koinobiont endoparasitoid of Tephritidae larvae. In its habitat of origin, *D. longicaudata* parasitizes larvae of several species of the genus *Bactrocera* [50,51], while they are feeding inside the fruit. Most of its hosts are polyphagous (for instance *Bactrocera dorsalis* Hendel attacks more than 150 fruit species), attacking several unrelated families of plants [51,52], including (Caricaceae, Moraceae, Myrtaceae, Rosaceae, and Solaneaceae, among the most important). Because larvae develop entirely in the same fruit, fruit species may be a reliable indicator of the quality of the larvae as hosts. Fruit species could also vary in the host encounter or parasitization rates, so the costs (energy and time allocated to host searching) would differ among host fruits [30]. These differences in potential rewards could have led to host fruit preference. In fact, innate preference for fruit species has been reported for this species [53,54]. Nonetheless, the evolutionary forces that forged these preference patterns have seldom been addressed because [as stated by 45] preference and performance associated to different host habitats have been studied separately [33,55,56] except for Eben et al. [48] and Ovruski et al. [57] who found no preference for fruit species that differentially affected female performance.

Parasitoids of polyphagous hosts, such as *D. longicaudata*, should profit from adjusting their foraging preferences to the distribution of their hosts. Associative learning has been

documented for fruit fly parasitoids [58,59], including *D. longicaudata* [60], however its role in modulating the host habitat preference has not been addressed. Likewise, the fact that the density of host larvae varies among different fruits [61,62] has not been considered.

In this work, we addressed the innate preference of *D. longicaudata* females among odours from four host habitats and, when a preference pattern was found, we tested both the PPH and the potential benefits in terms of parasitization rate. We also tested the hypothesis that *D. longicaudata* is able to adjust host habitat preferences as a function of the density of hosts in the available habitats. Finally, because *D. longicaudata* is capable of associative learning during host finding [60], we hypothesized that the preference for chemical cues of available fruit species is modified by experience to the point where innate preference will no longer drive parasitoid behaviour.

Methods

Insects and fruits

Parasitoids and fruit fly larvae were obtained from the rearing facility at Instituto de Genética "E. A. Favret" (IGEAF) [63]. *Diachasmimorpha longicaudata* colony was initiated with individuals coming from Centro de Investigaciones para la Regulación de Poblaciones de Organismos Nocivos (CIRPON), Argentina [64] in 2001. *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) larvae were used as host. Larvae were reared on artificial rearing medium (a mixture of carrot, sugar, brewer's yeast, corn flour, and food preservatives, according to Terán [65]) and were exposed to parasitoid females in small Petri dishes when they reached the third instar (following Viscarret et al. [63]). Assays were carried out with 5–7 days-old females with no oviposition experience (except in experiment 3). Females were maintained with males under controlled conditions (25 ± 1°C, 65 ± 5% R.H., and 14:10 L:D photoperiod), and were provided with honey and water ad libitum. Females had no contact with fruit or fruit odours until the test. Fruit showing no signs of insect infestation were obtained from the local market. All fruit were thoroughly washed with tap water, measured and maintained at 25 ± 1°C and 50 ± 5% R. H. until the tests.

Y-tube olfactometer

The olfactory preferences of *D. longicaudata* were tested using a Y-tube glass olfactometer, in which the females cannot see the fruit and they only perceive chemical cues. The air flow inside the Y-tube was generated by extracting air with a pump (AIR CADET Barnat, USA) connected to the end of the device. The air entered the system through a pair flowmeters (Bruno Schilling, Argentina) that were set at 300 ml/min (0.014 m/s). After entering the device the air was filtered (with glass wool and activated charcoal) and then bubbled in distilled water. Subsequently, the air stream entered two acrylic boxes (3.375 L) where the odour sources were held. Acrylic boxes were connected to the Y-tube which consisted in a two 15 cm-long arms (60° angle between arms, internal diameter 3cm) converging in a 15 cm-long central tube. The distal extreme of the central tube has a hole to introduce the insect. All parts were connected using colourless and odourless plunges and tubing (Dow Corning, Midland, Michigan, USA). The Y-tube assembly was illuminated by a fluorescent light tubes that provided a homogenous illumination of 1200 ± 100 luxes. The room was maintained at 26 ± 1°C and 70 ± 5% R.H.

Females were individually released inside the tube and only after they showed signs of acclimatization (i.e., exhibited host searching behaviours such as antennation) the air pump was turned on. Insects were given 10 min to choose an arm in the olfactometer. It was considered that a female made a choice when it walked into one of the arms, surpassed a distance of 4 cm from the end of the central tube and stayed beyond that limit for more than 30 s. The chosen

arm and the latency (time since the release to the decision) were recorded. If no choice was made in 10 min, the assay was concluded.

Every five tested females, the Y-tube and the acrylic boxes were cleaned with hot water, rinsed with ethanol, and left to air dry at room temperature. With each new experimental series of five females, the location of the odour sources was switched in the opposite arrangement. Forty females were evaluated for each treatment.

Experiments

Experiment 1. Preference of *D. longicaudata* females for different fruit species. Four fruit species were tested: peach (*Prunus persica* L., variety Elegant Lady), fig (*Ficus carica* L., variety Brown Turkey), orange [*Citrus sinensis* (L.) Osbeck, variety Valencia], and apple (*Malus domestica* Borkh, variety Red Delicious). These species represent three of the most important taxonomic families where *Bactrocera* larvae can be found in nature [52]. Fruit were offered to female parasitoids in every possible pair-wise combination. Forty females (replicates) were analysed per combination. Each acrylic box contained one apple, one peach, one orange or three figs. This way the amount of surface of each fruit was as similar as possible.

Experiment 2. Effect of the fruit infestation level on females' preference for host habitats. For this set of experiments, apple and orange were selected as study models and female choice was evaluated in four different scenarios: a) orange with high infestation vs. orange with low infestation; b) apple with high infestation vs. apple with low infestation; c) orange with high infestation vs. apple with low infestation; and d) apple with high infestation vs. orange with low infestation. Forty females (replicates) were analysed per combination. This pair of fruits was used as model based on the result of experiment 1 and the fact that are available in the Argentinean market throughout the year (opposite to fig and peach which are available for 1 and 3 months, respectively).

Infested fruit were obtained by placing 60 *C. capitata* females inside a rearing cage (64 L) with four fruit for 1 hour. After infestation, fruit was placed individually in plastic containers (1 L) and kept under controlled conditions (25 ± 1°C; 40 ± 5% R.H.). To estimate the infestation level without damaging the fruit, the number of larvae that exited the fruit was recorded the day after the first emerging larvae were detected. If one or two larvae exited the fruit within this 24h time frame, the fruit was assigned to the low infestation level; whereas if 5–10 larvae were recorded the fruit was assigned to the high infestation level. After the assay, the fruit were labelled and kept individually until all larvae emerged and could be counted. Highly infested fruits should have had 30–40 inside at the time of the experiment, whereas fruits with low infestation should have had 5–10 larvae. This procedure allowed obtaining infestation levels that resembled low and high infestation in nature. [66]. Those experimental series (i.e., five females) for which the infestation estimation method was not effective for predicting larval abundance, were removed from the dataset.

Experiment 3. Effect of previous experience on females' preference for host habitats. Host habitat preference was assessed using females that had been offered host larvae associated to a specific habitat during a conditioning period. Again, orange and apple were used as experimental models. Conditioning protocol followed Segura et al. [60], which proved effective to condition *D. longicaudata* females to a visual stimulus. During the conditioning period, 15 females were offered a small Petri dish (5 cm in diameter, 1 cm high, wrapped in *voile* fabric) containing 100 larvae of *C. capitata* immersed in orange or apple pulp for 6h. Exposed larvae were kept under controlled conditions (25 ± 1°C; 65 ± 5% R.H.) until adult emergence as to check that females had successfully parasitized them during the conditioning period. The procedure was repeated for three consecutive days. On the fourth day, conditioned females were

used in olfactometry assays where preference for orange or apple was assessed. Forty females (replicates) were analysed per combination.

Experiment 4. Quality of different fruit species as foraging substrates. In order to compare the foraging efficiency of *D. longicaudata* in different host habitats, infested oranges, apples, figs and peaches were exposed to naïve parasitoids. Infestation was achieved as described in experiment 2 (except for fig, in which 12 fruit were exposed to 60 *C. capitata* females). Fruit was then measured and placed in individual containers. When the 3rd larval instar was reached, fruit were individually exposed to *D. longicaudata* females for 6 h. Oranges, apples and peaches were exposed to 5 female parasitoids, whereas figs were exposed to 3 female parasitoids. This proportion allowed standardizing the area offered to the females (ca. 25 cm^2/female) among different fruit species. 120 replicates were carried out for each fruit species.

After exposure to parasitoids, fruit were placed in individual containers and kept under controlled conditions (25 ± 1°C; 40 ± 5% R.H.). Pupae were collected, counted, individually weighed to the nearest 0.1 mg using a precision scale (Denver Instrument, NY, USA). Afterwards, pupae were transferred to glass containers and maintained under controlled conditions (26 ± 1°C; 70 ± 10% R.H.) until adult (parasitoids or flies) emergence. The number of flies and parasitoids was recorded and the non-emerged pupae were dissected under a stereomicroscope to determine if the content corresponded to a fly or a parasitoid.

Experiment 5. Quality of host larvae reared on different fruit species. Several bionomic parameters were evaluated on parasitoids emerging from experiment 4. To this end, a female and a male parasitoid emerged from larvae developed in the same fruit species were placed in glass flaks (500 ml) with water and honey, and kept at 22 ± 1°C, 60 ± 5% R.H. and a 14:10 L:D photoperiod.

The mortality was recorded daily. Whenever a male died, it was replaced by another male to ensure continuous availability of sperm. One week after female emergence, 70 3rd instar *C. capitata* larvae, confined in small Petri dishes, were exposed to females for 6h. For each female, 9 larval exposures were performed every 48–72 h covering the period of higher fecundity [63]. Exposed larvae were transferred to a vial containing fresh artificial larval medium which in turn was placed inside a larger container with a layer of vermiculite and kept under controlled conditions (25 ± 1°C; 70 ± 5% R.H.). Every 48h, pupae were collected and counted, and then placed in glass flasks (450 ml). The number of emerged flies and parasitoids (males and females) was recorded. Non emerged pupae were dissected under a stereomicroscope to determine if the content corresponded to a fly or a parasitoid. For apple and orange, 40 couples (replicates) were evaluated, whereas 36 and 30 replicates were carried out for fig and peach, respectively.

Dead females were preserved in ethanol 70%. After all female died, a random sample was taken from each group (28, 32, 18 and 15 for apple, orange, fig and peach respectively) and the right front wing of each female was dissected and placed on a slide over a thin layer of silicone. Wings were photographed under a stereomicroscope (Motic Group Co., China) and the length of the wing was measured afterwards using Motic Images Plus 2.0 software (Motic Group Co., China).

Data analysis

In experiments 1 to 3, a G-test of goodness of fit (with Yates' correction for continuity) was used to compare the frequency of insects visiting each option. Latency was compared between options by means of a Student *t*-test (assumptions were met in all cases).

In experiment 4, parasitism rate was estimated as: (total number of parasitoids / total number of recovered pupae) x 100. For this rate, emerged and non-emerged insects (flies and

parasitoids) were considered. This variable was compared by means of a one-way ANOVA, followed by a post hoc Tukey's multiple comparisons test. As to address whether the different fruit species offered the same possibility of finding host larvae, the number of pupae recovered per fruit per unit area was compared by means of a one-way ANOVA and a post hoc Tukey's multiple comparisons test after the natural log transformation.

In experiment 5, the mean fecundity (number of offspring per female in each exposition), the total number of offspring per female (lifetime fecundity), the sex ratio (number of females in the progeny/ total number of offspring) and female right wing length were compared by means of a one-way ANOVA, followed by a post hoc Tukey's multiple comparisons test. Sex ratio was previously transformed to logit to meet ANOVA assumptions. The weight of *C. capitata* pupae recovered from different fruit species was compared through the estimation of the mean and the confidence intervals. For this variable, severe heteroscedasticity impeded to run an ANOVA or even a Kruskal-Wallis test, as no transformation allowed reducing differences in variance among treatments. Heteroscedasticity was due to a significantly larger variation in weight of pupae recovered from fig, so an exploratory ANOVA (followed by a post hoc Tukey's multiple comparisons test) was carried out with the remaining fruit species.

Longevity was compared among treatments by means of a Cox-Mantel survival analysis. Survival curves were computed using the Kaplan-Meier method.

Tests were performed using STATISTICA for Windows [67].

Results

Experiment 1. Attraction of *D. longicaudata* females' to different fruit species

Females showed a clear preference for figs compared to the other fruit (peach, orange and apple) ($G_{fig\text{-}peach} = 4.30$, $p = 0.038$; $G_{fig\text{-}orange} = 9.40$; $p < 0.01$; $G_{fig\text{-}apple} = 23.42$; $p < 0.01$). Peach was preferred over orange and apple ($G_{peach\text{-}orange} = 5.76$, $p = 0.016$; $G_{peach\text{-}apple} = 14.07$, $p < 0.01$). Finally, females showed a preference for orange over apple ($G_{orange\text{-}apple} = 7.46$, $p < 0.01$).These results showed a hierarchical pattern of female preference for different fruit species. The most attractive fruit was fig, then peach, then orange and finally apple (Fig 1). There were no significant differences in latency between options, except for the fig that was chosen more quickly than the orange (S1 Table).

Experiment 2. Attraction of *D. longicaudata* females' to fruit with different levels of infestation

When fruit of the same species were offered to the females in the Y-tube, a clear preference for the most infested fruit was shown ($G_{apple\ high\text{-}apple\ low} = 14.32$, $p < 0.01$; $G_{orange\ high\text{-}orange\ low} = 7.46$, $p < 0.01$) (Fig 2). Preference for the most infested fruit was also found when females were offered an orange with high infestation and an apple with low infestation ($G_{apple\ low\text{-}orange\ high} = 11.59$, $p < 0.01$) (Fig 2). However, apples with high levels of infestation were not more attractive than oranges with low levels of infestation ($G_{apple\ high\text{-}orange\ low} = 0.63$, $p = 0.428$) (Fig 2). Latency times did not differ between treatments (S2 Table).

Experiment 3. Effect of previous experience on *D. longicaudata* females' preference

In the Y-tube olfactometer, a significantly higher proportion of females oriented towards the fruit on which they were conditioned, irrespectively of the fruit species (Females conditioned on orange: $G_{apple\ -orange} = 16.85$, $p < 0.01$; females conditioned on apple: $G_{apple\ -orange} = 5.76$,

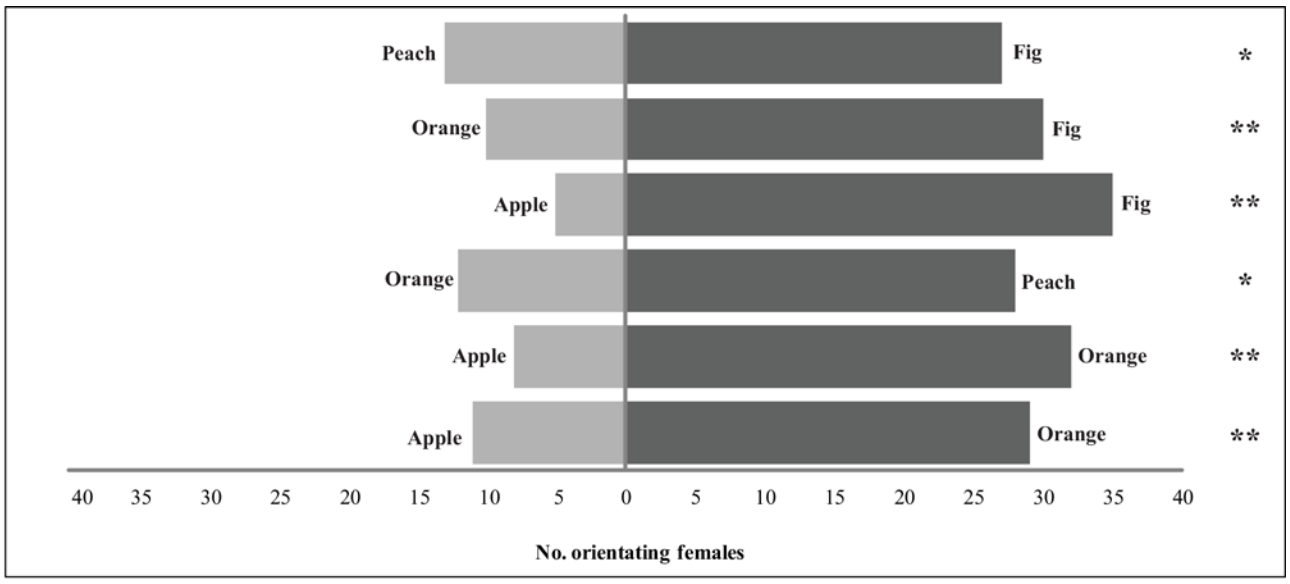

Fig 1. Preference for different fruit species by *Diachasmimorpha longicaudata* females in a Y-tube olfactometer. Bars show the number of females visiting each option in each pair-wise combination among the four fruit species (experiment 1). G-test level of significance: * $p < 0.05$; ** $p < 0.01$.

doi:10.1371/journal.pone.0152222.g001

$p = 0.016$) (Fig 3). Latency times did not differ between treatments (S3 Table). Parasitism controls showed that all the females used in experiment 3 had successfully parasitized larvae during the conditioning period.

Experiment 4. Quality of different fruit species as foraging substrates

While the number of fruit exposed to parasitoids was 120 for each fruit species, several replicates were lost for different reasons. In some cases, larvae were not recovered from some fruit that were considered to be infested (and were consequently offered to parasitoids). Likewise, some fruit were colonized by fungi and larval survival was severely affected. These cases were

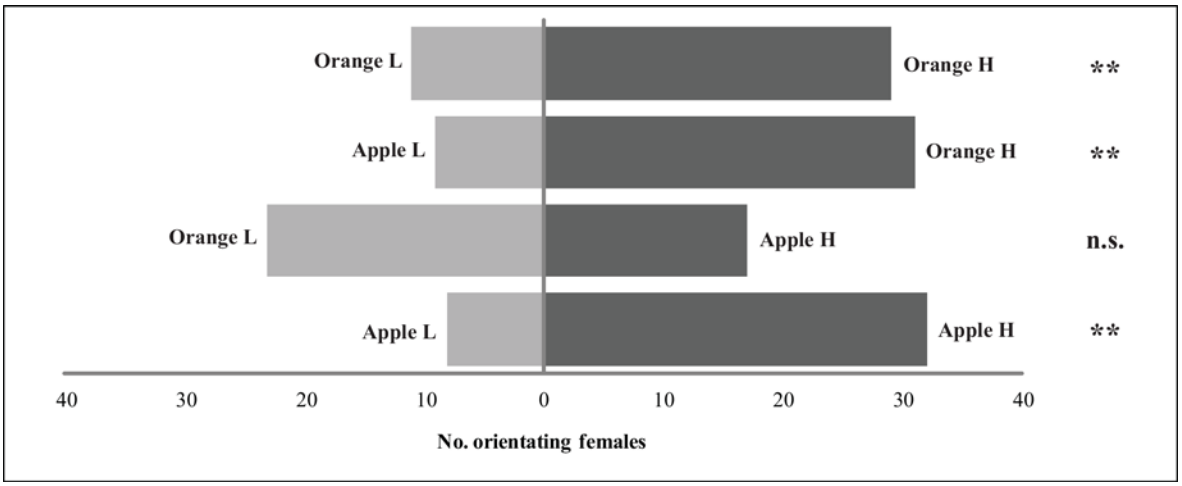

Fig 2. Preference of *Diachasmimorpha longicaudata* females for fruit with different levels of infestation by *Ceratitis capitata*. Bars show the number of females visiting each option in the experiment 2. H: high infestation level; L: low infestation level. G-test level of significance: n.s. = non-significant; * $p < 0.05$; ** $p < 0.01$. n.s.

doi:10.1371/journal.pone.0152222.g002

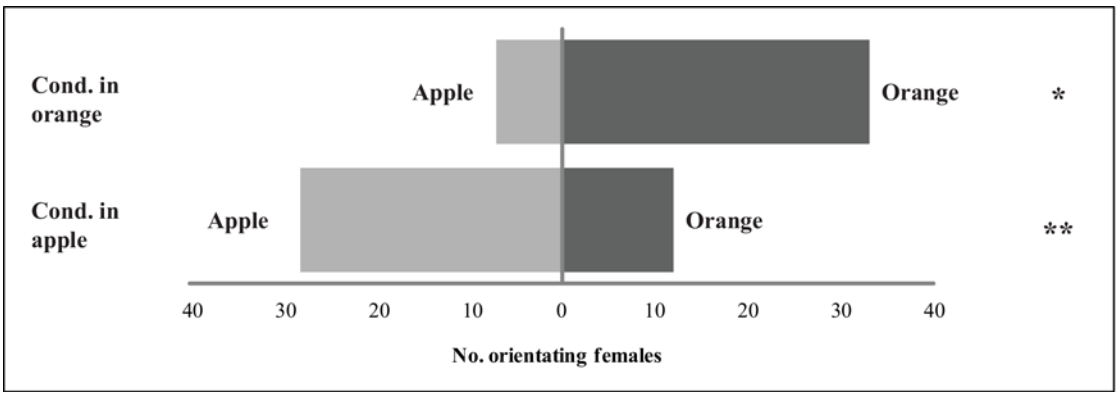

Fig 3. Effect of previous experience on the preference of *Diachasmimorpha longicaudata* females for different fruit species. Bars show the number of females (conditioned either on apple or orange) visiting each option in the experiment 3. G-test level of significance: * $p < 0.05$; ** $p < 0.01$.

doi:10.1371/journal.pone.0152222.g003

not included in the dataset and consequently the number of replicates was 295 (Apple: 58 fruit; Fig: 58 fruit; Orange: 64 fruit; Peach: 115 fruit).

The parasitism rate ranged between 16.76% and 55.66%, and significantly differed among fruit species (ANOVA: $F_{3, 291} = 28.43$; $p < 0.01$). Tukey's multiple comparisons showed that the parasitism rates in fig were higher than in apple, orange and peach (Fig 4A). In turn, apple showed higher rates than orange and peach, which showed no differences between them (Fig 4A).

The number of larvae per cm^2 of fruit surface offered to female parasitoids was significantly different among fruit species (ANOVA: $F_{3, 291} = 22.74$; $p < 0.01$) (Fig 4B). Multiple comparisons post hoc test showed that apple had significantly lower density of larvae than the other fruit species (Fig 4B), whereas no differences were found among the other three species (Fig 4B).

Experiment 5. Quality of host larvae reared on different fruit species

Mean daily fecundity ranged between 13.14 and 19.41 parasitoids/female and the total fecundity ranged between 106.13 and 160.12 parasitoids/female. Significant differences were found in the mean fecundity of parasitoid emerged from larvae that had fed on different fruit species (ANOVA: $F_{3, 142} = 32.08$, $p < 0.01$) (Table 1). The mean fecundity of females emerged from larvae reared on oranges was higher than the other fruit species, which in turn did not differ statistically (Table 1). The same pattern was observed for the lifetime fecundity (ANOVA: $F_{3, 142} = 31.80$, $p < 0.01$; Table 1). The sex ratio differed significantly among treatments (ANOVA: $F_{3, 142} = 6.26$, $p < 0.01$). Females reared on larvae that developed in oranges showed a higher proportion of females in the progeny than females reared in the other three fruit species, which showed no differences among them (Table 1). Survival analyses showed no differences in lifespan among treatments, both for females and males (Cox-Mantel test: Males: $Chi^2_3 = 2.81$, $p = 0.421$; Females: $Chi^2_3 = 6.28$, $p = 0.099$) (Table 1, Fig 5).

The weight of pupae reared in each fruit species was higher for peach and orange; fig showed intermediate values, and apple showed the lowest values (Table 2). The differences among the four fruit species could not be analysed statistically, and the ANOVA performed excluding the fig showed significant differences among the other three species (ANOVA: $F_{2, 234} = 54.78$, $p < 0.01$). Post hoc comparisons showed that pupae that developed in apple were lighter than pupae that developed in orange and peach, between which there were no significant differences in weight (Table 2).

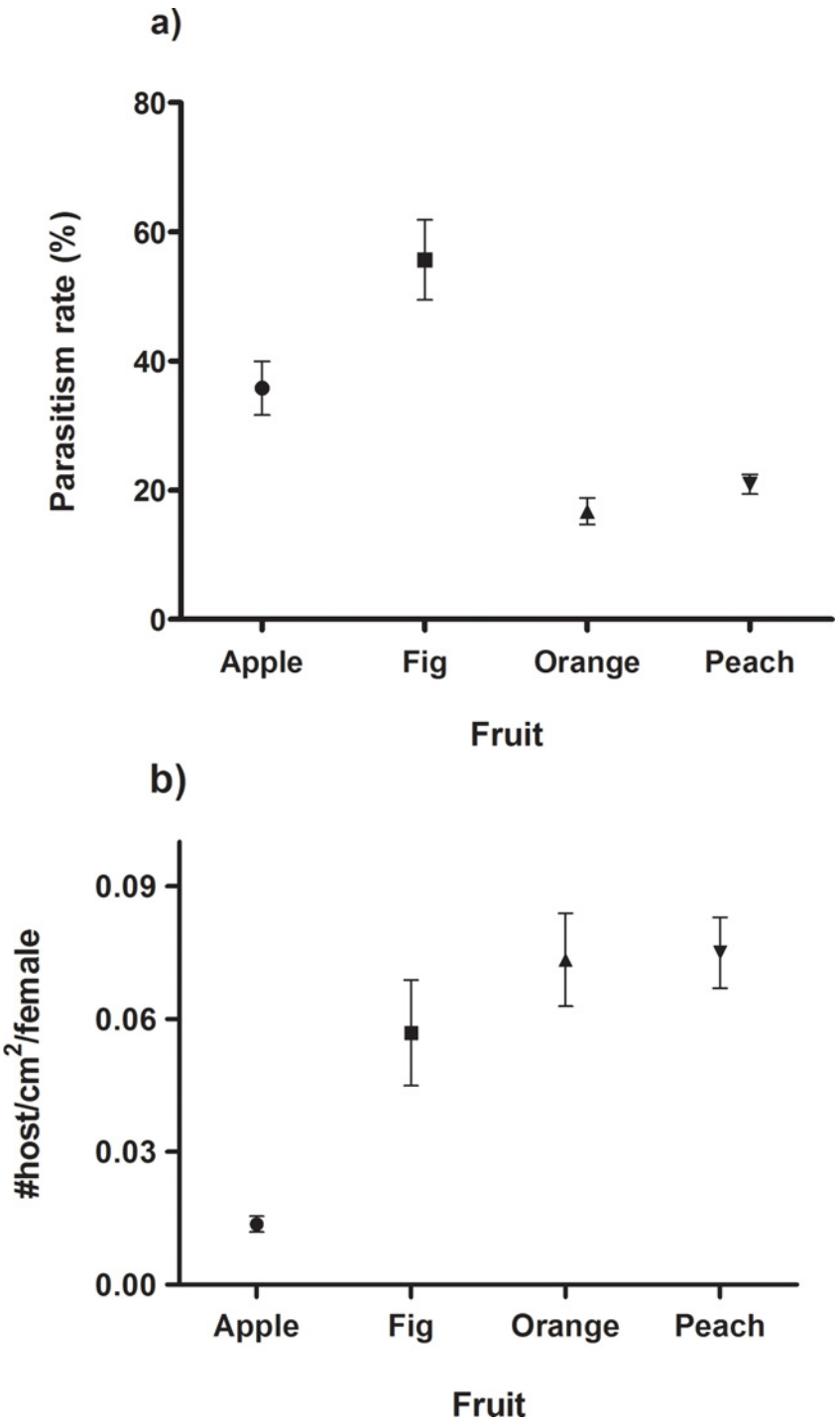

Fig 4. Quality of different fruit species as foraging substrates. a) Mean parasitism rate by *Diachasmimorpha longicaudata* recorded in different fruit species infested by *Ceratitis capitata* larvae. b) Mean density of larvae offered during infested fruit exposure in experiment 4. Bars show the standard error of the mean.

doi:10.1371/journal.pone.0152222.g004

Wing length was statistically different among females emerged from larvae reared in different fruit species (ANOVA: $F_{3, 89} = 5.37$, $p < 0.01$). Tukey's test showed that wings from females

Table 1. Mean fecundity (mean ± S.E.), total fecundity (mean ± S.E.), sex ratio (mean ± S.E.) and lifespan (mean ± S.E., in days) of *Diachasmimorpha longicaudata* reared in fruit fly larvae fed on different fruit species in experiment 5.

Fruit species	Mean fecundity	Total fecundity	Sex ratio	Female lifespan	Male lifespan
Apple	13.14 ± 1.03 a	106.13 ± 10.11 a	0.477 ± 0.019a	41.25 ± 2.62	26.56 ± 1.94
Fig	15.89 ± 1.23 a	128.89 ± 10.87 a	0.500 ± 0.026a	35.19 ± 2.56	27.50 ± 1.78
Orange	19.41 ± 1.11 b	160.12 ± 13.79 b	0.646 ± 0.021b	35.17 ± 2.03	24.08 ± 1.64
Peach	14.18 ± 0.90 a	125.18 ± 10.68 a	0.531 ± 0.029a	39.32 ± 2.04	26.80 ± 2.42

Means followed by the same letter within the same column do not differ statistically (α = 0.05; multiple contrasts).

doi:10.1371/journal.pone.0152222.t001

associated to oranges were larger than those associated to figs ($p = 0.017$) and apples ($p < 0.01$), but no differences were found among figs, apples and peaches ($p > 0.05$) (Fig 6).

Discussion

Innate preference for host habitats in parasitoids is expected to occur only if available habitats vary in profitability and/or host quality. In our study, naive *D. longicaudata* females showed a hierarchical preference pattern among non-infested fruit of four different species. This result can be partially explained by a differential reward in terms of fitness associated to each fruit species. Evidence show that the pattern of preference is not fixed and can be modified according to external factors, such as host density, and internal factors, such as experience.

Females preferred fig over the rest of the fruit species, then peach, orange and, finally, apple. This innate preference was not expected to be so strong for a parasitoid which hosts are so polyphagous. The innate response could be related, at least to some extent, to a differences in host density among different fruit species in nature [64,68,69]. Field and laboratory studies showed that the most frequent host fruit for *Bactrocera* species are mango (*Mangifera indica* L.), papaya (*Carica papaya* L.), guava (*Psidium guajava* L.), several species of *Cucurbita*, as well as native species of varying taxonomic families (e.g. *Psidium* sp., *Ziziphus* sp., *Syzypium* sp., etc.). Infestation of *Citrus* spp. by *Bactrocera* is rare [51,70,71]. Moreover, a survey performed of fruit flies (Diptera: Tephritidae) from wild and cultivated host plants species in Thailand and Malaysia found that *D. longicaudata* was frequently recovered from several species of Moraceae family (the same family of fig) [51]. According to White and Elson-Harris [52], apple is not an important host for *Bactrocera spp*. and only 10% of the species of this genus attack this fruit species. Although it is not possible to directly link these finding to the innate preference of *D. longicaudata* found in our experiments, a differential distribution of the native hosts among fruit species could account for the preference for fig over apple and orange.

The innate preference of *D. longicaudata* could also be related to differences among fruit species in the available enemy-free space [72]. Parasitism rates on fig were higher than those found on peach and orange. This might be related to the smaller size of figs which would disable larvae to escape from parasitism [73,74]. These differences could therefore explain the preference of *D. longicaudata* for fig within the olfactometer, but on the other hand no preference should be expected between peach and orange (on which the parasitism rate was not different), and olfactometer results showed a clear preference for peach. (Comparisons of parasitism rates between apple and the rest of the fruit species are limited because of its lower number of larvae per cm^2). The association between preference and realized parasitism rate has been studied in *D. longicaudata* by Leyva et al. [53] and Ovruski et al. [57]. Altogether, results so far seem to indicate no clear association between the preferred host fruit and the parasitism rate, at least not an association that completely explains the preference for host habitats.

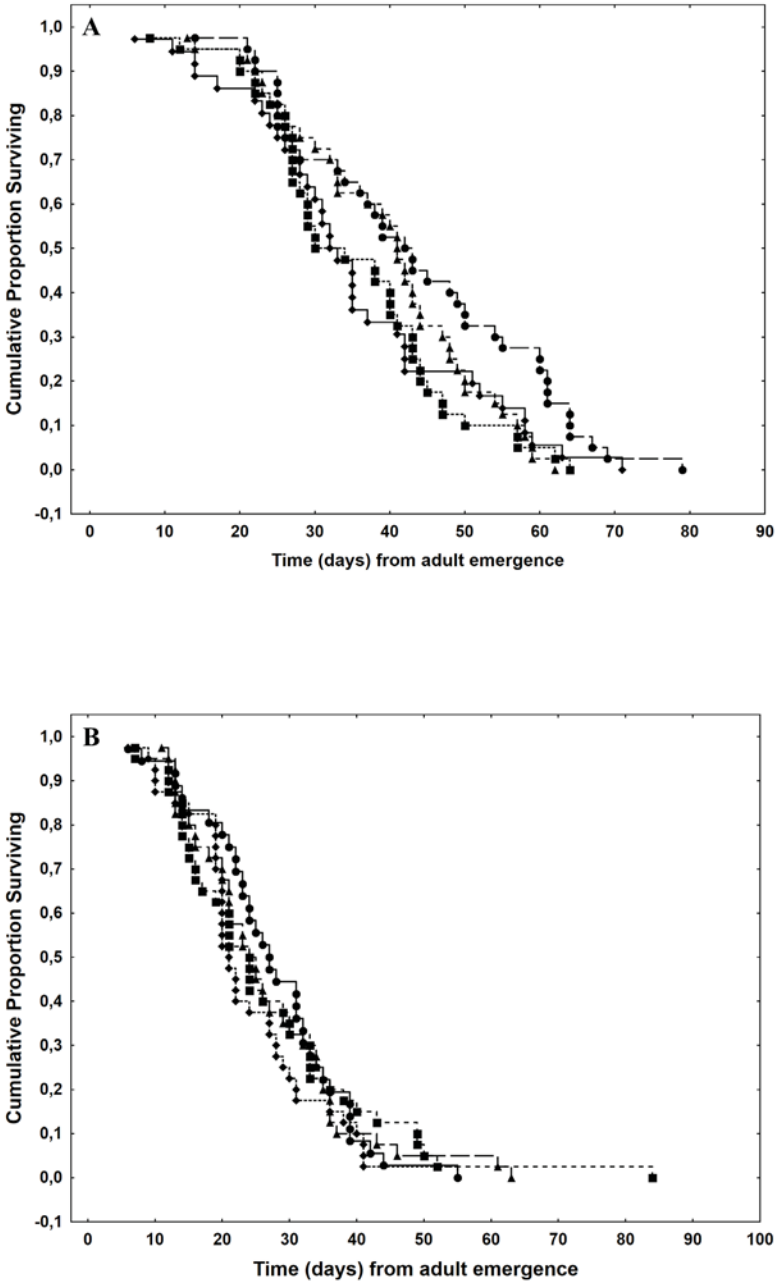

Fig 5. Survival curves for adult *Diachasmimorpha longicaudata* reared on *Ceratitis capitata* larvae that developed in different host fruit. Different fruit species are labelled with a different shape: Apple: circles; Fig: squares; Orange: triangles; Peach: diamonds. (a) males and (b) females.

doi:10.1371/journal.pone.0152222.g005

Several studies have focused on the PPH at the bitrophic scale, but the connection between preference and performance considering the first and third trophic level has seldom been studied [45]. In the present study, we did not detect a clear association between the preferences of female parasitoids for certain host habitats and their reward in terms of fitness. In fact fecundity and sex ratio showed that performance was higher for parasitoids associated to oranges, while survival was not affected by fruit species. This is in agreement with results obtained by Eben et al. [48] (who compared oranges and mangoes), which together with our results suggest

Table 2. Pupal weight (mean ± S.E.) of *Ceratitis capitata* reared on different fruit species.

Fruit species	Pupal weight (mg)	CI 95%
Apple	7.09 ± 0.17a	6.74–7.43
Fig	8.46 ± 0.36	7.74–9.19
Orange	9.16 ± 0.15b	8.86–9.45
Peach	9.34 ± 0.14b	9.06–9.62

Confidence intervals of 95% (CI 95%) are also presented.

Means followed by the same letter within the same column do not differ statistically (α = 0.05; multiple contrasts). Fig was not considered in the statistical analysis because of the larger variation around the mean.

doi:10.1371/journal.pone.0152222.t002

that orange would be a fruit that produces high quality hosts even when it is not the preferred host habitat. This lack of a perfect association between preference and performance when the first and third trophic levels are considered seems to be quite frequent [46,48,53,75] with cases in which PPH is supported being rare [45]. It could be argued that the correspondence between preference and performance fades away as the distance between trophic levels increases, and other factors, such as realized parasitism rate and the oviposition preference of the adult flies, might contribute to a larger extent to the parasitoid preference. This idea needs further research.

The size of the herbivore is a good estimator of its quality as host for koinobiont parasitoids that attack late instar larvae, like *D. longicaudata* [76–80]. We found an effect of the host fruit on the weight of *C. capitata* pupae, with apple showing the lowest weight. Similar results were obtained for adult size, which is considered an important component of parasitoid fitness [18,19, 81]. This could explain the fact that apple was the least chosen fruit within the olfactometer. Nonetheless, these parameters cannot entirely explain the pattern of preference for different fruits, because pupae that developed in peach and orange showed similar weights and adult size, with fig taking intermediate values. Again, preference for host habitats and performance associated to each one of them are not clearly correlated.

Despite an innate preference for host habitats, we found that *D. longicaudata* preference is modulated by the level of infestation (patch richness) and the experience acquired during foraging. *Diachasmimorpha longicaudata* is able to discriminate between infested and non-infested fruit based on infochemicals [48,82–84]. Our results showed that females are also capable of discerning between different levels of infestations, and orientate themselves to the more profitable patches. Moreover, chemical information about host abundance overrides the preference for a given fruit. This suggests that when females have a reliable cue of the host presence, orientation behaviour will no longer respond to innate preferences, something that has been documented in other host-parasitoid systems [13,85–87]. By doing so, parasitoids can adjust their host searching strategy and seek those habitats in which the probability of host encounter is higher.

Previous experience also affected the host habitat preference of *D. longicaudata*. This shows again that *D. longicaudata* can assimilate information about the distribution of its hosts and modify accordingly their foraging behaviour, in this case probably mediated by associative learning (because in the olfactometer trials fruit was not infested). Learning of chemical cues by insect parasitoids has been reported in several species and behavioural contexts, such as food [88–90] or host finding [14,91,92]. The effect of learning is so drastic for this species that it generates a preference for colours when there is no innate preference for such cue [60] and, as shown here, blurs the innate pattern of preference for fruit species based on chemical cues.

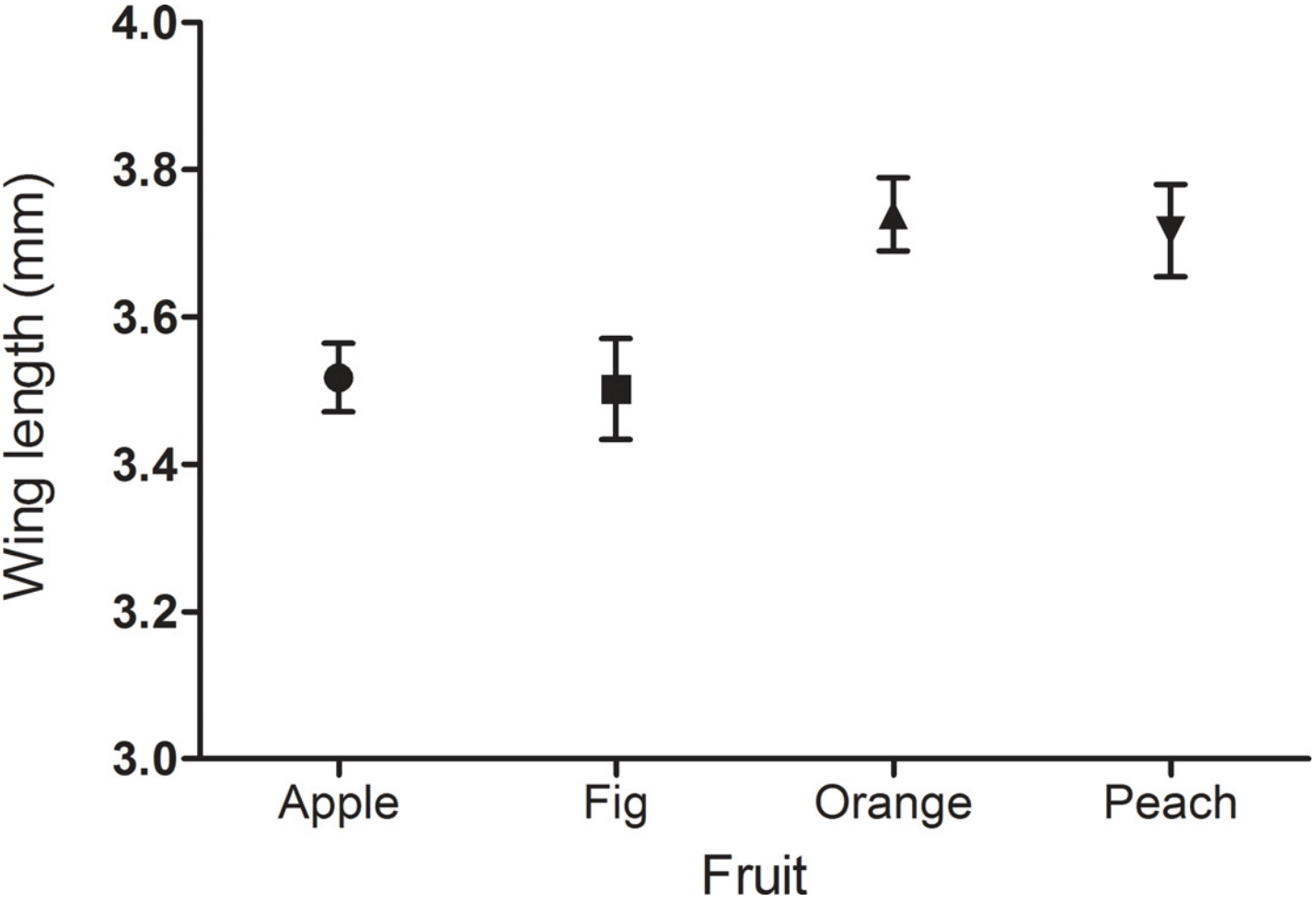

Fig 6. Effect of fruit species on parasitoid size. Mean wing length of *Diachasmimorpha longicaudata* reared on *Ceratitis capitata* larvae that developed in different host fruit. Bars show the standard error of the mean.

doi:10.1371/journal.pone.0152222.g006

Our results provide information about the host finding behaviour and, consequently, the effectiveness of *D. longicaudata* as a biological control agent of Tephritidae fruit fly pests. We demonstrated that female parasitoids can use chemical information both from the host and the host habitat during the host searching process. Females will orientate preferentially to specific host habitats in the absence of direct cues from their hosts, which would allow to reduce the searchable area and increases the probability of host encounter. However, if direct cues are perceived, females will orientate to the infested fruits [83] apparently in a dose-response manner, as they are able to detect different infestation levels. After finding suitable hosts, females are able to associate conspicuous chemical and visual cues from these host habitats that can be used in successive foraging bouts. The plasticity in the use of chemical and visual cues makes this species a good candidate to control hosts that can be encountered in fruits of different species, which are ephemeral habitats that change markedly in their chemical and physical properties along the season.

Supporting Information

S1 Table. Latency times (mean ± S.E.) recorded in the Y-tube olfactometer in experiment 1.
(DOCX)

S2 Table. Latency times (mean ± S.E.) recorded in the Y-tube olfactometer in experiment 2.
(DOCX)

S3 Table. Latency times (mean ± S.E.) for the selection in the Y-tube olfactometer in experiment 3.
(DOCX)

Acknowledgments

The authors thank Fabián Milla and Leonela Carabajal Paladino for supplying flies and parasitoids. We want to especially thank María Fernanda Rodríguez for her collaboration during the experiments. We also would like to thank anonymous reviewers for their helpful and constructive comments that greatly contributed to improving the final version of the paper.

Author Contributions

Conceived and designed the experiments: DFS FD MMV SMO JC JLC. Performed the experiments: DFS ALN MMV FD GEB. Analyzed the data: DFS GEB ALN MMV FD JC. Contributed reagents/materials/analysis tools: DFS SMO JC JLC. Wrote the paper: DFS GEB ALN FD MMV SMO JC JLC.

References

1. Turlings TC, Loughrin JH, Mccall PJ, Röse US, Lewis W J, Tumlinson J H. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proc Natl Acad Sci. 1995; 92: 4169–4174. PMID: 7753779
2. de Moraes CM, Lewis WJ, Pare PW, Alborn HT, Tumlinson JH. Herbivore-infested plants selectively attract parasitoids. Nature. 1998; 393: 570–573.
3. Dicke M, Vet LEM. Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. In: Olff H, Brown VK, Drent RH editors. Herbivores: between plants and predators. Oxford, UK: Blackwell Science; 1999. pp. 483–520.
4. Turlings TCJ, Tumlinson JH, Lewis WJ. Exploitation of herbivore-induced plant odors by host seeking parasitic wasps. Science. 1990; 250: 1251–1253. PMID: 17829213
5. Cortesero AM, De Moraes CM, Stapel JO, Tumlinson JH, Lewis WJ. Comparisons and contrasts in host-foraging strategies of two larval parasitoids with different degrees of host specificity. J Chem Ecol. 1997; 23: 1589–1606.
6. Ngumbi E, Chen L, Fadamiro HY. Comparative GC-EAD responses of a specialist (*Microplitis croceipes*) and a generalist (*Cotesia marginiventris*) parasitoid to cotton volatiles induced by two caterpillar species. J Chem Ecol. 2009; 35: 1009–1020. doi: 10.1007/s10886-009-9700-y PMID: 19802643
7. Turlings TC, Wäckers F. Recruitment of predators and parasitoids by herbivore-injured plants. Adv Insect Chem Ecol. 2004; 2: 21–75.
8. Dicke M. Behavioural and community ecology of plants that cry for help. Plant Cell Environ. 2009; 32: 654–665. doi: 10.1111/j.1365-3040.2008.01913.x PMID: 19021885
9. Dicke M, Baldwin IT. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends Plant Sci. 2010; 15: 167–175. doi: 10.1016/j.tplants.2009.12.002 PMID: 20047849
10. Hoffmeister TS, Gienapp P. Exploitation of the host's chemical communication in a parasitoid searching for concealing host larvae. Ethology. 1999; 105:223–232.
11. Vet LEM, Dicke M. Ecology of infochemical use by natural enemies in a tritrophic context. Annu Rev Entomol. 1992; 37: 141–172.
12. Turlings TCJ, Wäckers FL, Vet LEM, Lewis WJ, Tumlinson JH. Learning of host-finding cues by hymenopterous parasitoids. In: Papaj DR, Lewis AC, editors. Insect Learning. New York: Chapman y Hall; 1993. pp. 51–78.
13. Geervliet JBF, Vreugdenhil AI, Dicke M, Vet EM. Learning to discriminate between infochemicals from different plant-host complexes by the parasitoids *Cotesia glomerata* and *C. rubecula*. Entomol Exp Appl. 1998; 86: 241–252.

14. Stireman JO III. Learning in the generalist tachinid parasitoid *Exorista Mella* Walker (Diptera: Tachinidae). J Insect Behav. 2002; 15: 689–706.
15. Takemoto H, Powell W, Pickett J, Kainoh Y, Takabayashi J. Two-step learning involved in acquiring olfactory preferences for plant volatiles by parasitic wasps. Anim Behav. 2012; 83: 1491–1496.
16. Steidle JL, Van Loon JJ. Dietary specialization and infochemical use in carnivorous arthropods: testing a concept. Entomol Exp Appl. 2003; 108: 133–148.
17. Godfray HCJ. Parasitoids: behavioral and evolutionary ecology. Princeton, USA. Princenton University Press; 1994.
18. Harvey JA. Factors affecting the evolution of development strategies in parasitoid wasps: the importance of functional constraints and incorporating complexity. Ent Exp Appl. 2005; 117: 1–13.
19. Bukovinszky T, Gols R, Smid HM, Bukovinszkiné Kiss G, Dicke M, Harvey JA. Consequences of constitutive and induced variation in the host's food plant quality for parasitoid larval development. J Insect Physiol. 2012; 58: 367–375. doi: 10.1016/j.jinsphys.2011.12.017 PMID: 22233934
20. Stephens DW, Krebs JR. Foraging theory. Princeton: University Press; 1986.
21. Charnov EL, Stephens DW. On the evolution of host selection in solitary parasitoids. Am Nat. 1988; 132: 707–722.
22. Harvey JA, Gols R, Snaas H, Malcicka M, Visser B. Host preference and offspring performance are linked in three congeneric hyperparasitoid species. Ecol Entomol. 2015; 40: 114–122.
23. Levins R, MacArthur RH. An hypothesis to explain the incidence of monophagy. Ecology. 1969; 50: 9l0–911.
24. Jaenike J. On optimal oviposition behaviour in phytophagous insects, Theor. Popul. Biol. 1978; 14:350–356. PMID: 751265
25. Thompson JN. Coevolution and alternative hypotheses on insect/plant interactions. Ecology. 1988; 69: 893–895.
26. Mayhew PJ. Adaptive patterns of host-plant selection by phytophagous insects. Oikos. 1997; 79: 417–428.
27. Mayhew PJ. The evolution of gregariousness in parasitoid wasps. Proc R Soc Lond B. 1998; 265: 383–389
28. Gripenberg S, Mayhew PJ, Parnell M, Roslin T. A meta-analysis of preference–performance relationships in phytophagous insects. Ecol Lett. 2010; 13: 383–393. doi: 10.1111/j.1461-0248.2009.01433.x PMID: 20100245
29. van Alphen JJM, Vet LEM. An evolutionary approach to host finding and selection. In: Waage JK, Greathead D, editors. Insect Parasitoids London: Academic Press; 1986. pp. 23–61.
30. Rivero A. The relationship between host selection behaviour and offspring fitness in a koinobiont parasitoid. Ecol Entomol. 2000; 25: 467–472.
31. Luhring KA, Millar JG, Paine TD, Reed D, Hanks LM, Christiansen H. Oviposition preferences and progeny development of the egg parasitoid *Avetianella longoi*: factors mediating replacement of one species by a congener in a shared habitat. Biol Control. 2004; 30: 382–391.
32. Ode PJ, Berenbaum MR, Zangerl AR, Hardy ICW. Host plant, host plant chemistry and the polyembryonic parasitoid *Copidosoma sosares*: indirect effects in a tritrophic interaction. Oikos. 2004; 104: 388–400.
33. Cicero L, Sivinski J, Aluja M. Effect of host diet and adult parasitoid diet on egg load dynamics and egg size of braconid parasitoids attacking *Anastrepha ludens*. Physiol Entomol. 2012; 37: 177–184.
34. Jandricic SE, Dale AG, Bader A, Frank SD. The effect of banker plant species on the fitness of *Aphidius colemani* Viereck and its aphid host (*Rhopalosiphum padi* L.). Biol Control. 2014; 76: 28–35.
35. Li X, Niu Y, Liu T. The performance of *Oomyzus sokolowskii* (Hymenoptera: Eulophidae) parasitizing *Plutella xylostella* (Lepidoptera: Plutellidae) on different host plants. Appl Entomol Zool. 2014; 49: 67–75.
36. Benrey B, Denno RF. The slow-growth—high-mortality hypothesis: A test using the cabbage butterfly. Ecology. 1997; 78: 987–999.
37. Ode PJ. Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. Annu Rev Entomol. 2006; 51:163–85. PMID: 16332208
38. Gols R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA. Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild *Brassica* populations. J Chem Ecol. 2008; 34: 132–143. doi: 10.1007/s10886-008-9429-z PMID: 18231835

39. Blaul B, Ruther J. How parasitoid females produce sexy sons: a causal link between oviposition preference, dietary lipids and mate choice in Nasonia. Proc. R. Soc. B. 2011; 278: 3286–3293. doi: 10.1098/rspb.2011.0001 PMID: 21429922

40. Harvey JA, Gols R. Population-related variation in plant defense more strongly affects survival of an herbivore than its solitary parasitoid wasp. J Chem Ecol. 2011; 37: 1081–1090. doi: 10.1007/s10886-011-0024-3 PMID: 21987026

41. Stoepler TM, Lill JT, Murphy SM. Cascading effects of host size and host plant species on parasitoid resource allocation. Ecol Entomol. 2011; 36: 724–735.

42. Rutledge CE, Wiedenmann RN. Habitat preferences of three congeneric braconid parasitoids: implications for host-range testing in biological control. Biol Control. 1999; 16: 144–154.

43. Wyckhuys KAG, Heimpel GE. Response of the soybean aphid parasitoid *Binodoxys communis* to olfactory cues from target and non-target host-plant complexes. Entomol Exp Appl. 2007; 132: 149–158.

44. Belda C, Riudavets J. Attraction of the parasitoid *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) to odors from grain and stored product pests in a Y-tube olfactometer. Biol Control. 2010; 54: 29–34.

45. Gols R, Harvey JA. Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. Phytochem Rev. 2009; 8: 187–206.

46. Fathi SAA, Bozorg-Amirkalaee M, Sarfraz RM, Rafiee-Dastjerdi H. Parasitism and developmental parameters of the parasitoid *Diadegma majale* (Gravenhorst) in control of *Plutella xylostella* (L.) on selected cultivars of canola. BioControl. 2012; 57: 49–59.

47. Sarfraz M, Dosdall LM, Keddie BA. Influence of the herbivore host's wild food plants on parasitism, survival and development of the parasitoid *Diadegma insulare*. Biol Control. 2012; 62: 38–44.

48. Eben A, Benrey B, Sivinski J, Aluja M. Host species and host plant effects on preference and performance of *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae). Environ Entomol. 2000; 29: 87–94.

49. Ero MM, Clarke AR. Host location by the fruit fly parasitoid *Diachasmimorpha krausii*: role of fruit fly species, life stage and host plant. Agric For Entomol. 2012; 14: 101–110.

50. Wharton RA, Gilstrap FE. Key to and status of Opiinae Braconid (Hymenoptera) parasitoids used in biological control of *Ceratitis* and *Dacus* s.l. (Diptera: Tephritidae). Ann Entomol Soc Am. 1983; 76: 721–742.

51. Chinajariyawong A, Clarke AR, Jirasurat M, Kritsaneepiboon S, Lahey HA, Vijaysegaran S, et al. Survey of opiineparasitoids of fruit flies (Diptera: Tephritidae) in Thailand and Malaysia. The Raffles Bulletin of Zoology. 2000; 48: 71–101.

52. White IM, Elson-Harris MM. Fruit flies of economic significance; their identification and bionomics. Wallingford: CAB International; 1992.

53. Leyva JL, Browning HW, Gilstrap FE. Effect of host fruit species, size, and colour on parasitization of *Anastrepha ludens* (Diptera: Tephritidae) by *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae). Environ Entomol. 1991; 20: 1469–1474.

54. Messing RH, Jang EB. Response of the fruit fly parasitoid *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae) to host-fruit stimuli. Environ Entomol. 1992; 21: 1189–1195.

55. Ovruski SM, Oroño LE, Schliserman P, Nuñez-Campero S. The effect of four fruit species on the parasitization rate of *Anastrepha fraterculus* (Diptera: Tephritidae, Trypetinae) by *Diachamimorpha longicaudata* (Hymenoptera: Braconidae, Opiinae) under laboratory conditions. Biocontrol Sci Techn. 2007; 17: 1079–1085.

56. Cicero L, Sivinski J, Rull J, Aluja M. Effect of larval host food substrate on egg load dynamics, egg size and adult female size in four species of braconid fruit fly (Diptera: Tephritidae) parasitoids. J Insect Physiol. 2011; 57: 1471–1479. doi: 10.1016/j.jinsphys.2011.07.014 PMID: 21819991

57. Ovruski SM, van Nieuwenhove G, Bezdjian L, Albornoz-Medina P, Schliserman P. Evaluation of *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae) as a mortality factor of *Ceratitis capitata* (Diptera: Tephritidae) infesting *Citrus* species under laboratory and field-cage conditions. Biocontrol Sci Techn. 2012; 22: 187–202.

58. Dukas R, Duan JJ. Potential fitness consequences of associative learning in a parasitoid wasp. Behav Ecol. 2000; 11: 536–543.

59. Benelli G, Canale A. Learning of visual cues in the fruit fly parasitoid *Psyttalia concolor* (Szépligeti) (Hymenoptera: Braconidae). BioControl. 2012; 57: 767–777.

60. Segura DF, Viscarret MM, Carabajal Paladino LZ, Ovruski SM, Cladera JL. Role of visual information and learning in habitat selection by a generalist parasitoid foraging for concealed hosts. Anim Behav. 2007; 74: 131–142.

61. Birke A, Aluja M. *Anastrepha ludens* and *Anastrepha serpentina* (Diptera: Tephritidae) do not infest *Psidium guajava*, but *Anastrepha obliqua* occasionally shares this resource with *Anastrepha striata* in nature. J Econ Entomol. 2011; 104: 1204–1211. PMID: 21882684

62. Devescovi F, Liendo MC, Bachmann GE, Bouvet JP, Milla FH, Vera MT, et al. Fruit infestation patterns by *Anastrepha fraterculus* and *Ceratitis capitata* reveal that cross-recognition does not lead to complete avoidance of interspecific competition in nature. Agric For Entomol. 2015; 17: 325–335.

63. Viscarret MM, La Rossa R, Segura DF, Ovruski SM, Cladera JL. Evaluation of the parasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) reared on a genetic sexing strain of *Ceratitis capitata* (Wied.) (Diptera: Tephritidae). Biol Control. 2006; 36: 147–153.

64. Ovruski SM, Colin C, Soria A, Oroño L, Schliserman P. Introducción y establecimiento en laboratorio de *Diachasmimorpha tryoni* y *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae, Opiinae) para el control biológico de *Ceratitis capitata* (Diptera: Tephritidae, Dacinae) en la Argentina. Rev Soc Entomol Argent. 2003; 62:49–59.

65. Terán H. Comportamiento alimentario y su correlación a la reproducción en hembras de *Ceratitis capitata* Wied. Rev Agron del NOA. 1977; 14: 17–34.

66. Segura DF, Vera MT, Cladera JL. Dinámica de la infestación por estadios inmaduros de la mosca del Mediterráneo, *Ceratitis capitata* (Diptera: Tephritidae), en San Pedro, pcia. de Buenos Aires. Ecología Austral. 2004; 14: 3–17.

67. StatSoft, Inc. STATISTICA for Windows (program manual). Tulsa OK, USA 2000.

68. Mavrikakis PG, Economopoulos AP, Carey JR. Continuous winter reproduction and growth of the Mediterranean fruit fly (Diptera: Tephritidae) in Heraklion, Crete, Southern Greece. Environ Entomol. 2000; 29: 1180–1187.

69. Segura DF, Vera MT, Cagnotti C, Vaccaro N, de Coll O, Ovruski SM, et al. Relative abundance of *Ceratitis capitata* and *Anastrepha fraterculus* (Diptera: Tephritidae) in diverse host species and localities of Argentina. Ann Entomol Soc Am. 2006; 99: 70–83.

70. Clarke AR, Armstrong KF, Carmichael AE, Milne JR, Raghu S, Roderick GK, et al. Invasive phytophagous pests arising through a recent tropical evolutionary radiation: The *Bactrocera dorsalis* complex of fruit flies. Annu Rev Entomol. 2005; 50: 293–319. PMID: 15355242

71. Rwomushana I, Ekesi S, Gordon I, Ogol CKPO. Host plants and host plant preference studies for *Bactrocera invadens* (Diptera: Tephritidae) in Kenya, a new invasive fruit fly species in Africa. Ann Entomol Soc Am. 2008; 101: 331–340.

72. Denno RF, Larsson S, Olmstead KL. Host plant selection in willow-feeding leaf beetles (Coleoptera: Chrysomelidae): role of enemy-free space and plant quality. Ecology. 1990; 71: 124–137.

73. Sivinski J. The influence of host fruit morphology on parasitization rates in the Caribbean fruit fly, *Anastrepha suspensa*. Entomophaga. 1991; 36: 447–454.

74. Suárez L, Murúa F, Lara N, Escobar J, Taret G, Rubio J, et al. Biological control of *Ceratitis capitata* (Diptera: Tephritidae) in Argentina: releases of *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae) in fruit-producing semi-arid areas of San Juan. Nat Sci. 2014; 6: 664–675.

75. Ero MM, Hamacek E, Clarke A. Foraging behaviours of *Diachasmimorpha kraussii* (Fullaway) (Hymenoptera: Braconidae) and its host *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) in a nectarine (*Prunus persica* (L.) Batsch var. nectarina (Aiton) Maxim) orchard. Aust J Entomol. 2011; 50: 234–240.

76. Harvey JA, Harvey IF, Thompson DJ. Flexible larval growth allows use of a range of host sizes by a parasitoid wasp. Ecology. 1994; 75: 1420–1428.

77. Croft P, Copland MJ. The effect of host instar on the size and sex ratio of the endoparasitoid *Dacnusa sibirica*. Entomol Exp Appl. 1995; 74: 121–124.

78. Harvey JA, Bezemer TM, Elzinga JA, Strand MR. Development of the solitary endoparasitoid *Microplitis demolitor*: host quality does not increase with host age and size. Ecol Entomol. 2004; 29: 35–43.

79. Haeckermann J, Rott AS, Dorn S. How two different host species influence the performance of a gregarious parasitoid: host size is not equal to host quality. J Anim Ecol. 2007; 76: 376–383. PMID: 17302845

80. Sequeira R, Mackauer M. Nutritional ecology of an insect host-parasitoid association: the pea-aphid *Aphidus ervi* system. Ecology. 1992; 73: 183–189.

81. Visser ME. The importance of being large: the relationship between size and fitness in females of the parasitoid *Aphaereta minuta* (Hymenoptera: Braconidae). J Anim Ecol. 1994; 63: 963–978.

82. Silva JWP, Bento JMS, Zucchi RA. Olfactory response of three parasitoid species (Hymenoptera: Braconidae) to volatiles of guavas infested or not with fruit fly larvae (Diptera: Tephritidae). Biol Control. 2007; 41: 304–311.

83. Segura DF, Viscarret MM, Ovruski SM, Cladera JL. Response of the fruit fly parasitoid *Diachasmimorpha longicaudata* to host and host-habitat volatile cues. Entomol Exp Appl. 2012; 143: 164–176.

84. Carrasco M, Montoya P, Cruz-López L, Rojas JC. Responses of the fruit fly parasitoid *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae) to mango fruit volatiles. Environ Entomol. 2005; 34: 576–583.

85. Tentelier C, Wajnberg E, Fauvergue X. Parasitoids use herbivore-induced information to adapt patch exploitation behaviour. Ecol Entomol. 2005; 30: 739–744.

86. Tentelier C, Fauvergue X. Herbivore-induced plant volatiles as cues for habitat assessment by a foraging parasitoid. J Anim Ecol. 2007; 76: 1–8. PMID: 17184347

87. Uefune M, Nakashima Y, Tagashira E, Takabayashi J, Takagi M. Response of *Wollastoniella rotunda* (Hemiptera: Anthocoridae) to volatiles from eggplants infested with its prey *Thrips palmi* and *Tetranychus kanzawai*: prey species and density effects. Biol Control. 2010; 54: 19–22.

88. Lewis WJ, Takasu K. Use of learned odours by a parasitic wasp in accordance with host and food needs. Nature. 1990; 348: 635–636.

89. Patt JM, Hamilton GC, Lashomb JH. Response of two parasitoid wasps to nectar odors as a function of experience. Entomol Exp Appl. 1999; 90: 1–8.

90. Sato M, Takasu K. Food odor learning by both sexes of the pupal parasitoid *Pimpla alboannulatus* Uchida (Hymenoptera: Ichneumonidae). J Insect Behav. 2000; 13: 263–272.

91. Wäckers FL, Lewis WJ. Olfactory and visual learning and their combined influence on host site location by the parasitoid *Microplitis croceipes* (Cresson). Biol Control. 1994; 4: 105–112.

92. Kerguelen V, Cardé RT. Flight toward a learned odor and factors inducing landing of female *Brachymeria intermedia* (Hymenoptera: Chalcididae), a parasitoid of the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). J Insect Behav. 1998; 11: 221–234.

PLOS | ONE

RESEARCH ARTICLE

Phenotypic Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonizing Human Lungs

Jashanpreet Kaur[1,2], Shu Yao Duan[3], Lea A. I. Vaas[3,4], Anahit Penesyan[1,2], Wieland Meyer[3], Ian T. Paulsen[1,2], Helena Nevalainen[1,2]*

1 Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia, **2** Biomolecular Frontiers Research Centre, Macquarie University, Sydney, Australia, **3** Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Marie Bashir Institute for Infectious Diseases and Biosecurity, Sydney Medical School—Westmead Hospital, The University of Sydney, Westmead Millennium Institute, Sydney, Australia, **4** Bioinformatics Group, Centralbureau voor Schimmelculturen—Fungal Biodiversity Centre, Utrecht, The Netherlands

* helena.nevalainen@mq.edu.au

Citation: Kaur J, Duan SY, Vaas LAI, Penesyan A, Meyer W, Paulsen IT, et al. (2015) Phenotypic Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonizing Human Lungs. PLoS ONE 10(3): e0122354. doi:10.1371/journal.pone.0122354

Academic Editor: Dongsheng Zhou, Beijing Institute of Microbiology and Epidemiology, CHINA

Received: October 22, 2014

Accepted: February 13, 2015

Published: March 26, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Australian Research Council- Super Science Fellowship (ARC-SSF) was awarded to N.H. Packer, M.P. Molloy, H.K. Nevalainen, I.T. Paulsen and P.A. Haynes. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Genotyping studies of Australian *Scedosporium* isolates have revealed the strong prevalence of a recently described species: *Scedosporium aurantiacum*. In addition to occurring in the environment, this fungus is also known to colonise the respiratory tracts of cystic fibrosis (CF) patients. A high throughput Phenotype Microarray (PM) analysis using 94 assorted substrates (sugars, amino acids, hexose-acids and carboxylic acids) was carried out for four isolates exhibiting different levels of virulence, determined using a *Galleria mellonella* infection model. A significant difference was observed in the substrate utilisation patterns of strains displaying differential virulence. For example, certain sugars such as sucrose (saccharose) were utilised only by low virulence strains whereas some sugar derivatives such as D-turanose promoted respiration only in the more virulent strains. Strains with a higher level of virulence also displayed flexibility and metabolic adaptability at two different temperature conditions tested (28 and 37°C). Phenotype microarray data were integrated with the whole-genome sequence data of *S. aurantiacum* to reconstruct a pathway map for the metabolism of selected substrates to further elucidate differences between the strains.

Introduction

S. aurantiacum is a ubiquitous ascomycetous fungus found in diverse ecological niches including soil, sewage and polluted waters [1]. It has been recently added to the *S. boydii* species complex as a subset of isolates previously identified as *Scedosporium apiospermum* [2–5]. This emerging pathogen has been reported to be less susceptible to antifungals than other members of the *S. boydii* complex, such as *S. apiospermum* [6–8]. *S. aurantiacum* is an opportunistic pathogen capable of causing a wide variety of localized and superficial infections, such as malignant otitis externa, osteomyelitis, invasive sinusitis, keratitis and pneumonia [9, 10]. While

S. aurantiacum has been associated with airway colonization in Europe, *S. aurantiacum* related infections have been reported mainly in Australia [9, 11]. Recent population-based surveys have indicated that 17.4% of sputum specimens of Australian cystic fibrosis (CF) patients contain *S. aurantiacum*. This makes *S. aurantiacum* the second most common filamentous fungus associated with CF in Australia after *Aspergillus fumigatus* [12, 13]. CF, a genetically inherited disease, is characterized by defective mucociliary clearance, which provides an ideal environment for the growth of airborne fungal conidia in the lung [14]. The colonization of the respiratory tracts of Australian CF patients by *S. aurantiacum* can possibly be attributed to its relative high abundance in the Australian environment [2].

Considering the increasing incidences of *Scedosporium* infections, and high mortality rate associated with CF, there is a need to develop treatment strategies for these fungal infections [11]. The successful development of preventative strategies is limited by the similarity between the mammalian and fungal cell structures and metabolic pathways. The majority of the work reported on *S. aurantiacum* features clinical case studies and epidemiology research, with no published literature on the physiology and biochemistry of the organism [15]. Therefore, studies relating to cell growth, viability and general metabolism can provide a good starting point to facilitate the identification of novel targets to inhibit fungal growth without affecting the human host [15, 16].

As individual cell-based growth assays are relatively slow and can be used to test only a few phenotypes at a time [17], high throughput systems have been devised for the profiling of nutrient utilization in microorganisms [16]. One such method is a phenotype microarray (PM) carried out in 96-well microtitre plates, containing a variety of nutrients (*e.g.* sugars), where cell viability and respiration is automatically recorded [18]. Carbon utilization profiles in some filamentous fungi including *Aspergillus*, *Neurospora*, *Hypocrea* and *Acremonium* have been studied with this approach [16, 19–21].

In this study, we have evaluated the phenotypes of four *S. aurantiacum* strains isolated from clinical and environmental sources by recording their respiration rates on 94 substrates in microtitre plate assays. The results generated from respiration-based PM assays were validated by shake flask cultivation of the strains on selected carbon sources. Data obtained from the PM assays were compared against the *S. aurantiacum* draft genome for the presence or absence of particular metabolism related genes. The *Galleria mellonella* larvae models were used to assess the virulence levels of the four *S. aurantiacum* strains studied in this work.

Materials and Methods

Scedosporium aurantiacum strains

Scedosporium strains selected for the studies were obtained from the culture collection of the Medical Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia and included: (1) WM 06.482 isolated from the broncho-alveolar lavage of a cystic fibrosis patient in Australia; (2) WM 09.24 isolated from Sydney Circular Quay [7, 22]; (3) WM 08.202 (FMR8630; CBS116910) a type strain of *S. aurantiacum* isolated from a wound exudate of a patient in Santiago de Compostela, Spain and originally sourced from CBS culture collection (CBS-KNAW Fungal Biodiversity Centre, Netherlands), and (4) WM 10.136 (INS1120) isolated from a valley near Innsbruck, Austria. All the strains are a part of the Australian and global MLST (Multilocus Sequence Typing) network. Potential virulence of two of the *S. aurantiacum* strains addressed in this work (WM 06.482 and WM 08.202) has previously been assessed in an immunocompetent mouse model by Harun *et al* [7].

Growth measurements

All *S. aurantiacum* strains were cultured on Sabouraud dextrose agar plates (BD, Difco™, Australia) for 5 days at 37°C to achieve sufficient growth and conidiation. Three independent plate cultures were maintained for each strain in order to establish the differences in the appearance of colonies. Conidia were harvested from the cultures using 5 ml of sterile saline (0.9% w/v NaCl and 0.01% v/v Tween 80). The suspension was then filtered through sterile glass wool to separate conidia from the hyphae. The concentration was adjusted to $1x10^6$ conidia/ml for inoculation of liquid cultures each containing 50 ml of Sabouraud's broth (Sigma Aldrich, Australia) in a 250 ml conical flask. Different flasks were maintained for each time point and all the cultures were incubated at both 28 and 37°C on an orbital shaker at 250 rpm for a total of seven days. The mycelia were collected from the growing cultures by taking out one flask after every 12 hours, filtering the content through pre-weighed Whatman filter paper number 1 and drying in a vacuum oven at 70°C to a constant weight. Dry weight was calculated as the difference between the weight of the filter paper with and without the mycelia.

Pathogenicity testing using a *Galleria mellonella* larval model

Pathogenicity of the four *S. aurantiacum* strains was assessed using the invertebrate *G. mellonella* infection model [23]. Conidia were washed twice and diluted in PBS (phosphate-buffered saline) to a final concentration of 10^6 conidia/ml in an inoculum. *G. mellonella* larvae were obtained after the oviposition of the adult moths reared and maintained at 26°C and 60% relative humidity in the insectarium of the Westmead Hospital Animal Care Facility, Sydney, Australia. Ten similar sized larvae were weighed (about 3000 mg each) and placed in a 90 mm plastic Petri dish. Fungal inoculum (10 μl) was then injected into the last left pro-leg of the hemocoel of each larva using a 50 U syringe with a 29-gauge needle. Two different controls were also included in the assay: a group of 10 larvae inoculated with PBS to monitor potential effects on survival due to physical injury, and 10 untreated larvae. After injection, the larvae were incubated in Petri dishes at 35°C for 10 days and checked daily for survival. Larvae were considered dead when they were dark coloured and failed to respond to physical stimuli applied with a forceps. Survival of the larvae against each fungal strain was plotted after performing statistical analysis using Graph Pad Prism 6 (La Jolla, CA, USA).

Phenotype microarray

Biolog Phenotype analysis was carried out for all four *S. aurantiacum* strains using GEN III MicroPlate™ (Biolog Inc, USA) containing 94 assorted substrates (sugars, amino acids, hexose acids and carboxylic acids) and a positive and negative control (S1 Fig.). Fungal conidial suspensions ($1x\ 10^5$/ml) were prepared in the inoculating fluid (IF, Biolog, USA) and 100 μl of the inoculum was dispensed in three replicates into each well of the plate using a multichannel pipette (Biolog). After inoculation, the plates were incubated in the OmniLog incubator/reader (Biolog) for 72 hours at 28°C and 37°C. Cell respiration was recorded every 15 minutes by a charge-coupled device camera and plotted as a kinetic curve depicting reduction of the colorless tetrazolium blue dye to violet (formazan) by cell respiration. Raw values were imported from the OmniLog reader and analyzed using R package software opm [24]. This resulted in two datasets which comprised four strains x three replicates x two measurement temperatures x 94 substrates giving rise to 2256 individual cell respiration curves. Classification of phenotypes was performed based on the maximal curve height calculated as Omni Log units; an Omni Log value greater than 100 was considered as a positive phenotype. Comparison of substrate utilization in different strains was carried out using heat maps, which classified the strains based on estimation of the maximum height of the cell respiration curve [24].

Correlation of growth and respiration rates

Growth as biomass formation was determined for the four *S. aurantiacum* strains in shake flask cultivations on selected carbon sources also used in the Biolog respiration assay. Fungal conidia (1 x 10^5/ml) were inoculated into 50 ml of M9 minimal medium (Sigma), supplemented with a selected carbon source (1 mM) in a 250 ml shake flask and incubated at 37°C and 200 rpm for 60 hours. Carbon sources tested included maltose, D-trehalose, sucrose, D-turanose, D-salicin, D-glucose and D- fructose. All compounds were obtained from Sigma-Aldrich, dissolved in sterile water and filter sterilized. Cell dry weight was measured at the end of the incubation period as described above, and plotted against the OmniLog units obtained from the respiration curves. Each compound was tested in biological triplicate.

Metabolic pathway analysis and genome correlation

Sequencing of the genomes of the four *S. aurantiacum* strains used in this study has been completed recently with pending annotation [25]. Therefore, information on the putative pathways and enzymatic steps involved in the metabolism of the selected sugars was extracted from KEGG and MetaCyc and additional literature searches [26]. Amino acid translations of the gene sequences involved in the metabolic pathways of interest were identified from closely related reference organisms including *Trichoderma reesei*, *Aspergillus fumigatus*, *Penicillium chrysogenum and Neurospora crassa* and mapped back into the *S. aurantiacum* genome data using the tblastn program of BLAST algorithm [27]. Biological function was assigned for each gene encoding a particular enzyme in the predicted pathway based on the homology between the genome sequences for each *S. aurantiacum* strain and reference gene sequences [28, 29].

Results and Discussion

Growth pattern of *Scedosporium aurantiacum*

The first phenotypic feature that separated the four *S. aurantiacum* strains was the appearance of colonies on Sabouraud's agar. The color of the colonies varied from greyish white in WM 06.482, white in WM 08.202, suede-like in WM 10.136 and brownish-white in case of WM 09.24 (Fig. 1). All strains were slow-growing and produced a light yellow pigment on the reverse of the agar plates after 14 days of incubation.

Submerged cultures were grown in triplicate to establish a growth pattern for each strain. Growth of *S. aurantiacum* can be divided into four phases: lag, 0 to 36 hr; log, 36 to 48 hr; stationary, 48 to 72–84 hr and death after 84 hr (Fig. 2). A shorter lag phase (0–24 hours) was observed for the environmental strains (WM 09.24 and WM 10.136) whereas the clinical strains (WM 06.482 and WM 08.202) showed a longer lag phase, with the first significant change in the dry weight after 48 hours of incubation. As a general trend, the average mycelial dry weight reached its maximum in the log phase and decreased thereafter. WM 08.202 (type strain) was the slowest growing *S. aurantiacum* strain and also produced least biomass as compared to the others (Fig. 2).

Temperature had an impact on the growth of *S. aurantiacum* strains. WM 06.482 (clinical isolate) and WM 09.24 (environmental isolate) exhibited better growth at 37°C (as seen in Fig. 2), but were able to adapt to the lower temperature (28°C). The slowest growing strain WM 08.202 (clinical isolate) showed equal mycelial dry weights at 28°C and 37°C and strain WM 10.136 (environmental isolate) preferred 28°C, at which temperature the growth was fast and efficient. All four strains tested were able to adapt to the mammalian body temperature *i.e.* 37°C. Adaptation to various culture conditions, especially tolerance of mammalian

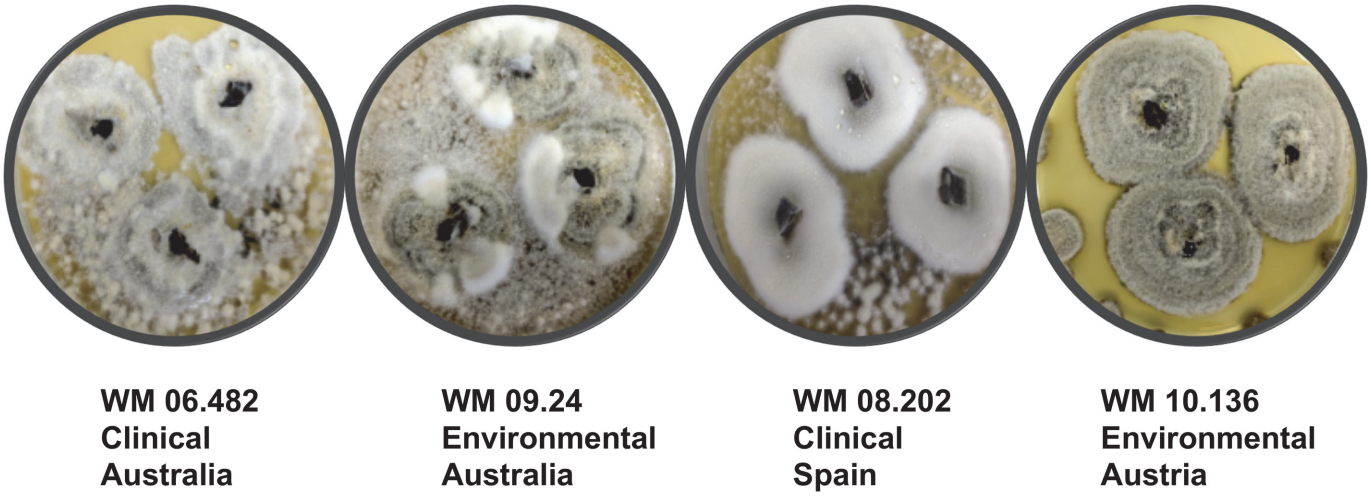

Fig 1. Colony morphology of the four different strains of *S. aurantiacum* growing on Sabouraud dextrose agar plates after 14 days of incubation.

doi:10.1371/journal.pone.0122354.g001

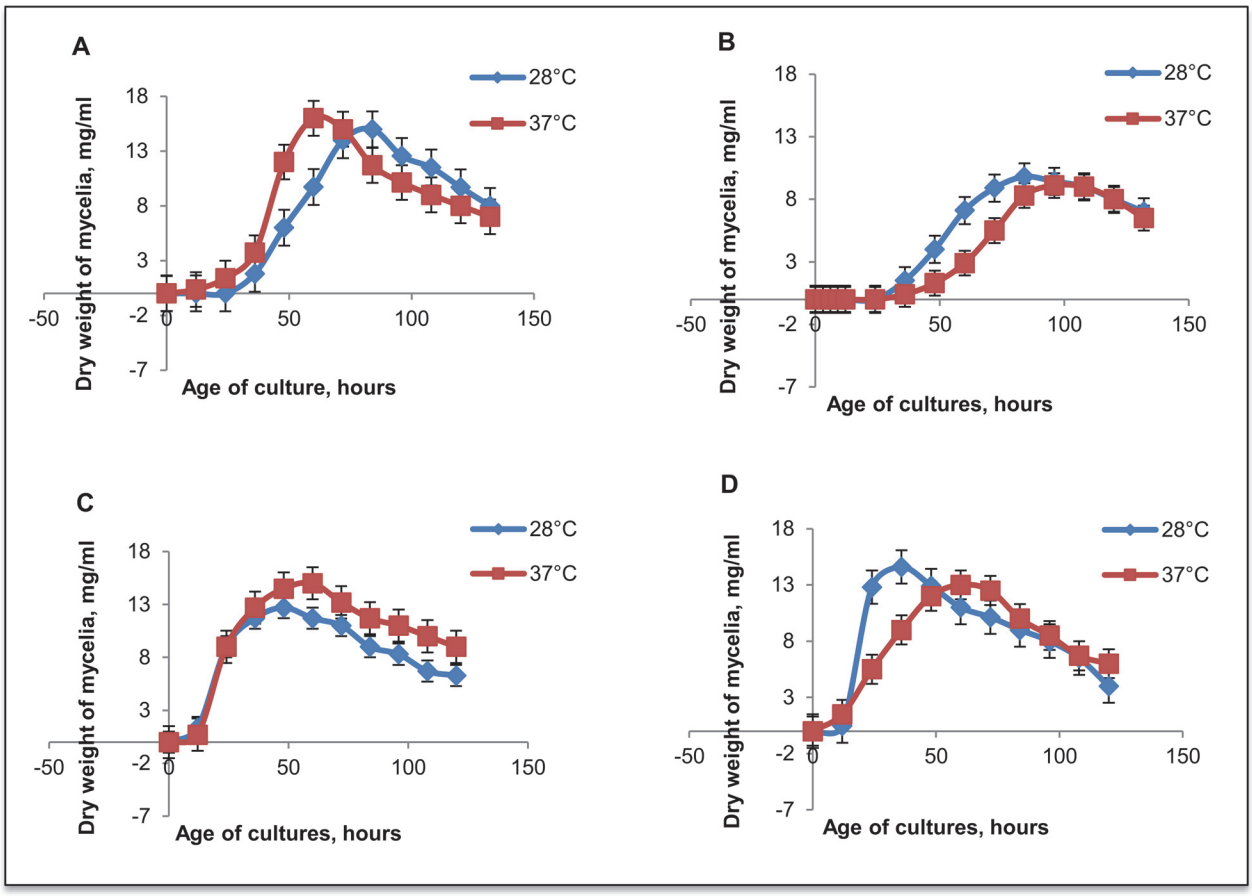

Fig 2. Growth of the *S. aurantiacum* strains in liquid culture in Sabouraud's broth, measured as change in the mycelial dry weight (mg/ml) over time. A) WM 06.482, B) WM 08.202, C) WM 09.24 and D) WM 10.136. Each experiment was repeated in triplicate, with bars representing ±1.25 standard errors, and 95% confidence interval.

doi:10.1371/journal.pone.0122354.g002

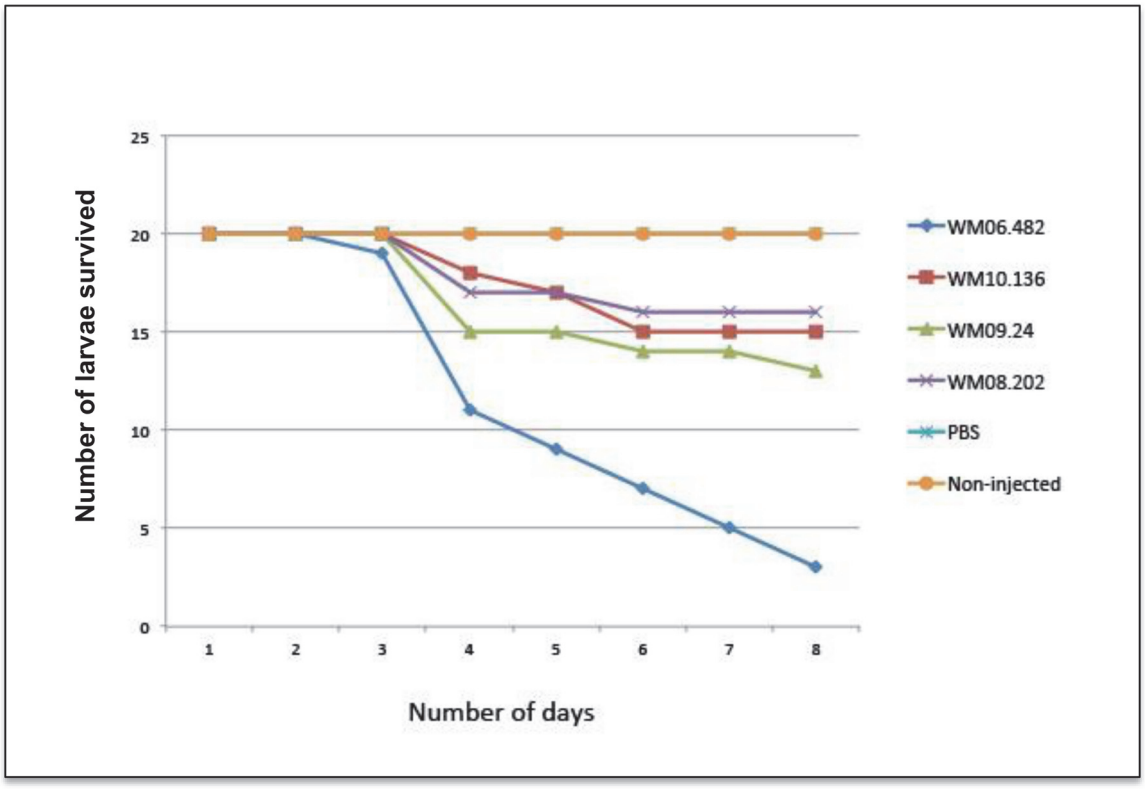

Fig 3. Survival of *G. mellonella* larvae infected with different strains of *S. aurantiacum*.

doi:10.1371/journal.pone.0122354.g003

temperatures (37°C) is a well-established phenomenon in other opportunistic fungal pathogens such as *Cryptococcus neoformans* [30].

Ranking the *S. aurantiacum* strains according to virulence

Virulence levels of the four *S. aurantiacum* isolates were explored using the *G. mellonella* larvae invertebrate model that has been previously used to assess the virulence of different strains of the human fungal pathogen *Candida albicans* [31]. Survival of larvae infected with the different strains is shown in Fig. 3. No larval death was observed in any of the control groups *i.e.* non-treated larvae and larvae inoculated with PBS.

As seen in Fig. 3, all *S. aurantiacum* strains were able to kill larvae; WM 06.482 (clinical isolate) was the most virulent among the strains tested, as the majority (85%) of the larvae failed to survive after eight days of infection and WM 08.202 (type strain isolated from a wound exudate) had the least effect on larval mortality (20%) The environmental strains, namely WM 09.24 and WM 10.136, killed approximately 40% and 25% of the larvae, respectively, within eight days.

While the *G. mellonella* model did clearly separate the strains with highest (WM 06.482) and lowest virulence (WM 08.202 and WM 10.136), the nature of WM 09.24 is less clear. Nevertheless, from this analysis, WM 09.24 was the second most virulent strain of the four *S. aurantiacum* strains tested. Similar to previous studies [11, 32] the virulence levels observed for different *S. aurantiacum* strains used in this study were independent of the origin of the strain as the environmental isolate WM 10.136 had similar virulence properties as the clinical strain WM 08.202.

Carbon utilization by *S. aurantiacum* strains

The virulence levels of *S. aurantiacum* strains can be attributed to the physiological differences [33], analysis at the phenotypic level can help to further understand the mechanisms of pathogenicity in this organism [34, 35]. Thus, utilization of a variety of nutrients (especially carbon sources) by the four *S. aurantiacum* strains was tested using an automated high throughput Biolog assay. The opm package for R was used for data analysis as it provides a range of benefits such as visualization and curve parameter estimation, metadata management, customizable plots and automated generation of tabular reports [36].

All four strains were able to respire on 54 out of the 94 substrates displayed on the GenIII microplates, including a range of sugars, a few amino acids and hexose acids at either 28 or 37°C. Temperature-based differences were observed specifically, when comparing the utilization of the most common substrate groups such as carbohydrates, carboxylic and amino acids. For example, maltose, D-salicin, D-fructose and lactose were utilized by two *S. aurantiacum* strains namely WM 08.202 and WM 10.136 only at 37°C. This is different from both WM 06.482 and WM 09.24, which could respire on these substrates at both 28 and 37°C thereby showing a similar metabolic response to the cultivation temperature (Fig. 4). Adaptation to the imposed temperature conditions has been described as an essential attribute of many highly virulent pathogens [37, 38].

Carbon acquisition and metabolism is central to the virulence and persistence of many lung pathogens and can be used to distinguish different strains within the species [34]. For example, difference in assimilation of five different carbon sources (ribitol, L-arabinitol, sucrose, maltose and ribose) was used to discriminate between the species. *S. aurantiacum* was distinguished from other members of the complex based on its inability to use sucrose as a substrate for growth [4]. In this study, two strains of *S. aurantiacum* (WM 06.482 and WM 09.24) were unable to metabolize sucrose but instead showed higher cellular respiration on its isomeric form turanose. While the outcome may seem surprising at the first glance, however there are previous studies proposing utilization of turanose as a potential indicator for virulence [39]. Turanose is known to promote high level of mycelial growth in the plant pathogen *Fusarium*

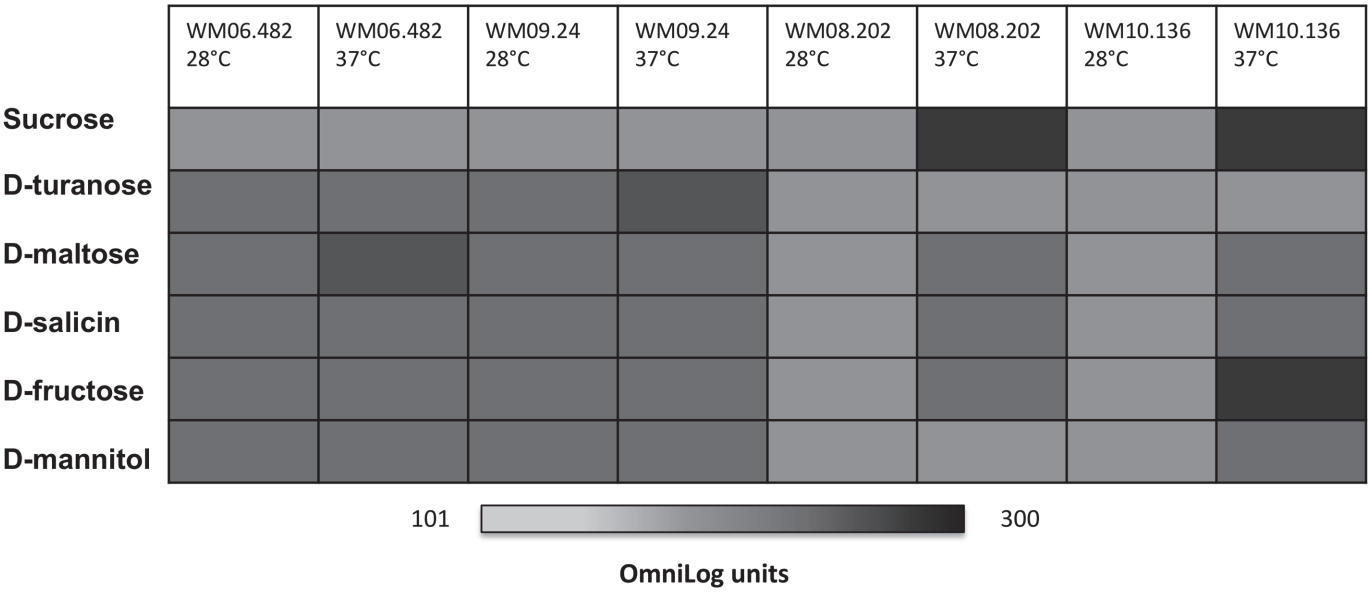

Fig 4. Different sugar utilization patterns of low and high virulent strains. OmniLog units with a minimum value of 100 depict a positive phenotype.

doi:10.1371/journal.pone.0122354.g004

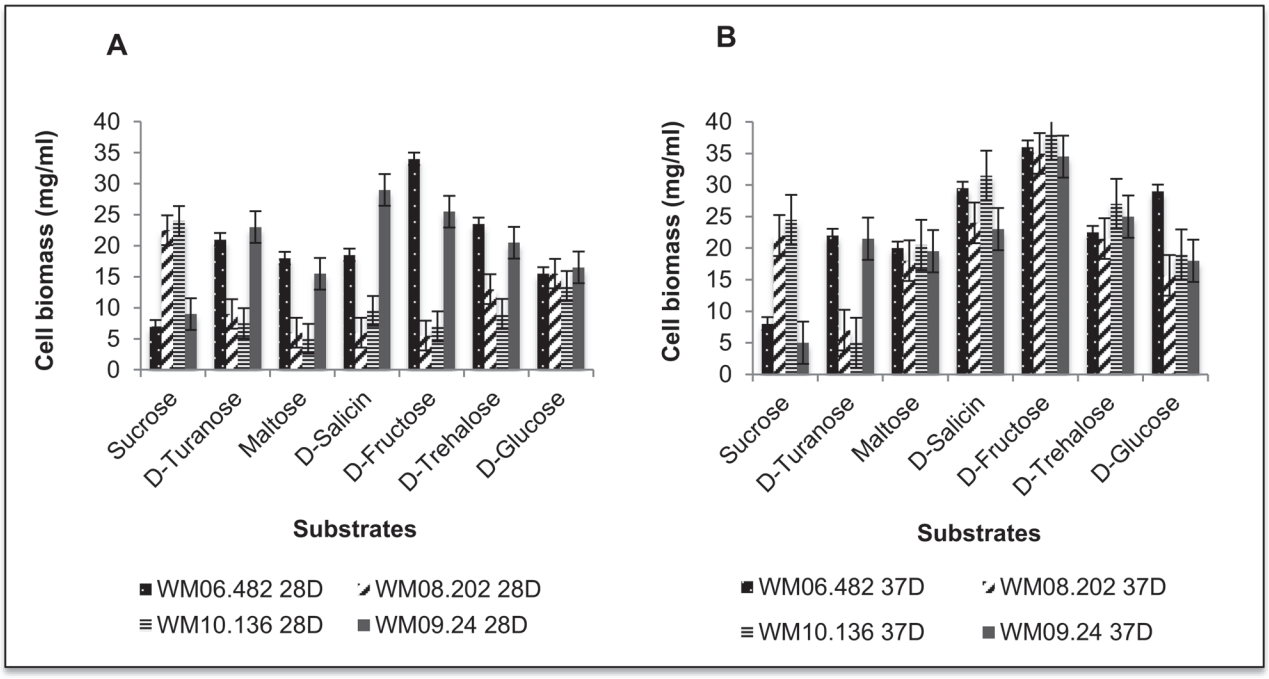

Fig 5. Relative growth of *S. aurantiacum* in minimal medium with different carbon sources, measured as average dry weight of mycelia at a) 28°C and b) 37°C. A different pattern is assigned to each strain as shown in the key below. The graphs clearly distinguish between the growth patterns of high and low virulent strains on selected carbon sources at both temperatures. (Weight of the inoculum was 10 mg/ml. Therefore biomass value above this limit was considered as significant growth). Each experiment was repeated in triplicate, with bars representing ±1.25 standard errors, and 95% confidence interval.

doi:10.1371/journal.pone.0122354.g005

virguliforme, and activates defense responses in higher plants suggesting a possible association between turanose assimilation and pathogenic properties [40–42]. High respiration rates on sucrose in WM 08.202 and WM 10.136 point towards the presence of a functional sucrose utilization pathway.

Correlation of growth and respiration assays

Cell respiration generally correlates with cell growth. In order to confirm the cellular respiration results obtained in the Biolog assay, growth of *S. aurantiacum* strains was determined in liquid cultures on a minimal medium supplemented with a panel of selected carbon compounds, incubated at 28°C (Fig. 5A) and 37°C (Fig. 5B). In accordance with the PM data, WM 06.482 and WM 09.24 showed growth on maltose, D-trehalose, D-salicin and D-fructose at both temperatures (28 and 37°C), whereas WM 08.202 and WM 10.136 were able to grow on these substrates only at high temperature (37°C).

Growth in the shake flasks was consistent with cell respiration on all six tested substrates as shown by plotting the cell biomass against the OmniLog units. Examples of this correlation at 37°C are shown in Fig. 6A for sucrose and Fig. 6B for turanose. *S. aurantiacum* strains WM 06.482 and WM 09.24 that showed no respiration on sucrose in the Biolog assay also did not show any growth in the minimal medium supplemented with sucrose. Instead, these strains grew well on turanose which is in accordance with efficient respiration measured in the PM assay on this substrate at 37°C. In contrast, the established low virulence strains WM 08.202 and WM 10.136 exhibited good respiration and growth on sucrose. Overall, the growth assays in liquid media were concordant with the Biolog phenotype microarray data, thus validating the respiration assay and illustrating its usability as a high-throughput phenotype assay for

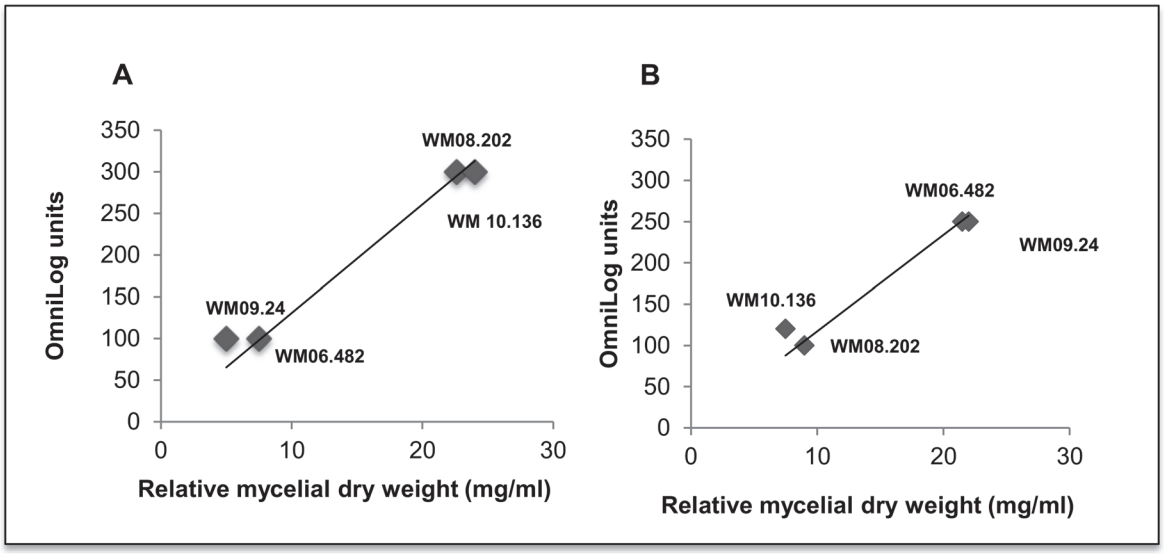

Fig 6. Correlation between cell growth and cell respiration for sucrose and turanose for *S. aurantiacum* at 37°C. Cell biomass was calculated for all the strains grown in sucrose and turanose supplemented minimal media and plotted against the respiration rates (OmniLog units). a) 1mM sucrose; b) 1mM turanose.

doi:10.1371/journal.pone.0122354.g006

Scedosporium. These findings suggest that common characteristics were shared between two groups of *S. aurantiacum* strains WM 06.482 and WM 09.24; and WM 08.202 and WM 10.136 as reflected in their substrate utilization patterns.

Resistance to selected chemicals

PM analysis revealed that all four strains of *S. aurantiacum* were highly halotolerant as high respiration rates were observed on high sodium chloride concentrations (4% and 8%). The salt tolerance capacity of *S. aurantiacum* is close to the optimum salt concentration required for the growth of the halophilic black yeast *Hortaea werneckii*, the most salt tolerant eukaryotic organisms reported to date [43]. Saline resistance could be one of the reasons for the persistence of *S. aurantiacum* in the salt-rich airway fluid of CF patients [44]. CF patients have abnormal salt transport across the airway epithelium, which causes defective mucociliary clearance and reduced clearance of the infectious agents [45]. Similarly, resistance of *S. aurantiacum* to other chemical treatments such as nalidixic acid and low pH under different temperature conditions can explain their pervasive nature and ability to survive under extreme environmental conditions [46].

The effect of amino acids on growth

The phenotype assays revealed some hexose acids, carboxylic acids, esters and fatty acids that resulted in slow or no growth. Examples of such compounds are aspartic acid, D-serine, L-histidine, L-pyroglutamic acid, D-galacturonic acid, L-galactonic acid-g-lactose, D-gluconic acid, D-glucuronic acid, mucic acid, D-saccharic acid, D-lactic acid methyl ester, a-keto-glutaric acid, D-malic acid and sodium formate. Some of these compounds such as D-lactic methyl ester that caused slow growth or no growth at all have been considered as inhibitors of the spore germination process e.g. in *Hypocrea jecorina* [21]. Given the high resistance of *S. aurantiacum* to many antifungal agents, similar combined strategies can be devised for screening the potential inhibitors against this pathogenic fungus.

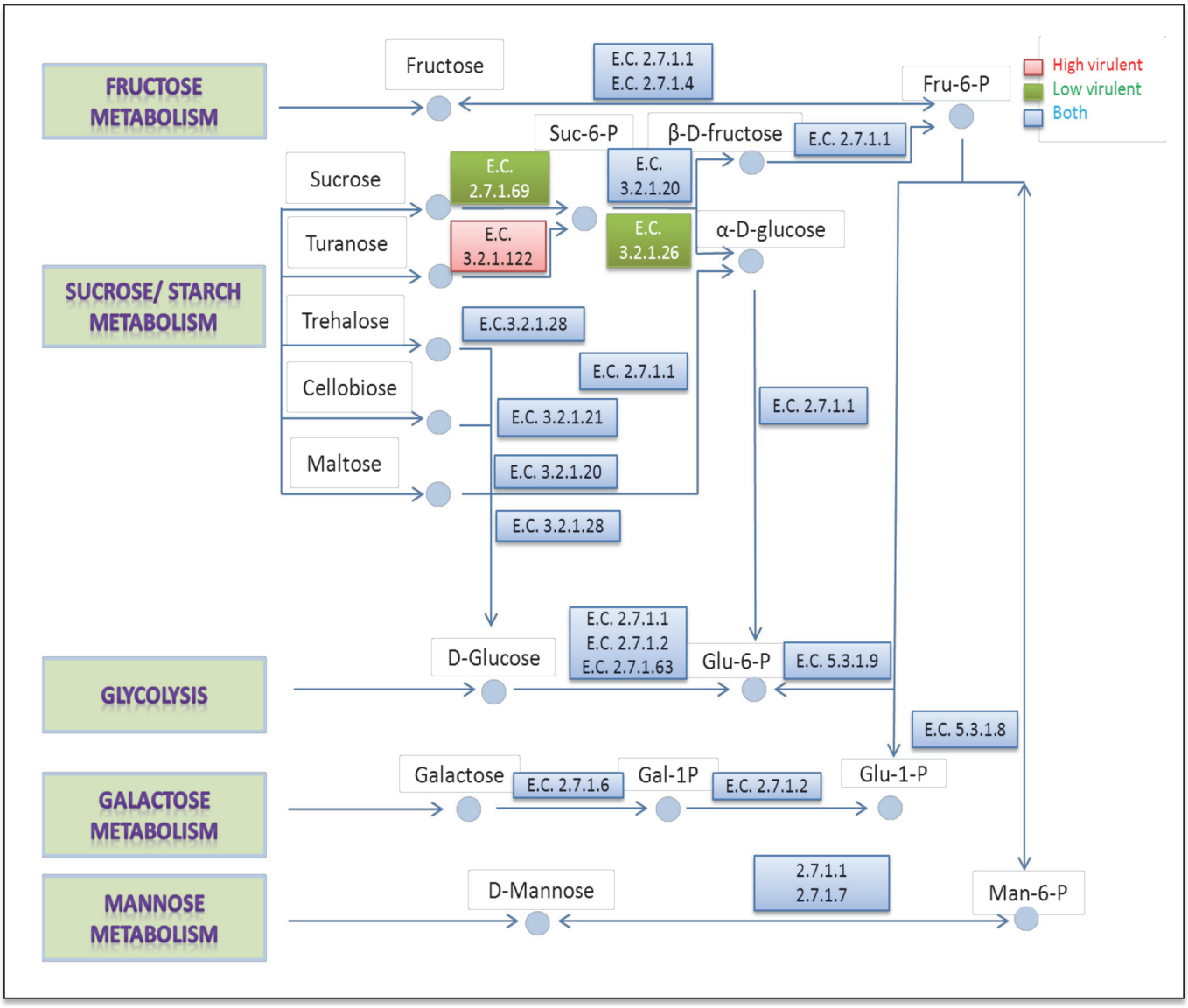

Fig 7. Schematic overview of various metabolic pathways present in *S. aurantiacum* obtained after superimposition of PM data with the assembled genome.

doi:10.1371/journal.pone.0122354.g007

Metabolic reconstructions using the *S. aurantiacum* draft genomes

Metabolic reconstructions were carried out for all the four *S. aurantiacum* strains by ascribing the observed phenotypic differences to the presence or absence of certain enzyme encoding genes in a particular carbohydrate utilization pathway. The main metabolic pathways found in the analysis were then used for the generation of biochemical maps (Fig. 7) that show the network of genome-encoded enzymes catalyzing the metabolism of different carbohydrates in the four different *S. aurantiacum* strains studied in this work. Five major carbohydrate metabolism pathways were revealed in the genome-wide analysis of *S. aurantiacum*, namely sucrose, fructose, mannose and galactose metabolism and glycolysis (Fig. 7).

Sucrose metabolism can involve either one-step or a two-step reaction depending on the enzymes involved in the overall process. In a two-step reaction common in many filamentous

fungi, sucrose is converted to sucrose-6-phosphate by the phosphotransferase enzyme and ultimately to the end product glucose in a reaction catalyzed by invertase [47, 48]. Alternatively, sucrose can be directly hydrolyzed to glucose solely by invertase. Based on the metabolic pathways reconstructed from genome data, only the low virulence strains WM 08.202 and WM 10.136 seemed to possess both the phosphotransferase (E.C. 2.7.1.69) and invertase (E.C. 3.2.1.26) enzymes which allowed them to utilize sucrose for growth and respiration.

On the other hand, inability of WM 06.482 and WM 09.24 to grow/respire on sucrose (as seen in Fig. 4 and 6) can be explained by the absence of sucrose-hydrolysing enzymes as revealed by the genome correlation. The assimilation of turanose in these strains can be attributed to the presence of phospho-alpha-glucosidase (E.C. 3.2.1.122) which was not found in the low virulence strains. The other three metabolic pathways studied including fructose, galactose and mannose metabolism were similar in all four *S. aurantiacum* strains. Thus phenotypic testing allowed us to specifically search for the genetic factors underpinning the phenotypes of different strains of *S. aurantiacum*.

In principle, the presence or absence of respective enzymes in different *S. aurantiacum* strains can be verified by amplification of the gene sequences from the genomic DNA. However, considering the non-availability of a fully annotated *S. aurantiacum* genome and the limited overall sequence homology (~40%) for the identified gene sequences between *S. aurantiacum* and the reference organisms, this methodology can lead to inconclusive results. Nevertheless, the ability of the strains to metabolize the discussed carbohydrates is a strong, yet indirect indication for the presence of genes encoding the required enzymes [49].

Conclusions

We have used morphological, physiological and metabolic assessment to characterize four different *S. aurantiacum* strains exhibiting different virulence levels. The analysis helped to identify metabolic differences between two groups of *S. aurantiacum* strains, WM 06.482 and WM 09.24; and WM 08.202 and WM 10.136. Correlation of the genome information with the metabolic assessment assisted in exposing five putative carbohydrate metabolism pathways of which sucrose and D-turanose utilization different between the above *S. aurantiacum* groups. While classification of the environmental strain WM 09.24 as a high or low virulence strain was not straightforward from the *Galleria mellonella* assay, it behaved similar to the high virulent strain WM 06.482 for which the virulence has also been established in a mouse model. Therefore we group WM 09.24 together with WM 06.482, which leads us to speculate on metabolic differences between high and low virulence strains, such as ability to utilize D-turanose. The differences can be investigated further with fully annotated genomes available in the near future.

Supporting Information

S1 Fig. Layout of biolog GenIII plate. Layout of the Biolog GenIII plate depicting various conditions/substrates used to detect substrate utilization of *S. aurantiacum* strains WM06.482, WM08.202, WM10.136 and WM09.24. The various substrates listed can be categorized as follows: 1) Control: A1. 2) Sugars: A2-A9, B1-B9 and C1-C9. 3) Hexose phosphates: from D06 and D07. 4) Amino acids: from E1-E9. 5) Hexose acids: from F1-F9. 6) Carboxylic acids, esters and fatty acids: G1-G9 and H1-H9. 7) Acidic pH: A11 and A12. 8) NaCl: B10-B12. 9) Lactic acids: C10. 10) Reducing agents: F11 and F12. 11) Gram negative/gram positive: F10 and G10. (TIFF)

Author Contributions

Conceived and designed the experiments: JK SD LV AP WM IP HN. Performed the experiments: JK SD. Analyzed the data: JK LV AP. Wrote the paper: JK HN.

References

1. Kaltseis J, Rainer J, De Hoog GS. Ecology of *Pseudallescheria* and *Scedosporium* species in human-dominated and natural environments and their distribution in clinical samples. Med Mycol. 2009; 47: 398–405. doi: 10.1080/13693780802585317 PMID: 19085459
2. Harun A, Gilgado F, Chen SC, Meyer W. Abundance of *Pseudallescheria*/*Scedosporium* species in the Australian urban environment suggests a possible source for scedosporiosis including the colonization of airways in cystic fibrosis. Med Mycol. 2010; 48 Suppl 1: S70–76. doi: 10.3109/13693786.2010.515254 PMID: 21067333
3. Alvarez E, Garcia-Hermoso D, Sutton DA, Cano JF, Stchigel AM, Hoinard D, et al. Molecular phylogeny and proposal of two new species of the emerging pathogenic fungus *Saksenaea*. J Clin Microbiol. 2010; 48: 4410–4416. doi: 10.1128/JCM.01646-10 PMID: 20926710
4. Gilgado F, Cano J, Gene J, Sutton DA, Guarro J. Molecular and phenotypic data supporting distinct species statuses for *Scedosporium apiospermum* and *Pseudallescheria boydii* and the proposed new species *Scedosporium dehoogii*. J Clin Microbiol. 2008; 46: 766–771. PMID: 18077629
5. Gilgado F, Cano J, Gene J, Guarro J. Molecular phylogeny of the *Pseudallescheria boydii* species complex: proposal of two new species. J Clin Microbiol. 2005; 43: 4930–4942. PMID: 16207945
6. Gilgado F, Cano J, Gene J, Serena C, Guarro J. Different virulence of the species of the *Pseudallescheria boydii* complex. Med Mycol. 2009; 47: 371–374. doi: 10.1080/13693780802256539 PMID: 18651312
7. Harun A, Serena C, Gilgado F, Chen SC, Meyer W. *Scedosporium aurantiacum* is as virulent as *S. prolificans*, and shows strain-specific virulence differences, in a mouse model. Med Mycol 48. 2010;Suppl 1: S45–51. doi: 10.3109/13693786.2010.517224 PMID: 21067330
8. Lackner M, de Hoog GS, Verweij PE, Najafzadeh MJ, Curfs-Breuker I, Klaassen CH, et al. Species-specific antifungal susceptibility patterns of *Scedosporium* and *Pseudallescheria* species. Antimicrob Agents Chemother. 2012; 56: 2635–2642. doi: 10.1128/AAC.05910-11 PMID: 22290955
9. Heath CH, Slavin MA, Sorrell TC, Handke R, Harun A, Phillips M, et al. Population-based surveillance for scedosporiosis in Australia: epidemiology, disease manifestations and emergence of *Scedosporium aurantiacum* infection. Clin Microbiol Infect. 2009; 15: 689–693. doi: 10.1111/j.1469-0691.2009.02802.x PMID: 19549223
10. Cortez KJ, Roilides E, Quiroz-Telles F, Meletiadis J, Antachopoulos C, Knudsen T, et al. Infections caused by *Scedosporium* spp. Clin Microbiol Rev. 2008; 21: 157–197. doi: 10.1128/CMR.00039-07 PMID: 18202441
11. Delhaes L, Harun A, Chen SC, Nguyen Q, Slavin M, Heath CH, et al. Molecular typing of Australian *Scedosporium* isolates showing genetic variability and numerous *S. aurantiacum*. Emerg Infect Dis. 2008; 14: 282–290. doi: 10.3201/eid1402.070920 PMID: 18258122
12. Blyth CC, Middleton PG, Harun A, Sorrell TC, Meyer W, Chen SC. Clinical associations and prevalence of *Scedosporium* spp. in Australian cystic fibrosis patients: identification of novel risk factors? Med Mycol 48. 2010;Suppl 1: S37–44. doi: 10.3109/13693786.2010.500627 PMID: 21067328
13. Pihet M, Carrere J, Cimon B, Chabasse D, Delhaes L, Symoens F, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis-a review. Med Mycol. 2009; 47: 387–397. doi: 10.1080/13693780802609604 PMID: 19107638
14. Cohen TS, Prince A. Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat Med. 2012; 18: 509–519. doi: 10.1038/nm.2715 PMID: 22481418
15. Santos AL, Bittencourt VC, Pinto MR, Silva BA, Barreto-Bergter E. Biochemical characterization of potential virulence markers in the human fungal pathogen *Pseudallescheria boydii*. Med Mycol. 2009; 47: 375–386. doi: 10.1080/13693780802610305 PMID: 19235547
16. Tanzer MM, Arst HN, Skalchunes AR, Coffin M, Darveaux BA, Heiniger RW, et al. Global nutritional profiling for mutant and chemical mode-of-action analysis in filamentous fungi. Funct Integr Genomics. 2003; 3: 160–170. PMID: 12898394
17. Fong SS, Joyce AR, Palsson BO. Parallel adaptive evolution cultures of *Escherichia coli* lead to convergent growth phenotypes with different gene expression states. Genome Res. 2005; 15: 1365–1372. PMID: 16204189

18. Atanasova L, Druzhinina IS. Review: Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi. J Zhejiang Univ Sci B. 2010; 11: 151–168. doi: 10.1631/jzus.B1000007 PMID: 20205302

19. Hoyos-Carvajal L, Orduz S, Bissett J. Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. Fungal Genet Biol. 2009; 46: 615–631. doi: 10.1016/j.fgb.2009.04.006 PMID: 19439189

20. Caddick MX, Peters D, Platt A. Nitrogen regulation in fungi. Antonie Van Leeuwenhoek. 1994; 65: 169–177. PMID: 7847882

21. Druzhinina IS, Schmoll M, Seiboth B, Kubicek CP. Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. Appl Environ Microbiol. 2006; 72: 2126–2133. PMID: 16517662

22. Blyth CC, Harun A, Middleton PG, Sleiman S, Lee O, Sorrell TC, et al. Detection of occult *Scedosporium* species in respiratory tract specimens from patients with cystic fibrosis by use of selective media. J Clin Microbiol. 2010; 48: 314–316. doi: 10.1128/JCM.01470-09 PMID: 19906904

23. Firacative C, Duan S, Meyer W. *Galleria mellonella* model identifies highly virulent strains among all major molecular types of *Cryptococcus gattii*. PLoS One. 2014; 9: e105076. doi: 10.1371/journal.pone.0105076 PMID: 25133687

24. Vaas LA, Sikorski J, Michael V, Goker M, Klenk HP. Visualization and curve-parameter estimation strategies for efficient exploration of phenotype microarray kinetics. PLoS One. 2012; 7: e34846. doi: 10.1371/journal.pone.0034846 PMID: 22536335

25. Bercoff AP, Papanicolaou A, Ramsperger M, Kaur J, Patel H, Harun A, et al. Draft genome of the opportunistic human pathogen *Scedosporium aurantiacum*—Australian environmental strain WM 09.24. Genome Announc. 2015; 3: e01526–14. doi: 10.1128/genomeA.01526-14 PMID: 25676755

26. Caspi R, Altman T, Billington R, Dreher K, Foerster H, Fulcher CA, et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids Res. 2014; 42: D459–471. doi: 10.1093/nar/gkt1103 PMID: 24225315

27. NCBI BLAST. Available: http://www.ncbi.nlm.nih.gov/blast/.

28. Loira N, Dulermo T, Nicaud JM, Sherman DJ. A genome-scale metabolic model of the lipid-accumulating yeast *Yarrowia lipolytica*. BMC Syst Biol. 2012; 6: 35. doi: 10.1186/1752-0509-6-35 PMID: 22558935

29. Smedsgaard J, Nielsen J. Metabolite profiling of fungi and yeast: from phenotype to metabolome by MS and informatics. J Exp Bot. 2005; 56: 273–286. PMID: 15618299

30. Steen BR, Lian T, Zuyderduyn S, MacDonald WK, Marra M, Jones SJ, et al. Temperature-regulated transcription in the pathogenic fungus *Cryptococcus neoformans*. Genome Res. 2002; 12: 1386–1400. PMID: 12213776

31. Cotter G, Doyle S, Kavanagh K. Development of an insect model for the *in vivo* pathogenicity testing of yeasts. FEMS Immunol Med Microbiol. 2000; 27: 163–169. PMID: 10640612

32. Harun A, Perdomo H, Gilgado F, Chen SC, Cano J, Guarro J, et al. Genotyping of *Scedosporium* species: a review of molecular approaches. Med Mycol. 2009; 47: 406–414. doi: 10.1080/13693780802510240 PMID: 19085455

33. Llorens A, Hinojo MJ, Mateo R, Gonzalez-Jaen MT, Valle-Algarra FM, Logrieco A, et al. Characterization of *Fusarium* spp. isolates by PCR-RFLP analysis of the intergenic spacer region of the rRNA gene (rDNA). Int J Food Microbiol. 2006; 106: 297–306. PMID: 16246443

34. Morales CA, Porwollik S, Frye JG, Kinde H, McClelland M, Guard-Bouldin. Correlation of phenotype with the genotype of egg-contaminating *Salmonella enterica* serovar *Enteritidis*. Appl Environ Microbiol. 2005; 71: 4388–4399. PMID: 16085829

35. Lan CY, Newport G, Murillo LA, Jones T, Scherer S, Davis RW, et al. Metabolic specialization associated with phenotypic switching in *Candida albicans*. Proc Natl Acad Sci U S A. 2002; 99: 14907–14912. PMID: 12397174

36. Vaas LA, Sikorski J, Hofner B, Fiebig A, Buddruhs N, Kenk HP, et al. opm: an R package for analysing OmniLog (R) phenotype microarray data. Bioinformatics. 2013; 29: 1823–1824. doi: 10.1093/bioinformatics/btt291 PMID: 23740744

37. Bochner BR. New technologies to assess genotype-phenotype relationships. Nat Rev Genet. 2003; 4: 309–314. PMID: 12671661

38. Kraus PR, Boily MJ, Giles SS, Stajich JE, Allen A, Coz GM, et al. Identification of *Cryptococcus neoformans* temperature-regulated genes with a genomic-DNA microarray. Eukaryot Cell. 2004; 3: 1249–1260. PMID: 15470254

39. Loesch A, Hutwimmer S, Strasser H. Carbon utilization pattern as a potential quality control criterion for virulence of *Beauveria brongniartii*. J Invertebr Pathol. 2010; 104: 58–65. doi: 10.1016/j.jip.2010.01.007 PMID: 20123102

40. Tang E, Hill CB, Hartman G. Carbon utilization profiles of *Fusarium virguliforme* isolates. Can J Microbiol. 2010; 56: 979–986. doi: 10.1139/W10-085 PMID: 21164567

41. Khalil S, Alsanius BW. Utilisation of carbon sources by *Pythium*, *Phytophthora* and *Fusarium* species as determined by Biolog (R) microplate assay. Open Microbiol J. 2009; 3: 9–14. doi: 10.2174/1874285800903010009 PMID: 19294012

42. Sinha AK, Hofmann MG, Romer U, Kockenberger W, Elling L, Roitsch T. Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. Plant Physiol. 2002; 128: 1480–1489. PMID: 11950996

43. Gunde-Cimerman N, Zalar P, de Hoog S, Plemenitaš A. Hypersaline waters in salterns—natural ecological niches for halophilic black yeasts. FEMS Microbiol Ecol. 2000; 32: 235–240. PMID: 10858582

44. Wine JJ. The genesis of cystic fibrosis lung disease. J Clin Invest. 1999; 103: 309–312. PMID: 9927490

45. Chmiel JF, Davis PB. State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? Respir Res. 2003; 4: 8. PMID: 14511398

46. Guarro J, Kantarcioglu AS, Horre R, Rodriguez-Tudela JL, Cuenca Estrella M, Berenguer J, et al. *Scedosporium apiospermum*: changing clinical spectrum of a therapy-refractory opportunist. Med Mycol. 2006; 44: 295–327. PMID: 16772225

47. Mandels GR. Metabolism of Sucrose and Related Oligosaccharides by Spores of the Fungus *Myrothecium Verrucaria*. Plant Physiol. 1954; 29: 18–26. PMID: 16654607

48. Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK. Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot. 2003; 54: 513–524. PMID: 12508062

49. Jun H, Kieselbach T, Jonsson LJ. Enzyme production by filamentous fungi: analysis of the secretome of *Trichoderma reesei* grown on unconventional carbon source. Microb Cell Fact. 2011; 10: 68. doi: 10.1186/1475-2859-10-68 PMID: 21861877

P L o S One

BACK TO RESULTS

Click for Open Access

Basic Description | Abstracting/ Indexing & Article Access | Publisher & Ordering Information | Advertising, Rights, Demographics

JCR Web

Click highlighted text for a new search on that item.

ISSN:	1932-6203
Title:	P L o S One Additional Title Information
Publishing Body:	Public Library of Science
Country:	United States
Status:	Active
Start Year:	2006
Frequency:	Irregular
Document Type:	Journal; Academic/Scholarly
Refereed:	Yes
Abstracted/Indexed:	Yes
Media:	Online - full text
Language:	Text in English
Price:	Free
Subject:	SCIENCES: COMPREHENSIVE WORKS MEDICAL SCIENCES
Dewey #:	500, 610
LC#:	Q179.9
Editor(s):	Damian Pattinson
Publisher(s):	Peter Binfield
URL:	http://www.plosone.org/home.action
Description:	Covers primary research from all disciplines within science and medicine.

ADDITIONAL TITLE INFORMATION

Acronym Description:	Public Library of Science
Alternate Title:	Variant format: PLoS ONE

Back to Top

Add this item to: (select a list) + ADD

Request this title: I'd like to request this title. GO

Corrections: Submit corrections to Ulrich's about this title. GO

Publisher of this title? If yes, click GO! to contact Ulrich's about updating your title listings in the Ulrich's database. GO

Print • Download • E-mail

Back to Top