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Detecting a difference in reaction norms for size and time at maturation: pheromone strains of the European corn borer (*Ostrinia nubilalis*: Lepidoptera, Crambidae)

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ABSTRACT

Background: Sibling herbivore species or host strains specialized to different food plants frequently evolve specific adaptations to their hosts, including host-specific differences in developmental traits (body mass and development time). Such differences may (1) be a consequence of an evolutionary change in relative quality of different hosts, or (2) reflect host-specific changes in the reaction norms for size and time at maturation *per se*.

Aim: Detect a difference in reaction norms for size and time at maturation among the host strains of an herbivorous insect.

Organism: European corn borer *Ostrinia nubilalis*, a polyphagous pest moth with two distinct host plant strains – E and Z – feeding on hop/mugwort and on maize, respectively.

Methods: A laboratory growth trial in which the larvae from these two strains were reared on an artificial diet that was either neutral or included the native host plant of the respective strain. The growth of the larvae was monitored on a daily basis.

Results: Larvae from strain Z developed over a longer period and attained higher pupal masses than larvae from strain E, the strains thereby showing systematic differences in reaction norms for time and size at maturation.

Conclusion: Examining the sign of the correlation between size and time at maturation at the level of among-strain comparison is recommended as a tool for detecting host-specific changes in the reaction norms for size and time at maturation.

Keywords: *Artemisia vulgaris*, body size, evolution, growth rate, host race, insect, *Ostrinia nubilalis*, phenology, *Zea mays*.

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INTRODUCTION

Specialization to different host plants is thought to be one of the primary forces behind evolutionary divergence in herbivorous insects (Berlocher and Feder, 2002; Dres and Mallet, 2002; Schoonhoven *et al.*, 2005). Studies on adaptation to a new host plant primarily have focused on documenting and explaining the change in *relative quality* of the plants from the perspective of the herbivore. In particular, in the course of evolutionary host shift, the new plant species becomes more suitable for growth and development; the plant previously used can become a lower quality host if trade-offs exist between performances on the different hosts (Bernays and Chapman, 1994). Typically, the quality of the plant is reflected in host-specific juvenile growth rates, which, in turn, depend on physiological adaptations to physical and chemical properties of particular hosts (Chown and Nicolson, 2004). According to the prediction of predominantly negative environmental correlations between size and time at maturation (Roff, 1992; Stearns, 1992; Day and Rowe, 2002), host-specific growth rates result in host-specific final masses and developmental periods so that greater final masses and shorter development times are typically observed on the higher quality host (Bernays and Chapman, 1994; Tammaru, 1998; Schoonhoven *et al.*, 2005; Price *et al.*, 2011) (Fig. 1a, b). Such host quality dependent effects form an important source of among-host differences in developmental variables of herbivorous insects.

Nevertheless, and perhaps more interestingly, following a host shift herbivores may evolve adaptations that go beyond the changes in relative quality of different host plant species. In particular, host races may also differ in maturation decisions (*sensu* Roff, 1992; Stearns, 1992), i.e. a growing juvenile of a particular host race may ‘decide’ to pupate earlier, and at a lower mass than individuals of another race (Fig. 1c). In other words, an evolutionary change in maturation decision would imply a change in the reaction norm for size and time at maturation in itself, not just a ‘choice’ of a different position on the reaction norm (for a similar approach, see Tammaru *et al.*, 2000). In contrast to the patterns caused by variation in host quality (Fig. 1a,b), differences in maturation decision must result in a positive correlation between size and time at maturation (Fig. 1c), a feature potentially useful to distinguish between these two types of evolutionary changes associated with host shift.

Documenting an evolutionary change in maturation decision provides information relevant to the study of body size evolution. Indeed, in contrast to recent advances in our understanding of the developmental physiology of growth in an evolutionary context (D’Amico *et al.*, 2001; Davidowitz *et al.*, 2005, 2012), selective forces determining optimal size of an organism are still poorly understood (Blanckenhorn, 2000; Kingsolver and Pfennig, 2004; Amarillo-Suarez *et al.*, 2011; Kingsolver and Diamond, 2011). The cases in which an evolutionary change in body size is a by-product of a host plant shift have a specific advantage: many cases are sufficiently recent events (Singer *et al.*, 1993; Carroll *et al.*, 1998; Agrawal, 2000). Selective pressures on different variables can therefore be measured with reasonable confidence (e.g. Mira and Bernays, 2002; Messina and Karren, 2003; Diamond and Kingsolver, 2010), without the need to extrapolate to the remote and unrecorded evolutionary past. Unfortunately, however, such cases are rarely exploited in the context of assessing selective pressures on body size (see, however, Messina, 2004), perhaps partly due to methodological difficulties in distinguishing changes in maturation decision from the manifestations of an evolutionary change in relative quality of different hosts.

Here, we report a case in which an observed difference in developmental variables between two host strains of a moth species could be ascribed to an evolutionary change in maturation decisions, and not just changes in relative quality of different host plants. In particular, in a laboratory rearing, larvae representing two host strains were reared on

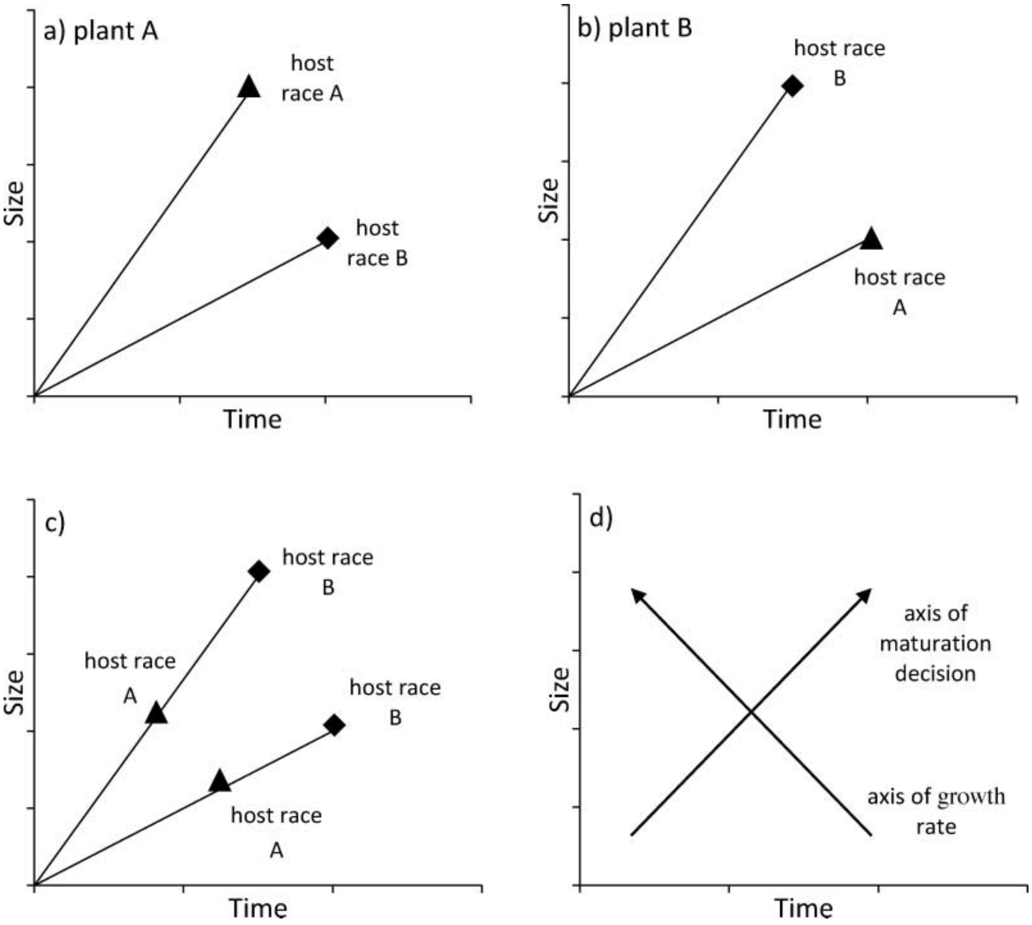

Fig. 1. Schematic representation of two types of potential adaptation to hosts (plants A and B) by two host races of herbivorous insect. Solid points denote size and time at maturation under particular growth conditions. Panels (a) and (b) represent adaptations to ensure high growth performance on the particular host plant to which the herbivore is adapted to: on 'own' plant, both growth rate and size at maturation is higher. (c) The two host races have evolved different size and time at maturation independent of growth rate. Panels (a) and (b) can be interpreted as a shift along the axis of growth rate, whereas (c) depicts a shift along the axis of maturation decision or a change in the reaction norm itself (d). For further discussion, see Tammaru *et al.* (2000, 2010).

different diets under a common garden design. The strains did not show a difference in larval growth rates but differed consistently in both time and size at maturation.

METHODS

Study species

The widespread European corn borer moth (*Ostrinia nubilalis*: Lepidoptera: Crambidae) is widely studied because it is a serious maize pest (Haeussler, 1952), and because of interest in its sympatric pheromone strains (E and Z), which show different patterns of host specialization (Frolov, 1998; Pelozuelo *et al.*, 2004): strain Z, which feeds almost exclusively on maize (*Zea*), and strain E, which feeds on mugwort (*Artemisia*) and hop (*Humulus*) (Pelozuelo *et al.*, 2004), with very limited gene flow between the two strains (Martel *et al.*, 2003). There are five larval instars in both strains.

Experimental design

European corn borer larvae used in the rearing experiment represented two pheromone strains (E and Z), with two populations per strain. Moths of strain Z originated from Mortmoulin, Central France (48°51′N, 1°58′E; hereafter population Z1) and Magneraud, Western France (46°9′N, 0°41′W; population Z2), the ancestors of the laboratory strain having been collected from maize. The number of larvae per population in the rearing experiment was 77 (a few generations in the laboratory before the experiment) and 107 (several generations in the laboratory, but wild-caught individuals added annually), respectively. Moths of strain E were obtained from Cornell University, originating from the Eastern USA (population E1; $n = 80$, several generations in the laboratory), and from the Swedish University of Agricultural Sciences, originating from Slovenia (population E2; $n = 76$, several generations in the laboratory). For each population, the larvae represented 4–5 different broods (i.e. offspring of individual females).

Larvae were reared individually in 25 mL plastic vials at 24°C under a 16:8 h light/dark photoperiod, which corresponds to natural conditions during the moth's larval period in Central Europe. Rearing vials were randomized on trays with respect to food treatment, brood, and population; the position of the trays in the rearing chamber was changed randomly on a daily basis. All larvae were fed an artificial diet throughout the experiment; fresh food was provided at least every third day. Overall mortality was less than 5%.

Up to the beginning of the last instar, all larvae from both strains were reared on a diet of *wheat*, which was assumed to be a neutral food, unprecedented in nature or in the laboratory for either strain. The artificial diet was prepared using the recipe described by Gahukar (1976), except that maize flour was replaced with wheat germ flour.

On the first day of the last instar, larvae from all four populations were randomly divided into two treatment groups: larvae that continued to be fed with the neutral (wheat) diet and larvae that were fed a diet that included their respective native host plant (i.e. *mugwort* diet for strain E and *maize* diet for strain Z). As a consequence, there were 8 treatment groups (4 populations × 2 diets) in the fifth instar rearing. For the *maize* diet, the standard recipe (Gahukar, 1976) was used. For the *mugwort* diet, ~50% of maize flour was replaced with dried and ground mugwort stalks.

Traits recorded and data analysis

The larvae were weighed at the end of their fourth instar, and pupae were weighed and sexed. For all larvae, development time in the fifth instar was recorded by daily inspection. Growth rate of the last instar was calculated as $[((\text{pupal mass})^{1/3} - (\text{mass at end of fourth instar})^{1/3})/\text{development time}]$. Considering the allometry of larval growth rates in Lepidoptera (Tammaru and Esperk, 2007), the cubic-root transformation appears most appropriate for the linearization of growth trajectories. In addition to this instar-based estimate of growth rate, an approximation of instantaneous growth rate (Tammaru and Esperk, 2007) was calculated for the last instar, using the equation [((mass on day 3)$^{1/3}$ – (mass on day 1)$^{1/3}$)/number of days]. The advantage of this measure is in capturing the 'pure' growth phase of the larvae, which is not confounded by the effects of moulting (Esperk and Tammaru, 2004), and thus presumably best reflects environmental quality.

Larval traits were analysed by mixed analysis of variance with three fixed factors (population, diet, and sex) and one random factor (brood, i.e. the offspring of one particular female). With just two populations per strain, we did not include 'strain' in the models. If we had, it would have implied a model with a random factor (population nested within strain) with just two levels, which is not considered meaningful (Crawley, 2002). Instead, we base our conclusions about between-strain differences on the consistency of the among-population patterns. The Kenward-Roger adjustment was applied to avoid overestimation of degrees of freedom by treating a brood, not an individual, as an independent observation on a population. Analyses were conducted with SAS v. 9.2 (SAS Institute, Inc., 2009).

RESULTS

Growth rate of the last instar (either instantaneous or instar based) did not differ between the populations; however, significant differences were noted between the diets (Table 1). Unexpectedly, for both strains, the larvae showed higher growth rates on the neutral diet than on diets that included the respective native host plant. However, the diet the larvae had been reared on did not affect pupal mass (Fig. 2a). Differences in last instar duration were significant (Table 1); the larvae on the native diet developed over a longer period, although only by 3.3% on average. There were no significant differences in pupal mass or development time between the populations within strains (Table 2).

Table 1. Results of mixed-model ANOVAs (brood as a random variable) for the effects of diet and population on larval growth parameters

Parameter	Effect	d.f.	*F*	*P*-value
Instantaneous growth rate[a]	population	16.2	1.16	0.356
	diet	270	9.38	0.003
	sex	273	0.56	0.455
Instar based growth rate[b]	population	16	2.80	0.073
	diet	285	6.29	0.013
	sex	287	179.68	<0.001
Pupal mass	population	14.7	19.55	<0.001
	diet	287	2.51	0.114
	sex	291	339.78	<0.001
Last instar duration	population	10.5	10.12	0.002
	diet	293	5.44	0.02
	sex	295	33.39	<0.001

Note: None of the population × diet interactions attained statistical significance ($P > 0.4$), and were omitted from the models.

[a] Growth phase of the larvae not confounded by the effects of moulting.

[b] Calculated over the whole time spent in the last instar.

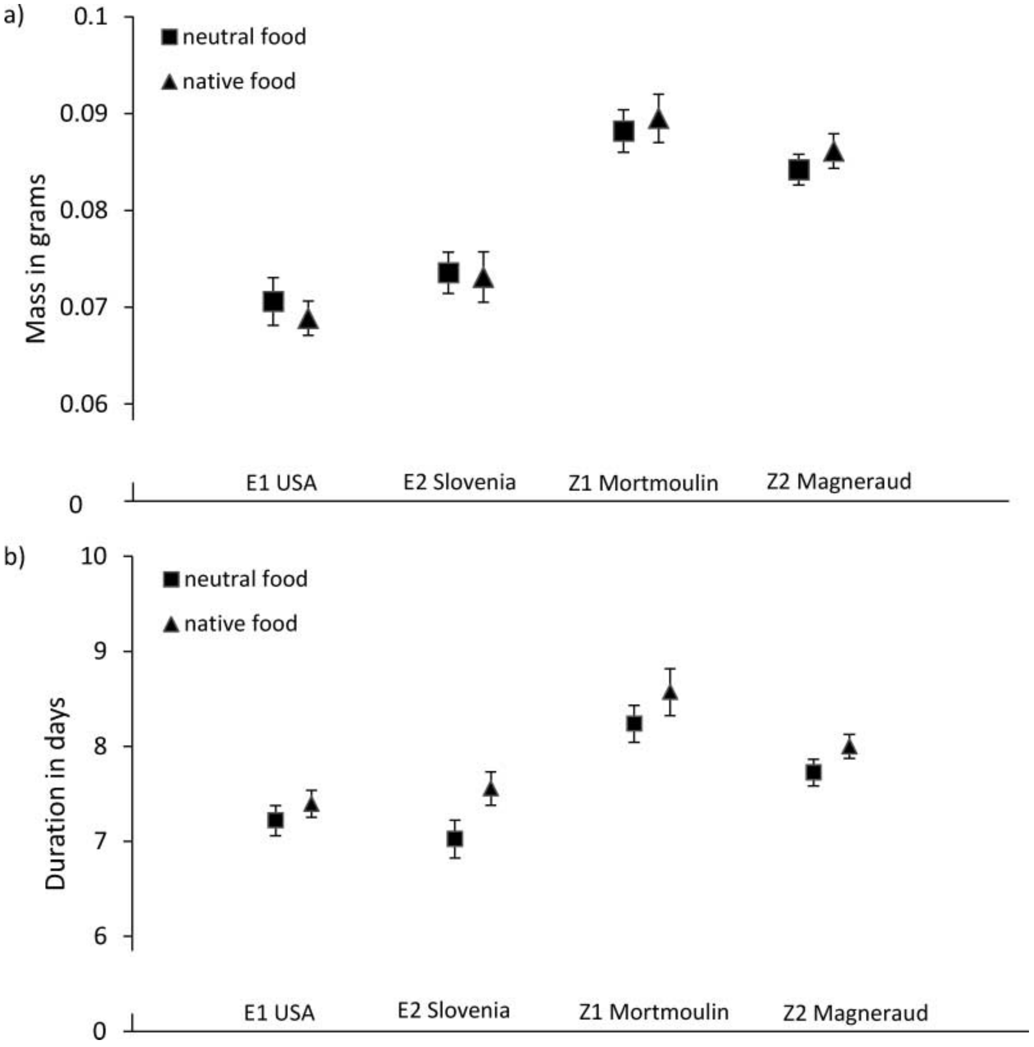

Fig. 2. (a) Pupal mass and (b) duration of last instar in four populations of *Ostrinia nubilalis*. 'Neutral food' refers to the neutral wheat diet (see Methods) whereas in the 'native food' treatment, the larvae were reared on a diet that included their native host plant during their fifth instar, i.e. maize for strain Z and mugwort for strain E. Error bars denote standard errors.

Importantly, there were consistent differences in time and size at maturation between the populations representing different pheromone strains. Insects representing strain Z were heavier by 18.2% in terms of pupal mass (Table 1, Fig. 2a). Last instar duration also was significantly different, with larvae of strain Z growing over a longer period (Fig. 2b). Plotting these two variables on one graph, we obtain a positive relationship between size and age at maturation at the among-strain level (Fig. 3).

Table 2. Results of mixed ANOVAs (brood as a random variable) with Tukey-Kramer pairwise comparisons between treatment groups (population × diet) of last instar durations and pupal masses

Duration / Pupal mass		E1		E2		Z1		Z2	
		Wheat	Mugwort	Wheat	Mugwort	Wheat	Maize	Wheat	Maize
E1	Wheat		1.000	0.879	0.939	0.500	0.109	0.239	**0.019**
	Mugwort	0.653		0.848	0.906	0.387	0.064	0.141	**0.006**
E2	Wheat	0.817	0.106		0.151	**0.018**	**0.001**	**0.002**	**< 0.01**
	Mugwort	0.998	0.549	0.938		0.991	0.693	0.950	0.394
Z1	Wheat	**< 0.01**	**< 0.01**	**0.021**	**0.001**		0.983	1.000	0.929
	Maize	**< 0.01**	**< 0.01**	**0.019**	**0.001**	1.000		0.991	1.000
Z2	Wheat	**< 0.01**	**< 0.01**	**0.022**	**0.001**	0.999	0.999		0.927
	Maize	**< 0.01**	**< 0.01**	0.062	**0.003**	0.998	0.993	0.999	

Note: P-values are presented. Significant differences are in **bold**.

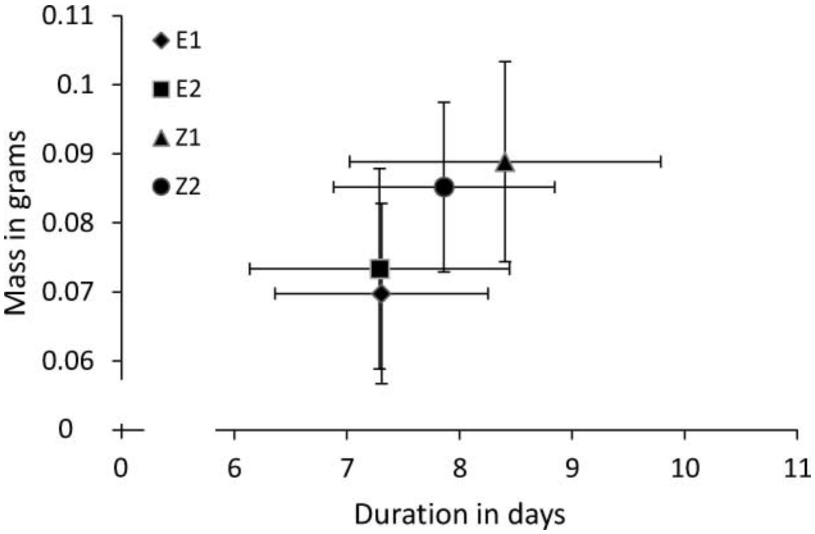

Fig. 3. Development time in last instar and size at maturation in four populations of European corn borer. Average values of a population are used, as there were no significant differences between neutral and native food groups. Error bars denote standard deviation.

DISCUSSION

Our results indicate an among-strain difference in developmental traits in *O. nubilalis*. Individuals of strain Z, compared with those of strain E, matured at a larger size, having spent more time growing. This difference was consistent among the two populations representing each of the strains and the diets used: the difference was observed both when the two strains were reared on a neutral food (i.e. a diet not likely to resemble any of the native host plants of the strains) and also when the strains were reared on artificial substrates that included a mix of their native host plant.

The case of *O. nubilalis* can be interpreted as an evolutionary change in the reaction norm for size and time at maturation *per se* (Fig. 1c) rather than just a shift along an invariable reaction norm (Fig. 1a, b), an interpretation that is supported by two lines of evidence: First, there were among-strain differences in developmental traits despite no differences in growth rates. Second, there was a positive rather than a negative correlation for the strain-specific means of size and time at maturation (Fig. 3). Both these patterns are consistent with an evolutionary shift along the axis of maturation decision but inconsistent with a shift along the axis of growth rate. Indeed, examining the sign of the correlation between size and time at maturation at the level of among-strain comparison is recommended as a tool for detecting host-specific changes in the reaction norms for size and time at maturation

Larval growth rates, irrespective of the way in which they were expressed, did not differ between strains or populations. As growth rate is widely recognized as an index of individual quality in herbivorous insects, we may conclude that the populations did not differ in 'overall quality' (caused by, for example, different degrees of inbreeding depression, or different levels of adaptation to laboratory conditions). This result is encouraging, as it allows us to interpret any observed differences as genetic differences, negating the possibility of an across-generations environmental difference due to different conditions possibly experienced by the ancestral generations.

Differences in reaction norms for time and size at maturation among host strains of herbivorous insects have rarely been documented. Nevertheless, one such case is the well-studied *Rhagoletis pomonella* (Diptera: Tephritidae), a model for sympatric speciation in progress. Indeed, Filchak *et al.* (1999) showed that hawthorn flies specialized on haws have been selected for to complete larval development faster, most likely because haws rot faster and become lethal to maggots. A similar example comes from *Eurosta solidaginis* (Diptera: Tephritidae), where two host plant races differ in emergence time by 10–14 days (Craig *et al.*, 1993). Our study shows that controlled laboratory studies have the potential to reveal further such cases, accumulation of which would allow studies on host plant specialization in herbivores to make a contribution to the evolutionary ecology of body size.

Returning to *O. nubilalis*, despite considerable research focusing on the biology of this species, few studies have been conducted on adaptation to the host of different strains of the moth. As an example, Calcagno *et al.* (2007) found some evidence of host-specific adaptations: the larvae of the mugwort race survived better on mugwort and the larvae of the maize race survived better on maize; pupal mass tended to be higher on native hosts as well, although the difference was not clear-cut for females. The design of that study did not, however, allow one to identify proximate developmental traits responsible for the difference, or to classify the host-associated evolutionary change according to the scheme presented above (Fig. 1).

A likely adaptive explanation for the among-strain differences in maturation decisions by the European corn borer moth rests on the difference in the phenology of the two primary host plants, mugwort and maize. It is possible that the nutritional properties of mugwort decline faster than those of maize, so *O. nubilalis* larvae need to complete their development on mugwort faster. Indeed, consistent with the results of the present study, Thomas *et al.* (2003) showed that under laboratory and field conditions, strain E moths emerged on average 3 days and 10 days earlier, respectively. In addition, Dopman *et al.* (2010) found some seasonal difference in the occurrence of strain Z and strain E in North America, but it was not consistent over years and locations. Perhaps an even more simple explanation may be related to the size of the plants: the amount of food provided by a maize plant is unlikely to be exhausted, whereas that might not be the case for the much smaller mugwort plant. Indeed, Losey *et al.* (2002) found that stem diameter (artificial tubes and several plant species) influences larval survival, with a larger diameter being more favourable.

A further question to be addressed is the rate of different evolutionary changes related to host specialization. The answer is often not forthcoming, as the timing of specialization to different host plants is not usually known with sufficient accuracy. Further studies on *O. nubilalis* life history could be valuable in the context of studying the rate of host-related adaptive changes. For *O. nubilalis*, the divergence between E and Z strains is estimated to have occurred between 75,000 and 150,000 years ago (Malausa *et al.*, 2007). Although *O. nubilalis* is native to Europe, the primary host of strain Z – maize – is not. Maize was introduced to Europe about 500 years ago, so host specialization on maize must have evolved more recently than separation of the pheromone strains (Thomas *et al.*, 2003). This knowledge should allow one to place the associated evolutionary changes on an absolute time scale, and encourages further use of the European corn borer as a model system for the evolutionary ecology of plant–herbivore interactions.

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