Divergence in behaviour between the European corn borer, *Ostrinia nubilalis*, and its sibling species *Ostrinia scapularis*: adaptation to human harvesting?

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Divergent adaptation to host plant species may be the major mechanism driving speciation and adaptive radiations in phytophagous insects. Host plants can differ intrinsically in a number of attributes, but the role of natural enemies in host plant specialization is often underappreciated. Here, we report behavioural divergence between the European corn borer (ECB, *Ostrinia nubilalis*) and its sibling species *Ostrinia scapularis*, in relation to a major enemy: humans. Harvesting maize imposes selective mortality on *Ostrinia* larvae: those located above the cut-off line of the stalk face almost certain death. We show that ECB larvae diapause closer to the ground than those of *O. scapularis*, which is sympatric but feeds mainly on weeds. The difference in diapause height results from genetically determined differences in geotactic behaviour. ECB larvae descend towards the ground specifically at harvest time, increasing their chances of surviving harvesting by about 50 per cent over *O. scapularis* larvae. Natural enemies appear as a major driver of host-plant specialization in this example, stressing the need to consider 'tri-trophic' ecological niches to understand insect diversification. Our results also strongly suggest that geotaxis evolved as a singular instance of behavioural resistance in a major agricultural pest.

Keywords: agricultural pest; geotaxis; harvesting selection; host-plant specialization; maize; phytophagous insects

1. INTRODUCTION

Insect–plant interactions provide many of the clearest instances of ecological specialization and speciation (Drès & Mallet 2002; Futuyma & Agrawal 2009). Phytophagous insects are often specialized on a limited number of host plant species, through suites of morphological, physiological and behavioural traits (Jaenike 1980; Rausher 2001; Frantz *et al.* 2009; Singer & McBride 2010). An important fraction of the tremendous diversity of phytophagous insects is thought to have originated from host-shifts: plant species offer distinct ecological niches to which diverging insect species may adapt (Schluter 2001). Divergent ecological specialization can contribute, directly or indirectly, to the build-up of reproductive isolation (Rundle & Nosil 2005). Although the source of divergent selection is traditionally looked for at the level of insect–plant interactions (e.g. plant secondary metabolites, Ehrlich & Raven 1964; or phenology, Feder & Filchak 1999), it is increasingly recognized that natural enemies can play an important role in driving

insect diversification on host species, stressing the need to consider 'tri-trophic' ecological niches (Singer & Stireman 2005). For instance, different host-plant species can select for contrasting anti-predator cryptic patterns (Nosil *et al.* 2002) or differ in the nature and abundance of the parasitoid fauna (Brown *et al.* 1995; Feder 1995).

Here, we study the behaviour of two host-affiliated sibling species: *Ostrinia nubilalis* Hübner and *Ostrinia scapularis* Walker (Lepidoptera: Crambidae), in relation to a major natural enemy: humans. *Ostrinia nubilalis* (the European corn borer, ECB) is a cosmopolitan pest of maize (*Zea mays* L.). It first appeared after the introduction of maize to Western Europe, approximately 500 years ago (Rebourg *et al.* 2003) and was accidentally introduced in the Americas in the early twentieth century (Thompson & Parker 1928). The ECB is thought to have originated from *O. scapularis*, its extant closest relative, which is sympatric with ECB in Europe but does not feed on maize: its main host plant is mugwort (*Artemisia vulgaris* L., Bourguet *et al.* 2000; Malausa *et al.* 2007a). The two sibling species are morphologically indistinguishable at all stages, weakly genetically differentiated and readily produce fertile hybrids (Thomas *et al.* 2003; Malausa *et al.* 2005; Calcagno *et al.* 2007). Both can develop successfully on either maize or mugwort (Calcagno *et al.* 2007). They were regarded as conspecific

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2010.0433> or via <http://rspb.royalsocietypublishing.org>.

host races until recent reclassification (Frolov *et al.* 2007; Malausa *et al.* 2007b). Hybridization in the field is rare, possibly because of the use of different stereoisomers of the main female pheromone (Bontemps *et al.* 2004; Malausa *et al.* 2005; Dopman *et al.* 2010).

Maize and mugwort differ greatly in structure, physiology and phenology: maize is a C4 annual Poaceae, whereas mugwort is a C3 perennial Asteraceae (Bourguet *et al.* 2000). Accordingly, ECB larvae were found to perform better than *O. scapularis* larvae on maize, and conversely to perform less well on mugwort, indicative of physiological specialization (Calcagno *et al.* 2007). In addition, the two *Ostrinia* sibling species differ with respect to development time (Thomas *et al.* 2003), but see Malausa *et al.* 2005). Natural enemies could also have promoted the divergence, since both the identity of parasitoids and their prevalence differ between maize and mugwort (Thomas *et al.* 2003; Péliissié *et al.* 2010).

Previous studies have nonetheless ignored a fundamental difference between the natural (or unnatural) enemies associated with the two host species. In both Europe and North America, maize fields are harvested by cutting-off the stalks 15–40 cm above ground level (Caffrey & Worthley 1927; Colbert 2000; Carraretto 2005), while mugwort is neither harvested by humans nor grazed by mammals. Although harvesting techniques have varied over the last 500 years, from hand-harvesting to modern combined-harvesting, most shared the common feature of removing and disposing of the upper parts of the stalks (by burning, shredding or using them as fodder or silage for cattle; Brindley & Dicke 1963; Colbert 2000; Carraretto 2005). Since *Ostrinia* larvae develop and diapause throughout winter in the stems of their host plant (Thompson & Parker 1928), those located in the upper part of maize stalks are likely to die. Destroying the stalks after harvesting has indeed been advocated to control ECB populations (Caffrey & Worthley 1927; Brindley & Dicke 1963). Hence, humans have consistently acted as predators on maize-feeding *Ostrinia*, selectively killing those larvae above the harvest cut-off line.

This difference in the vertical distribution of mortality risk between maize and mugwort may select for divergent behaviours on the two host species. After shifting to maize, *Ostrinia* larvae that descended towards the ground prior to harvest time increased their chances of survival. Any variation affecting larval position at harvest would have therefore become a target of selection. Consequently, we hypothesized that the ECB and *O. scapularis* should exhibit divergent geotactic behaviours, more specifically that the ECB should be positively geotactic (i.e. move towards the ground) at the end of the growing season. To test this hypothesis, we compared the behaviours of the two sibling species, in both field and controlled laboratory conditions, and quantified the selection pressure exerted on geotactic behaviour by modern harvesting techniques. We show that (i) the ECB consistently diapauses closer to the ground than *O. scapularis* in field conditions, be it on maize or on mugwort; (ii) this difference is caused by an innate tendency of ECB larvae to move down at the end of their development, a behaviour for which we found no evidence in *O. scapularis* larvae; and (iii) under conditions of human harvesting on maize, the geotactic behaviour

that we document in ECB confers a strong survival advantage (close to 50%) over the behaviour we measured in its sibling species. Our results indicate that selective harvesting by humans is a major agent in the divergent specialization of the ECB and *O. scapularis* to their host species, comparable in intensity, if not stronger, to other agents (such as physiological differences between plants). They also strongly suggest that the ECB adapted its behaviour in response to human agricultural techniques, in a singular instance of behavioural resistance.

2. MATERIAL AND METHODS

(a) Field experiment

In June 2003, we introduced first instars of either ECB or *O. scapularis* onto maize and mugwort plants in an experimental field. Fifty plants of each species were infested with ECB larvae, and 50 with *O. scapularis* larvae (20 larvae per plant on average). The experiment took place in Grignon (France, 48.850° N, 1.915° E), in the centre of the area where the two sibling species are sympatric. First instars were obtained from individuals sampled the preceding year at several places in Northern France (see Calcagno *et al.* 2007) for detailed sampling locations and protocol). Larval development was left to proceed in natural conditions, and the vertical distribution of diapausing larvae within the plants were recorded in October 2003 (the average harvest time in that area). All surviving larvae were assigned to one of the six height classes—1, ground level or below; 2, less than 20 cm above ground; 3, 20–30 cm; 4, 30–50 cm; 5, 50–100 cm; 6, greater than 100 cm. They were also dissected for sex determination.

(b) Laboratory experiment

In 2006, we conducted a behavioural assay to study and compare the geotactic behaviours (i.e. gravity-related movements) of the two sibling species. *Ostrinia* larvae of both species (*ca* 1000 per species) were sampled in autumn–winter 2005 from several populations in Northern France: Versailles (48.800° N, 2.133° E), Chartres (48.450° N, 1.480° E), Montdidier (49.650° N, 2.567° E), Beauvais (49.433° N, 2.083° E) and Amiens (49.905° N, 2.300° E). They were placed under diapause-breaking conditions (high humidity and ambient temperature; Glover *et al.* 1992) in May 2006 and yielded adults in June 2006. These were placed into mating cages to produce first generation (G₁) eggmasses. After hatching, larvae were grown in usual laboratory conditions, on a standard diet (Gahukar 1975), throughout summer. About 50 G₁ adults per species were obtained in September 2006, and placed in mating cages to produce G₂ first instars used for the experiment.

Larval movements were monitored from hatching to diapause (12 weeks in total) by growing larvae in ‘artificial plants’ (specially designed vertical stacks of plastic vials, see electronic supplementary material, figure S1). These stacks were assembled from 11 small plastic vials (5 cm diameter × 3 cm high each), maintained together with water-based glue. Partitions between adjacent vials were perforated six times in random places (holes 5 mm diameter), to allow for vertical movements of larvae. Each box was filled with a standardized amount (two spoons) of diet. On 18 October 2006, G₂ first instars from ECB or *O. scapularis* (drawn randomly from all produced larvae) were placed in the central box of the stacks. Forty larvae were placed in each stack, and 20

replicate stacks were used per species, half of them set upside-down to control for possible effects of their asymmetrical inner shape (electronic supplementary material, figure S1B). Stacks were placed in a climatic chamber (60% of humidity), in total darkness, to rule out light-related movements (phototaxis). The number of larvae present in each vial of each stack was recorded weekly for six weeks. Each time, surviving larvae were transferred individually, maintaining their vertical position, into a new stack. The new stack retained the same orientation as the former, but was re-assembled from new vials (washed and frozen before use) and refilled with diet (electronic supplementary material, figure S1C). The location of the stacks in the climatic chamber was shuffled every week. To simulate natural conditions and induce diapause, temperature and thermoperiod, initially set at 20°C : 25°C/8 h : 16 h, were gradually decreased so as to reach 13°C : 16°C/12 h : 12 h in the sixth week. At this stage, most larvae were entering diapause and no longer moved or fed. They were kept in constant conditions for six more weeks, and on 16 January 2007, their final positions were recorded. The sex of 80 randomly chosen larvae of each species was determined by PCR amplification of a female-specific locus (R. Streiff 2007, unpublished data).

(c) Statistical analysis

In both experiments, vertical positions of larvae were analysed by bootstrap, since larvae in the same stalk/stack could not be considered independent. Stalks/stacks were resampled 2000 times, with correction for bias and acceleration, to generate 95% confidence intervals and *p*-values for pairwise comparisons (Di Ciccio & Efron 1996). The null hypothesis was that the two species had the same average vertical position, and the alternative that ECB was lower. Performing two-tailed tests did not change statistical significance in any case.

3. RESULTS

(a) Behaviours in the field

The vertical distributions of ECB and *O. scapularis* larvae on maize were different, ECB larvae being more concentrated in the lower parts of the stalks (figure 1a). As a result, the average diapausing position of ECB larvae was significantly lower than that of *O. scapularis* larvae (figure 1b). The same result was observed on mugwort (figure 2b). This clear pattern on both their natural host-plant and the weed they do not use in natural conditions reveals a robust tendency of ECB larvae to diapause at a lower position than its sibling species, regardless of the precise characteristics of the habitat. The vertical distribution of ECB larvae observed on maize is in good agreement with observations from 1924 in the US, at similar times in the harvesting season (figure 1a; data extracted from Caffrey & Worthley 1927). Although our observed distribution appears more concentrated at very low positions (below 30 cm; figure 1a), this cannot be tested properly since the two datasets are very different. Sex-ratios were identical in the two species (electronic supplementary material, S1).

(b) Behaviours in the laboratory

Figure 2a plots the mean vertical position of the two sibling species in the stacks throughout the course of the laboratory experiment. Both species, initially introduced

in the middle of the stacks (position 0), moved up in early stages, reaching similar positions—with the mean around position +3—after three weeks (figure 2b). Although unexpected, this can be understood in light of *Ostrinia* feeding habits: before boring the stalks, early instars graze young plant tissues that are mostly apical (Caffrey & Worthley 1927; V. Calcagno 2005, personal observation).

Subsequently, the behaviours of the two sibling species differed significantly. Whereas the mean position of *O. scapularis* slowly reverted to the middle of the stacks, the ECB reversed its initial climbing behaviour and diapaused at a lower position, well below 0 ($p < 10^{-4}$; figure 2c). The final distribution of *O. scapularis* (observed at week 12) is almost perfectly U-shaped and symmetrical around zero (figure 2c; Fisher's symmetry test, $p = 0.981$). This pattern can be simply explained if larvae, after their initial tendency to climb, adopted random movements with no average vertical preference; no positive geotaxis has to be invoked. This is not the case for ECB, whose larvae accumulated in the lower vials in the stacks. About 25 per cent of ECB larvae diapaused in the lowermost vial (twice as much as *O. scapularis*, figure 2c; $p < 0.01$). The resulting final distribution at week 12 was significantly skewed downward (symmetry test, $p < 10^{-4}$), which can only be explained by positive geotactic movements. Again, sex ratios did not differ between the two species (electronic supplementary material, S1).

(c) Adaptive significance in relation to human harvesting

It has been measured experimentally that combined-harvesting reduces ECB adult emergence the next spring by 64 per cent on average, compared with non-harvested fields (Umeozor *et al.* 1985). We have independently estimated the proportion of larvae found below the cut-off line at harvest time between 30 and 50% (figure 1a; a value of 28% obtained by direct measurement was reported in Caffrey & Worthley (1927)). These two results imply that harvesting kills almost all larvae above the cut-off line, their survival being reduced by 89 per cent to 100 per cent (electronic supplementary material, S2). Taking into account that natural mortality might be intrinsically lower in the lower part of the stalks or that harvesting might reduce survival in the maize stubbles (e.g. because of higher exposure to predators) does not reduce this estimate importantly (electronic supplementary material, S2 and figure S2).

How does the difference in behaviour between the ECB and *O. scapularis* translate in terms of expected survival on maize? ECB larvae are 68 per cent, 46 per cent or 43 per cent more likely to be below the cut-off line, depending on its height (figure 1a; the three values are for 20 cm, 30 cm and 50 cm, respectively). Hence, the behaviour of ECB means it is approximately 50 per cent (in the range 40–70%) more likely to survive harvesting when compared with the behaviour of *O. scapularis*, every year in harvested fields. This difference is quite high, comparable to the biggest difference in survival probability on maize that has been observed as a result of physiological specialization (60%; Calcagno *et al.* 2007).

Figure 1. Behaviours in the field. (a) Vertical distribution of ECB (black; 2002, $n = 68$) and *O. scapularis* (grey; 2002, $n = 78$) larvae in maize stalks at harvest time. Note the six height classes used. For comparison, distributions of ECB observed in US maize fields at two different times in the harvesting season are also shown (dotted lines; 1924, $n = 135$ and $n = 110$; data extracted from Caffrey & Worthley 1927). At harvesting, maize stalks are cut-off around 25 cm above ground (black rectangle). (b) Mean vertical position of ECB (black) and *O. scapularis* (grey), on maize and mugwort plants. Positions are in units of the height classes used in the field experiment (visible in (a)). Mugwort stands are typically shorter than maize stalks (about 1.5 versus 2.5 m), which explains why both species diapaused lower on mugwort. 95% confidence intervals and p -values were obtained by bootstrap (maize, $n = 78$ and 68; mugwort, $n = 155$ and 58).

4. DISCUSSION

Phytophagous insects have provided influential models of divergent ecological specialization and speciation (Drès & Mallet 2002; Futuyma & Agrawal 2009). Host-shift associated speciation may have contributed a significant proportion of species richness on the Earth and is supported by abundant theory (Maynard-Smith 1966; Fry 2003). Nonetheless, there is a concern that studies of insect specialization may have overlooked important dimensions of the process by focusing on limited aspects of the life cycle (Scheirs *et al.* 2005). In particular, most studies have been concerned with the insect–plant

interaction, at the expense of third-party actors such as natural enemies (Singer & Stireman 2005). Novel host species can not only free insect populations from some natural enemies, facilitating the process of host-shift (the enemy-free space hypothesis; e.g. Price *et al.* 1980; Brown *et al.* 1995; Feder 1995), but also may conversely harbour new enemies to which the populations must adapt.

The ECB (*O. nubilalis*) shifted to maize when the latter was introduced into Europe 500 years ago, presumably from mugwort, the host plant of its sibling species *O. scapularis*. When shifting to maize, it was confronted

Figure 2. Behaviours in the laboratory. (a) Mean positions of larvae in the stacks throughout the course of the experiment. Grey, *O. scapularis*; black, ECB. Initially all larvae were placed in the central box (position zero). After 12 weeks, all larvae had entered diapause and no longer moved. 95% confidence intervals and p -values were obtained by bootstrap ($-p > 0.05$; ** $p < 0.01$; *** $p < 0.001$). (b) Vertical distribution of larvae in the stacks after two weeks. Light grey, *O. scapularis* ($n = 450$); dark grey, ECB ($n = 532$). (c) Final distributions after 12 weeks. Light grey, *O. scapularis* ($n = 227$); dark grey, ECB ($n = 312$).

with a new plant environment, while escaping some natural enemies (such as *Macrocentrus cingulum*, a braconid wasp; Pélissié *et al.* 2010). It also encountered a new enemy: humans. Traditional maize-harvesting techniques in Europe involved cutting-off the stalks in autumn, thereby reducing survival of those larvae found above the cut-off line (Carraretto 2005). This selective mortality has persisted, and probably culminated, with the spread of combined harvesters around 1900 (Colbert 2000; Carraretto 2005).

Here, we have shown that the ECB and *O. scapularis* differ with respect to their height at harvest time: the ECB consistently diapauses closer to the ground than its sibling species in similar field conditions, be it on maize or on mugwort. Even though insect movements within plants are potentially affected by several interacting environmental cues, such as light or tactile stimuli (Jander 1963; Perkins *et al.* 2008), we have demonstrated in the laboratory that this difference can be explained simply: ECB larvae exhibit positive geotaxis (i.e. a tendency to move down in a gravity field) when they are

about to enter diapause, whereas *O. scapularis* larvae do not. We have shown that this genetically determined behaviour is strongly adaptive on maize: by increasing the chance of being below the harvest cut-off line, it confers ECB a survival advantage of about 50 per cent over *O. scapularis* in harvested fields.

The survival advantage conferred by positive geotaxis on maize is similar to, and perhaps exceeds, the survival difference between the ECB and *O. scapularis* throughout larval development, and attributable to physiological specialization (Calcagno *et al.* 2007). On the contrary, there is no obvious reason to think that positive geotaxis is advantageous on mugwort. This weed is not harvested, not grazed by mammals and mugwort shoots are fragile, often breaking into fragments (D. Bourguet 2000, personal observation): this would make gravity-based placement unreliable. Divergence in geotactic behaviour is therefore a major facet of host-plant specialization in this system, adding to the argument that natural enemies should be included as a dimension of ecological niche differentiation (Singer & Stireman 2005).

Although there is little doubt that human harvesting of maize has been the dominant selective agent on geotaxis for the last 100 years, its effect may have been weaker further in the past. Other factors could have played a role in the evolution of geotaxis. For instance, as maize stalks desiccate in autumn, larvae may be selected to move down to feed on more succulent plant portions (Caffrey & Worthley 1927). We nonetheless consider this unlikely to explain the observed divergent behaviours, since similar desiccation occurs in mugwort, and also because positive geotaxis is expressed at a stage where larvae essentially cease to feed. Even though our hypothesis is that geotaxis evolved as a detailed response to human harvesting, we cannot rule out that some other factor has been involved, possibly preadapting the ECB to modern harvesting. Clarifying how positive geotaxis developed historically opens a challenging but promising research avenue with this model system. In particular, independent evolution of similar adaptive behaviours could be looked for in related species. *Ostrinia furnacalis* in Asia is an interesting candidate for such an investigation of parallel evolution, since it may result from an independent host shift to maize (Frolov *et al.* 2007). If, as we strongly suspect, geotaxis proves to have evolved as a specific response to harvesting techniques, this would constitute a very singular instance of behavioural resistance to agricultural management techniques other than insecticide application (Levine *et al.* 2002; Mochizuki *et al.* 2002; Miller *et al.* 2009).

The authors thank P. Audiot and S. Meusnier for help with laboratory work, F. Rousset for statistical advice and M. Hochberg and S. Ponsard for comments. This work was funded by the IFB—AO Biodiversité et Changement Global—and the CNRS—AO Impact des Biotechnologies sur les Agrosystèmes. V.C. and Y.T. were supported by a grant from the French Ministère de l'Éducation et de la Recherche.

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