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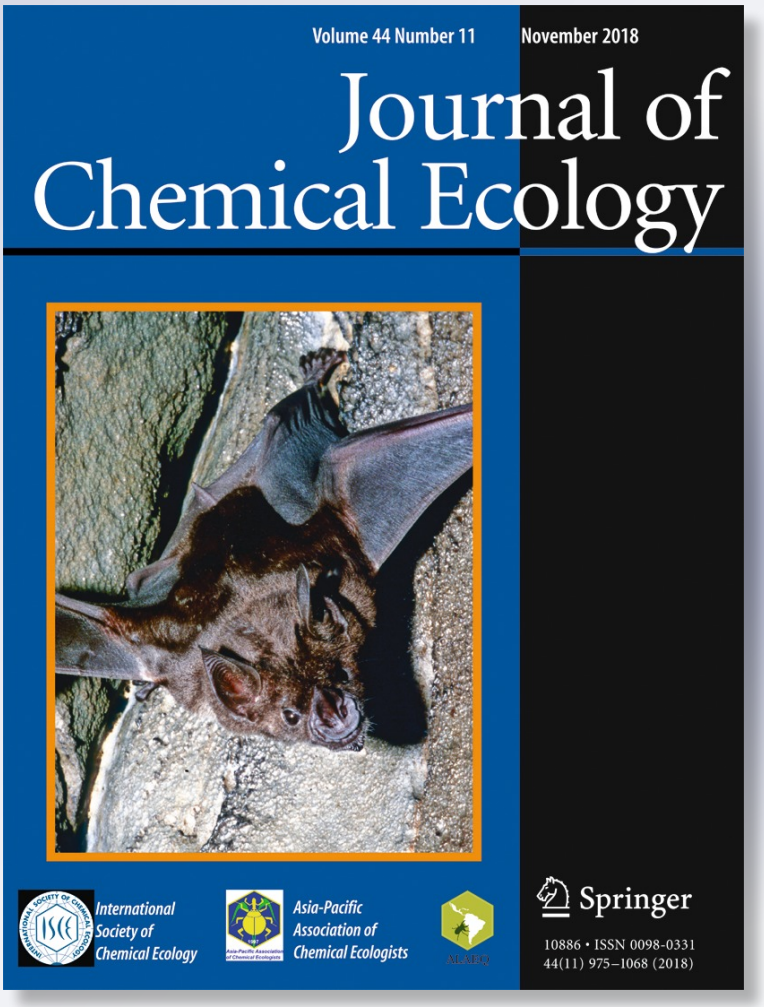

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α-Amylase Mediates Host Acceptance in the Braconid Parasitoid *Cotesia flavipes*

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Abstract

Foraging parasitoids use chemical signals in host recognition and selection processes. Although, the volatiles play a relevant role in the localization by parasitoids of their hosts feeding on plants, the host identification process for acceptance occurs mainly during contact between the parasitoid and its host where host products related to feeding activities, fecal pellets and oral secretions, play a crucial role. The purpose of this study was to identify the nature of the contact kairomone(s) that mediate the acceptance for oviposition of the parasitoid *Cotesia flavipes* Cameron (Hymenoptera, Braconidae), which was released in Kenya in 1993 to control the invasive crambid *Chilo partellus* (Swinhoe). Using host and non-hosts of *C. flavipes*, we showed that it is mainly the oral secretions of the larvae that harbour the active compound(s) that mediate host acceptance for oviposition by *C. flavipes*. Using an integration of behavioral observations and biochemical approaches, the active compound of the oral secretions was identified as an α-amylase. Using synthetized α-amylases from *Drosophila melanogaster* (an insect model for which syntheses of active and inactive α-amylases are available), we observed that the conformation of the enzyme rather than its catalytic site as well as its substrate and its degradation product is responsible for host acceptance and oviposition mediation of *C. flavipes* females. The results suggest that the α-amylase from oral secretions of the caterpillar host is a good candidate for an evolutionary solution to host acceptance for oviposition in *C. flavipes*.

Keywords Biological control · Pest insects · Lepidoptera stemborers · *Chilo partellus* · *Cotesia flavipes* · Kairomone · α-amylase · Host recognition · Parasitoids · Host oral secretion · Multitrophic interactions · Semiochemicals

Introduction

The ability of parasitoids to successfully utilize cues that allow the host's habitat location, and to discriminate between suitable and unsuitable hosts is vital for their efficiency in the field (Wajnberg et al. 2008; Wajnberg and Colazza 2013). During location of hosts, parasitoids typically exploit long and short-range stimuli emanating from the host habitat (Godfray 1994; Vinson 1975, 1976), followed by stimuli directly associated with the host and its products (Godfray 1994; Vet and Dicke 1992; Vinson 1976, 1985). The host's habitat location is often mediated by volatile organic compounds (plant VOCs) of the plant resulting from the elicitation of plant defence metabolic pathways produced constitutively in response to salivary enzymes produced by the insect when feeding on the plant (Dicke 2016; Erb et al. 2011; Heil 2008; Turlings et al. 1990). However, VOCs do not convey reliable information on the suitability of the caterpillar species but rather act as indicators of the presence of herbivores (Ngi-Song and Overholt 1997; Obonyo et al. 2008). It is only when approaching the host that reliable information on the host's identity is perceived via contact-chemoreception by the parasitoid; this information consists of fecal pellets and oral

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secretions produced by the host's feeding activities (Ngi-Song and Overholt 1997; Obonyo et al. 2010a, b) and see Kaiser et al. [2017a] for a recent review. To ensure that they will parasitize a suitable host, the parasitoids need to be able to discriminate between different species and particularly between hosts and non-hosts. Among parasitoids, some are generalists able to parasitize a wide range of herbivorous hosts while others such as many endoparasitoids are restricted to parasitize only one or a few related host species (Harvey et al. 2005). Among parasitoids, *Cotesia* is one of the most diverse genera (Kaiser et al. 2017b). Many *Cotesia* species may appear generalists but careful ecological studies revealed a hidden complexity with an assemblage of populations with restricted host ranges (Kaiser et al. 2017b).

In sub-Saharan Africa, lepidopteran stemborers of the Crambidae, Pyralidae and Noctuidae families are economically important pests of maize and sorghum (Harris 1990; Kfir et al. 2002; Polaszek 1998). The most cited species are the crambid *Chilo partellus* (Swinhoe), the noctuids *Busseola fusca* (Fuller) and *Sesamia calamistis* Hampson, and the pyralid *Eldana saccharina* (Walker) (Polaszek 1998). With the exception of *C. partellus*, which was accidentally introduced from Asia into Africa before the 1930s (Kfir 1992), they are indigenous to Africa. During the early 1990s, the International Centre of Insect Physiology and Ecology (*icipe*) initiated a project on the biological control of *C. partellus* with the introduction of *Cotesia* into Kenya from Asia. *Cotesia flavipes* parasitizes the larvae of more than 30 Lepidoptera species including *C. partellus*, *Chilo suppressalis* (Walker), *S. calamistis* and *Spodoptera mauritiana* Boisduval (Boisduval) (Lep.: Noctuidae) (https://www.cabi.org/isc/datasheet/5951). It was first released in the coastal area in 1993 (Overholt et al. 1994), where it reduced *C. partellus* densities by over 50% (Jiang et al. 2006; Zhou et al. 2001). However, its presence and efficiency appear variable according to the location and the type of the crop cultivated (Cugala et al. 2001). There was therefore a need to understand the relationships between this parasitoid species and its host for a better biological control management (Bichang'a 2013).

The present study first tries to clarify the role of fecal pellets and oral secretions in mediating host acceptance for oviposition in *C. flavipes*. Thereafter, an integration of behavioral observations and biochemical approaches were used to identify the active compounds.

Materials and Methods

Insects *Cotesia flavipes* adults were obtained from laboratory-reared colonies established at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. The colony originated from individuals collected in the field in the coastal region of Kenya in 1998. Field-collected *C. flavipes* were added twice a year to regenerate the colony. The parasitoid was reared on *C. partellus* larvae according to Overholt et al. (1994). Parasitoid cocoons were kept in a Perspex cage (30 cm × 30 cm × 30 cm) until their emergence. Adult parasitoids were fed on a 20% honey/water solution, put under artificial light and left for 24 hr to mate. In the behavioral bioassays, only 1-day-old naïve (i.e. without oviposition experience), mated females were used. Experimental conditions were maintained at 25 ± 2 °C, 50–80% relative humidity (RH), and a 12:12 hr (L:D) photoperiod (Overholt et al. 1994).

The natural host *C. partellus*, the suitable new association host *S. calamistis* as well as the non-hosts *B. fusca* and *S. nonagrioides* were used in this study (Obonyo et al. 2008).

Chilo partellus and *S. calamistis* larvae were collected from maize grown in coastal regions of Kenya, and *B. fusca* larvae from maize grown in Western Kenya (Kitale), while *S. nonagrioides* larvae were collected from *Typha domingensis* in Makindu. The larvae of *C. partellus* were reared on the artificial diets described by Ochieng et al. (1985) whereas the larvae of the other species were reared on the artificial diet described by Onyango and Ochieng'-Odero (1994). For each host species, feral stemborer larvae from their respective region were added twice a year to rejuvenate the colonies.

Acceptance of host larvae for oviposition by *C. flavipes* was reported to be enhanced when the host larvae were fed on maize stems for 24 hr prior exposure to parasitism (Inayatullah 1983; Mohyuddin et al. 1981; Overholt et al. 1994; Potting et al. 1993; Van Leerdam et al. 1985). Therefore, for isolation of the semiochemicals produced during feeding of larvae of suitable and unsuitable borer species that could be involved in host acceptance by *C. flavipes*, larvae previously fed for 24 hr on maize stems were used.

Collection of Maize Stem Juice and Fecal Pellets of Chilo Partellus The purpose of this study was to confirm that the active compound(s) mediating *C. partellus* acceptance came from the host's products related to feeding activities and not from the host's food. In this context, a portion of maize stem was crushed and the resulting juice was collected and directly used in behavioral bioassays. Thereafter, fresh fecal pellets collected from larvae previously fed for 24 hr on maize stems were also directly used.

Collection of Oral Secretions from Host and Non-host Larvae Since the oral secretions were found to harbour the active compound(s) mediating host acceptance, the remainder of the study focused on larval oral secretions. For the collection of oral secretions, a single larva held with soft forceps was squeezed behind the head and a capillary tube was used to collect oral secretions, which were placed directly on ice. The process was repeated with at least

100 larvae. The volume of oral secretion was estimated by weighting. The samples were either used directly in behavioral bioassays or preserved at −80 °C for later use. Different types of oral secretions were used:

- Secretions from larvae of *C. partellus*, *S. calamistis*, *B. fusca,* and *S. nonagrioides* previously fed for 24 hr on maize stems to confirm the involvement of oral secretions in the specificity of host-parasitoid associations.
- Oral secretions of *C. partellus* previously fed 24 hr on stems of *Pennisetum purpureum* Schumach. (Poaceae), which is often found in the surroundings of maize fields, as well as the artificial diet as described by Ochieng et al. (1985) to test if the active compounds depended on the host plant or the type of food.
- The oral secretions from larvae starved for 48 hr to verify if production of these semiochemicals were induced by larval feeding.
- In a previous study, it was hypothesized that the semiochemicals from oral secretions involved in host recognition by *C. flavipes* might include enzymes or thermolabile proteins (Obonyo et al. 2010b). Therefore, the oral secretions from larvae fed on maize stems and previously treated with proteinase K (Sigma product P2308) that destroys the proteins present in the oral secretions, were used. Hence, about 40 μl of oral secretion of larvae of *C. partellus* previously fed on maize stems was treated with 1.25 units/mg of proteinase K at 37 °C for 1 hr.

Behavioral Bioassays In previous studies, the parasitic wasps exhibited host recognition and acceptance by antennating the surface of the host body followed by at least one stinging attempt indicating acceptance of a caterpillar as a host for oviposition (Obonyo et al. 2010a, b). Therefore, these two behavioral steps (Fig. 1 and Appendix 1) were used here as evidence for host acceptance when offering fresh fecal pellets from *C. partellus* or the different extracts (i.e. plant juice and different type of oral secretions, as well as electrophoretic bands obtained in the next section, known proteins and other compounds). The sample to be tested was placed at the centre of a 8 cm diameter Petri dish and presented to a single female wasp. For each replication, about 0.01 g of fresh fecal pellet or about 0.5 to 1 μl of the extract to be tested were deposited on a 2 mm cotton wool ball. A single female wasp was introduced in the arena near the fresh fecal pellet or the cotton wool ball and were both covered with a transparent circular Perpex lid (3 cm diameter, 1 cm height) to prevent the parasitoid from flying off.

The behaviour of the wasp in the Petri dish was then monitored for a maximum of 120 sec. For each wasp, both antennation and stinging attempt were recorded. The percentage of positive responses was calculated from 10 or 30 wasps tested per electrophoretic band or per type of sample tested (see previous and next sections), respectively. The wasp, the cotton wool ball with tested extracts, and the arena were replaced after each wasp had been tested.

All behavioural experiments were carried out in a room at 26 ± 1 °C between 10 to 14 h00 with a constant source of light to maintain an optimal temperature for the behavioural activities of the female wasps.

Electrophoresis and Isolation of Proteins from the Polyacrylamide Gel The oral secretions from *C. partellus* were first centrifuged at a maximum speed of 14,000×g for 5 min in order to remove any debris such as undigested food materials. This was followed by desalting and concentrating the samples using Amicon® Ultra-0.5 centrifugal filter devices (Merck Millipore). The samples were then quantified using the Pierce BCA protein assay Kit (Thermo Scientific No. 23227) based on bicinchoninic acid (Smith et al. 1985). All the measurements were carried out using Eppendorf-Biospectrometer fluorescence machine (SN 667).

Electrophoresis was then conducted under non-denaturing conditions (native PAGE electrophoresis, Ornstein-Davis discontinuous buffer system) according to Chrambach and Jovin (1983) and Niepmann and Zheng (2006). The gels were cast in two sections using the Bio Rad Mini-PROTEAN® Electrophoresis System and Hoefer™ Mini Vertical Electrophoresis Systems (Fisher Sci.com). A stacking gel

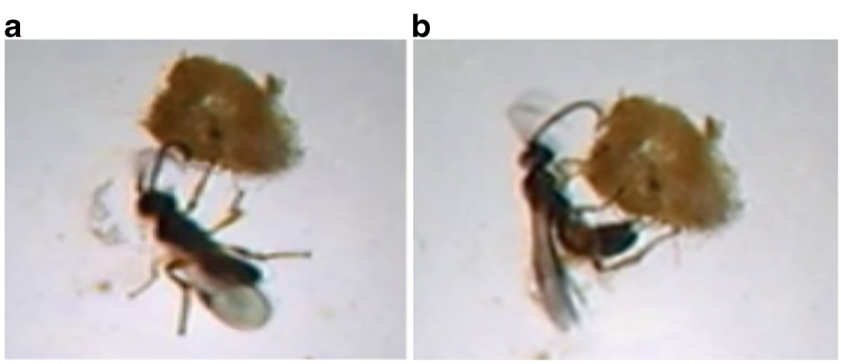

Fig. 1 The parasitic wasps exhibited a host recognition and acceptance by typically antennating (i.e. antennal drumming) the surface of the host body (A) followed by at least one stinging attempt (B) (see also the video in Appendix 1). These two behavioural steps are used as evidence for host acceptance by the parasitoid

(4%T, 2.7%C, 0.125 M Tris-Cl pH 6.8) was cast on top of a resolving gel of (7.5%, T4.4%C, 0.125 M Tris-Cl pH 6.8). Electrophoresis was conducted (running buffer: 0.025 M Tris, 0.192 M glycine pH 8.3) immediately after loading the samples at a constant voltage of 150 V and current of 25 mA for 1-2 hr in a cold room. At the end of the run, gels were immediately removed and stained for 30 min in a staining solution consisting of 0.2% Coomassie Brilliant Blue R250. The gels were then destained with a solution of methanol, glacial acetic acid and water at the ratio of 4:1:5. The stained proteins were compared to a molecular mass standard (Sigma Aldrich) containing albumin from bovine serum (Sigma A8654, 132 kDa), urease from jack bean (Sigma U7752, 272 and 545 kDa), α lactalbumin from bovine milk (Sigma L4385, 14.2 kDa) and albumin from chicken egg white (Sigma A8529, 45 kDa).

For the isolation of electrophoretic bands, the protein bands were manually excised from the gel before staining process following the method of Kurien and Scofield (2012) with some modifications. The excised gel fragments containing the protein of interest were frozen overnight at −80 °C. Each frozen gel fragment was ground using a mortar into fine powder under liquid nitrogen and the resulting gel powder transferred to the upper chamber of the Costar® column (centrifuge tube filters, Costar lot No. 22304012 Corning incorporated, NY 14831-USA). The protein trapped in the gel powder was eluted using native elution buffer 0.25 M Tris HCl buffer pH 6.8, or normal saline depending on subsequent application. After 10 min of centrifugation at 13000×g, 300 to 350 μl of the filtrate was recovered and stored for further concentration and desalting. A second elution was performed with fresh elution buffer and a filtrate of approximately 250–300 μl was collected and combined with the previous one. Each protein eluted was concentrated 25–30 × folds using an Amicon centrifugal device equipped with 30 K MWCO omega membrane. The concentrated protein eluents were assayed for protein content with the aforementioned Pierce BCA protein assay Kit. For each protein eluent, the purity and elution efficiency were checked by native PAGE electrophoresis. Proteins in the gel were Coomassie-stained as described above. All the 7 major bands revealed in the oral secretion of maize-fed *C. partellus* (see Fig. 1) were separated and purified as described above for use in behavioural assays (see previous section).

Protein Identification The gel purified protein eluent inducing parasitoids' host recognition and oviposition were identified using LC-MS/MS. The protein eluents were first denatured in Laemmli buffer and then concentrated using a short electrophoretic migration, which also allowed removal of any contaminants that could interfere with the trypsic digestion. Electrophoretic bands were excised and the gel pieces were washed in successive baths consisting of 50 mM ammonium bicarbonate and acetonitrile. Proteins were then reduced by 10 mM of 1.4 dithiothreitol (DDT) and alkylated with 55 mM of iodoacetamide to block the sulfide bonds of cysteines. After rinsing to remove residues of DTT and iodoacetamide, proteins were hydrolyzed by the addition of 0.125 μg trypsin for 7 hr. After hydrolysis, the resulting peptides were extracted from the gel pieces with 50% acetonitrile acidified with 0.5% of trifluoroacetic acid (TFA). After complete speed vac drying, peptides were resuspended in a solution of 2% acetonitrile, 0.05% formic acid and 0.05% trifluoroacetic acid. Peptide mixes were then analyzed by LC-MS/MS using a nanoRSCL (thermoFinnigan) coupled with a LTQ Orbitrap Discovery (Thermo). The samples were then loaded onto a PepMap100C18 trap column for 5 min with 2% acetonitrile (ACN), 0.08% TFA qsp H2O. Two buffers systems were used to elute the peptides: 2%ACN and 0.1% formic acid in water (buffer A); 98% ACN and 0.1%formic acid in water (buffer B). Peptide separation was performed using a linear gradient from 4 to 38% of buffer B in 15 min. The nanoHPLC was connected to the mass spectrometer using a nano electrospray interface (non-coated capillary probe 10 μ I.d. New objective). Peptide ions were analyzed using Thermo Xcalibur (version 2.0.7) using the following data dependant steps: (1) full MS scan with a 300 to 1400 m/z range in the Orbitrap with a resolution of 15,000; (2) fragmentation by CID in the linear trap with a normalized energy at 35%. Step 2 was repeated for the three most intense ions with a minimum intensity of 500. Dynamic exclusion was set to 30 sec.

Raw files were converted to the mzxml format using msconvert (3.0.9576 http://proteowizard.sourceforge.net/tools.shtml). Database search was performed using X!tandem JACKHAMMER (Craig and Beavis 2004). Tolerance was set to 10 ppm for precursor ions and 0.5 Th for fragment ions. Cys-carboxyamidomethylation was set to static modification. Methionine oxidation, Nter acetylation of proteins, glutamine Nter deamidation and glutamic acid Nter water loss were set to variable modifications. Three databases were used: the *Spodoptera frugiperda* (Smith) EST database (http://www.ncbi.nlm.nih.gov/nucest version 2015, translated in the six reading frames and filtered to a minimum of 80 amino acids; 392,538 entries); the *Zea mays* database (from maizegdb, version v5a; 136,770 entries) and a standard contaminant database (55 entries). Identified peptides were filtered using X!tandemPipeline v3.3.4 (Langella et al. 2016) with the following criteria: peptide E-value less than 0.03, minimum 2 peptides per protein, protein E-value less than 10^{-4}. Unassigned spectra were subjected to *de novo* identification using denovopipeline v1.5.1 (http://pappso.inra.fr/bioinfo/denovopipeline/), that allows the selection of unassigned spectra of good quality and their submission to pepnovo (v2010117, Frank and Pevzner 2005). Spectrum quality score was set to 0.2 and pepnovo score to 70. *De novo* sequences

were then aligned to the same databases as for X!Tandem search using Fasts.v36.06 (Mackey et al. 2002). Proteins with a homology score of less than 10^{-4} were validated. The biological and analytical reproducibility were addressed by quantitative western blot (see next section).

Identified EST sequences obtained from digested peptides were submitted to a BLAST procedure (BLASTX, NCBI). The resulting protein was characterized by the name, the source and the molecular weight and a E-value/log E-value coverage. In order to calculate the coverage per cent of a peptide, the EST sequence was translated into a protein sequence using the Expasy Translate tool (http://www.expasy.org/tools/dna.html).

Western Blot Analysis of the Protein Eluent Inducing Parasitoid Oviposition In order to confirm that the proteins purified and identified were indeed α-amylases, a western blot using an antibody specific to *Drosophila melanogaster* Meigen α-amylase was performed. Ten microliters of each heat denatured protein sample (of about 500 ng/μl) were loaded on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and electrophoresis conducted for 1 hr at 200 V in MOPS buffer. The proteins were then transferred to an iBlot Gel Transfer Nitrocellulose membrane (Invitrogen) using the iBlot Gel Transfer Device (Invitrogen). The membrane was washed in 1X PBS for 20 min, after which it was incubated for 90 min in a milk solution (1X PBS, 0.1% Tween, 5% milk) in order to saturate the membrane with proteins. The membrane was then incubated with the primary anti *Drosophila melanogaster* α-amylase antibody (gift from Dr. B. Lemaitre) according to Chng et al. 2014, it was diluted 1000-fold in a solution of 1X PBS, 0.1% Tween, 1% milk) for several hours. After this step, the membrane was washed six times in 1X PBS, 0.1% Tween before incubating with the secondary antibody (Anti guinea pig IgG Peroxidase, Sigma A7289), 1000-fold diluted in a solution of 1X PBS, 0.1% Tween, 1% milk, for 1 hr. The membrane was then washed 3 times in 1X PBS, 0.1% Tween. The peroxidase activity was detected with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and recorded on an Odyssey FC imager.

Sources of Different α-Amylases Assayed To confirm the identity of the protein (α-amylase) mediating host acceptance and oviposition by *C. flavipes*, we used synthesized α-amylases from different organisms commercially available or from those found in our laboratory at Gif-sur-Yvette: the microorganism, *Aspergillus oryzae* (Ahlburg) E. Cohn, the insects, *Drosophila melanogaster* and *Chilo suppressalis* (Walker), and the pig as a mammal (porcine pancreas). The α-amylases from *A. oryzae* and porcine pancreas were obtained from Sigma No A9857 and A3176, respectively. The α-amylase from *Drosophila melanogaster* was produced on the yeast *Pichia pastoris* (Guillierm) Phaff, as described by Commin et al. (2013). The α-amylase of *C. suppressalis* was also produced in *P. Pastoris*; the coding sequence of the *C. suppressalis* amylase gene 108,827 was synthesized (Eurofins MWG), with replacement of the signal peptide by the one of *D. melanogaster* amylase (Appendix 2). We assayed an amylase from *C. suppressalis*, because its genome was available, as opposed to the one of *C. partellus*. In addition, to check if the behavioural activities of *C. flavipes* triggered by α-amylase (see results) was due to the structural conformation and/or the catalytic activity, we synthesized an inactive α-amylase with no change in its structural conformation. An inactivated α-amylase of *D. melanogaster* was obtained by a single replacement of the crucial catalytic residue Asp186 by an asparagine, which does not change the structural conformation (Aghajari et al. 2002). Another inactive α-amylase, named amyrel, with a change in its structural conformation differing by 42% from the classical α-amylase protein of *D. melanogaster* was also synthesized (Da Lage et al. 1998). A colorimetric activity test (Infinity Amylase Reagent, Thermo Fisher) was used to confirm that these inactive α-amylases of *D. melanogaster* had no catalytic activity.

As controls, the following extracts were also used in behavioral bioassays: the buffer used to solubilize the α-amylases; the glycogen (at 17 g/L) used to purify the synthesized α-amylases of *D. melanogaster* and *C. suppressalis*; the corn starch (at 17 g/L); the inactivated α-amylase of *D. melanogaster* and amyrel (cf. glycogen-amylase complex precipitation method of Loyter and Schramm 1962); and the degradation product of the α-amylase, i.e. maltose (at 34 g/L).

Statistical Analysis The Marascuilo's procedure, i.e. a pairwise comparison after Pearson's Chi-square test to test the overall significance differences, was used to separate the proportions of wasps that exhibited positive responses (i.e. antennation + stinging attempts) (Marascuilo 1966).

Results

The oral secretions of larvae fed on maize stems mediated a significantly higher response (i.e. antennation and stinging attempt) of *C. flavipes* than the fecal pellets, whereas no reaction was observed with maize stem juice (Table 1). The strongest response by *C. flavipes* was obtained with oral secretions of *C. partellus* followed by those of *S. calamistis*, and the non-hosts *B. fusca* and *S. nonagrioides* (Tables 2). The oral secretions from *C. partellus* larvae previously fed on *P. purpureum* triggered as many responses as those from maize-fed larvae (Table 3). By contrast, oral secretions of larvae fed on artificial diet did not elicit any behavioral activity. Likewise, the oral secretions from larvae starved for 48 hr and the oral secretions from larvae fed on maize stems treated with proteinase K did not elicit any response.

Table 1 Responses of *Cotesia flavipes* to maize stem juice, fresh fecal pellets and oral secretions of *Chilo partellus* larvae fed on *Zea mays*

Type of sample	Antennation + stinging attempt (%*, $n = 30$)
Maize stem juice	0 (0) a
Fresh fecal pellets	40.0 (12)b
Oral secretions	93.3 (28)c

The percentages of individuals that exhibited antennation and stinging attempt are given followed in parenthesis by their total number over thirty individuals tested

*After Pearson's Chi-squared test (Chi-square = 21.773; $df = 2$; $p < 0.0001$), percentages with different letter are significant at 5% level according to the Marascuilo's procedure (multiple proportions comparison)

The electrophoretic analyses of the active oral secretions yielded more intense electrophoretic bands (i.e. higher quantities of proteins) than those of the inactive oral secretions (Fig. 2a).

In a one-dimension gel electrophoresis under non-denaturing conditions, the oral secretion of larvae fed on maize stems showed seven major electrophoretic bands (Fig. 2a). Each major band was manually excised from the gel, extracted (Fig. 2b) and tested for further behavioral responses as shown in Table 2. Out of these seven protein bands, only two bands elicited activity, particularly band no 4 (≈ 50 kDa), which triggered a response in 90% of the parasitoids (Table 4). It was thus subjected to further analysis and identification.

In order to identify the active protein band that induced the highest behavioral response, proteins from band No 4 were digested and the resulting peptide mixture analyzed by liquid chromatography-mass spectrometry. Database search allowed the identification of two distinct maize proteins with 5 and 2 peptide sequences respectively, while *de novo* sequencing allowed the identification of 22 peptides that matched the accession gi|295,290,041|gb|FP379314.1|FP379314| of the *S. frugiperda* database of mid gut cDNA sequences (Appendix 3).

Table 2 Response of *Cotesia flavipes* to oral secretions of host and non-host larvae feeding on *Zea mays*

Oral secretions of different species	Antennation + stinging attempt (%*, $n = 30$)
Chilo partellus (host)	90.0 (27)c
Sesamia calamistis (host)	46.7 (14)b
Busseola fusca (non-host)	16.7 (5)a
Sesamia nonagrioides (non-host)	13.3 (4)a

The percentages of individuals that exhibited antennation and stinging attempt are given followed in parenthesis by their total number over thirty individuals tested

*After Pearson's Chi-squared test (Chi-square = 15.348; $df = 3$; $p = 0.001542$), percentages with different letter are significant at 5% level according to the Marascuilo's procedure (multiple proportions comparison)

Table 3 Response of *Cotesia flavipes* to oral secretions of its host larvae, *Chilo partellus*

Type of sample	Antennation + stinging attempt (%*, $n = 30$)
Larvae fed on *Zea mays* stems	90.0 (27)c
Larvae fed on *Pennisetum purpureum* stems	86.7 (26)c
Larvae fed on artificial diet	0 (0)a
Starved larvae	0 (0)a
Larvae fed on maize stems treated by proteinase K	0 (0)a

The percentages of individuals that exhibited antennation and stinging attempt are given followed in parenthesis by their total number over thirty individuals tested

*After Pearson's Chi-squared test (Chi-square = 57.14; $df = 4$; $p < 0.0001$), percentages with different letter are significant at 5% level according to the Marascuilo's procedure (multiple proportions comparison)

The protein sequence blasted significantly with α-amylase superfamilies (Appendix 4). The confirmation of α-amylase assignation of the electrophoretic band 4 was done by western blot analysis (Fig. 3). The anti-α-amylase of *D. melanogaster* linked mostly with the band no 4 (≈ 50 kDa) of the oral secretion of *C. partellus* and with that extracted from the gel.

The activity elicited by different α-amylases from different origins confirmed the involvement of this enzyme in *C. flavipes* antennation and stinging attempts (Table 5). In contrast, the protein BSA, for example, did not induce any behavioral response in the wasp, and neither did amyrel with a change in structural conformation in contrast to classical α-amylase protein of *D. melanogaster*. In addition, the buffer, the glycogen, the corn starch as well as the maltose did not induce any behavioural response in the wasp. The α-amylases from *D. melanogaster* and *C. suppressalis* induced the highest behavioral responses in *C. flavipes* antennation and stinging attempt (Table 5). To check if the behavioral activity of *C. flavipes* triggered by α-amylase was due to the structural conformation and/or the catalytic activity, we used an inactivated α-amylase from *D. melanogaster* which showed no change in its structural conformation. This inactivated α-amylase still induced behavioral activities of *C. flavipes* indicating that the conformation rather than the catalytic activity of α-amylase is responsible for the host acceptance process by *C. flavipes*.

Discussion

The present findings show that the oral secretions of the larvae of lepidopteran stem borers harbor active compound(s) that mediate host acceptance for oviposition in *C. flavipes*. These secretions allow *C. flavipes* females to discriminate between hosts and non-host larvae. The most

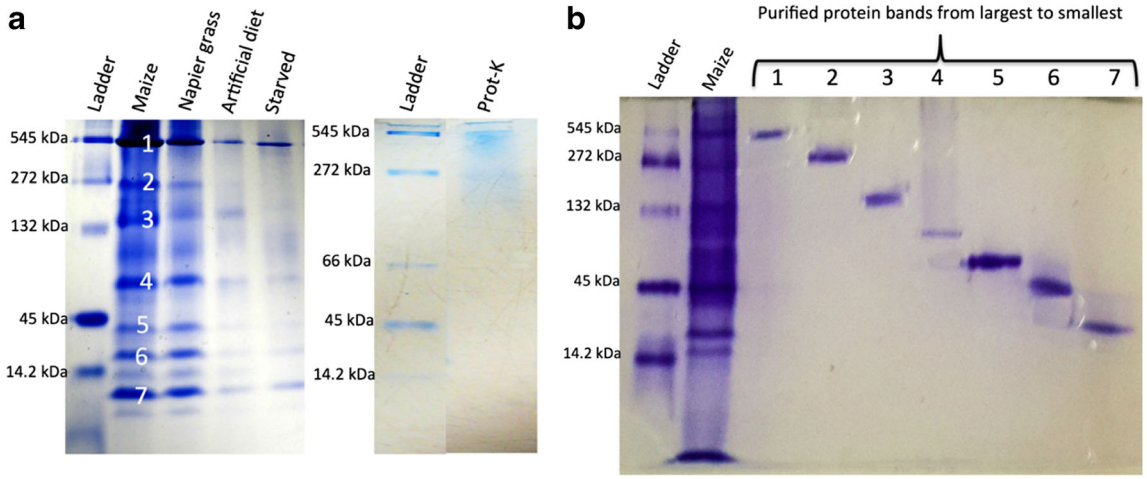

Fig. 2 Analysis of oral extracts in a native gel system. Protein samples were separated by 1D gel, 7% native Onstein-Davis discontinuous (Tris-glycine) PAGE before Coomassie staining. A) Comparison of *Chilo partellus* oral extract fed on different diet. Ladder: Sigma molecular weight markers; lane 1: oral secretion from *Chilo partellus* larvae fed on *Zea mays* stems (Maize) (each main electrophoretic band [noted 1 to 7 on the gel] were individually extracted from the gel (see Fig. 1b) under non-denaturing conditions and tested towards *Cotesia flavipes* (see Table 2); lane 2: oral secretion from *Chilo partellus* larvae fed on *Pennisetum purpureum* stems (Napier grass); lane 3: oral secretion from *Chilo partellus* larvae fed on artificial diet (Artificial diet); lane 4: oral secretion from starved larvae of *Chilo partellus* (Starved). For each lane, 15 µl of the oral secretion was loaded after concentrating and before quantification of the samples (Bio Rad Mini-PROTEAN® Electrophoresis System). After proteinase K treatment no band was obtained (Prot-K). B) Individual protein band purified from the gel of regurgitant of *Chilo partellus* fed on maize. Lanes: 1 molecular weight marker (sigma Aldrich), 2 regurgitants from *Chilo partellus* fed on maize (Maize); lanes 1–7 bands purified and tested for activity against *Cotesia flavipes* (Hoefer™ Mini Vertical Electrophoresis Systems (Fisher Sci. com) (see Table 2)

active compound isolated from the oral secretion from the larvae of the natural host *C. partellus* was identified as an α-amylase. Polypeptides and proteins have previously been reported as chemical signals in the host selection process of hymenopteran parasitoids (Bénédet et al. 1999; Gauthier et al. 2004; Weseloh 1977), but they have never been identified in details.

The use of synthesized α-amylases in this study allowed us to confirm the identity of the active proteins present in the oral secretion of *C. partellus* that mediates host acceptance for oviposition. The α-amylases that induced behavioral responses of *C. flavipes* possessed a similar molecular weight as those of *D. melanogaster* (51 kDa), *C. suppressalis* (≈ 50 kDa), *A. oryzae* (51 kDa), and pig (50 kDa, suggesting that the size of the active protein is important. However, a different protein such as BSA with a similar molecular weight did not induce any behavioral response suggesting that the conformation of the protein rather than its weight is involved in host acceptance for oviposition of the parasitoid. In fact, amyrel, an inactive α-amylase of *D. melanogaster,* with a change in the structural conformation of the classical α-amylase protein of *D. melanogaster*, did not induce any behavioral response in *C. flavipes* whereas another inactive α-amylase of *D. melanogaster*, with a similar conformation of the active α-amylase, did. This indicates that it is the conformation of the α-amylase rather than its catalytic site that induces this activity and suggests that *C. flavipes* can perceive this protein through its sensorial equipment, antennae or tarsi (Obonyo et al. 2011).

However, our results showed that the *C. flavipes* parasitoid response to α-amylase is not a host specific response since the parasitoid responded also to α-amylases from *D. melanogaster*, *A. oryzae*, *C. suppressalis* and in a lower trend to α-amylase from pigs. This is probably due to the fact that *C. flavipes* is a generalist, parasitizing larvae of more than 30 Lepidoptera species including *C. suppressalis* (https://www.cabi.org/isc/datasheet/5951). However, in its natural habitat, *C. flavipes* has no chance to be exposed to

Table 4 Response of *Cotesia flavipes* to the seven main electrophoretic bands (see Fig. 1) obtained from the oral secretions of its host, larva of *Chilo partellus*

Band tested	Antennation + stinging attempt (%*, $n = 10$)
1	0 (0)a
2	0 (0)a
3	30 (3)a
4	90 (9)b
5	0 (0)a
6	0 (0)a
7	0 (0)a

The percentages of individuals that exhibited antennation and stinging attempt are given followed in parenthesis by their total number over ten individuals tested

*After Pearson's Chi-squared test (Chi-square = 25.61; $df = 6$; $p = 0.00026$), percentages with different letter are significant at 5% level according to the Marascuilo's procedure (multiple proportions comparison)

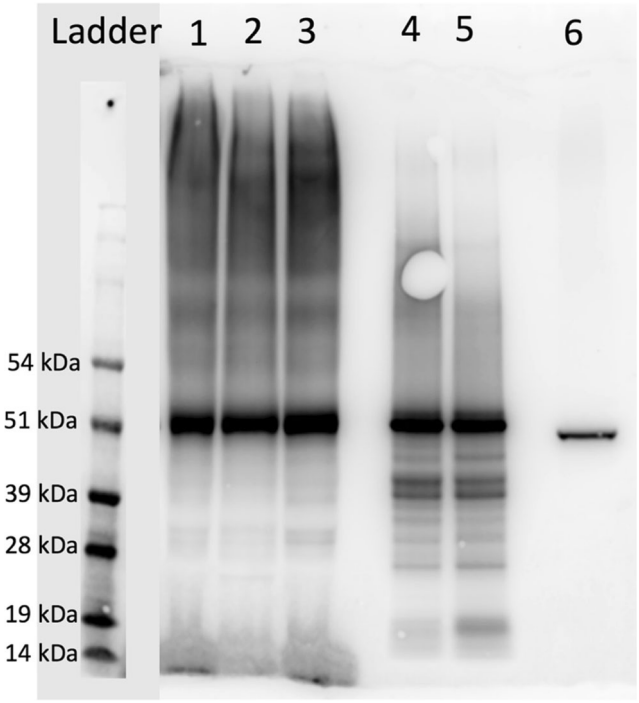

Fig. 3 Western blot performed using a *Drosophila melanogaster* α-amylase-specific antibody. Ladder: molecular weight markers (pre-stained SeeBlue Plus2, Thermo Fischer); 1, 2 and 3: oral secretions from *Chilo partellus* larvae fed on maize stems; 4 and 5: band n°4 of Fig. 1 which has been extracted from the gel and used for Western Blot analysis; 6: α-amylase from *Drosophila melanogaster*

the α-amylases of *D. melanogaster*, *A. oryzae* or pigs. In addition, the response of *C. flavipes* females to α-amylase is not binomial (yes or no) but gradual according to the origin of the enzyme and, in our study, strongest in insects such as *D. melanogaster* and *C. suppressalis*. Consequently, the parasitoid's response to α-amylase should allow them to discriminate between hosts, being more intense towards their natural hosts as compared to their non-hosts. The sequences of α-amylases of animals show high variability at the protein level (Da Lage et al. 2002). This diversity of α-amylase proteins may have adaptive or functional significance in the diversity of use by insects. The stemborers use their own α-amylase proteins for feeding process; thus, the variation of α-amylase proteins may be linked to the different host plants on which they used to feed and the parasitoids use this variation of the α-amylase proteins as a way to discriminate stemborer hosts in different habitats where the parasitoids live. In our study, we observed the strongest behavioral response of *C. flavipes* to the oral secretions of its natural host *C. partellus,* a weaker response to the oral secretions of *S. calamistis* and the weakest responses to the oral secretions of the non-hosts, which might be due to the different α-amylases present.

In addition, the *Cotesia flavipes* complex is composed of four species, namely *C. chilonis* (Matsumura), *C. flavipes* Cameron, *C. nonagriae* (Olliff) and *C. sesamiae* (Cameron), all gregarious endoparasitoids of a few families of lepidopteran stem borers (Crambidae, Pyralidae, and Noctuidae) of Poales (Poaceae, Typhaceae and Cyperaceae) (Kaiser et al. 2017b). It was described a high diversity of *Cotesia* spp. with a strong host specificity, particularly on *Busseola* spp. and *Chilo* spp. (Mailafiya et al. 2009). Consequently, the parasitoid's response to host kairomone such as α-amylase should allow them to discriminate between hosts. In this context, our study suggests α-amylases from oral secretions of the caterpillar hosts are good candidates for an evolutionary solution to host acceptance for oviposition in *C. flavipes* complex. Additional works are needed to demonstrate first if this enzyme can explain the specific host-parasitoid association in the *Cotesia flavipes* species-group.

Table 5 Response of *Cotesia flavipes* to different proteins (at 300–500 ng/μl) as well as to buffer, maltose, glycogen and corn starch

Proteins tested	Antennation + stinging attempt (%*, $n = 30$)
α-amylase from *Aspergillus oryzae*	40 (12)bc
α -amylase from pig	20 (6)b
α -amylase from *Drosophila melanogaster*	63.3 (19)c
α -amylase from *Chilo suppressalis*	46.7 (14)bc
Inactive α-amylase from *Drosophila melanogaster*	43.3 (13)bc
Amyrel	0a
BSA	0a
Buffer	0a
Maltose	0a
Glycogen	0a
Corn starch	0a

The percentages of individuals that exhibited antennation and stinging attempt are given followed in parenthesis by their total number over thirty individuals tested

*After Pearson's Chi-squared test (Chi-square = 71.92; $df = 1$; $p < 0.0001$), percentages with different letter are significant at 5% level according to the Marascuilo's procedure (multiple proportions comparison)

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