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Salivary α-Amylase of Stem Borer Hosts Determines Host Recognition and Acceptance for Oviposition by *Cotesia* spp. (Hymenoptera, Braconidae)

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Foraging insect parasitoids use specific chemical cues to discriminate between host and non-host species. Several compounds have been identified in "host location and acceptance." However, nothing is known about the molecular variations in these compounds that could account for host-range differences between parasitoid species. In a previous study, it was shown that during the host-finding process, contact between the braconid *Cotesia flavipes* and its host is crucial, and that α-amylase of oral secretions from the host plays a key role for host acceptance and oviposition by the parasitoid. The present study sought to establish whether the variations in this enzyme could explain specific host recognition in different host-parasitoid associations. Different species and populations of the *C. flavipes* complex specialized on graminaceous lepidopteran stemborers were used. Electrophoresis of α-amylase revealed different isoforms that mediate the parasitoid's oviposition acceptance and preference for a specific host. This discovery opens up new avenues for investigating the evolutionary processes at play in chemically-mediated host specialization in the species-rich *Cotesia* genus.

Keywords: parasitic wasp, *Cotesia flavipes*, *Cotesia sesamiae*, *Cotesia typhae*, protein perception, host specificity, oviposition

INTRODUCTION

Parasitoids comprise the major biological control agents of pest insects (Pimentel et al., 1992; Tilman et al., 2001; Lazarovitz et al., 2007; Godfray et al., 2010). Among them, the Hymenoptera order contains the most diversified species: 50,000 in Hymenoptera, compared with only 15,000 in Diptera, and 3,000 in other orders (Quicke, 1997). To reproduce successfully, the parasitoids need to overcome the behavioral and physiological defenses of their hosts (Kaiser et al., 2017a). The hosts' defense mechanisms, which co-evolved with the parasitoids, may be linked to host range changes and the appearance of host races within different parasitoid species (Kaiser et al., 2017a). These underlying mechanisms provide insight into evolutionary biology, and they might improve the selection of parasitoids in bio-control.

The ability of parasitoids to efficiently utilize cues from their habitat and to efficiently distinguish suitable from unsuitable hosts, determines their field efficiency (Wajnberg et al., 2008;

Wajnberg and Colazza, 2013). When locating hosts, they first use long (i.e., from a distance) and short-range chemicals coming from the host habitat, and secondly those directly present on the host and on its feeding products (Wajnberg et al., 2008; Wajnberg and Colazza, 2013). However, long-range chemicals from the habitat do not generally give them sufficiently reliable information about the host's suitability (Vet, 1999). In contrast, those directly present on the host and on its feeding products are directly used during host-contact evaluation by the parasitoids. These chemicals generally allow them to assess the quality and status of the herbivore's suitability (Lewis and Martin, 1990; Vinson, 1991; Godfray, 1994; Wajnberg et al., 2008; Wajnberg and Colazza, 2013). Moreover, the structure and quantity of these semiochemicals, which vary according to the host's species, the developmental stage of the host, the host's size, condition, and diet, influence host acceptance and selection by the parasitoids (Vinson, 1991; Röse et al., 1997; Wajnberg et al., 2008; Wajnberg and Colazza, 2013).

Among parasitoids, *Cotesia* is one of the most diverse genera in the Braconidae family (Kaiser et al., 2017a). Many *Cotesia* species may appear to have broad host ranges, but careful ecological studies have revealed a hidden complexity with an assemblage of populations with more restricted host ranges (Branca et al., 2011; Kaiser et al., 2017b). Whereas recent studies revealed that variations in virulence genes account for differences in host range and in the degree of specialization toward a host (Gauthier et al., 2018), almost nothing is known about the variations in functions involved in specific host recognition and acceptance.

The *Cotesia flavipes* species-monophyletic group is composed of four sister species: *C. chilonis* (Matsumura), *C. flavipes* Cameron, *C. nonagriae* (Olliff), and *C. sesamiae* (Cameron). They are all gregarious endoparasitoids of crambid, pyralid and noctuid stem borers feeding on Poaceae, Typhaceae, and Cyperaceae species (Kaiser et al., 2017b). These small wasps, after mating, lay egg(s) in a host's body (generally a caterpillar). To inhibit the immune response of the caterpillars, they use a domesticated virus called bracovirus (PolyDNA virus). These viruses are located in the wasp ovaries and are integrated into the genome of the wasp and injected into the caterpillar together with the eggs during the parasitism process (see Kaiser et al., 2017a for review).

Cotesia flavipes Cameron is widespread in Asia and was introduced into Africa to control the invasive Asian crambid *Chilo partellus* Swinhoe (Overholt et al., 1994a,b). It parasitizes the larvae of more than 30 Lepidoptera species, including the crambids *C. partellus* and *Chilo suppressalis* (Walker), as well as the African noctuid *Sesamia calamistis* Hampson, a new association host (https://www.cabi.org/isc/datasheet/5951). The *C. flavipes* population brought into Africa for classical biological control was specific to *C. partellus* in Asia (Muirhead et al., 2012). *Cotesia sesamiae* is widespread in Sub-Saharan Africa and is commonly found on *Busseola fusca* and *S. calamistis* (Kfir, 1995; Kfir et al., 2002), but its parasitism success greatly depends on the host species and parasitoids populations (Mochiah et al., 2002; Gitau et al., 2010). Two factors contribute to the differences and hence to the performance of *C. sesamiae* populations on stem borer pests across Africa, namely, the symbiotic polyDNA viruses, which are responsible for the differences in virulence of *C. sesamiae* population on *B. fusca* (Gitau et al., 2010), and the bacteria *Wolbachia,* by creating cytoplasmic incompatibilities between populations of *C. sesamiae* populations (Mochiah et al., 2002). In contrast to the *C. sesamiae* population from Mombasa/coastal Kenya (*Cs*-Coast), the *C. sesamiae* population from Kitale/inland Kenya (*Cs*-Inland) is able to develop in *B. fusca*, which is predominant in the highlands, whereas both are able to develop in the noctuid *S. calamistis,* the main host of *C. sesamiae* population from Mombasa/coastal Kenya (Ngi-Song et al., 1995). The *Cs*-Inland is mostly present in the highlands and wet regions, where its host *B. fusca* occurs, and is absent in the dry and warmer regions, where *Cs*-Coast and *C. flavipes* predominate (Mailafiya et al., 2010; Mwalusepo et al., 2015). The genetic diversity of these *C. sesamiae* populations, especially regarding their relationships with spatial, biotic, and abiotic ecological factors, is reported by Branca et al. (2018). The authors highlighted the importance of host forces in the evolution of the diversity of parasitoid-host interactions.

Cotesia sesamiae and *C. flavipes* locate their host at a distance by the emission of volatiles from the plants infested by their hosts. However, these volatiles do not convey reliable information on host suitability but are simply indicators of the presence of herbivores in the plant. As a result, *C. sesamiae* and *C. flavipes* might be attracted to plants infested by unsuitable Lepidoptera stemborers (Potting et al., 1993, 1995; Ngi-Song et al., 1996; Obonyo et al., 2008). It is only when approaching and touching the host that *C. sesamiae* and *C. flavipes* are able to identify their hosts properly, relying on specific host-produced signals. The signals particularly arise from oral secretions, which give reliable information on the host identity perceived by the tactile and contact-chemoreception of the parasitoid (Obonyo et al., 2010a,b). These authors observed that host selection and acceptance by the parasitoid females for parasitism is characterized by two behavioral steps: drumming the body of the host with the antennae (antennation), followed by an attempt to oviposit into the host. Recently, Bichang'a et al. (2018) showed that α-amylase present in the oral secretions of *C. partellus* larvae mediates these behavioral responses of *C. flavipes*. The present study investigates whether α-amylase presents variations which allow for recognition and selection of host species or population in *Cotesia* spp. using the two populations of *C. sesamiae* living in Kenya with their respective hosts *B. fusca* and *S. calamistis*, as well as a new species of *Cotesia* described recently as *C. typhae* Fernandez-Triana sp., parasitizing *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera, Noctuidae) (Kaiser et al., 2017a), and the introduced *C. flavipes* and its old association host *C. partellus*.

MATERIALS AND METHODS

Insect Rearing

Females of *C. flavipes,* an inland and coastal population of *C. sesamiae* (hereafter named *Cs*-Inland and Cs-Coast, respectively), as well as that of *C. typhae,* came from laboratory-reared colonies established at *icipe*, Nairobi, Kenya. *Cotesia flavipes* was initially obtained in 2005 from *C. partellus* larvae collected

TABLE 1 | Suitability of lepidopteran stem borer species to different Cotesia species and strains based on field observations and the literature.

	Chilo partellus	*Busseola fusca*	*Sesamia calamistis*	*Sesamia nonagrioides*
Cotesia flavipes	o	w	new	non
Cotesia sesamiae				
Cs-Inland	w	o	o	non
Cs-Coast	new	w	o	non
Cotesia typhae	non	non	w	o

A code was attributed to indicate the level of host suitability, where non, non-host; w, "weak" host association; new, new host association; o, old host association.

from maize fields in Mombasa, coastal Kenya. *Cs*-Inland was initially obtained in 2006 from *B. fusca* larvae infesting maize fields in Kitale, Western Kenya, while the *Cs*-Coast was initially obtained in 2007 from *S. calamistis* larvae infesting maize fields in Mombasa (coastal Kenya). *Cotesia typhae* was initially obtained in 2013 from *S. nonagrioides* larvae infesting *Cyperus dives* at Kobodo near Lake Victoria, Kenya.

Cotesia flavipes, *Cs*-Inland, *Cs*-Coast, and *C. typhae* were continuously reared on larvae of *C. partellus*, *B. fusca*, *S. calamistis,* and *S. nonagrioides*, respectively, as previously described by (Overholt et al., 1994a). Twice a year, all colonies were rejuvenated by field-collected parasitoids.

For each colony, the cocoons were kept until emergence. After emergence, adult parasitoids were fed on a 20% honey/water solution and placed under artificial light for 8 h to mate. In all the behavioral bioassays, 1-day-old naïve (i.e., without oviposition experience), mated females were used. Similar to Overholt et al. (1994a), experimental conditions were at 25 ± 2°C, at 50–80% relative humidity (RH) and with a 12:12 h (L:D) photoperiod.

Different host species that varied in their suitability according to the *Cotesia* species and strains were used (**Table 1**). Old host association (=natural host) was defined according to both the origin of the parasitoid and the host (**Table 1**). For example, *C. partellus* is considered an old host association, since this host is from the same origin of the parasitoid in Asia (Overholt et al., 1994b) and was parasitizing this host before its introduction into Africa, whereas the African *S. calamistis* is a new association.

Chilo partellus and *S. calamistis* were initially collected from maize fields in coastal regions of Kenya, and *B. fusca* from maize fields in Western Kenya (Kitale), while *S. nonagrioides* were initially collected from *Typha domingensis* in Makindu, Kenya. The larvae of *C. partellus* were continuously reared at *icipe* on artificial diets of Ochieng et al. (1985), whereas the larvae of the other species were fed on the artificial diet of Onyango and Ochieng'-Odero (1994). Twice a year, all host's colonies were rejuvenated by field-collected stemborer larvae. **Table 1** gives the host-parasitoid species and strains associations.

Collection of Oral Secretions From Host Larvae

Acceptance of host larvae for oviposition by *Cotesia* parasitoids is enhanced when the host larvae are fed on maize stems for 24 h prior to exposure to parasitism (Mohyuddin et al., 1981; Inayatullah, 1983; Van Leerdam et al., 1985; Potting et al., 1993; Overholt et al., 1994a), most likely because more α-amylase can be found in the oral secretion from larvae after feeding on maize stems than on artificial diets (Bichang'a et al., 2018). Therefore, α-amylase was isolated from third and fourth instar larvae previously fed for 24 h on maize stems. Each larva was squeezed by soft forceps behind the head to collect its oral secretion into a capillary tube and was immediately transferred to an Eppendorf tube which had been placed on ice. This was repeated for at least 100–200 larvae per species to get a sufficient amount of oral secretion (about 500–800 μL per species), estimated by weighing. All samples were preserved at −80°C until further use.

Purification of the α-Amylases

The oral secretions were first centrifuged at 11,000 × g for 5 min in order to remove the undetected debris (grass and undigested food materials). About 600–800 μL of supernatant was transferred to a clean tube and the proteins precipitated using ammonium sulfate salt. To the supernatant, ammonium sulfate salt was gradually added to a final salt saturation of 90% and precipitated overnight at 4°C. The proteins were subsequently pelleted by centrifugation at 12,000 × g for 1 h at 4°C and were then resuspended in HEPES-NaCl buffer (HEPES 20 mM, NaCl 20 mM, $CaCl_2$ 1 mM, pH 7.5) and dialyzed (MWCO 12–14000 Da) overnight at 4°C in the same buffer.

The α-amylase was purified using the glycogen-amylase complex precipitation method described by Loyter and Schramm (1962) with some modifications. Briefly, ice-cold absolute ethanol was added dropwise (2/3 v/v) to the dialyzed samples placed on ice and mixed for 40 min at 4°C. This mixture was centrifuged at 20,000 rpm for 30 min at 4°C to pellet the nucleic acids. To the supernatant, glycogen (Sigma Aldrich) was added to a final concentration of 2.4 mg/ml per sample and mixed for 20 min for *S. calamistis* and *S. nonagrioides*, and 5 min for *B. fusca* and *C. partellus* at 4°C (As observed in previous assays; the different timings allowed for optimum yield of α-amylases). Subsequently, the mixtures were centrifuged for 20 min at 20,000 rpm at 4°C to pellet the amylase-substrate complex, and the pellets were dissolved in the aforementioned HEPES-NaCl buffer. The amylase-substrate complexes were left on the bench for 3 h at room temperature to digest the glycogen in the complexes. The remaining α-amylases were dialyzed (MWCO 12-14000 Da) overnight against the same buffer and kept at −20°C for electrophoresis and bioassays.

Native PAGE and α-Amylase zymogram

For the α-amylases of each host species, electrophoresis was conducted under non-denaturing conditions (native PAGE electrophoresis) as follows: For each host species, ten microliters of purified α-amylase were mixed separately with 10μL buffer (50 mM tris-HCl, pH 6.8, 10% glycerol (v/v), and 1% bromophenol blue) and electrophoresed in the Ornstein-Davis discontinuous buffer system on a 7.5% native polyacrylamide gel at 4°C according to Schrambach and Jovin (1983) and Niepmann and Zheng (2006). After running the gel at a

constant voltage of 150 V and a current of 25 mA for 1 h, and when the dye-containing sample reached the bottom of the glass, the polyacrylamide gel was stained according to Nagaraju and Abraham (1995) with minor modifications. The gel was incubated for 1 h at 37°C in 1% soluble potato starch (Sigma Aldrich) and 1 M $CaCl_2$, washed thoroughly with ddH_20 and subsequently stained with 0.1% of Lugol's iodine solution (I_3K) until white bands against a blue background were visible. The proteins were compared to a molecular mass standard (Sigma Aldrich) containing albumin from bovine serum (Sigma A8654, 132 kDa), albumin from chicken egg white (Sigma A8529, 45 kDa), and α lactalbumin from bovine milk (Sigma L4385, 14.2 kDa). The gel images were acquired using the myECL™ Imager (Thermo) and analyzed using myImageAnalysis™ Software (Thermo).

It was previously observed that the concentration of α-amylase in the extract conditioned the behavioral response of the wasp (Bichang'a, 2018; Bichang'a et al., 2018).

For each host species, the concentration of α-amylase was estimated using a calibration electrophoretic migration obtained from increasing concentrations of between 50 and 1000 μg/mL of α-amylase of *Aspergillus oryzae* from Sigma No A9857 and of *D. melanogaster* produced on the *Pichia pastoris* yeast (**Figure S1**). This calibration electrophoretic migration did not lead us to a precise amount of α-amylase but rather to a range of concentrations. Moreover, it was observed that the optimal range of concentrations of α-amylase to induce host recognition and acceptance for oviposition behaviors by the parasitoids was 300–500 μg/ml (Bichang'a, 2018; Bichang'a et al., 2018). For each of the host species, the concentrations of α-amylase used for the subsequent bioassays was adjusted at 300–500 μg/ml.

Western Blot Analysis of the Purified α-Amylases of Each Host Species

In order to confirm for each stem borer species that the purified proteins were indeed α-amylases, after being used for all bioassays, a western blot was performed using an antibody specific to *Drosophila melanogaster* Meigen α-amylase using the similar protocol of Bichang'a et al. (2018). Ten microliters of each heat denatured protein sample (about 500 ng/μl) were loaded on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and electrophoresis conducted for 1 h at 200 volts in a 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 1,000-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), diluted 1,000-fold in a solution of 1X PBS, 0.1% Tween, 1% milk, for 1 h. The membrane was then washed 3 times in 1X PBS, 0.1% Tween. The peroxidase activity was detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and recorded on an Odyssey FC imager.

Behavioral Bioassays

In this study, the two behavioral steps (antennation + stinging attempt), as shown by Obonyo et al. (2010a,b), were used to confirm host acceptance by *Cotesia* females for oviposition. To test the behavioral activities triggered by different α-amylases, 300–500 μg/ml of α-amylases [the minimal concentration found to mediate a positive response of *C. flavipes* (Bichang'a et al., 2018)] were placed on small pieces of cotton wool and presented to female parasitoids. A small piece of cotton wool was rolled into a spherical shape of around 2 mm in diameter and placed at the center of a Petri dish of 8 cm in diameter without a cover. About 0.5–1 μL of α-amylase was deposited on the cotton wool ball. A single female wasp was introduced near the cotton wool and both were covered with a transparent circular Perpex lid (3 cm wide, 1 cm high) to prevent the parasitoid from flying off, and to allow for observations.

The behavior of the parasitoid in the Petri dish was monitored for a maximum of 120 s. For each female, both antennation and stinging attempts were recorded. The percentage of positive responses (i.e., antennation + stinging) was calculated from 30 females tested per type of α-amylase. The females, the cotton wool balls with tested α-amylase and the arena were replaced after each observation.

According to Obonyo et al. (2010a), all behavioral experiments were carried out in a room at 26 ± 1°C between 10 a.m. and 2 p.m. with a constant source of light to maintain an optimal temperature for the behavioral activities of the female parasitoids.

Statistical Analysis

For each bioassay, Marascuilo's procedure, that is, a pairwise comparison after Pearson's Chi-square test to check 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 α-amylase exhibited species-specific electrophoretic migrations showing different numbers of isoforms using the Lugol test (**Figure 1**). The α-amylase of *C. partellus* exhibited mostly 1 band, whereas α-amylase of *B. fusca* appeared to have two main different isoforms, while that of *S. calamistis* exhibited two thick, highly visible isoforms, and three thinner bands between and three faint bands, which migrated much faster than the others. α-Amylase of *S. nonagrioides* had three thick groups of isoforms, one thin band and a pair of highly visible thin bands migrating faster. We confirmed by Western blot analysis for *S. nonagrioides*, *S. calamistis* and *B. fusca* that these were alpha-amylase proteins (**Figure 2**). In the non-denaturing gels stained using iodine at **Figure 1**, which show white bands where active amylases have migrated, proteins are separated by their electric

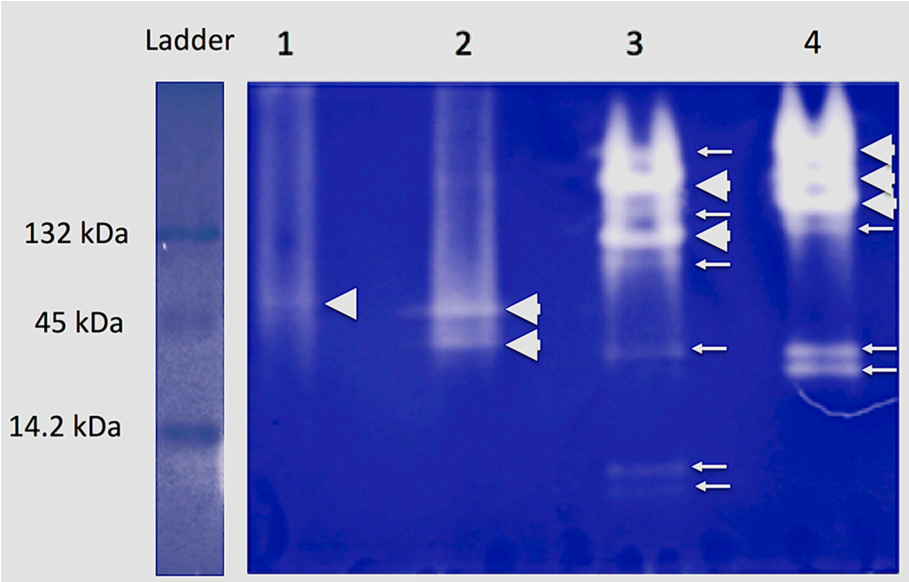

FIGURE 1 | Non-denaturing gel electrophoresis of the amylolytic activity of the purified α-amylases from the oral secretions of larvae of *Chilo partellus* (1), *Busseola fusca* (2), *Sesamia calamistis* (3), and *Sesamia nonagrioides* (4). For each species, the arrows highlight the main isoforms obtained.

charge, which is mostly the result of the difference between (Lys and Arg) and (Asp and Glu) residue numbers. A single gene may exhibit two bands if the two alleles differ in charge. If there are more than two bands, especially if they are separated, e.g., as two pairs of bands, one can infer that there are two active copies. In contrast, in the SDS-PAGE (denaturing) used for Western blot, all the proteins migrate to the same position because they have the same molecular weight. This is the reason why a single labeled band was observed in **Figure 2**. For a mixture of various proteins, migration of **Figure 1** depends on both electric charge and molecular weight (as well as conformation, shape, etc.); but as far as amylases only are concerned, since they all have similar molecular weight, the differences observed in migration distances are due to the differences in electric charges (electromorphs). However, no band was obtained for *C. partellus,* although α-amylase activity was seen in these sample type in **Figure 1**. The amount of protein sample of the *C. partellus* used for western blot was lower compared to amounts of the other species. The limit of protein detection was therefore attained for this sample type by Western blot.

For each parasitoid species and strains used in this study, parasitoid females exhibited different behavior according to the origin of the α-amylase (*C. flavipes*: Chi-square = 13.43; df = 3, $P = 0.0038$; *Cs*-Inland: Chi-square = 27.548; df = 3, $P < 0.0001$; *Cs*-Coast: Chi-square = 8.2458; df = 3 and $P = 0.04119$ and *C. typhae:* Chi-square = 15.239; df = 3 and $P = 0.001623$) (**Figure 3**). For *C. flavipes* females, α-amylases from the larvae of the old association host *C. partellus* and the new association host *S. calamistis* induced the highest positive responses followed by those from *B. fusca,* whereas those from *S. nonagrioides* larvae did not induce any behavior (**Figure 3**). For *Cs*-Inland females, α-amylases from the preferred host *B. fusca* induced the highest positive response, followed by those from the suitable *S. calamistis*, whereas those from the unsuitable hosts *C. partellus* and *S. nonagrioides* did not induce any response (**Figure 3**). For the *Cs*-Coast females, α-amylases from the suitable new association hosts *C. partellus* and the natural host *S. calamistis* induced higher responses than those from the unsuitable *B. fusca* and *S. nonagrioides* (**Figure 3**). For the more specific *Cotesia* species, α-amylase from the suitable host *S. nonagrioides* induced a higher response than those from the unsuitable species (**Figure 3**).

In summary, for each parasitoid species and population there was a strong co-relationship between the behavioral response toward α-amylases of the larvae by the parasitoid female (**Figure 3**) and the level of host suitability (**Table 1**).

DISCUSSIONS

This study revealed that the response of female *Cotesia* to the α-amylase from larval oral secretions depended on both the host and parasitoid species or population, with a strong relationship between the level of response and host preference/suitability. Highest responses were observed with the proteins of the old association host (i.e., most suitable host), whereas protein of unsuitable species triggered little or no response. Variations of host α-amylase between host species would thus allow specific host recognition and acceptance by the parasitoids.

Lepidopteran stemborers in Africa present high ecological and genetic diversity (Le Ru et al., 2006a,b), characterized by a large number of closely related plant-specific species (Le Ru et al., 2006a,b; Moolman et al., 2014; Ong'amo et al., 2014; Goftishu et al., 2018). Correspondingly, Mailafiya et al. (2009) found a

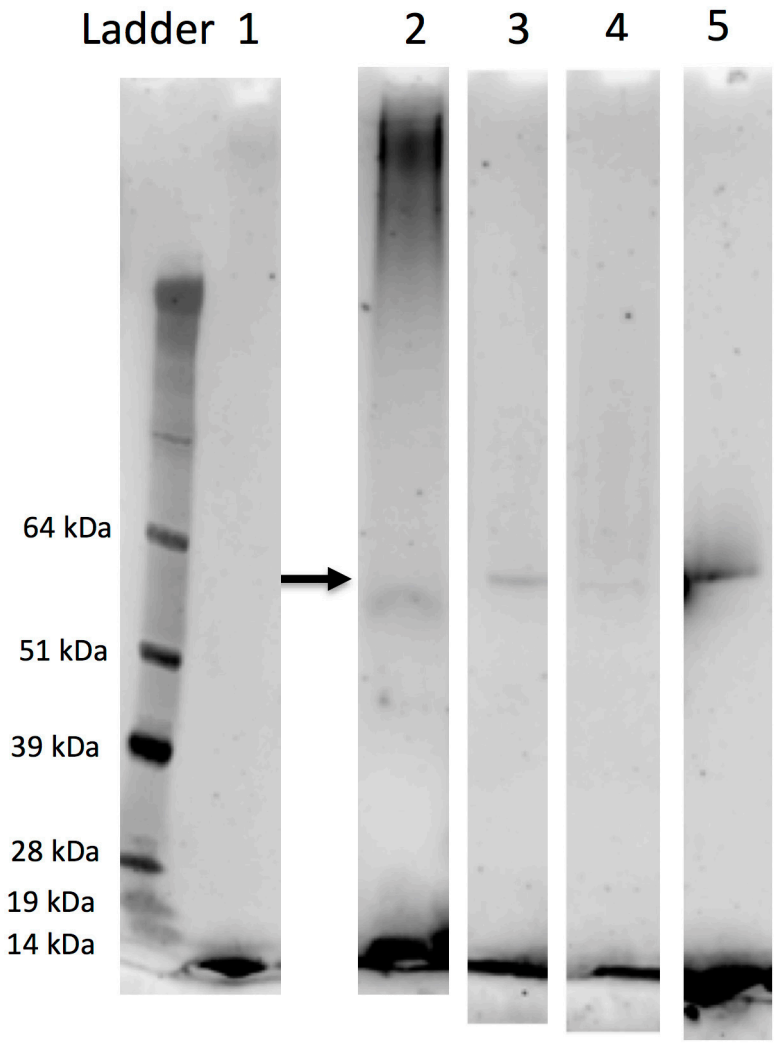

FIGURE 2 | Western blot performed using a *Drosophila melanogaster* α-amylase-specific antibody toward the purified α-amylases from the oral secretions of larvae of *Chilo partellus* (1), *Busseola fusca* (2), *Sesamia calamistis* (3), and *Sesamia nonagrioides* (4). Ladder: molecular weight markers (pre-stained SeeBlue Plus2, Thermo Fischer). 5: α-amylase from *Drosophila melanogaster*.

high diversity of the *Cotesia* spp., particularly among *Busseola* spp. and *Chilo* spp., which also revealed a strong host-parasitoid specificity. This suggests that the chemical(s) involved in host recognition and acceptance by these parasitoids must be specific to the host species involved, as verified in the present study. However, the response of parasitoid females to α-amylase is not binomial (yes or no), and becomes more intense with the α-amylase of its natural host. Some behavioral responses still occurred with α-amylases of unsuitable hosts, nonetheless. The probability of an encounter between *B. fusca* with *C. flavipes* and *Cs*-coast, as well as between *C. partellus* with *Cs*-Inland, is very low, however, due to the different geographical distribution of their respective hosts: *B. fusca* is mostly present in the highlands, whereas *C. partellus* is mostly found in the lowlands (Mailafiya et al., 2010; Mwalusepo et al., 2015). Such ecological patterns of the host-parasitoid associations suggest that their preference for the α-amylase of their host results from adaptation (even recent adaptation, e.g., for *C. flavipes* toward *S. calamistis*) to local hosts, as shown for the virulence function for *C. sesamiae* populations (Dupas et al., 2008; Gauthier et al., 2018).

α-Amylases are among the important classes of digestive enzymes used by the insects to hydrolyse starch in various plant tissues to oligosaccharides. Thus, they play a critical role in insect survival by providing energy (Franco et al., 2000). They have also been identified in most insect orders, such as Orthoptera, Hemiptera, Heteroptera, Hymenoptera, Diptera, Lepidoptera, and Coleoptera (Kaur et al., 2014). In Lepidoptera, several α-amylase genes commonly occur (e.g., Özgür et al., 2009; Pytelkova et al., 2009; Da Lage et al., 2011). In our study the same enzyme had different isoforms in electrophoresis that exhibited species-specific migration patterns. Since isoform migration distance depends on the molecule electric charge, it is

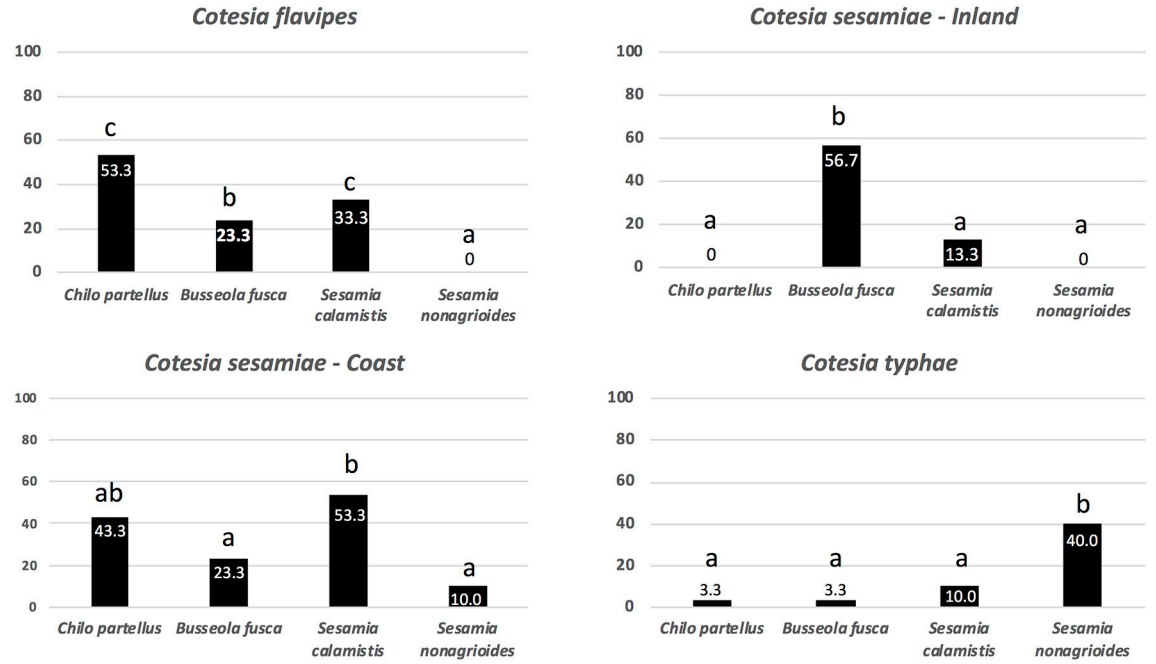

FIGURE 3 | Response of *Cotesia flavipes*, *Cotesia sesamiae*-Inland, *Cotesia sesamiae*-Coast, and *Cotesia typhae* females to purified α-amylase from different host species. The percentages of females ($n = 30$) that exhibited antennation and stinging attempts are given for each bar. After Pearson's Chi-squared test, bars headed with a different letter are significant at a 5% level according to the Marascuilo procedure (multiple proportions comparison).

not obvious whether different bands represent allelic variation or if they duplicate gene copies. However, in species showing well-separated groups of bands, such as the two species of *Sesamia*, it is likely that at least those groups reflect different gene copies. It can be hypothesized that within these species, individuals can express different isoforms of the α-amylase. To confirm this hypothesis, it would be necessary to look at the α-amylase expression in each individual. Up to now, only one α-amylase gene sequence has been identified in *S. nonagrioides* (actually a cDNA; Da Lage J.-L., unpublished study), but given that most Lepidoptera with published genomes harbor several α-amylase genes (Da Lage, 2018 for a review), it is quite likely that this is the case in *S. nonagrioides*. Several α-amylase gene copies are expressed in a species close to *C. partellus*, *Chilo suppressalis*; and three α-amylase gene copies in *Ephestia kuhniella* (Pytelkova et al., 2009). Nevertheless, all these studies indicated that the insects express multiple α-amylase at the same time; suggesting that no individual variation in α-amylase genes expression might occur within the same species. Therefore, the α-amylase gene expression is species-specific.

The two *Sesamia* species have different ranges of host plants (Le Ru et al., 2006a,b), so genes coding for digestive enzymes like α-amylase may have evolved under different selective pressures. Tri-dimensional amylase structures may vary according to the species or even to the isoform if significant sequence differences exist, such as presence or absence of some disulfide bonds, or particular loops (Da Lage et al., 2002). Those structural differences might be discriminated by the sensory equipment of the parasitoid wasp.

For *C. flavipes* it was shown that it is the conformation of the α-amylase rather than its catalytic activity that induces the parasitoid responses (i.e., antennation + stinging attempts; Bichang'a et al., 2018). Therefore, the existence of different α-amylase isoforms specific to each stem borer species as shown in **Figure 1** corroborates the variable behavioral responses obtained in relation to the host-parasitoid association.

The question arises of how the parasitoids access host α-amylase in nature. Lepidopteran stemborer larvae spend their lives and feed inside plant stems. Before entering the feeding tunnel of the host larvae, the wasp first contacts the fecal pellets left by the larvae pushed outside of the stem. These pellets act as a marker of the status of the larva inside the stem tunnel as being host or non-host (Obonyo et al., 2010b), and shows whether they are actively feeding or not. It is most probable that the fecal pellets already contain the stimulatory compounds, since the pellets induced oviposition (Bichang'a et al., 2018). However, the parasitoid is able to definitely recognize the host and accept to oviposit in it only when it is in contact with the host body (Obonyo et al., 2010a,b). We hypothesized that it is during this final step that the parasitoid can confirm the identity of the host larva by detecting the same stimulatory compounds found in the previous fecal pellets and also present on the body of the larva deposited by its feeding activity. These stimulatory compounds need to give quick and appropriate information to the parasitoid on the suitability of the larva (both host and health status) because host larvae often bite the attacking wasps inside the tunnel created by the borer, causing a 50% mortality risk

(Takasu and Overholt, 1997). The high selection pressure due to the high mortality at oviposition should favor wasps that can recognize hosts with a minimal risk of injury (Ward, 1992). In this context, the parasitoid response to α-amylase needs to be specific to the host involved. In addition, this supposes that the parasitoids can perceive the α-amylase through their sensorial equipment.

Obonyo et al. (2010a) observed that female parasitoids use the tip of their antennae to recognize and accept their host larvae for oviposition. They identified the presence of specific sensilla known to have gustatory functions in insects on the last antennal segment (Obonyo et al., 2011). Mailhan (2016) showed that these sensilla chaetica are able to detect the α-amylase. However, this result was not confirmed until recently by Tolassy (2018), who suggested that other sensilla from other sensorial organs, such as from the tarsi, might be involved.

There is no physiological evidence that the parasitoid can detect the α-amylase, since gustation in insects is known to be influenced generally by small compounds such as sugars, free amino acids and water-soluble alkaloids (Thiéry et al., 2013 for review). Nevertheless, it is well-known that hymenopterans are able to detect large molecules such as long chain cuticular hydrocarbons of more than 60 carbons (Cvacka et al., 2006; Blomquist and Bagnères, 2010) and that non-volatile long-chain hydrocarbons can been detected by olfactory sensilla (Ozaki et al., 2005, 2012). We cannot therefore rule out the detection of α-amylase by sensilla specialized in olfaction on *Cotesia* spp. antennae.

In conclusion, this study shows that α-amylase is a key protein for host acceptance and oviposition by species of the *C. flavipes* complex, and that its variation is involved in the specificity of host-parasitoid association. These findings open new routes for the investigation of evolutionary processes at play in Lepidoptera stem borers-*Cotesia* and their interactions.

In addition, these findings highlight some issues in biological control perspectives. The ecosystem service provided by biological control relies to a large extent on the natural adaptive abilities of biological control agents. Pest resistance is less frequent in biological control than in chemical control (Holt and Hochberg, 1997). One reason advanced for this better protection against host resistance is that biological control agents can co-evolve and adapt to host resistance, whereas chemical control agents cannot. The link between α-amylase isoforms and *Cotesia* species and population in this study gives a strong insight into such adaptive processes of the parasitoid to its host. In the near future the main relevance in agriculture will be to deliver more efficient parasitoid strains against pest insects. The identification of α-amylase's receptors involved in host acceptance mediation will help in targeting the genes of these receptors with the aim of carrying out genetic improvements on them.

AUTHOR CONTRIBUTIONS

P-AC made conception and design of the study. GB and J-LD contributed to purify and isolate the α-amylase. KS performed the bioassays. SM performed the α-amylase purifications. P-AC wrote the first draft of the manuscript. GB, J-LD, BL, and LK wrote sections of the manuscript. EM corrected the English. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00228/full#supplementary-material

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