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Assortments of Digestive Enzymes Induced in First Instar Larvae of *Busseola fusca* Feeding on Different Plants

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ABSTRACT: The stem borer *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) is an important pest of maize and sorghum in sub-Saharan Africa. This insect has oligophagous feeding habits, feeding mostly on maize and sorghum with a narrow range of wild Poaceous plant species. We hypothesised that first instar *B. fusca* larvae, the critical stage for successful establishment on a host plant, can establish and then grow on a particular plant as a result of induction of a complement of digestive enzymes that mediates host acceptance at first instars. A fast semi-quantitative analysis of potentially digestive enzymatic activities present in the first larvae previously fed for 4 days on leaves of host and non-host plants was performed using the API-ZYM kit system able to detect a multiplex of enzyme activities. Regardless of the plant species, the larvae exhibited higher activities of the carbohydrate metabolising enzymes than of aminopeptidases and proteases. In addition, highest activities of carbohydrates degrading enzymes were exhibited by larvae that consumed leaves of the most preferred plant species of *B. fusca*. Conversely, esterases were only detected in neonate larvae that consumed leaves of the less preferred and non-host plants. No alkaline phosphatase and lipase activities were detected. The significance of these results was discussed in terms of food requirements of first instar larvae when settling on a plant.

KEYWORDS: Lepidoptera, stem borers, maize pest, sub-Saharan Africa, digestive enzymes

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Introduction

The evolutionary success of phytophagous insects depends on their ability to utilise specific plants as food sources. Phytophagous insects are largely influenced by host-plant chemistry.[1,2] Each plant has a specific phytochemical profile consisting of both primary and secondary metabolites that form the basis of host selection and discrimination by an insect.[3] These chemicals inform the foraging insects about the suitability of a plant as food source[1,2] and determine an insect's food choice and its subsequent performance.[4] They are thus important in host-plant adaptation.[1,5,6]

Following the adaptation of a particular host-plant insects partly depend on the efficiency of their digestive physiology to use chemically diverse host plants as food source,[7–10] helping them for settlement on a particular plant. In most cases, this usually involves the induction of a cocktail of digestive and detoxifying gut enzymes that permit the exploitation of toxic plant allelochemicals encountered during foraging.[11,12] Digestive plasticity to plant chemical toxicants has been previously reported in a number of insect species as an adaptive mechanism to noxious chemical containing host plants. For example, a number of insect species including the grasshopper, *Melanoplus sanguinipes* (Fabricius),[13] the gypsy moth, *Lymantria dispar* L.,[14] the cotton bollworm, *Helicoverpa armigera* (Hübner),[9,15] and the beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae)[10] have been reported to modify the activity of their gut enzymes in response to the chemical composition of the food. Therefore, a complement of digestive enzymes quantitatively or qualitatively expressed in the larvae on insect feeding should reflect the type of chemicals present and exploited by an insect to start to feed and survive on a particular plant.

In sub-Saharan Africa, the oligophagous stem borer *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) is an important pest of economically important food crops such as maize, *Zea mays* L. (Poaceae), and sorghum, *Sorghum bicolor* (L.) Moench (Poaceae).[16] The oligophagic feeding habit of *B. fusca* larvae can be associated with possible plastic biochemical, physiological, or evolutionary mechanisms that allow the insect to confront a variety of chemical complexities posed by diverse food plants. Similar studies on plastic responses to nutritive stresses have been reported for other insect species,[12,17] and they provide basic information on the mechanisms of host-plants' specialisation. Thus, knowledge of the assortments of digestive enzymes induced in larvae when they start to feed on different plants can aid in the understanding of the physiological mechanisms used by *B. fusca* larvae to choose a particular plant species characterised by diverse chemicals. In addition, this will help to understand the mechanisms used by the larvae that disarm secondary metabolites to enable them to adapt on a variety of plants.

This study focused on first instar *B. fusca* larvae, which among Lepidoptera is the critical stage for successful establishment on a host plant;[4,8,18–20] they are more discriminative and selective in their food choice than older larvae.[8] Among wild

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Poaceae plant species frequently found in *B. fusca* natural habitat,[21] with the exception of *Panicum deustum* Thunb (Poaceae) from which *B. fusca* larvae have never been recovered, all other plant species were reported to host *B. fusca* albeit at differing abundances[21–24] and suitability.[25,26] Thereby, wild sorghum, *Sorghum arundinaceum* (Desv.) Stapf., was the most suitable hosts followed by *Megathyrsus maximus* (Jacq.) B.K. Simon & S.W.L. Jacobs, Napier grass *Pennisetum purpureum* Schumach, and *Arundo donax* L., whereas *P. deustum* and *Setaria megaphylla* (Steud.) Th. Dur. & Schinz have been shown to be completely unsuitable.[25,26] *Pannicum deustum* and *S. megaphylla* are therefore considered as non-hosts. Among these 7 plant species, it was also found that during the first instar life of *B. fusca* (ie, about during the first 7 days after hatching), the relative growth rate (RGR) of *B. fusca* larvae was 0.13 mg/d on *S. arundinaceum* (compared with the cultivated crop, *Z. mays*, which was 0.15 mg/d), whereas they were 0.09 and 0.05 mg/d, respectively, on *P. purpureum* and *A. donax*; and only 0.06, 0.03, and 0.03 mg/d, respectively, on *M. maximus, P. deustum*, and *S. megaphylla*.[26] Moreover, very few first instar larvae (from 1% to 8%) were able to survive on *A. donax, M. maximus, P. deustum*, and *S. megaphylla*.[26]

In this context, we hypothesised that first instar *B. fusca* larvae can establish and then grow on a particular plant (to establish on it) as a result of induction of a complement of digestive enzymes that mediates host acceptance at first instars. To provide evidences for this hypothesis, we estimated larval physiological responses in relation to the differential assortments of digestive enzymes, by a semi-quantitative analysis, in the first instar larvae reared on host and non-host plants used in previous studies.[25,26]

Materials and Methods

Insects

The *B. fusca* larvae stemmed from laboratory-reared individuals maintained on the artificial diet of Onyango and Ochieng'-Odero[27] from the Animal Rearing and Containment Unit (ARCU) of the International Centre of Insect Physiology and Ecology (*icipe*, Nairobi, Kenya). Feral individuals collected from maize fields in western Kenya were added to the colony thrice a year to rejuvenate the laboratory culture. One-day-old neonates were used in the experiments.

Plants

The maize cultivar 511 and 6 wild Poaceae species including wild sorghum *S. arundinaceum*, Napier grass *P. purpureum, A. donax, S. megaphylla, M. maximus*, and *P. deustum* were used. Maize was grown in 4-L pots (1 plant per pot) from seeds provided by Simlaw, Kenya Seeds Company, Nairobi. The other plant species were obtained from their natural habitats and propagated from tillers or stem cuttings in 4-L pots (1 plant per pot) in a greenhouse at *icipe*. The environmental conditions for growth were approximately 31°C/17°C (day/night) with 12:12 h (L:D) photoperiod. Plants were watered 3 times weekly and once with a solution of calcium ammonium nitrate (26% N). All plants were 3 weeks old, when used in the experiments, with exception of *S. arundinaceum*, which, due to its slow growth, was 5 weeks old. Each plant was infested with approximately 100 first instar larvae and allowed to feed for 4 days. Previous study indicated a high mortality for longer feeding periods on plant species with low suitability and even no survival on *P. deustum* and *S. megaphylla*.[26]

Enzyme induction in larvae on feeding on different plant species

A fast semi-quantitative analysis of 19 enzymatic activities present in first instar larvae previously fed on leaves of 7 plant species was performed using the API-ZYM system from the API® test kit of BioMérieux (Marcy l'Etoile, France), a test kit that can detect a multiplex of 19 specific enzyme activities present in a sample as described by Rahbé et al.[28] The API-ZYM system consists of a gallery of 20 micro-cups (Figure 1): 19 of each with a specific substrate for enzyme detection and 1 micro-cup empty (ie, with no substrate) as control. The kit was proposing to detect the following 19 enzyme activities: alkaline phosphatase (pH 8.5), esterase (C4), esterase lipase (C8), lipase (C14), leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin (*N*-benzoyl-DL-arginine-2-naphtylamidase), α-chymotrypsin (*N*-glutaryl-phenylalanine-2-naphtylamidase), acid phosphatase (pH 5.4), naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, α-glucosidase, *N*-acetyl-beta-glucosaminidase, α-mannosidase, and α-fucosidase. These enzymes are commonly found in the guts of lepidopteran larvae.[29]

First, 4-day-old larvae were recovered from the infested plants. It was, however, not possible to isolate the midgut for enzyme analysis due to the tiny size of the recovered larvae. Thus, the entire larval body without the head (to prevent contamination by salivary enzymes) was used; it was assumed that the resulting larval homogenates contained mostly digestive enzymes as the fatty tissues at this developmental stage are still not developed.

From each plant species, 40 larvae were homogenised in 1300 μL distilled water and centrifuged (18 000*g*, 10 min, +4°C). A 65-μL aliquot of each of the resultant supernatant was then pipetted to each of the 20 porous plastic micro-cups of the API system (ie, 19 micro-cups dispersed with specific chromogenic substrate and 1 used as a control) and then incubated at 37°C for 4 hours. A similar protocol was used for the control experiment using larvae that were starved but water-fed for 4 days.

For each micro-cup, a coloured product whose intensity is directly proportional to the amount of substrate hydrolysed is formed following the specific enzyme-substrate reaction. For the control micro-cup, no coloration was formed. The

Figure 1. A picture showing the differential staining of the APIZYM plate with ZYM A and ZYM B after 4 hours of incubation of larval homogenates with substrates dispersed on the porous plate. Each coloured well gives a rank score based on the intensity of colour formed.

approximate amount (in nmol) of the enzyme is given by comparison with a colour scale included in the kit, as compared with the control.

Enzyme activities in the tissue extracts were detected by the cleavage of a chromogenic substrate (naphthyl derivatives) dispersed dry on a porous plastic micro-cup (about 100 µL) of the API-ZYM system. Enzymatic reactions were enhanced by application of an sodium dodecyl sulphate (SDS)-based acid Tris buffer (Zym A) as a drop in each well following plate incubation. The reactions were then visualised following the addition of a Fast Blue BB solution (Zym B) to each of the micro-cups. Each test experiment was repeated 3 times and visible colour changes of the medium in the micro-cups were considered positive. The enzyme activity in each micro-cup was visually determined by using a ranking expressed on a scale from 0 (no enzyme reaction) to 5 (very high enzyme concentration) corresponding to the intensity of the coloured product produced on comparison with the colour scale provided with the kit (from ⩽5 to ⩾40 nmol: 1 corresponded to 5 nmol, 2 to 10 nmol, 3 to 20 nmol, 4 to 30 nmol, 5 to 40 nmol and above of substrate released according to the manufacturer's specification; Figure 1). Rank values reflecting enzyme activity from water-fed larvae served as baseline. The final feeding score value was obtained by subtracting the rank values of this group from the rank values obtained for the experimental groups. Score values greater than 0 indicated induction of an appreciable amount of enzyme activities in all larval homogenates.

Statistical analysis

Rank values for enzyme activities were generated following the Kruskal-Wallis test and their means separated using Tukey-Kramer test (Proc GLM). Statistical analyses were done in R version 3.3.1.[30]

Results

Of the 19 enzymes tested with the Api Zym kit, 14 enzymes' activities were evident in the larval homogenates (Table 1). Regardless of the plant species, the carbohydrate metabolising enzymes were more active than aminopeptidases and proteases. No alkaline phosphatase and lipase activity were detected, whereby esterases were only found in larvae that consumed leaves of *M. maximus, P. deustum*, and *S. megaphylla*.

The activities of the aminopeptidases and proteases were not varying significantly between plant species. By contrast, the activities of most of the carbohydrate metabolising enzymes varied significantly. This included β-galactosidase, β-glucuronidase, α-glucosidase, and β-glucosidase. Highest activities of carbohydrates degrading enzymes were exhibited by larvae that consumed leaves of *Z. mays, S. arundinaceum*, and *P. purpureum*, whereby β-glucosidase activity was significantly highest for larvae fed only on *Z. mays* and *S. arundinaceum*.

Discussion

Although quantitative enzymatic assays with zymographic detections are important to complement this study, this semi-quantitative analysis is a good first overview of the assortments of digestive enzymes induced in first instar larvae after their first feeding on a particular plant that might help the larvae to establish on it. Moreover, this assortment might be directly linked to the chemical composition of foliage consumed by larvae. The low performance of first instar larvae of *B. fusca* on *M. maximus, P. deustum*, and *S. megaphylla* reported in a previous study by Juma et al[26] may suggest the presence of antibiotic properties in these plants, possibly as a result of plant secondary metabolites or low nutritional quality, which cannot allow first instar larvae of *B. fusca* to establish. C4 esterase activity was only detected in homogenates of larvae fed on *M. maximus, P. deustum*, and *S. megaphylla*, which have been shown plant of low suitability.[26] Induction of esterases in insect midgut has

Table 1. Semi-quantitative analysis of enzyme activities in the homogenate of entire neonate larvae (with heads decapitated) reared for 4 days on different plants species. Enzyme activities (means ± SE, n = 5) correspond to the activities found in the crushed larval homogenate (40 larvae per replicate). Activities correspond to the release of 5, 10, and 20 nmol of substrate per 4 hours of incubation at 37°C (rate 0: activity not detected).

ENZYMES	*ZEA MAYS*	*SORGHUM ARUNDINACEUM*	*PENNISETUM PURPUREUM*	*ARUNDO DONAX*	*MEGATHYRSUS MAXIMUS*	*PANICUM DEUSTUM*	*SETARIA MEGAPHYLLA*
Alkaline phosphatase	–	–	–	–	–	–	–
Esterase (C4)	–	–	–	–	0.2 ± 0.2a	1.0a	0.2 ± 0.2a
Esterase lipase (C8)	–	–	–	–	0.2 ± 0.2	–	–
Lipase (C14)	–	–	–	–	–	–	–
Leucine aminopeptidase	–	–	–	–	–	–	–
Valine aminopeptidase	0.7 ± 0.2a	0.6 ± 0.2a	1.0a	0.4 ± 0.2a	–	–	0.6 ± 0.2a
Cystine aminopeptidase	–	0.2 ± 0.2a	–	–	0.2 ± 0.2a	0.2 ± 0.2a	–
Trypsin	–	–	–	–	0.5 ± 0.3	–	–
α-chymotrypsin	–	–	0.7 ± 0.2a	–	0.5 ± 0.3a	–	–
Acid phosphatase	–	–	–	–	–	–	–
Naphthol-AS-BI-phosphohydrolase	1.0b	0.6 ± 0.2ab	1.0b	1.0b	0.2 ± 0.2a	1.0b	0.8 ± 0.2ab
α-galactosidase	–	1.2 ± 0.5a	–	–	–	0.5 ± 0.3a	–
β-galactosidase	1.2 ± 0.3b	1.2 ± 0.2b	1.7 ± 0.2b	0.8 ± 0.2a	0.5 ± 0.3a	–	0.2 ± 0.2a
β-glucuronidase	1.2 ± 0.2a	2.0 ± 0.5b	2.0b	1.0a	1.0a	–	0.8 ± 0.2a
α-glucosidase	1.8 ± 0.2b	1.6 ± 0.2b	2.0b	1.2 ± 0.2a	–	0.5 ± 0.3a	1.0 ± 0.3a
β-glucosidase	1.8 ± 0.5c	1.4 ± 0.2c	1.0b	0.4 ± 0.2a	0.7 ± 0.2ab	–	–
N-acetyl-beta-glucosaminidase	–	–	–	–	–	–	–
α-mannosidase	–	1.0 ± 0.3a	1.5 ± 0.3a	–	0.7 ± 0.2a	–	–
α-fucosidase	–	0.4 ± 0.2	–	–	–	–	–

Means within a line followed by different letters are significantly different at 5% level (Tukey-Kramer test).

been shown to be positively correlated with resistance to plant allelochemicals by several Lepidoptera species.[31–37] *Panicum* sp. and *Setaria* sp., for example, are known to possess beta-phenylethylamine alkaloids (such as *N*-methyltyramine) and a phenolic acid, setarin (4-allyloxycoumarin), respectively.[38]

The level of activities of glycosidases including β-galactosidase, β-glucuronidase, α-glucosidase, and β-glucosidase was significantly induced in homogenates of larvae fed on maize, wild sorghum, and *P. purpureum*. These plants were previously demonstrated to support the best larval performance in the laboratory over the plant species used in this study.[26] This may be related to higher content of disaccharides and polysaccharides in young, developing leaves of these plants as compared with the other plant species used as it has been observed for secondary metabolites in young maize leaf whorls by Bergvinson et al.[39] This indicates that these disaccharides and polysaccharides might mostly be hydrolysed by these enzymes into simple sugars for first instar larvae energy metabolism.

In addition, β-glucosidases, that were high in larvae fed on maize and sorghum, are also involved in the detoxification of a wide range of other plant-derived β-o-glycosyl containing allelochemicals such as the benzoxazinoids (DIMBOA and MBOA) present in maize[40–42] and dhurrin, the cyanogenic glycoside present in sorghum.[43] This suggests a possible physiological adaptation of *B. fusca* to toxic allelochemicals of these host plants as reported in other lepidoteran species including European corn

borer, *Ostrinia nubulalis* (Hübner) (Lepidoptera: Pyralidae)[44,45] and in *Spodoptera frugiperda* (J.E. Smith).[42] Similarly, specialised *Heliconius* caterpillars (Lepidoptera: Nymphalidae) are reported to efficiently convert sorghum-specific cyanogenic glycosides to soluble and harmless thiols[29] preventing the latter to release harmful cyanide and even allowing the caterpillars to utilise the toxic compounds as a nitrogen source.

Proteases including trypsin and chymotrypsin and aminopeptidases are the most predominant enzymes in the midgut of most lepidopteran insects.[46–49] Although third instar larvae of *B. fusca* were found to harbour digestive proteases,[50] the activity of proteases (aminopeptidases and serine proteases) in larval homogenate of first instar of *B. fusca* on all 7 plants was, however, lower than that of the carbohydrate-hydrolysing enzymes, suggesting that leaves of the plant species tested might be a poor source of proteins at least for first instar larvae of *B. fusca*, and therefore, the protein calorific value of the studied plants did not significantly induce the first instar larvae gut proteolytic activity. However, the level of free amino acids and soluble low-molecular-weight proteins in leaves might be sufficient for the development of young *B. fusca* larvae, and therefore, they did not induce gut extra proteolytic activities.

No lipase activity involved in fat digestion was found in first instar larvae of *B. fusca*. This indicates that *B. fusca*, at this initial stage of development, does not utilise fat for initial growth on plant.

In conclusion, our results indicated that first instar larvae of *B. fusca* generally relies on simple carbohydrates rather than proteins as food source. This might be linked to its specialisation to *Z. mays* and *S. arundinaceum*.[22,23] In fact, the advantage for host plant specialisation by herbivorous insects has been hypothesised to involve an increase in energetic efficiency.[51] Therefore, maize and wild sorghum should have a more suitable composition, level, or ratio of different carbohydrates required by *B. fusca* first instar larvae for energy unlike the other plant species considered in this study, thus allowing a better settlement of first instar of *B. fusca* on these plants than on the others.

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Author Contributions

The research was conducted by GJ under the supervision of PAC listed as co-author. GJ and PAC participated in defining the research methodology and all co-authors participated in writing/editing the paper.

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