“The European corn borer (*Ostrinia nubilalis*, Hbn.), its susceptibility to the Bt-toxin Cry1F, its pheromone races and its gene flow in Europe in view of an Insect Resistance Management“

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Chapter 1: General introduction

The European corn borer (ECB), *Ostrinia nubilalis* (Lepidoptera: Crambidae) is known as one of the most important pest of maize (*Zea mays*), causing worldwide crop losses estimated at 7 %. *O. nubilalis* is native to Europe, Northern Africa, and Western Asia (Mutuura and Monroe 1970) and was introduced into North America in the early 20th century probably in shipments of broom corn from Austria, Hungary, and Italy (Smith 1920). Meanwhile ECB has spread to all corn-producing areas of North America east of the Rocky Mountains (Willet and Harrison 1999) resulting in crop losses of up to 1 billion US dollars (Huang et al. 1999a). Today ECB is common on all continents. In Germany the most highly infested maize areas can be found in the southwestern and eastern parts (Fig.1). Schmitz et al. (2002) reported that *O. nubilalis* is spreading up to 10-12 km per year in northerly direction. In Germany, ECB has not reached all of its potential infestation areas in the northern parts of Germany (Kluge et al. 1999, Gathmann and Rothmeier 2005), (Fig.1). Apart from maize, there are more than 200 plants which can serve as hosts for ECB, e.g. mugwort (*Artemisia vulgaris*) and hop (*Humulus lupulus*) (Lewis 1975). *O. nubilalis* will attack almost any herbaceous wild or cultivated plant with stems large enough for the larvae to enter (Hudon et al. 1989). In Germany the infestation of hop with considerable yield losses was described for the first time in the years 1879/1880 (Nickerl 1880). It was reported that afterwards high infestation rates occurred at several locations in 1903 (Wilke 1925) and again in 1925-1941 (Schlumberger 1940; Hampp 1940). The latest incidence of high infestation of hop by ECB was observed in 2002 and caused a yield loss of 5-25% in the main infestation areas.

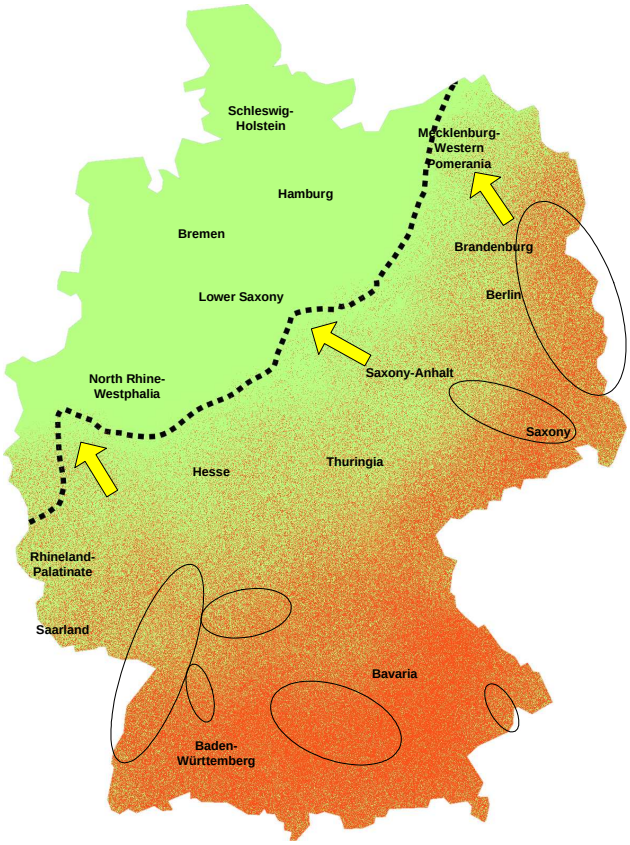

Figure 1. Northern borderline of dispersal of *O. nubilalis* in Germany, direction of dispersal and main infestation areas. (Source: Klune et al. 1999; modified by Liebe 2004; Detlef Bartsch, pers. comment; Pflanzenschutzdienst Rockstock 2007).

An effective method to prevent an infestation and damage to maize is the cultivation of Bt-maize. Larvae of *O. nubilalis* die when feeding on these plants, because Bt-maize carries a modified gene of the soil bacterium *Bacillus thuringiensis* which enables the expression of a toxin specific against Lepidopterans during the whole cultivation period. The gene coding for the toxin Cry1Ab was introduced into maize plants for the first time in 1995 in North America and since then the cultivation of Bt-maize increased rapidly in some countries.

In consequence, the risk of an adaption of the target species to the toxin specific for their control has risen. A sustainable use of Bt-toxin expressing plants is only possible when insect resistance will be prevented for a long period (Schuphan et al. 2002). Therefore, a resistance management is required to delay or even avoid a resistance development of *O. nubilalis* in the field. An appropriate resistance management, however, can only be developed based on an understanding of the genetic basis and the modes of action of pest adaption (Hawthorne 2001). Therefore it is crucial to consider information on the genetic background of the respective insect population, and on its reaction and degree of susceptibility towards the toxin of the genetically modified crop. Furthermore, there is a need for more information on the dispersal amd migration behaviour, the levels of gene flow between populations and alternative host plants of *O. nubilalis* since these data contribute to the adoption of Insect Resistance Management (IRM) plans. The following information is already known:

1. 1 Life cycle of *O. nubilalis*

Figure 2. a. Egg mass, b. Larva, c. Adult (female) of *O. nubilalis.* (Source: a./c. C. Gaspers; b. Harald Zimmermann; www.schmetterling-raupe.de).

In Figure 2 the different life stages of *O. nubilalis* are shown. Adults of *O. nubilalis* emerge from June to August and are mostly active at dawn and during the night. The lifespan of ECB adults averages approximately 10 days (Hill 1987). High humidity and the ingestion of food increase the fertility and lifespan of adults (Leahy and Andow 1994). Females oviposit egg

masses with a mean of 20 eggs, commonly at the underside of the host plant leaves. Emerging larvae at first feed cursorily from the leaves of their home plants and then start spreading to neighbouring plants. After a few days they burrow into the stalks and cobs of maize. In the course of the vegetation period the larvae feed downwards through the stalk. ECB larvae pass four molting phases (L1- stage – L5- stage) and overwinter as L5- larvae in the stalks close to the plant roots (Fig. 3).

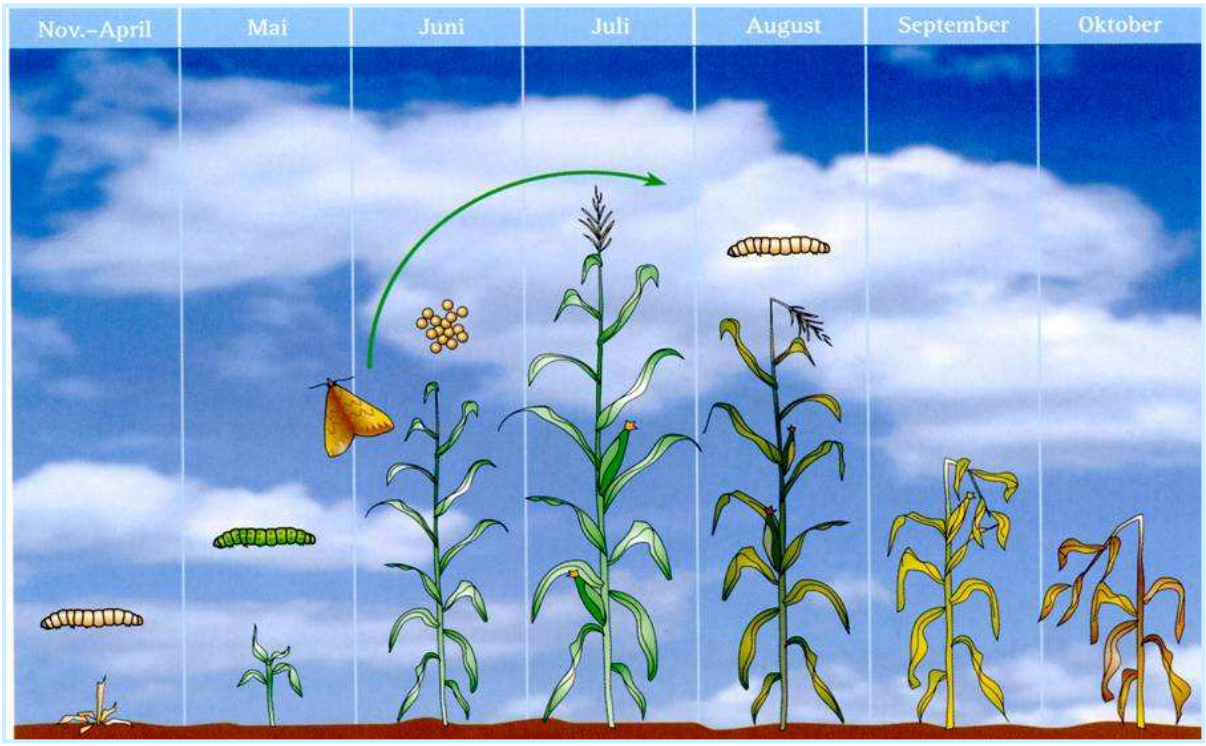

Figure 3. Univoltine life cycle of *O. nubilalis* (Source: Siber Communications GmbH; www.transgen.de).

The onset of the diapause occurs in late summer, when the mean photophase is more than 15 hours per day (Çagan 1998). During the diapause the larvae accumulate glycerol and become cold resistant. The elicitor of the increasing glycerol level is probably the incipient cold stress in autumn (Nordin et al. 1984). In spring the glycerol concentration is decreasing in the larvae. In Germany, pupation of the larvae commonly starts in the second half of May. The soil temperature primarily controls the increasing activity of the larvae. The first adults emerge in June.

1. 2 Population structure of *Ostrinia nubilalis*

1. 2. 1 Voltinism and pheromone races

ECB has been shown to be variable concerning its voltinism and its pheromone communication system (Klun et al. 1975; Anglade et al. 1984). A phenotypical differentiation of pheromone and voltinism types is not possible. Based on the geography of early infestations and on the characteristics of those ECB populations (voltinism and pheromone races) introduced into North America, it was suggested that ECB has been introduced several times. A bivoltine (two generations per year) population was first described in Massachusetts, and an univoltine (one generation per year) one in New York near Lake Erie. An additional area of early infestation was centered around the cities of Amsterdam and Albany, New York. In Germany so far only univoltine populations have been found, but in warmer regions in Southern Europe more than one generation per year has been observed (Engel 1971).

Like many other moths, ECB uses a chemical communication system for long-distance mate attraction. E- and Z-populations of ECB have been distinguished in which females produce and the males respond to different ratios of E- and Z- isomers of 11-tetradecenyl acetate (11-14:OAc) (Fig. 4). In Z-strain borers, the female moths produce and males respond to a 97:3 ratio of Z11-14:OAc to E11-14:OAc, whereas in the E-strain borers, the opposite blend of isomers is used (1:99 ratio of Z11-14:OAc to E11-14:OAc) (Roelofs et al. 1985).

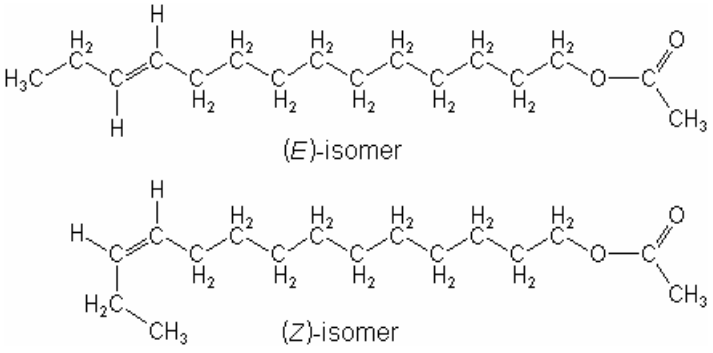

Figure 4. E- and Z- isomers of 11-tetradecenyl acetate (11-14:OAc).

Hybridization of the two pheromone types is readily achieved in the laboratory (Liebherr and Roelofs 1975), and the analysis of hybrid females from reciprocal crosses has shown that they produce the intermediate molar E/Z pheromone blend of 65:35 (Klun and Maini 1979). The analysis of wild female moths from the United States (Klun and Maini 1979; Roelofs et al.

1985) and Europe (Klun and Maini 1979) has indicated that a low level of natural hybridization occurs in areas of sympatry. It was reported that the pheromone differences between the two strains are determined by three major genes (Roelofs et al. 1987; Hansson et al. 1987; Klun and Maini 1979; Zhu et al. 1996). The inheritance patterns for sex pheromone production in females, the pheromone detection on male antennal olfactory receptor cells, and the male pheromone behavioral responses were studied in pheromonally distinct populations of ECB from New York State (Roelofs et al. 1987). Pheromone production as well as the organization of the male pheromone receptor are autosomally inherited and male behavior is determined by a sex-linked gene (Z-chromosome). Dopman et al. (2004) suggested that a reductase represent a candidate gene for the observed variation in pheromone blend production. Differences in the pheromone blend produced by female ECB are most likely due to changes in the specificity of the reactions in which Δ11-14-carbon-precursor acids are reduced and acetylated to produce the E- and Z-acetates, which are the pheromone components. Both, hybrids and "pure" strain ECB females produce an approximate 70:30 mixture of E/Z precursor acids. Because the acetylation of the alcohol precursors is not selective (Zhu et al. 1996) the 99:1 E/Z; 65:35 E/Z; 3:97 E/Z pheromone blends are most likely generated by the differential specifity of alleles at a locus encoding a reductase (Roelofs et al. 1987; Roelofs and Wolf 1988). Regarding the organization of the male pheromone receptor no candidate gene was suggested, but it was described that the two pheromone components were detected by different specialized receptors in the olfactory sensilla on the male antennae (Hansson et al. 1987). In each sensillum Z-race males have a receptor cell characterized by a large spike amplitude tuned to the Z-isomer. In E-race males the situation is the reverse, a large spike amplitude cell is tuned to the E-isomer and a small spike amplitude cell to the Z-isomer. In hybrids of the E- and Z-race the spike amplitudes are of equal height. Sex-linked control of the behavioral responses in crosses of E and Z corn borers was confirmed by demonstrating complete linkage of a sex-linked TPI (triose phosphate isomerase) locus and the locus controlling the response to the sex pheromone (Glover et al. 1990). All three genes were inherited independently (Löfstedt et al. 1989).

1. 2. 2 Spatial distribution and adaption of the pheromone races to different host plants

Regarding the distribution of the different pheromone races it is postulated that the Z-race predominates in most of the range in Europe and North America, whereas the E-race is found in Switzerland, Italy and Eastern North America, from Massachusetts to South Carolina (Klun and Huettel 1988; Mason et al. 1996). E-race ECB appear to be the predominant strain in

parts of Italy and Switzerland and are rare or absent elsewhere (Willet and Harrison 1999). In North America (e.g. Glover et al. 1990) and Switzerland (Pena et al. 1988) both E- and Z-populations feed on maize. Analysing 27 females collected from maize in Lombardia (Italy) Marçon et al. (1999) also found Z- and E-race individuals. Most studies on the adaptation of the pheromone races to different host plants were performed in France. There, it seems that ECB feeding on maize predominantely belong to the Z-race, whereas ECB feeding on alternative host plants such as mugwort or hop belong mostly to the E-race (Thomas et al. 2003; Bethenod et al. 2004; Pelozuelo et al. 2004). A similar situation is described for the larch budmoth, *Zeiraphera diniana*. In this species, two sympatric host races have been reported, one feeding on larch (*Larix decidua*), the other one feeding on cembran pine (*Pinus cembra*) (Emelianov et al. 1995). Females of each race produce a blend of E11-14OAc and E9-dodecenyl acetate (E9-12:OAc), but in inverse ratios. Larch-race females produce close to 100% E11-14OAc with traces of E9-12:OAc, whereas pine-race females produce a 1:1000 ratio of E11-14OAc to E9-12:OAc. Males responded strongest to the pheromone blend of their own race (Priesner and Baltenweiler 1987).

1. 3 Damage caused by *O. nubilalis* and control strategies against it

Maize yield losses caused by *O. nubilalis* in Germany can amount to 20% in areas with high infestation (Magg et al. 2002). In 2005 approximately 370,000 ha of the total acreage were affected nationwide and a clear increase of infestation could be observed especially in North Rhine-Westphalia, Thuringia, Saxony and Brandenburg (Degenhardt et al. 2003). The first incidence of ECB in Lower Saxony was observed in 2006. The threshold of commercial damage will be reached with 10-15 egg masses or 60-80 larvae per 100 plants. It was estimated that one larva per plant can cause 4% yield loss (Hugger 1998). According to estimates of the Federal Biological Research Center for Agriculture and Forestry ECB causes a damage of 11-12 Mio Euros per year. The damage occurs mainly in the form of broken stems and bend maize cobs (Fig. 5). The disruption of the vascular tissue can inhibit the maturation of the cobs (Engel 1971). Additionally, infestation with *O. nubilalis* increases the susceptibility of the plants to fungal decay, because moulds such as *Fusarium* species can enter the maize plants via the perforations caused by the larvae feeding on the stalks (Mastel 2003). This can result in high levels of mycotoxins produced by *Fusarium* species which cause rot of stalks and cobs (Magg et al. 2002; Fig. 5). Currently no fungicides are permitted to control the moulds (Mastel 2003).

Figure 5. Damage caused by *O. nubilalis* (Source: C. Gaspers).

Different strategies are possible to control *O. nubilalis*. A mechanical provisional method of plant protection is to chaff the maize plants very well during harvest. Stalks and stubbles need to be smashed with rotation tillage machines to destroy the larvae (Ackermann et al. 2003). Finally, it is necessary to plough the maize splits deep into the soil to increase the mortality of diapausing larvae over the winter (Zellner 2001). Another reasonable strategy is crop rotation. A frequently used biological method is the application of ichneumonids such as *Trichogramma evanescens* and *Trichogramma brassica*, which has been used against ECB in Germany since the eighties. The advantage here is that ichneumonids are egg parasites, so that already the damage caused by emerging larvae is avoided. Disadvantages are the high costs and the complexity of the treatment. There are also potential effects of *Trichogramma* on non-target lepidoptera. Furthermore, the control success depends on the weather (Degenhardt et al. 2003). A direct control of *O. nubilalis* with insecticides (synthetic formulations or biological preparations) is only possible within a short time-period when the larvae have not yet burrowed into the maize stalks. However, the production of egg masses and the emergence of larvae ranges over a longer timespan. Therefore it is necessary to spray the insecticides several times, resulting in higher costs and more work.

Over the past years an alternative method for controlling ECB has been established and is particularly used in the United States, but also in Europe. The cultivation of genetically modified insecticidal crops is an effective method for plant protection. For this purpose single genes from the soil bacterium *Bacillus thuringiensis* (Bt) were introduced into maize plants, so that lepidopteran-specific Bt-toxins are produced by the plants during the whole growing period.

1. 4 *Bacillus thuringiensis* and the Bt-toxins

The gram-positive spore-forming soil microorganism *B. thuringiensis* is an entomopathogenic bacterium commonly used as a biopesticide (Schnepf et al. 1998). It belongs to the *Bacillaceae* family and to the *Bacillus cereus* group, which also includes the highly pathogenic bacterium *B. anthracis* and the non-pathogenic bacterium *B. mycoides*. *B. thuringiensis* strains have a genome size of 2.4 to 5.7 million bp (Carlson et al. 1994) and produce insecticidal crystal proteins (ICPs) during sporulation. The ICPs, also termed δ-endotoxins or Cry proteins, accumulate in the bacterial mother cell to form a crystal inclusion that is released into the medium at the end of sporulation (Lereclus et al. 2000). These crystalline inclusions are toxic when ingested by susceptible insects. Many *B. thuringiensis* strains with different ranges of insect target species have been identified (Burges 1981).

1. 4. 1 Mode of action and structure

The mode of action of the *B. thuringiensis* Cry proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry toxin to midgut receptors, and the insertion of the toxin into the apical membrane to create ion channels or pores. Crystals are comprised of protoxins of 125-140 kDa.

In lepidopterans, the protoxins are solubilized under the alkaline conditions of the insect midgut (Hofmann et al. 1988). After solubilization, many proteins must be processed by insect midgut proteases (Lecadet and Dedonder 1967; Tojo and Aizawa 1983) to become activated toxins of 55-65 kDa (Chestukhina et al. 1982; Choma et al. 1990; Huang et al. 1999b). The major proteases of the lepidopteran insect midgut are trypsin-like (Lecadet and Dedonder 1966; Milne and Kaplan 1993) or chymotrypsin-like (Johnson et al. 1995; Novillo et al. 1997; Peterson et al. 1995). Activated Cry toxins have two known functions, receptor binding and ion channel activity. The activated toxin binds readily to specific receptors on the brush border membrane of the midgut epithelium of susceptible insects (Hofmann and Lüthy 1986; Hofmann et al. 1988). Insertion into the apical membrane of the columnar epithelial cells follows the initial receptor-mediated binding, rendering the toxin insensitive to proteases and monoclonal antibodies (Wolfersberger et al. 1986) and inducing ion channels or nonspecific pores in the target membrane. In the alkaline midgut, the toxin may function as a cation channel (Schwartz et al. 1993), taking advantage of the large K^+ gradient, which causes a disruption of the osmotic balance of midgut epithelial cells. An influx of water, along with ions, cause the cell lysis of epithelial cells. By subsequent midgut paralysis a blending of

hemolymph and gut tissue occurs, resulting in a sepsis in the larvae. In general, the insect stops feeding 24 hours after the intake of the toxin, followed by death in three to five days (Chambers et al. 1991).

The genes encoding for ICPs have been localized in large plasmids (>30 MDa, González et al. 1981, 1982). Most Bt-strains produce a number of related toxins, each coded for by a single gene (Lereclus et al. 1993). Each toxin has a specific target site within the insect (Gill et al. 1992). Most of the Cry proteins are protoxins with a length of approximately 125-140 kDa (Van Rie 2000). Cry proteins have three structural domains of approximately 200 residues each (Fig. 6). Domain I is formed by a bundle of seven anti-parallel α-helices where a central amphipatic helix (α-5) is surrounded by the others. The function of this domain has been associated with pore formation in lipid rafts and membrane vesicles (Li et al. 1991; Grochulski et al. 1995). Domain II consists of three anti-parallel β-sheets and domain III is formed by two β-sheets. Both domain II and III have been associated with the recognition and binding of a receptor in midgut cells (Schnepf et al. 1998). Domain III has also been associated with the regulation of pore activity (Schnepf et al. 1998).

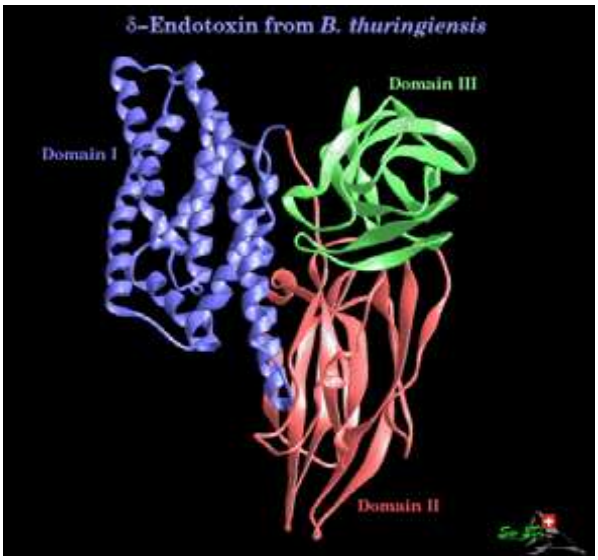

Figure 6. Structure of δ-Endotoxins (Source: Swiss-prot; Li et al. 1991: Nature 353, 815-821).

1. 4. 2 Classification

Since the first cloning of an insecticidal crystal protein gene from *B. thuringiensis* (Schnepf and Whiteley 1981), many such genes have been isolated. The first systematic attempt to organize their genetic nomenclature relied only on the insecticidal activities of crystal proteins classified by the primary ranking of the corresponding genes (Höfte and Whiteley 1989). The *cryI* genes encoded proteins toxic to lepidopterans; *cryII* genes encoded proteins toxic to both lepidopterans and dipterans, *cryIII* genes encoded proteins toxic to coleopterans, and *cryIV* genes encoded proteins toxic to dipterans only (Crickmore et al. 1998). Since then the number of sequenced *cry* genes has steadily increased, so that a new nomenclature was introduced. Meanwhile 169 proteins have been identified and classified into 28 Cry-classes according to the latest system of nomenclature, which was based on the respective aminoacid sequences of the toxins (Crickmore et al. 1998). Using this system, genealogical trees could be created by

means of sequence homologies of the Cry proteins. There are four different ways of ranking which are based on the percentage of aminoacid homology. The first ranking shows up to 45% sequence identity and is marked with an Arabic number (e.g. Cry1), followed by an uppercase letter (e.g. Cry1A) for the second ranking which shows a sequence identity of 45-78%. The further classification contains 78-95% sequence identity and is designated with a lowercase letter for the third ranking (e.g. Cry1Aa) and finally another Arabic number for more than 95% sequence identity (quaternary ranking), (e.g. Cry1Aa1).

1. 4. 3 Use of Bt-toxins as insecticides

In Germany, Bt-formulations for spraying have been authorized since 1964. They are used particularly for the cultivation of maize, potatos, fruits and vegetables and are also allowed in organic farming. Commercially available Bt-preparations consist of dehumidified spores of the bacterium and the crystal toxins. For a use against lepidopterans they are typically composed of five toxins: Cry1Aa, Cry1Ab, Cry1Ac, Cry2A und Cry2B (Tabashnik et al. 1997). Disadvantages of the application of Bt-spraying formulations are the rapid degradation of the spores by UV-light, the loss through rainfall and the low degree of efficiency against larvae which have already burrowed into maize stems (High et al. 2002).

For the production of genetically modified crops it is advantageous that single toxins are extremely toxic to specific pest insects. For the control of *O. nubilalis* the toxins Cry1Ac and Cry1Ab were primarily used. For the first Bt-maize line approved in 1995 (event Bt176) a synthetic gene for the truncated active Cry1Ab toxin was introduced into maize embryos by microprojectile-bombardement. To obtain an improved expression of the gene in the maize-plant, the Guanine/Cytosine-concentration of the native *Cry1Ab* gene was increased, so that only 65% of the nucleotide sequence corresponded to the native gene (Koziel et al. 1993). Additionally, a gene for herbicide tolerance (*bar*-gene) and a selective marker gene coding resistance to the antibiotic ampicillin (*bla*-gene) were inserted.

Maize containing the *cry1Ab*-gene from *B. thuringiensis* was introduced for commercial cultivation in the United States in 1996. At that time the acreage of Bt-maize represented less than 5% of the total cultivation area of maize. In 2007, maize was cultivated in the United States on 37.5 million hectares, of which 27.5 million hectares was Bt-maize. Hence the overall proportion of Bt-maize is now 73%. Spain was the only country to commercially cultivate Bt-maize for several years within the EU. Bt-maize was first grown in Spain in 1998,

and by 2004 the production had risen to 60,000 hectares. In 2007, Bt-maize was cultivated on more than 75,000 hectares.

1. 5 Development of resistance

One major problem with insect control via insecticides is the natural evolution of genes conferring resistance to these insecticides. These resistance genes mediate a selection advantage for insects on fields treated with insecticides, causing a higher reproduction of these insects in contrast to susceptible insects of the same population. In consequence there will be an accumulation of the rare resistance gene after some time. Insects have developed resistance to all major classes of chemical insecticides, and because of the increasing cultivation of Bt-crops it is suspected that resistance against several Bt-toxins are likely to occur. Reasons for the development of Bt-resistance can be loss or modifications of the proteases in the midgut, which are necessary for the activation of the protoxin (Ferré and Van Rie 2002), higher proteolytic activity, which will cause a degradation of the toxin, or a reduction of the binding-affinity at the membran of the midgut epithelial cells (Avisar et al. 2004).

In 1985 the first incidence of resistance against Bt-spray formulations was observed in the USA in the case of the diamondback moth *Plutella xylostella*. The reason for this was the widespread use of Bt as a biological plant-protective pesticide for cabbage monoculture on Hawaii (McGaughey 1994b). This was the only documented case of a resistance development of an insect in the field (Tabashnik et al. 1997) until 2006. Recently, however, three cases of field-evolved resistance to Bt-corn and Bt-cotton were documented, which concern the American cotton bollworm *Helicoverpa zea*, the maize stalk borer *Busseola fusca* and the fall armyworm *Spodoptera frugiparda* (Ali et al 2006; Van Rensburg 2007; Matten et al 2008; Moar et al. 2008; Tabashnik et al. 2008b).

Resistance to Bt and its toxins has also been reported from selection studies in laboratory populations of several insect species (Bauer 1995; Schnepf et al. 1998; Frutos et al. 1999; Ferré and Van Rie 2002; Pereira et al. 2008; Crespo et al. 2009), suggesting that pest insects can develop resistance to Bt-toxins (Siqueira et al. 2004). Laboratory- and field-selected resistance may be due to different factors. Laboratory development of resistance is more likely to involve polygenes (multiple genes, each having a small impact on the selected trait), since they are prone to be selected under conditions where biological and environmental stress

factors are minimized. In contrast, the development of resistance in the field is more likely to involve single major genes, which can be selected from a much wider genetic pool under more stressful conditions (Roush and McKenzie 1987).

The most common type of lepidopteran resistance to Bt-toxins entails a high level of resistance to at least one Cry1A toxin, recessive inheritance, reduced binding of at least one Cry1A toxin, and little or no cross-resistance to Cry1C (Tabashnik et al. 1998). This type of resistance occurs in some strains of diamondback moth (*Plutella xylostella*), the Indian meal moth (*Plodia interpunctella*), the tobacco budworm (*Heliothis virescens*), and the pink bollworm (*Pectinophora gossypiella*). The simplest explanation for this resistance is that modifications of target sites reduce or eliminate binding of Cry1A toxins in homozygous resistant individuals, but have little effect on the susceptibility of heterozygous individuals to Cry1A toxins. The resistance of a laboratory-selected *P. interpunctella* strain and of field populations of *P. xylostella* from Hawaii is due to a change in the membrane ICP (insecticidal crystal proteins) receptors (Ferré et al. 1991). The best characterized ones of these receptors have been identified for lepidopterans, and two major receptor classes have emerged: the aminopeptidase N (APN) receptors and the cadherin-like receptors. Currently, 38 different APNs have been reported for 12 different lepidopterans (Pigott and Ellar 2007). Pink bollworm resistance to Bt-cotton in at least four laboratory-selected strains is a recessive trait associated with mutations in a gene encoding a cadherin protein binding Cry1Ac (Morin et al. 2003, 2004; Tabashnik et al. 2002, 2004). In *Manduca sexta*, the tobacco hornworm, the Cry1Ab receptor is believed to be a cadherin-like membrane protein (Francis and Bulla 1997; Kreeton and Bulla 1997; Vadlamudi et al. 1993), while the Cry1Ac and Cry1C receptors have been identified as APN (aminopeptidase N) proteins (Knight et al. 1994; Luo et al. 1996). Alkaline phosphatase has also been proposed to be a Cry1Ac receptor (Sangadala et al. 1994). In gypsy moth (*Lymantria dispar*), the Cry1Ac receptor also seems to be APN, (Valaitis et al. 1995, 1997). In *P. xylostella* (Luo et al. 1997) and *Bombyx mori* (Yaoi et al. 1997) APN also appears to function as a Cry1Ac binding protein. APN consists of a short cytoplasmic tail, a transmembrane region, a Seronin/Threonin rich region, and a zinc metalloproteinase domain. Widely expressed in many cells, tissues, and species, APN cleaves the N-terminal amino acids from bioactive peptides, leading to their inactivation or degradation. It has putative involvement in several biological processes e. g. cell adhesion, and it also serves as a receptor. Cadherins are transmembrane glycoproteines which effect cell junctions and form part of the cell adhesion receptors. Because of these two important proteins which have several functions, it is questionable how much the fitness of pests will be affected or whether even a

survival will be possible if resistance is inherited as a dominant trait. In the case of *P. xylostella* Tabasnik et al. (1994) reported, relating to computer simulations, that a decreased fitness is associated with reduced binding of Cry1Ac. They suggested, that the reduction in binding that confers resistance to Bt interfers with the normal function of the receptor. There are further alternative hypotheses that the observed reduction in fitness may reflect inbreeding depression or genetic drift (Groeters et al. 1994).

1. 6 Insect Resistance Management (IRM)

Widespread pesticide use has resulted in the selection of insecticide resistance alleles within a very short period of time (Raymond et al. 1991). Therefore, it is generally accepted that one of the most important elements in the cultivation of genetically modified crops producing *B. thuringiensis* toxins is the development and implementation of effective resistance management plans in order to delay the incidence of resistance to Bt in target pests (Gould 1998). The most widely accepted resistance management strategy is the high-dose/refuge (HDR) model, which has been implemented in North America (Alstad and Andow 1995). Refuges are defined as non-Bt-plants that can be used by the target pest and should be planted and maintained in close proximity to the Bt-crops (Gould 1998). The principle underlying this system of resistance management is that any resistant insect emerging from Bt-crops is more likely to mate with one of the much larger number of susceptible pest insects emerging from the refuges than with each other, thereby decreasing the selection of Bt resistance alleles. If the resistance is recessive, heterozygous offspring produced by a mating of resistant with susceptible individuals is susceptible to the Bt-toxins, delaying the evolution of resistance (Gould 1988; McGaughey and Whalon 1992; Tabashnik 1994). In order for the HDR strategy to be effective, four main components are required: 1. The toxin expression in the plant has to be very high to make sure that all homozygous sensitive as well as all heterozygous resistant individuals are killed. It was suggested that the concentration has to be 25-fold higher than the lethal concentration which kills 99% of the individuals of sensitive strains in order to kill all heterozygous resistant individuals (Andow and Hutchison 1998). 2. The effectiveness of this strategy assumes that resistance is inherited as a recessive trait (Sayyed et al. 2000), and 3. that the frequency of the resistance alleles is less than 1×10^{-3} (Andow et al. 2000). 4. A refuge of non-Bt-plants needs to provide enough homozygous susceptible individuals, which can randomly mate with the few homozygous resistant individuals. The resulting progeny would consist of heterozygous resistant individuals only (Alstad and Andow 1995).

An insect resistance management also implies the monitoring of the baseline susceptibility of the target pest by carrying out bioassays. To develop a concept for a worldwide resistance monitoring, it is important to know the range of susceptibility of ECB to the Bt-toxin. Therefore the baseline susceptibility has to be established for different ECB populations in monitoring areas. On the basis of these data and in doing repeated testing it is then possible to detect a resistance development at an early stage, e.g. by deviations from the baseline susceptibility, in following long term tests.

1. 7 Aim of this study

With respect to an IRM of *O. nubilalis* different research areas should be explored in this study.

First, for a prospective cultivation of Bt-maize expressing the Cry1F toxin in Europe, the baseline susceptibility against this toxin should be established for several ECB populations from the main maize cultivation areas. The compilation of these data will provide the basis for further susceptibility testing to detect changes in the baseline susceptibility over time. Furthermore, the found range of variability in the susceptibility of European populations towards Cry1F will be a point of reference to judge the necessary amount of future testing. The obtained data for the European ECB populations will also be compared with existing baseline susceptibility data from American populations, where Bt-maize containing the *cry1F* gene was cultivated from 2003. Possible differences in susceptibility against Cry1F toxin between populations from European and American countries could indicate genetic differences in the proneness to a resistance development. If the susceptibility was lower in American populations this may be evidence for a rapid adaptation of ECB to the Cry1F toxin so that a cultivation of Cry1F Bt-maize in Europe would not be advisable.

A second research area investigated in this study is the population structure of ECB regarding the different pheromone races, their spatial distribution and the adaptation of the races to different host plants which may be used as alternative refuges for the HDR strategy. Pheromone race individuals of different populations from several countries and host plants were to be analysed by gas chromatography analysis. These results are meant to give an indication of the mating behaviour of individuals from the two different pheromone types, mainly by the found number of hybrids. Until now there are contradictory results and

hypotheses on whether assortative or random mating occurs between different pheromone types. In this context, the frequency of *Tpi* alleles as a marker (see section 1.2.1) should be ascertained for the different populations using allozyme analyses.

Third, the level of gene flow among ECB populations will be estimated as the strategies proposed for delaying resistance development to Bt-toxins require high levels of gene flow between individuals feeding on genetically modified and refuge plants (Bourguet et al. 2000a, 2000b). Allozyme polymorphism is well suited for population studies and has been used to investigate the genetic population structure in several migrant Noctuidae species (Daly and Gregg 1985; Pashley et al. 1985; Korman et al. 1993; Buès et al. 1994). In this study allozyme analyses of ECB will provide evidence on the population structure and the genetic differentiation of different populations within one country, of populations from different countries across Europe and of the two pheromone races, as well as populations from different host plants in Germany. A low overall genetic differentiation at the European scale would suggest overall high levels of gene flow between ECB populations.

Chapter 2: Methods

2. 1 ECB Sampling

Field samples of ECB for all experimental studies during this project consisted either of diapausing larvae and pupae, or of egg masses and adults collected in light trap cages. A list of all sampling locations together with the sampling size and year, and the collected ECB life stages is given in Table 1.

Egg masses were collected in June and July using light traps linked to cages which contained maize plants (Fig. 7) or both hop and maize plants (only location 'Laimerstadt'). The cages consisted of a metal frame sized 2 x 1.5 x 2 m covered by a mosquito net. The light traps were constructed according to a model developed at the department of pest management at Karlsruhe University (Germany) using a black light neon lamp (Philipps TLD 15W/05). Light traps were switched on from 10 pm to 1 am each night. Trapped female ECB laid their eggs onto the maize leaves in the cages. The leaf sections containing the egg masses were cut out once a week and transferred to the laboratory.

Egg masses from Muret (France) provided by EU-partners had been sampled as diapausing larvae in September and October 2004 and reared over several generations. Late instar larvae were collected in autumn from infested maize stems, which could be recognized by perforation holes through which the larvae had entered the maize plant. Larvae from Novara, Pola, Serres, Komotini (Greece) and Georgia were provided by EU-partners. Corn stalks containing about 300 ECB pupae were collected in May in an area near Padua and from Camposampiero, both Italy. Adults from Laimerstadt were sampled in a light trap cage.

Figure 7. Light trap linked to a cage.

Table 1. ECB sampling sites, sampling size and year, and life stage of the collected corn borers. All ECB populations, except one population each from Bonn (mugwort) and Laimerstadt (hop), were collected from maize plants, (x: unknown sample size as population was collected by a third party).

Population	Year	Sample size	Life stage	Location
Germany:				
Heilbronn	2004	200	egg masses	N 49° 05'/ E 09° 12'
Upper Rhine Valley	2004	x	egg masses	N 48° 26'/ E 08° 30'
Oderbruch	2004	1000	diapausing larvae	N 52° 29'/ E 14° 13'
Bonn	2001	x	egg masses	N 50° 38'/ E 06° 55'
Bonn (mugwort)	2001/2005	x/40	diapausing larvae	N 50° 38'/ E 06° 55'
Walldorf	2005	1000	egg masses	N 49° 18'/ E 08° 37'
Bodensee	2001	x	egg masses	N 47° 46'/ E 08° 46'
Laimerstadt (hop)	2005	80/56	adults/egg masses	N 48° 52'/ E 11° 42'
Italy:				
Padua	2005	300	pupae	N 45° 21'/ E 11° 49'
Novara	2005	x	diapausing larvae	N 45° 29'/ E 08° 36'
Lacchiarella	2005	500	diapausing larvae	N 45° 21'/ E 09° 08'
Biandrate	2006	200	diapausing larvae	N 45° 27'/ E 08° 27'
Pombia	2006/2007	100/200	diapausing larvae	N 45° 40'/ E 08° 38'
Camposampiero	2007	300	pupae	N 45° 34'/ E 11° 55'
France:				
Grignon	2005	500	diapausing larvae	N 48° 46'/ E 02° 23'
Poitiers	2005	500	diapausing larvae	N 46° 45'/ E 00° 00'
Muret	2005	x	diapausing larvae	N 43° 28'/ E 01° 19'
Spain:				
Ebro	2004	250	diapausing larvae	N 41° 52'/ E 00° 45'
Badajoz	2004	1000	diapausing larvae	N 39° 04'/ E 05° 34'
Greece:				
Serres	2006	x	diapausing larvae	N 41° 07'/ E 23° 29'
Komotini	2006	x	diapausing larvae	N 41° 04'/ E 25° 25'
Serbia:				
Pola	2005	x	diapausing larvae	-
Croatia:				
Vinkovci	2007	100	diapausing larvae	N 45° 17'/ E 18° 48'
Austria:				
Tullen	2007	100	diapausing larvae	N 48° 12'/ E 16° 22'
Georgia:	2006	x	diapausing larvae	-

2. 2 ECB Rearing

ECB rearing procedures were based on Wyniger (1974). The rearing diet consisted of crouched maize, wheat germ and yeast, to which a vitamin mixture, antibiotics and preserving agents were added (Table 2)**.** Fumidil B (Ceva Ltd, Watford, Herts) is added to the diet to suppress microsporids (Microsporida: Nosematidae), a group of unicellular protozoans, which often occur as intracellular parasites in insects (Undeen 1997). *O. nubilalis* is infested mainly

by the species *Nosema pyrausta*. Microsporidis can have a considerably negative influence on the amount and quality of egg masses, as well as the general fitness of *O. nubilalis* (Lewis and Lynch 1970; Solter et al. 1990; Ohnesorge 1992). Benzoic and sorbic acid inhibit the growth of fungi like *Aspergillus niger*, *A. flavus* and *Cladosporium avellaneum*. The addition of the antibiotic Chlortetracyclin controls the incidence of bacteria (Guthrie et al. 1985). Agar powder was used to solidify the diet.

For the preparation of the diet, water was mixed with agar powder and then heated in a microwave to boiling point. All other ingredients were mixed separately with heated water in a second container. The agar solution was then added to this mixture, stirred thoroughly, poured into a plastic storage box and left to cool down. The diet was stored in a fridge at 4°C.

Table 2. Ingredients of the ECB diet

Ingredients	**Amount**
H_2O	340 ml
Crouched maize	112 g
Wheat germs	28 g
Brewers' yeast	30 g
Benzoic Acid	2 g
Ascorbic Acid	6 g
Fumidil B	1 g
Vitamin supplement	2 g
Sorbic Acid	0.8 g
Chlortetracycline	0.4 g
H_2O	340 ml
Agar powder	16 g

All life stages of ECB were kept in climate chambers at a photoperiod of 16:8 (L:D) and 70% humidity, except larvae which were reared under conditions intended to break the diapause. Details are given in Table 3.

Table 3. Conditions for rearing ECB larvae and adults in climate chambers (modified from Wyninger 1974).

Life stage	Photoperiod	Temperature	Relative humidity
1. Larvae	L:D 16:8 700 luces	25°C day ± 1°C 22°C night ± 1°C	70 %
2. Larvae for breaking diapause	L:D 24:0 700 luces	28°C day ± 1°C -	80 %
3. Adults	L:D 16:8 4200 luces	22°C day ± 1°C 20°C night ± 1°C	70 %

In order to rear egg masses collected from the light traps leaf sections with approximately five egg masses each were placed in plastic petri dishes together with a block of diet. After the hatching of larvae, the leaf sections were removed and the larvae were kept in a climate chamber at 25° ± 1°C. After pupation, pupae were moved to plastic boxes with 7 cm diameter and 6 cm height lined with filter paper. Emerging moths were transferred to mating cages and maintained at 22° ± 1°C. As nutrition for the adults a piece of cotton in a glass dish soaked with 10% honey solution in water was added (Leahy and Andow 1994; Fadamiro and Baker 1998). For oviposition a transparent plastic foil was attached to the top of the cages which attracted females to oviposit. The foil was exchanged every second day and the egg masses obtained were cut out, transferred to petri dishes and kept in a climate chamber.

Infested corn stalks collected at Oderbruch, Badajoz, Ebro Valley, Biandrate, Pombia and Vinkovci were dissected and the obtained larvae were transferred to plastic petri dishes supplied with diet. To break their diapause, the larvae were kept at 28 ± 1°C at constant light 24:0 (L:D) and approximately 1 ml water was added every day to each petri dish (Çagan 1998). Infested corn stalks from Grignon, Poitiers and Lacchiarella were kept outside until the end of October. Thereafter they were dissected and obtained live larvae were transferred to plastic dishes. For a shortened diapause of about 12 weeks they were kept in a fridge at 4°C. Then the larvae were put into petri dishes supplied with diet and water and kept in a climate chamber at 28 ± 1°C with constant light. Pupation commonly started about two weeks later. Pupae from Padua and Camposampiero sampled in May were placed directly into plastic boxes for emergence and the adult individuals were then transferred to the mating cage.

Populations from different locations as listed in Table 1 were strictly reared separately from each other.

2. 3 Bioassays

For the bioassays neonates of the F_2- or F_3-generation were commonly used. Tests were performed at room temperature in bioassay trays ("Bio-Ba-128", Color-Dec Italy, Capezzano Pianore, Italy). Each well of the tray was filled with 1 ml ECB rearing diet prepared without the antibiotics (Table 2). Six different Cry1F toxin concentrations were used (1 ng/cm^2, 2 ng/cm^2, 4 ng/cm^2, 8 ng/cm^2, 16 ng/cm^2 and 32 ng/cm^2), prepared in 0.1% Triton^R X-100 buffer. Onto the diet surface of each well 100 µl of the different toxin doses and, for the control treatment, of a toxin-free buffer solution were applied. A single neonate larva was placed into each well and maintained at a constant temperature of 25°C for 7 days at a photoperiod of 16:8 L:D hours. After 7 days of exposure the number of dead larvae and the weight of each live larva was recorded. Moribund larvae, larvae not molting to the L2-stage and L2-larvae with a weight less than 0.1 mg were classified as dead. The bioassays were performed four times per population. For most of the populations, all trials were performed using individuals from the same ECB generation except for the Ebro Valley, Novara and Muret populations.

2. 4 Gas Chromatographic Analyses

Gas chromatographic analyses were used to determine the sex pheromone type of ECB females. In order to condition female corn borers for the production of the sex pheromone individuals not older than 1-2 days were kept individually at darkness for 18-24 h in 10 ml glass tubes covered with plastic caps (Dopman et al. 2004). From each female the tip of the abdomen was cut off at a length of about 3-4 mm because sex pheromone glands of female Lepidoptera are commonly found between the 8th and 9th abdominal segments (Götz 1951; Percy-Cunningham and MacDonald 1987). For dissolving of pheromone components, the truncated abdomen was incubated for at least 1h in 60 µl hexane and room temperature (RT). Then the abdomen tip was removed with a gage-needle, and after centrifugation (10500 rpm/8 min/20°C) the supernatant was transferred into gas chromatographic vials. The obtained sample was then analysed by gas chromatography (GC) on a 30 m x 0.25 mm i. d. fused silica capillary column containing a 0.25 µm film of 95% Polysiloxan (Varian VF-5MS). An Agilent 6890 N gas chromatograph (Agilent Technologies) equipped with a split/splitless injector, autosampler and mass selective (MS) detector was used for all analyses. MSD was operated in single ion modus (SIM, m/z: 68, 82, 96, 194). The column oven was programmed at 80°C (initial temperature), 80-190°C at 4°/min and 190-270°C at 30°C/min. The injector

temperature was 250°C, the interface temperature was 280°C, and helium flow was 1 ml/min. As ECB pheromone standards E11-tetradecenyl acetate (E11-14:OAc) and Z11-tetradecenyl acetate (Z11-14:OAc) were purchased from Trifolio, Germany. Hexane solutions of the standards were used for the GC analyses. The two standard pheromone isomers eluted at approx. 26 min, with peak separation of approx. 0.1 min. for Z11-14:OAc and E11-14:OAc isomers. E- and Z- race females were identified by comparing their retention times and masses with those of the synthetic external standards of the isomers (Fig. 8). External standard measurements were repeated at least after each 12th sample.

In order to verify the obtained pheromone peaks and to avoid any mismatching, samples with a single peak (E- or Z-isomer) were reanalysed spiked with 60 µl EZ-standard (0.05 µl/ml) or the opposite pheromone standard (0.05 µl/ml). Samples with clear EZ-pheromone peaks were spiked either with 60 µl E- or Z-standard (0.05 µl/ml).

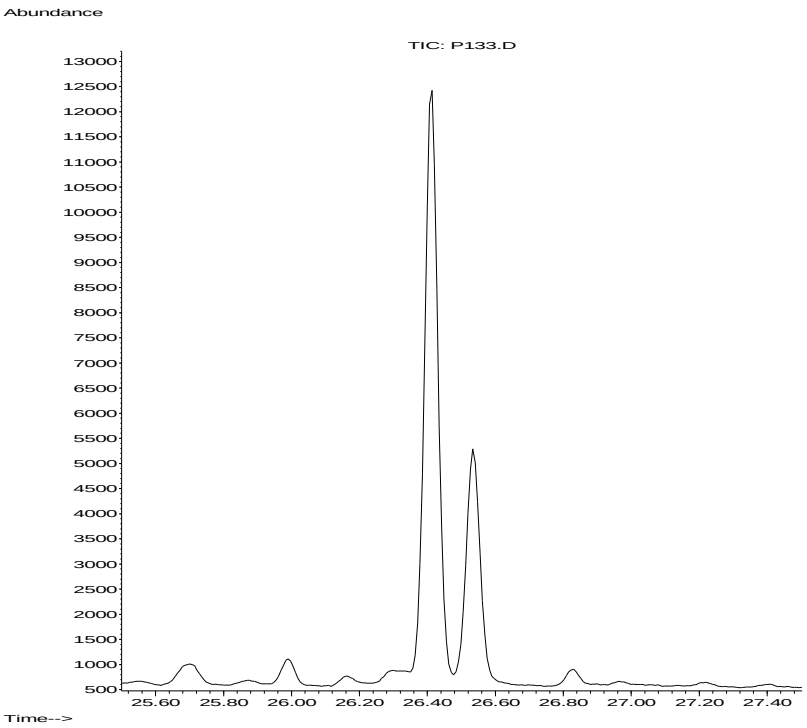

Figure 8. Scaled-up sections of a chromatogram typical for an ECB hybrid female (65:35; E:Z). The abscissa shows the retention times, the ordinate shows the peak size. The retention times of the associated peaks correspond to those of the respective external standards.

2. 5 Allozyme Analyses

The application of protein electrophoresis to estimate the degree of genetic variation was first demonstrated by Hubby and Lewontin (1966) and Harris (1966). Proteins can be separated in

a gel medium on the basis of their net charge, size and shape and can be classified as isozymes or allozymes. Isozymes are different molecular forms of an enzyme that are encoded by different loci, and allozymes are molecular forms of an enzyme encoded by different alleles of the same locus.

For the allozyme analyses of the European corn borer, adult individuals were frozen at –50°C to prevent any decomposition of their enzyme systems.

Horizontal starch gels were prepared by heating a mixture of 44.5 g hydrolysed starch in 350 ml of a solution containing an ionic buffer in an Erlenmeyer flask. Once the heated starch solution became noticeably viscous just below boiling point, vacuum pressure from a water tap aspirator was applied to remove any gas from the fluid. After all gas bubbles were removed, the gel solution was poured into a mould of appropriate size, and allowed to cool and solidify for at least one hour. Afterwards the gel was covered with polythene foil to prevent dehydration and stored at 4° C overnight before use (Baker 2000).

For the gel runs each corn borer individual was homogenized individually in 100 µl Tris-EDTA buffer (pH 6.8) after removing the head and the wings. From each homogenate 12 µl were soaked onto watman paper stripes (about 1 x 10 mm), which were placed onto the starting line of the gel across its whole depth. The loaded gel was again covered with polythene foil and then placed onto the electrophoresis apparatus bridging between two buffer chambers. Ice packs were placed on top of the gel to prevent a temperature increase during the gel run. An electrical current was then applied to the gel, mediated by a buffer solution in the cathodal and anodal chambers. Running conditions were 2.5 h / 200 V / 120 mA. When the run was completed, the gel was removed from the apparatus and sliced horizontally with a special slicer to produce 5-6 slices of 1 mm thickness. Each slice was used to stain a different enzym and reveal its allele pattern (Baker 2000).

Five polymorphic allozymes could be analysed: Glucose-phosphate-isomerase (GPI), Triose-phosphate-isomerase (TPI), Phosphogluco-mutase (PGM), Aspartate-amino-transferase (AAT) and 3-Hydroxybutyrate-dehydrogenase (HBDH). Only PGM is a monomeric enzyme, whereas all others are dimeric enzymes. Monomeric enzymes consist of one unit thus exhibiting one band for a homozygous and two bands for a heterozyous individual during electrophoresis (Fig. 9a). Dimeric enzymes consist of two subunits and therefore show one band for a homozygous and three bands for a heterozyous individual, whereby the middle band will show double intensity (Fig. 9b).

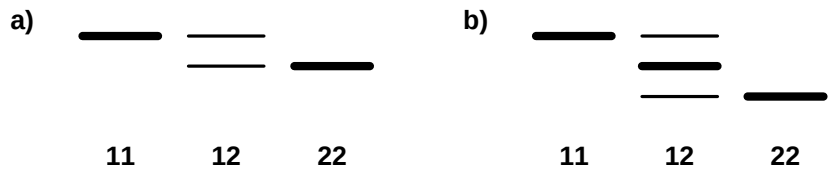

Figure 9. Electrophoretic pattern for a monomeric enzyme (a), and a dimeric enzyme (b). Homozygotes are represented by 11, 22 and heterozygotes are represented by 12.

GPI allele patterns (Fig. 10) were revealed by staining with 10 ml Tris A (pH 8), 1 ml $MgCl_2$ (1%), 15 mg fructose-6-phosphate, 1 ml NAD (1%), 0.5 ml NADP (1%), 1 ml NBT, 0.5 ml PMS and 17 units (17 µl) glucose-6-phosphate dehydrogenase.

AAT allele patterns were revealed by incubating the gel slice for 20 min with 40 ml Tris A, 200 mg aspartatic acid, 100 mg α-ketoglutaric acid, 10 mg 5-phosphate-pyridoxal. Afterwards the solution was removed and the slice was stained with 10 ml Tris A (pH 8) and 50 mg Fast Blue.

HBDH allele patterns were revealed by staining with 10 ml Tris A, 100 mg D,L-β-Hydroxy-butyrate, 1 ml NAD (1%), 0.25 ml MTT and 0.25 ml PMS.

PGM allele patterns were revealed by staining with 10 ml Tris A (pH 8), 1 ml $MgCl_2$, 100 mg glucose-1-phosphate, 1 ml NAD (1%), 0.5 ml NADP (1%), 0.5 ml NBT (1%), 0.5 ml MTT (1%), 0.5 ml PMS and 17 units (17 µl) glucose-6-phosphate dehydrogenase.

TPI allele patterns were revealed by staining with 1.5 ml Tris A (pH 8), 15 mg EDTA, 5 ml distilled water, 1.2 ml NAD (1%) and 1 ml sodium arsenate acid (1%), 1 ml DHAP (5mg/ml H_2O), 0.5 ml MTT, 0.3 ml PBT and 80 Units (12.5 µl) of glyceraldehyde-3-phosphate dehydrogenase.

All staining ingredients and buffer solutions are given in Table 4. After mixing all ingredients for a specific staining reaction 10 ml agar solution was added. The whole mixture was then applied onto the surface of the gel slice where it solidified. In order to promote staining the gel slices were incubated at 37°C until the enzyme allele patterns became visible. Staining was stopped by adding 3 ml of a fixing-solution.

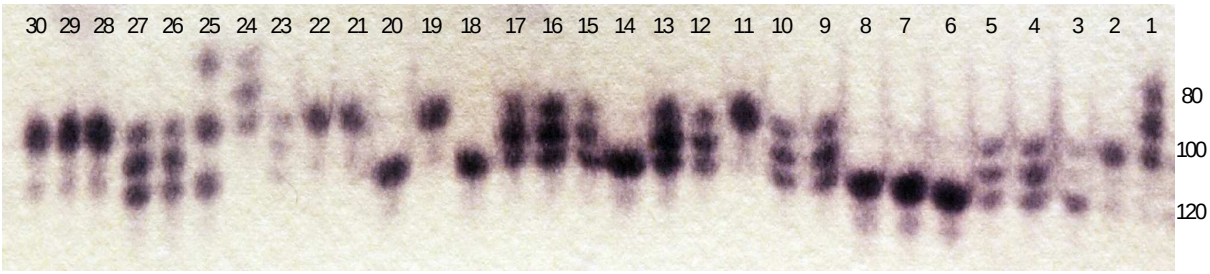

Figure 10. Gpi allele pattern of 30 ECB adults from the Padua population with three different alleles (80, 100, 120; 120-band = fastest allele).

Table 4. Buffers and staining ingredients

Tris-EDTA buffer (pH 6.8):	Tris	0.12 g
	EDTA	0.037 g
	H_2O	100 ml
		→ pH = 6.8 (with HCl-dilution)
	NADP 1%	40 µl
Tris-borate-EDTA (TBE) (pH 8.6) buffer:	Tris	109 g
	Boracic acid	30.9 g
	EDTA	7.6 g
	H_2O	1000 ml
		→ pH = 8.6 (with boracic acid, 1M)
Electrode buffer (1L):	TBE-buffer	250 ml
	H_2O	750 ml
Gel buffer (350 ml):	TBE-buffer	8.75 ml
	H_2O	341.25 ml
Tris A (1L):	EGTA	0.4 g
	Tris	24.2 g
	H_2O	1000 ml
		→ pH = 8.0 (with HCl-dilution, 35%)
Fixing solution:	Ethanol 95%	500 ml
	Acetic acid	100 ml
	H_2O	400 ml
Agar solution:	Agar agar	6 g
	H_2O	400 ml

Chapter 3: Susceptibility of European and North American Populations of the European Corn Borer to Cry1F Endotoxin

3. 1 Introduction

The European corn borer (ECB), *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), is one of the most important maize pests throughout Europe and the U.S.A. with up to 20% yield loss caused by larval damage (Mason et al. 1996). ECB-resistant Bt-maize can effectively control the European corn borer, at the same time reducing the environmental costs associated with the use of conventional insecticides (Shelton et al. 2002).

In the United States, cultivation of Bt-crops targeting ECB began in 1996. Since this time, US growers have rapidly adopted Bt-maize, and in 2007 approximately 49% of the total 37.6 million ha of maize was planted with Bt-maize targeting ECB or corn rootworm (*Diabrotica* spp.) (USDA NASS 2007). Cultivation of Bt-maize in Europe started two years later. Only 62,187 ha of Bt-maize was grown in the European Union in 2006, thereof 53,667 ha was located in Spain. In 2007, cultivation of Bt-maize increased to 110,077 ha in seven EU Member States (Spain, France, Czech Republic, Portugal, Germany, and Slovakia) over last year (EuropaBio, The European Association for Bioindustries, October 2007; (http://www.europabio.org/index.htm), and it is to be expected that the cultivation area will increase continually, especially in areas with high ECB infestation rates.

However, season-long and high expression levels of Bt-toxins in transgenic crops is thought to place considerable selection pressure for resistance on target pest populations and the risk for resistance evolution is perceived to be high. Insect resistance to Bt-toxins has been identified in a number of different lepidopterans, and has been selected for a number of laboratory strains of European corn borer exposed to high doses of Bt-toxins (Bolin et al. 1999; Huang et al. 1999; Siqueira et al. 2004; Pereira et al. 2008; Ferré and Van Rie 2002). Only the diamondback moth *Plutella xylostella* (L.), the American bollworm *Helicoverpa zea* and the fall armyworm *Spodoptera frugiparda* have developed resistance to Bt-toxins in field populations, (Tabashnik et al. 1997; Moar et al. 2008; Tabashnik et al. 2008b).

To maintain the effectiveness of Bt-toxins for ECB control, it is necessary to monitor the susceptibility of the target pest and to use resistance management strategies to prevent or at least delay adaptation (Gould 1998). The ability to detect insecticide resistance in pest populations is necessary to determine whether control failures are due to the presence of resistant insects or to some other factors affecting product performance. It is also crucial to assess the

extent and distribution of resistant populations and to test the effectiveness of management programs designed to reduce the frequency of resistant individuals.

One of the basic resistance management strategies is to monitor the susceptibility of the target populations in order to detect a possible resistance development at an early stage.

If resistance to a specific toxin occurs in the field, resistance management will most likely involve alternating or stacking of multiple toxins that are unaffected by cross-resistance (Siqueira et al. 2004). Cross-resistance can occur, if two or more different Bt-toxins share the same membrane receptor, which is widespread among lepidopteran species. Previous studies showed that Cry1Ac effectively competes for the Cry1Ab binding site in ECB midgut membrane receptors (Denolf et al. 1993; Hua et al. 2000), and that Cry1Ac and Cry1Ab share binding sites as a general pattern for all insect species tested to date (Ferré and Van Rie 2002). Some lepidopteran species exhibit a common binding site for as many as five different Bt-toxins, among them Cry1Ab, Cry1Ac and Cry1F (Granero et al. 1996; Herrero et al. 2001; Hernandez and Ferré 2005). In ECB, however, Cry1Ab and Cry1F seem to have seperate high-affinity binding sites, but also bind with low affinity to a common receptor (Hua et al. 2000; González-Cabrera et al. 2006). Consequently there are only low levels of cross-resistance of Cry1Ab-selected strains to Cry1F (Siqueira et al. 2004), so that Cry1Ab and Cry1F are suitable candidates for a multiple-toxin resistance strategy.

Although a number of baseline susceptibility studies of ECB to Cry1Ab and Cry1Ac endotoxins have been performed (e.g. Siegfried et al. 1995; Bolin et al. 1999; González-Núñez et al. 2000; Farinos et al. 2003; Saeglitz et al. 2006), there is hardly any information on the susceptibility of ECB to Cry1F. Bt-maize producing the Cry1F insecticidal protein was commercially available in 2003 in the USA and is pending regulatory approval in the EU since 2005. The objective of this study was to establish the baseline susceptibility of ECB to Cry1F prior to widespread commercial use of Cry1F Bt-corn in Europe and the USA. Generally, there are difficulties in comparing susceptibility estimates from different studies, because of a variety of bioassays procedures, and because of other influencing factors, such as laboratory conditions and toxin characteristics. Hence this study also aimed to evaluate bioassay procedures and to identify critical flaws which may bias the obtained susceptibility estimates.

In a joint study of the Department of Entomoloy at the University of Nebraska and the Institute of Environmental Research at Aachen University (RWTH) the intra- and inter-population variation in the susceptibility of ECB neonates to Cry1F protein in diet bioassay

was characterized for the major maize cultivation areas in Europe (Germany, Italy, France and Spain) and the north-eastern maize cultivation areas in the U.S.A.

There are different methodologies applied in various countries mostly due to historical and logistic reasons. ECB sampling strategies and design of susceptibility testing is performed according to the local availability of ECB, laboratory equipment and biochemicals supply. Sources of variation that may apply for the work presented in this study were examined in more detail for the parameters a) test temperature, b) generation, and c) larval developmental stage. Tests and comparisons in this study were designed to have – if at all – only a minor impact on the results.

3. 2 Materials and Methods

3. 2. 1 ECB Sampling and Rearing

Field samples of ECB in the European countries, performed in 2004 and 2005, consisted either of diapausing larvae and pupae, or of egg masses collected in light trap cages. Egg masses from Muret (France) and larvae from Novara (Italy) were provided by EU-partners. In total 11 European ECB populations were tested, originating from Spain (N = 2), France (3), Italy (3) and Germany (3). Sampling sites are illustrated in Figure 11. Field sampling in the United States, performed from 1999 to 2001, consisted of adults obtained by light trapping or sweep net sampling, second generation egg masses or diapausing larvae. In total 24 ECB populations were tested in 2000-2001, originating from Nebraska (N = 4), South Dakota (2), Iowa (7), Kansas (3), Illinois (5) and Minnesota (3), the population from Warren County, Illinois, was tested in both years. The American sampling sites are illustrated in Figure 12.

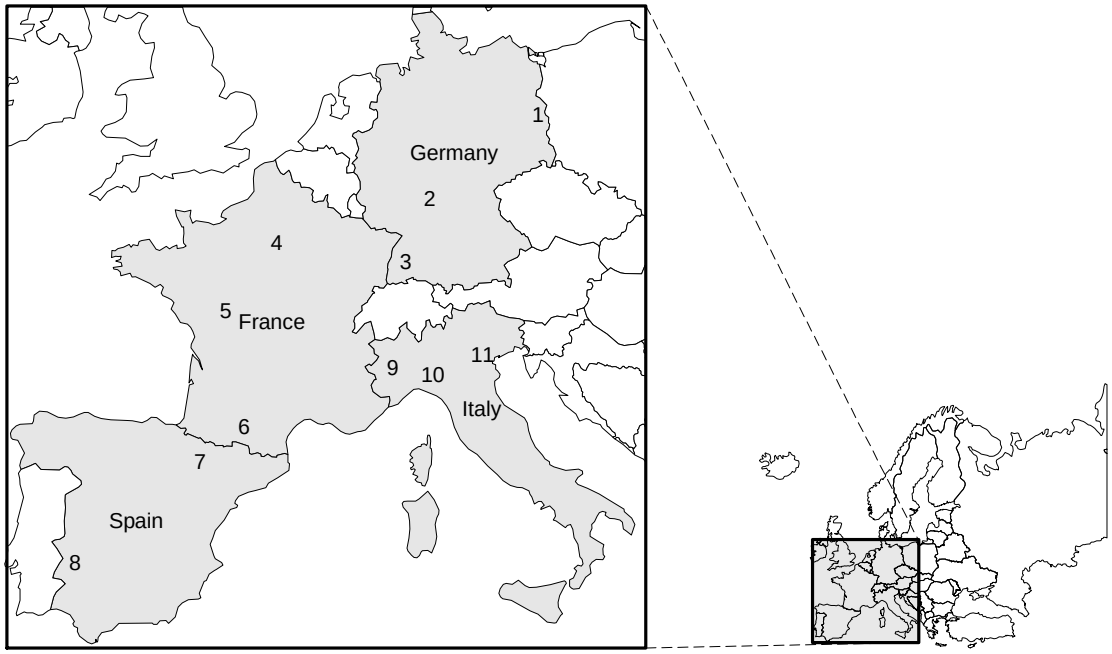

Figure 11. European sampling sites for ECB egg masses, larvae and pupae in 2004 – 2005 (1: Oderbruch; 2: Heilbronn; 3: Upper Rhine Valley; 4: Grignon; 5: Poitiers; 6: Muret; 7: Ebro; 8: Badajoz; 9: Novara; 10: Lacchiarella; 11: Padua).

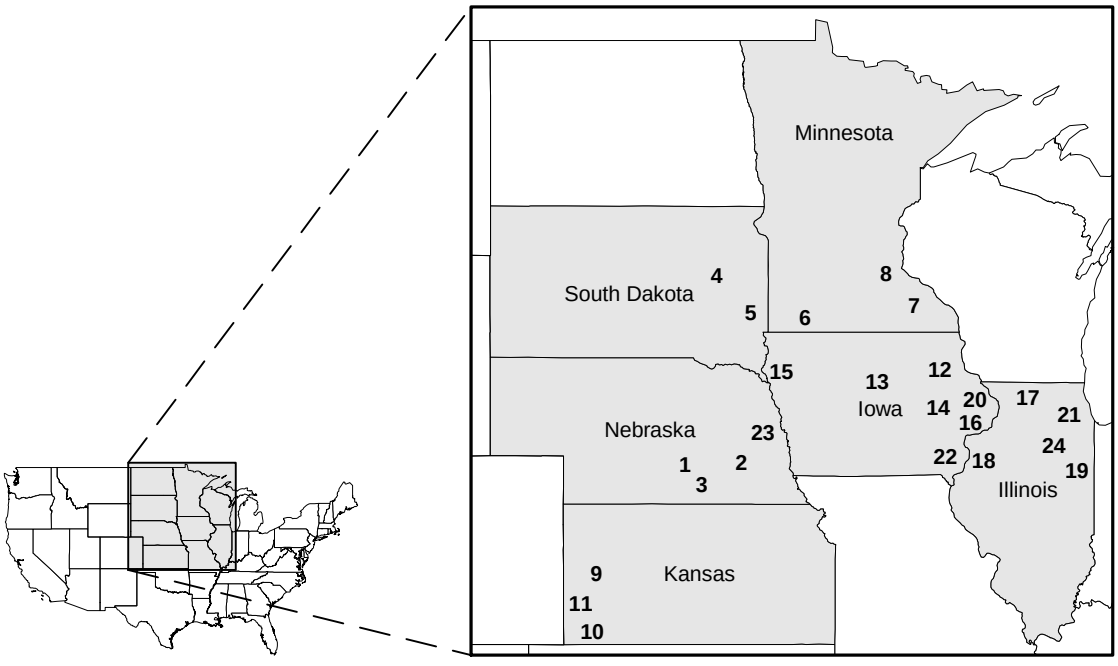

Figure 12. Sampling sites for ECB egg masses, larvae and adults in the U.S.A. in 1999 – 2001 (1: Aurora; 2: Saunders County; 3: Hamilton County; 4: Beadle County; 5: Moody County; 6: Nobles County; 7: Dakota County; 8: Goodhue County; 9: Scott County; 10: Cent. Finney County; 11: So. Finney County; 12: Marion County; 13: Story County; 14: Polk/Tama County;15: Sioux County; 16: Linn County; 17: Warren County; 18: Henderson County; 19: Champaign County; 20: Clinton County; 21: Dekalb County; 22: Henry County; 23: Mead; 24: Waterman).

ECB rearing procedures were based on Wyniger (1974) and Guthrie et al. (1965). Larvae were reared on a wheat germ based diet (Lewis and Lynch 1969), (see Chapter 2). For breaking or shortening diapause slightly different procedures were used. European larvae were kept at 28 ± 1°C and 24 h light, and water was added every day to each petri dish (Çagan 1998). Diapausing larvae from Grignon, Poitiers and Lacchiarella were kept outside until the end of October. Thereafter they were kept at 4°C for a shortened diapause of about 12 weeks. Then larvae were moved into petri dishes with diet and water, and kept in a climate chamber at 28 ± 1°C and 24 h light until pupation. All following life stages as well as collected egg masses and pupae were kept in climate chambers at 25° ± 1°C at a photoperiod of 16:8 light:darkness (L:D) and 70% relative humidity (RH). After pupation, pupae were moved to plastic boxes (7 cm diameter, 6 cm height) provided with filter paper. Emerging moths were transferred to mating cages. A piece of cotton in a glass dish was soaked with 10% honey solution in water to provide nutrition for the adults (Leahy and Andow 1994; Fadamiro and Baker 1998).

Diapausing larvae collected in the U.S. were reared at 27°C in 24 h light and 80% RH. At pupation, insects were moved to mating cages where adults were maintained at 8:16 L:D at 18.3°C and 27°C, respectively, with RH at 80%. Egg masses of the mated females were collected and kept in plastic petri dishes provided with filter paper moistened with sterile water to prevent desiccation, and incubated at 27°C until hatching.
Based on the experience of experts from US and Europe, there is currently no reason to beleave that the sampling strategy (overwintering of larvae versus sampling of fresh egg masses in spring) would have an influence on L1-/L2-larvae susceptibility in the toxicity tests.

3. 2. 2 Bioassays

For the European bioassays predominantly F_2 or F_3 neonates of the field collected insects were used. Most trials were performed using individuals from the same ECB generation except for bioassays of the Ebro, Novara and Muret populations. The bioassays of the American ECB populations were performed with the F_1 generation and in some instances with individuals from both, the F_1 and F_2 generation.
The protein used for the bioassays consisted of chromatographically purified and proteolytically truncated Cry1F Bt-toxin (provided by Dow AgroSciences; Indianapolis, IN). Four bioassays were performed for each European population whereby six different Cry1F

toxin concentrations prepared in 0.1% Triton^R X-100 buffer were used (1 ng/cm^2, 2 ng/cm^2, 4 ng/cm^2, 8 ng/cm^2, 16 ng/cm^2 and 32 ng/cm^2). The American bioassays were conducted in duplicate and included at least five Cry1F toxin concentrations. For both, the European and American bioassays, selective concentrations yielded mortality rates above zero and below 100 percent.

All European tests were prepared at room temperature in bioassay trays ("Bio-Ba-128", Color-Dec Italy, Capezzano Pianore, Italy and CD International, Pitman, NJ). Each well of the bioassay tray was filled with 1 ml diet. The different Cry1F concentrations and a buffer solution without Cry1F as the control treatment were applied onto the diet surface of each well. A single neonate larva was placed into each well and maintained at a constant temperature of 25°C at a photoperiod of 16:8 L:D hours. American ECB larvae were maintained at a constant temperature of 27°C. After 7 days of exposure the number of lethally affected larvae and the weight of each live larva were recorded.

European and American bioassays were evaluated according to slightly different mortality criteria. In the European trials moribund larvae, all larvae not molting to the L2-instar independent of their weight, and L2-larvae with a weight less than 0.1 mg were classified as lethally affected. L2-instar could be detemined, when a degusted head capsule from the molted L1-larva could be found in the diet. In the American trials, larvae that had not grown beyond first instar were considered to be lethally affected, whereas first instar larvae were defined as larvae with a weight of ≤ 0.1 mg. Therefore, the definition of survivors is different for the European and American bioassays. It was analyzed further in this study whether this difference would be of biological relevance for susceptibility screening. To confirm the direct comparability of the methods, three European populations (Grignon, Poitiers and Lacchiarella) were tested repeatedly using both mortality criteria.

3. 2. 3 Statistical Analyses

Baseline susceptibility estimates for each population were calculated by probit analyses (Finney 1971, LeOra Software 1987, ToxRat 2003). For the European populations two lethal concentrations (LC_{50}, LC_{95}) and growth inhibition value (EC_{50}) were estimated with their 95% confidence limits. For each population, the mortality response model was fitted to the percent mortality estimates for all toxin concentrations and replicates. For the American populations the observed mortality was corrected for the mortality in the control treatments, and thus lethal concentrations with 95% confidence limits were calculated. Larval weights of European and American populations were transformed to % growth inhibition relative to the controls

and these data were then analysed by non-linear regression (SAS Institute Inc. 1988). Larvae which were considered to be lethally affected, were recorded as 100% inhibition of larval growth.

Differences in the susceptibility of all tested populations were analysed by pairwise comparisons using 95% confidence intervals. When the confidence limits of two populations did not overlap, they were considered to be significantly different from one another ($P < 0.05$; Robertson and Preisler 1992; Payton et al. 2003).

3. 3 Results

3. 3. 1 Lethal Concentrations

Susceptibility values of all tested European populations calculated as lethal concentrations to Cry1F toxin are shown in Table 5. The LC_{50} values ranged from 2.72 ng/cm^2 (Padua, Italy) to 6.11 ng/cm^2 (Poitiers, France), the LC_{95} values from 10.43 ng/cm^2 (Novara, Italy) to 30.95 ng/cm^2 (Oderbruch, Germany). Hence, the difference between the most tolerant and most susceptible population was 2- and 3-fold, respectively. Based on the associated confidence limits significant differences were observed among some of the studied populations. However, these differences were not consistent for the different LC levels, nor did they reflect the geographic pattern of the populations.

Table 5. Susceptibility of European corn borer neonate larvae to Cry1F toxin in terms of estimates of lethal concentrations. Susceptibility of European ECB neonate larvae to Cry1F toxin in terms of estimates of lethal concentrations (LC_{50}, LC_{95}: concentrations causing 50% and 95% mortality; N: number of larvae; G: generation; CL: confidence limits; df: degrees of freedom; χ^2: Chi square).

Population	N	G	LC_{50} [1,2] (95%CL)	LC_{95} [1,2] (95%CL)	Slope ± SE	df	χ^2
Heilbronn (Germany)	448	F_2	3.08 [abc] (2.57 – 3.70)	15.47 [abc] (10.84 – 22.09)	2.35 ± 0.07	3	1.53
Upper Rhine Valley (Germany)	448	F_3	3.18 [abc] (2.63 – 3.84)	18.49 [abc] (12.52 – 27.32)	2.15 ± 0.06	3	4.26
Oderbruch (Germany)	448	F_2	3.59 [abcd] (2.86 – 4.52)	30.95 [cd] (17.76 – 53.95)	1.76 ± 0.05	4	0.74
Badajoz (Spain)	448	F_2	4.15 [cd] (3.60 – 4.79)	13.96 [abd] (10.66 – 18.28)	3.12 ± 0.10	3	4.74
Ebro (Spain)	448	$F_{3/4}$	3.78 [bcd] (3.22 – 4.43)	13.90 [abd] (10.34 – 18.69)	2.91 ± 0.10	3	2.07
Novara (Italy)	448	$F_{7/8}$	3.09 [ab] (2.69 – 3.55)	10.43 [a] (8.14 – 13.38)	3.11 ± 0.09	4	2.04
Padua (Italy)	448	F_2	2.72 [a] (2.30 – 3.20)	13.00 [ab] (9.66 – 17.46)	2.42 ± 0.05	4	4.22
Lacchiarella (Italy)	448	F_3	5.93 [e] (5.06 – 6.94)	25.41 [c] (18.90 – 34.17)	2.60 ± 0.06	4	5.00
Muret (France)	448	$F_{10/11}$	3.33 [abc] (2.87 – 3.86)	10.50 [a] (8.12 – 13.58)	3.30 ± 0.13	4	1.97
Grignon (France)	448	F_3	4.68 [de] (4.09 – 5.35)	15.09 [abc] (11.84 – 19.23)	3.23 ± 0.09	4	0.62
Poitiers (France)	448	F_3	6.11 [e] (5.31 – 7.03)	19.35 [bc] (15.20 – 24.64)	3.29 ± 0.10	4	4.96

[1] ng Cry1F/cm^2

[2] Values superscribed by the same letter within a column are not significantly different

For two European populations there were significant differences between bioassay replicates (Table 6). The LC-values of the Upper Rhine Valley population represent a 3.5-fold and 7-fold variation in susceptibility to Cry1F to both the LC_{50} and LC_{95}, respectively. For the Lacchiarella population, a 2.5 – 3-fold variation was detected.

Table 6. Mean LC estimates for different bioassay replicates for the population from the Upper Rhine Valley and from Lacchiarella (LC: lethal concentration; CL: confidence limits; replicates I – IV).

	I	II	III	IV
Upper Rhine Valley				
LC_{50} (95%CL) [1,2]	1.73 [a] (1.16 – 2.60)	1.76 [a] (1.40 – 2.20)	5.89 [b] (4.33 – 8.01)	4.05 [b] (2.96 – 5.54)
LC_{95} (95%CL) [1,2]	10.06 [b] (5.26 – 19.23)	4.07 [a] (2.64 – 6.26)	29.31 [b] (15.96 – 53.84)	20.67 [b] (11.36 – 37.62)
Lacchiarella				
LC_{50} (95%CL) [1,2]	6.13 [ab] (4.37 – 8.58)	6.53 [b] (4.74 – 9.01)	8.77 [b] (6.57 – 11.70)	3.48 [a] (2.65 – 4.57)
LC_{95} (95%CL) [1,2]	26.31 [a] (14.07 – 49.18)	36.34 [a] (18.70 – 70.61)	25.70 [a] (15.32 – 43.13)	12.58 [a] (7.64 – 20.72)

[1] ng Cry1F/cm^2
[2] Values followed by the same letter within a column are not significantly different.

Lethal concentrations for all tested American populations are listed in Table 7. Overall the LC_{50} values ranged from 1.23 (Champaign County, Illinois) to 6.26 ng/cm^2 (Henderson County, Illinois), the LC_{95} ranged from 4.59 (Champaign County, Illinois) to 21.25 ng/cm^2 (Goodhoe County, Minnesota). Hence, the differences between the most tolerant and most susceptible populations was approximately 5-fold (LC_{50}) and 4.5-fold (LC_{95}). Based on the associated confidence limits there were significant differences in the susceptibility of some of the studied American populations. However, as with the European results, the differences did not indicate a geographical pattern in susceptibility.

Table 7. Susceptibility of USA ECB neonate larvae to Cry1F toxin in terms of estimates of lethal concentrations (Co: County; SD: South Dakota; MN: Minnesota; NE: Nebraska; KS: Kansas; IA: Iowa; IL: Illinois; LC_{50}, LC_{95}: concentrations causing 50% and 95% mortality; N: number of larvae; CL: confidence limits).

Population	N	LC_{50} [1,2] (95%CL)	LC_{95} [1,2] (95%CL)	Slope ± SE
2000				
Beadle Co, SD	766	4.11 [b] (3.51 – 4.62)	7.87 [a] (6.90 – 9.54)	4.54 ± 0.62
Moody Co, SD	766	2.35 [a] (1.86 – 2.84)	7.21 [a] (5.90 – 9.33)	2.64 ± 0.28
Nobles Co, MN	767	2.37 [ab] (1.51 – 3.33)	10.37 [ab] (7.12 – 18.19)	2.00 ± 0.16
Saunders Co, NE	768	3.91 [b] (3.20 – 4.61)	12.18 [b] (10.17 – 15.30)	2.59 ± 0.25
Hamilton Co, NE	768	2.88 [ab] (1.94 – 3.84)	9.80 [ab] (7.25 – 15.20)	2.41 ± 0.22
Scott Co, KS	768	2.55 [a] (2.22 – 2.91)	8.33 [ab] (7.00 – 10.34)	2.49 ± 0.18
Cent. Finney Co, KS	764	4.12 [bc] (2.99 – 5.38)	18.59 [b] (13.53 – 29.07)	1.96 ± 0.16
So. Finney Co, KS	768	4.96 [bc] (4.25 – 5.67)	12.48 [b] (10.66 – 15.29)	3.20 ± 0.31
Marion Co, IA	753	2.57 [ab] (1.81 – 3.41)	10.29 [ab] (7.37 – 16.82)	2.12 ± 0.17
Story Co, IA	1151	3.17 [ab] (1.95 – 4.19)	9.12 [ab] (6.95 – 14.48)	2.80 ± 0.34
Polk/Tama Co, IA	1533	4.74 [bc] (3.30 – 6.26)	16.83 [b] (12.21 – 27.62)	2.33 ± 0.20
Sioux Co, IA	767	3.23 [ab] (2.70 – 3.79)	11.16 [ab] (9.29 – 14.03)	2.38 ± 0.20
Linn Co, IA	384	3.04 [ab] (2.40 – 3.70)	8.51 [ab] (6.80 – 11.61)	2.86 ± 0.26
Warren Co, IL	768	3.81 [b] (3.34 – 4.33)	14.25 [b] (11.84 – 17.84)	2.24 ± 0.14
Henderson Co, IL	128	6.26 [c] (5.15 – 7.46)	17.55 [b] (14.02 – 23.96)	2.86 ± 0.22
2001				
Aurora, NE	764	3.68[c] (2.69 – 4.77)	20.93 [bc] (14.88 – 34.53)	2.18 ± 0.17
Champaign Co, IL	764	1.23 [a] (0.96 – 1.49)	4.59 [a] (3.74 – 6.09)	2.88 ± 0.33
Clinton Co, IA	761	2.26 [abc] (1.38 – 3.06)	11.55 [bc] (8.14 – 21.40)	2.32 ± 0.25
Dakota Co, MN	768	2.38 [bc] (1.99 – 2.76)	10.28 [b] (8.37 – 13.53)	2.59 ± 0.24
DeKalb Co, IL	761	1.47 [ab] (1.00 – 1.95)	9.17 [b] (6.49 – 15.35)	2.07 ± 0.18
Goodhue Co, MN	760	2.20 [abc] (1.03 – 3.65)	21.25 [bc] (10.97 – 83.89)	1.67 ± 0.13
Henry Co, IA	765	1.98 [bc] (1.57 – 2.40)	11.36 [b] (8.99 – 15.47)	2.17 ± 0.20
Mead, NE	762	2.28 [bc] (1.70 – 2.90)	21.22 [c] (15.90 – 31.07)	1.70 ± 0.16
Warren Co, IL	764	1.42 [ab] (0.79 – 2.08)	17.55 [bc] (6.47 – 20.24)	1.95 ± 0.19
Waterman, IL	768	2.92 [c] (2.17 – 3.71)	13.14 [bc] (9.44 – 22.12)	2.52 ± 0.20

3. 3. 2 Growth Inhibition

Growth inhibition values for the European ECB populations, estimated as EC_{50}, are presented in Figure 13. The obtained values ranged from 1.42 ng/cm^2 (Padua, Italy) to 3.22 ng/cm^2 (Grignon, France). Significant differences among the local populations in terms of larval growth inhibition are given only between the populations of Padua (Italy) and Lacchiarella (Italy) / Poitiers (France) (Fig. 13). The range of variation in susceptibility indicated by larval growth inhibition was similar to that indicated by mortality (Table 5); 2.5-fold for EC_{50}.

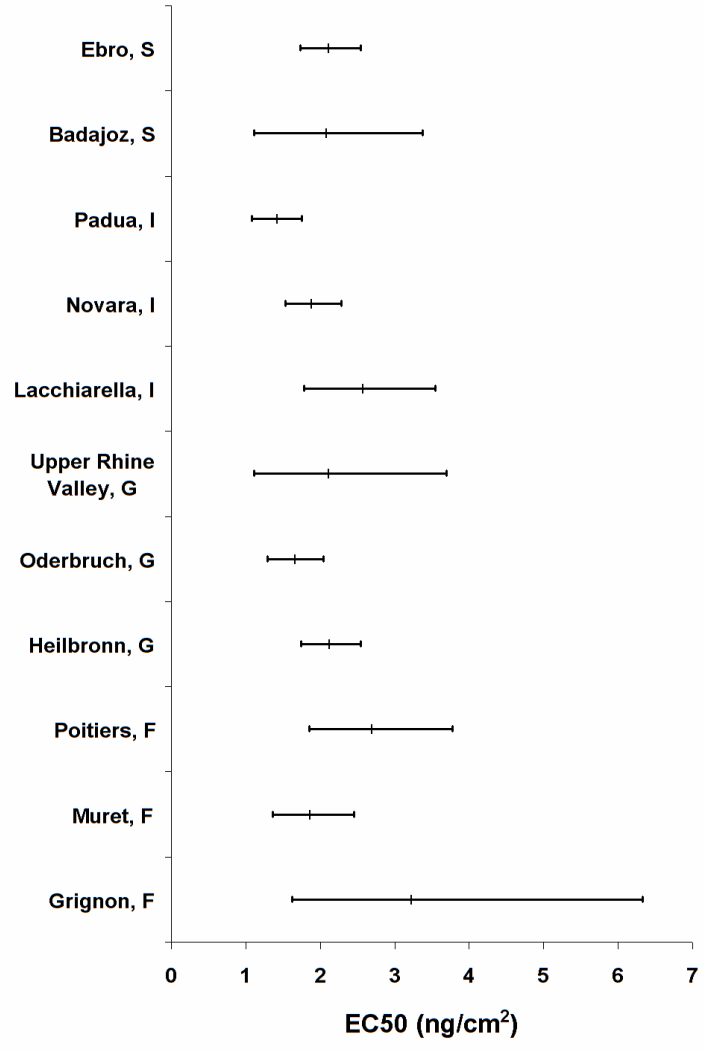

Figure 13. EC_{50} values (concentration causing 50% growth inhibition) of European ECB neonate larvae exposed to Cry1F Bt-toxin. Horitonzal bars indicate 95% confidence limits (F: France; I: Italy; S: Spain; G: Germany)**.**

Growth inhibition estimates for the American ECB populations, estimated as EC_{50}, are presented in Figure 14. The obtained values ranged from 0.4 ng/cm^2 (DeKalb County, Illinois) to 1.0 ng/cm^2 (Beadle County, South Dakota). Significant differences among the local populations in terms of larval growth inhibition are given between some populations (Fig. 14).

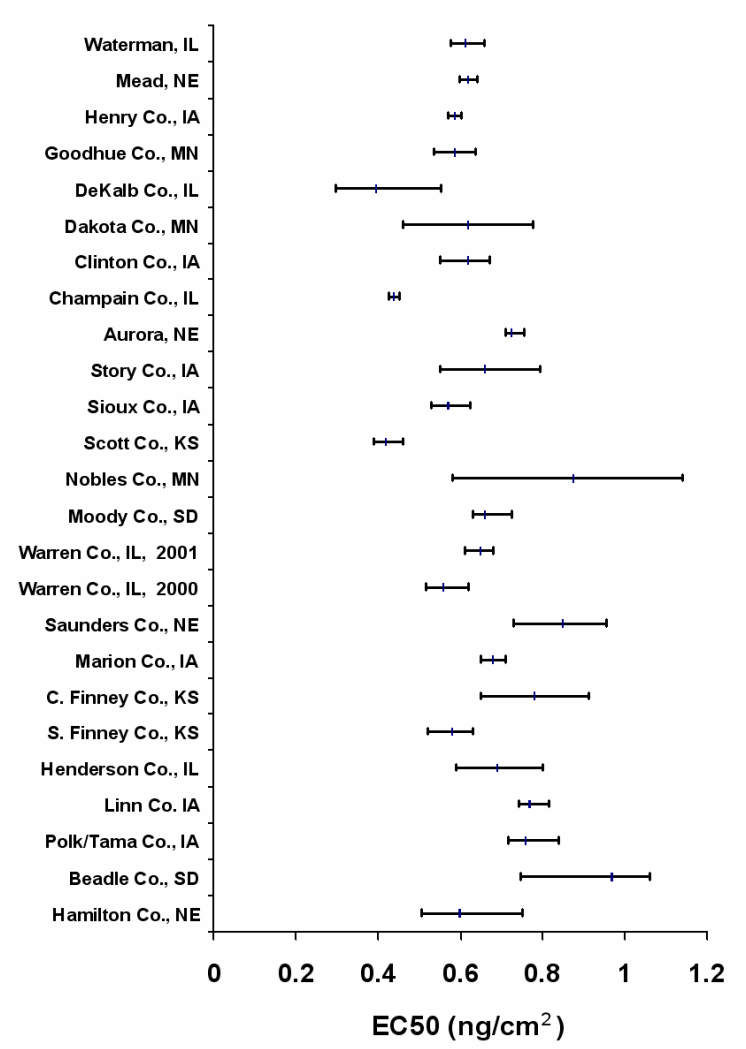

Figure 14. EC_{50} values (concentration causing 50% growth inhibition) of European corn borer neonate larvae exposed to Cry1F Bt-toxin. Horizontal bars indicate 95% confidence limits (Co: County; IL: Illinois; NE: Nebraska; IA: Iowa; MN: Minnesota; KS: Kansas; SD: South Dakota).

3. 4 Discussion

At first sight European and American susceptibility estimates obtained in this study were fairly similar concerning the LC_{50} and LC_{95} values (Table 5 and 7) indicating a similar susceptibility of the ECB larvae even on a continental scale. A comparison of the EC_{50}-values between the European and American populations represent an approximately 3.5-fold higher

EC_{50} between the two most tolerant and the two most susceptible populations (Fig. 13 and 14). However, it is to be considered that a different definition of survivors and therefore different mortality criteria were applied to the two continental data sets, and that the mortality criteria used could influence the comparability of the European and American LC-values. In fact, using molting to the L2-stage as a criterium for surviving Bt-toxin exposure led to marked differences in the estimates: in the European data analyses fewer larvae were classified as surviving resulting in lower LC- and EC-values. On the other hand, using the 0.1 mg weight treshold as the mortality criterium in the American analyses increased the number of survivors and hence the LC estimates. It did also result in more survivors with lower weights regarding the EC estimates.

Table 8 illustrates the mortality values (LC_{50}) for the populations from Grignon, Poitiers (both France) and Lacchiarella (Italy) when the two different mortality criteria were applied. Not surprisingly, compared to the assessment used for the European populations, the LC-values increase by using the American method of only setting a 0.1 mg weight threshold for the definition of the L1-stage. Using the 0.1 mg weight threshold led to an increased LC estimate since the overall mortality rates decreased.

Table 8. LC_{50} values obtained from analyses without L1-larvae and with L1-larvae > 0.1 mg (LC: lethal concentration; EC: effective concentration; CL: confidence limits; n.d.: not defined).

	Mortality criteria = larva moribund, not molting to L2 stage, or L2 with larval weight < 0.1 mg	Mortality criteria = larva moribund or larval weight < 0.1 mg
Population	LC_{50} (95%CL)[1]	LC_{50} (95%CL)[1,2]
Lacchiarella	5.93 (5.06 – 6.94)	18.37 (13.79 – 24.47)
Grignon	4.68 (4.09 – 5.35)	8.69 (7.33 – 10.31)
Poitiers	6.11 (5.31 – 7.03)	16.98 (13.08 – 22.05)

[1] ng Cry1F/cm^2

From recent observations, it is known that most larvae with 7-day weights above 0.1 mg were still alive after ten to fourteen days of exposure to Cry1F. They later also molded into the L2-stage (C. Gaspers, data not shown). Therefore it is concluded that the application of the 0.1 mg weight treshold without the molting criterium is the more realistic evaluation of the bioassays.

The European baseline susceptibility may increase only up to 2-3-fold compared to the susceptibility to Cry1F of the American populations (using the data presented in Table 8) if

the same mortality criteria or the same definition of survivors are used. These significant differences between populations are most likely due to the natural variation as described by Robertson et al. 1995, rather than an adaptation to the exposure to Bt-toxins, and are in line with the results of other susceptibility studies (e.g. Siegfried et al. 1995; Marçon et al. 1999). This was also reported by González-Núñez et al. (2000), who obtained even greater differences between LC_{50} values for Spanish ECB populations and those from previous American studies for Cry1Ab toxin. However, González-Núñez et al. (2000) used yet other mortality criteria, e.g. larvae were classified as lethally affected, when they did not show any reaction when prodded. Additionately, it is to be considered that also replicates of the same population can produce significant differences in the susceptibility (Table 6).

Overall it is generally difficult to compare the susceptibility estimates from different studies, because there is a number of external factors as well as methodological issues which could bias the obtained estimates and may most likely explain the found significant differences. First of all, the choice of bioassay methods in terms of toxin application, namely using surface application or incorporation method, seems to make a crucial difference. In this study most larvae surviving after seven days of exposure in higher toxin concentrations, especially those with relatively high weights, were found below the toxin layer deep in the diet. This may be due to using the surface application method with its thin toxin layer, which is located only on the surface of the diet. The ability of larvae to avoid exposure by burrowing below the toxin layer has previously been described by Bolin et al. (1999), who pointed out that using the incorporation method instead of the surface application method resulted in a considerable increase in mortality. A test trial for some European populations to compare these two methods (Gaspers, data not shown; Saeglitz et al. 2006) confirmed these findings and showed a large mortality increase when using the incorporation method. A critical point relating to this problem is the manual preparation of this kind of bioassay. When the diet is filled into the compartments of the trays, there may be a slight rise in the middle of the well if the texture is too solid. If the texture is too liquid, capillary forces will cause a lifting of the diet where it touches the well. In both cases the toxin will not cover the diet surface uniformly. Therefore it is possible that larvae may find a point in the diet with a thinner layer of the toxin and manage to avoid exposure by feeding below the toxin layer. This poses the question whether ‘heavy-weight’ L2-larvae were really less susceptible than others or whether they may have avoided the toxin layer and should be excluded from the analyses. In general, it is assumed that the

significant differences in susceptibility to Cry1F even in replicates of the same population (Table 6) could be caused by the manual preparation of the surface application method.

There was also a temperature gradient in larval weights. In Table 9 the mean larval weights of the European populations are given for each toxin concentration together with the season when the bioassays were performed. It is obvious that the mean weights increased with increasing temperature. This was most likely caused by higher developmental rates of larvae in the control treatment and low toxin concentrations (1 ng/cm^2 and 2 ng/cm^2), some of which even entered the third instar within 7 days. Besides, it was obvious, that the last three series of bioassays (4 replicates each for Grignon, Poitiers, both France, and Lacchiarella, Italy) showed the highest LC_{50} values. These bioassays were performed during a heat wave which caused temperatures of around 30°C in the laboratory. The bioassays for most other populations were performed at moderate laboratory temperatures of about 20°C.

Table 9. Mean weights of neonates from all European populations in each Cry1F toxin concentration after 7 days of exposure (URV: Upper Rhine Valley; H: Heilbronn; O: Oderbruch; B: Badajoz; E: Ebro; N: Novara; Pa: Padua; L: Lacchiarella; M: Muret; G: Grignon; Poi: Poitiers; W: winter 2005/2006 (10-15°); Sp: spring (15 - 20°C); Su 05: summer 2005 (20°C); Su 06: summer 2006 (> 30°C)).

	Population										
	URV	H	O	B	E	N	Pa	L	M	G	Poi
ng/cm^2	(W)	(W)	(W)	(W)	(W)	(Sp)	(Su05)	(Su06)	(W)	(Su06)	(Su06)
0	0.56	0.50	0.65	0.50	0.66	0.83	0.63	1.11	0.69	1.11	0.91
1	0.54	0.48	0.52	0.55	0.49	0.68	0.49	0.89	0.60	1.12	0.76
2	0.46	0.41	0.50	0.33	0.46	0.52	0.36	0.78	0.44	0.85	0.66
4	0.44	0.37	0.43	0.32	0.42	0.39	0.32	0.64	0.37	0.80	0.47
8	0.34	0.31	0.39	0.29	0.41	0.39	0.28	0.54	0.28	0.49	0.40
16	0.33	0.41	0.36	0.29	0.31	-	0.25	0.34	0.35	0.82	0.31

In order to find out if there was an influence of temperature on the results of the bioassays, a series of bioassays was performed in a 35°C and in a 20°C climate chamber. During the seven days of exposure all bioassays were kept in a climate chamber at 25°C ± 1°C at a photoperiod of 16:8 (L:D). The LC estimates from the bioassays performed at 20°C were in a similar range as the previous results. The bioassays performed at 35°C, however, yielded a significant increase up to 7-fold of the LC_{50} and LC_{95} estimates compared to the estimates given in Table 5.

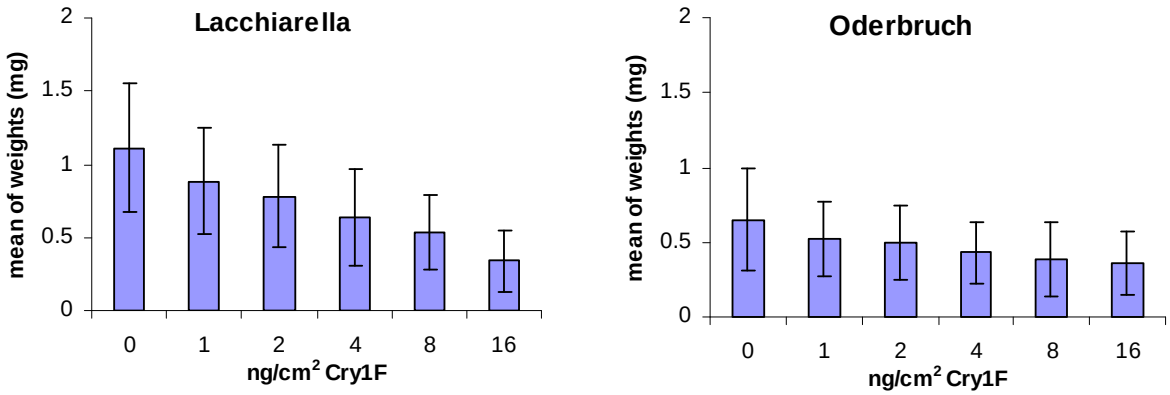

Figure 15. Mean weights of larvae from Lacchiarella (Italy) and Oderbruch (Germany)

The influence of temperature on the mean weights of two populations is shown in Figure 15. For the first population (Lacchiarella) all bioassays were performed with neonates from adults reared in summer 2006 (>30°C during feeding) and for the second (Oderbruch) the bioassays were performed with neonates from adults reared at winter conditions (10-15°C during feeding). For the Lacchiarella population a much steeper decrease in the mean weights along the toxin concentrations could be observed than for the Oderbruch population. This is due to a fairly big difference between the mean weights of larvae in the control after the exposure. Surprisingly, both populations converge to a very similar mean weight in the 16 ng/cm^2 Cry1F concentration.

Additionally the possibility of instability of Cry1F during storage should keep clearly in mind, because Hernandez and Ferré (2005) reported that the Cry1F toxin deteriorated during storage. But this could not be the only explanation for increasing LC_{50} values because in Table 8 it is shown that also the larvae in the control reached higher weights in bioassays performed during a period of very hot weather.

As the bioassays in this study were performed with different generations of ECB reared from the original field populations, there was a theoretical chance that this may influence the results. Saeglitz et al. (2006) and Marçon et al. (1999) have shown for the Cry1Ab toxin that there was no significant difference between the results of bioassays using advanced generations compared to those using earlier generations. Hence the same was expected for the Cry1F toxin. In order to test a possible "generation" effect, the 2nd generation bioassays for the Padua population were repeated with the 10th generation in July 2006. The result was a

2.5-fold higher LC_{50}, but only a 1.5-fold higher LC_{95}, compared to the first trials. Such negligible differences could also be observed between susceptibility estimates from the 2nd generation versus the 14th generation of the Heilbronn population (1.7-fold higher LC_{50}) and even for the 4th versus the 3rd generation of the Grignon population. Therefore the obtained differences seemed to represent the usual variability between different bioassay trials or to a possible instability of Cry1F during storage. Finally the use of different toxin batches may cause differences in baseline susceptibility to Bt-toxins as described in Saeglitz et al. (2006). The protein batches used in the two studies were different and they were performed at different times. Bioassays run on different batches of Cry1F at the same suggested differences in specific activity of these batches (Blair Siegfried, unpublished data). The protein batch used in the baseline determination for Europe will be used in the future monitoring program; similarly, the protein batch used in the US monitoring program is consistent across years. Periodic re-evaluation of the protein batches is conducted to ensure it does not become degraded in storage.

In order to render baseline susceptibility studies on the basis of bioassays a reasonable method to evaluate a possible resistance development at an early stage, it is very important to do this under strictly standardised conditions. This concerns factors such as climate, bioassay procedure, toxin charges and criteria for mortality to allow a comparison among different populations. Otherwise it may not be possible to compare the baseline susceptibility estimates from different studies. However, by using standard procedures the variability will be small enough to base susceptibility testing on only a small number of European populations for a efficient monitoring of the resistance development of Bt-toxins.

Chapter 4: Distribution of *Ostrinia nubilalis* (Lepidoptera: Crambidae) pheromone races in Europe

4. 1 Introduction

The European corn borer (ECB), *O. nubilalis*, is one of main target organisms of transgenic Bt-maize. The continuous expression of *Bacillus thuringiensis* (Bt-) toxins in these crops is expected to enhance selection pressure for a resistance development in ECB field populations (Gould 1998), hence strategies for a resistence management need to be devised. So far the most widely used agricultural practice to delay resistance is the high-dose/refuge strategy (Alstad and Andow 1995). Refuges are defined as non-Bt-crop plants in proximity to the Bt-crops that can be used by the target pest (Gould 1998). Any resistant insects emerging from the Bt-crop should be able to mate with one of the much larger number of susceptible adults emerging from the refuge. The resulting offspring will be heterozygous in terms of their resistance genes and therefore susceptible to Bt.

Because ECB is feeding on more than 200 plants (Lewis 1975), other host plants instead of maize may serve as refuges. However, it needs to be ensured that individuals emerging from other host plants will mate randomly with individuals from Bt-maize. This assessment is important as generalist herbivores usually have a preference for one or a few host species even when the plants are closely related (Tikkanen et al. 1999). The European corn borer seems to show a preference for specific host plants due to a polymorphism regarding the sex pheromone communication system. Two ECB sex pheromone races have been identified based on two different isomers of 11-tetradecenyl acetates. In the Z-race, females emit and males respond to a 3:97 sex pheromone blend of (E)/(Z)-11-tetradecenyl acetates (E11-/Z11-14:OAc) (Klun et al. 1973), and in the E-race, females emit and males respond to the opposite 99:1 E/Z pheromone blend (Glover et al. 1987). Hybrid individuals produce the intermediate E/Z molar pheromone blend of 65:35 (Klun and Maini 1979). They can be produced in the laboratory and were found at low rates in areas where the races occur in sympatry (Buechi et al. 1982; Roelofs et al. 1985; Klun and Huettel 1988). Field and laboratory data indicated that hybridization occurs when E-males court and mate with Z-females (Liebherr and Roelofs 1975; Glover et al. 1991). The disposition of pheromone production and the male pheromone receptor are autosomally inherited, whereas the male behavioral response is determined by a sex-linked gene (Z-chromosome) (Roelofs et al. 1987). All three genes are inherited independently (Löfstedt et al. 1989) and the genetic factors responsible for major differences in female pheromone blend production and male behavioral response to those blends, exhibit

simple Mendelian inheritance (Roelofs et al. 1987; Glover et al. 1990; Klun and Maini 1979; Dopman et al. 2004). Sex-linked control of the behavioral responses in crosses of pure E- and Z-ECB was confirmed by demonstrating complete linkage of a sex-linked triose phosphate isomerase (TPI) locus with the locus controlling the response to the sex pheromone (Glover et al. 1990). According to this, E-race populations are fixed for the *Tpi-1* allele, whereas Z-race populations are segregating for both *Tpi-1* and *Tpi-2* at intermediate frequencies, with *Tpi-2* being the more common allele (Glover et al. 1991).

Regarding the distribution of the two pheromone races it was observed that on maize the Z-race predominates in most of the ECB range in Europe and North America, whereas both races occur in Switzerland, Italy and Eastern North America, and from Massachusetts to South Carolina (Klun and Huettel, 1988; Pena et al. 1988; Glover et al. 1990; Mason et al. 1996; Marcon et al. 1999). In France, a host speciation of the two pheromone races was postulated (Thomas et al. 2003; Pelozuelo et al. 2004) whereby E-race populations occur in northern France and feed on mugwort (*Artemisia vulgaris*) but not on maize (Thomas et al. 2003). At the same sampling site in northern France females from mugwort produced the E-pheromone blend and females from maize produced the Z-pheromone blend. No hybrid was detected (Thomas et al. 2003). In other parts of France, the E-phenotyp was found only in mugwort and hop (*Humulus lupulus*) ECB populations, whereas the Z-phenotyp was identified only in maize populations. On each host plant, ECB individuals expressing the hybrid phenotyp were rare (< 1%), (Pelozuelo et al. 2004). In contrast to this findings, it is reported, that in other moths (Löfstedt 1993) the female pheromone and male response in ECB are genetically driven and independent of the host plant on which larvae were feeding (Roelofs et al 1985; Glover et al. 1990). According to this, host speciation, as postulated for France, and strictly assortative mating of the different pheromone types cannot be generalized for other geographical areas.

Overall there are still many open questions concerning ECB pheromone types, their mating behavior, host plant choice and geographical distribution. In this study 17 European ECB populations from maize were analysed by gas chromatographic analyses to identify their pheromone blend and the frequency of hybrids. Consequently this study should give insights on the mating behaviour regarding assortative or random mating. Additionally two German populations, one from mugwort and one from hop, were tested for a comparison with the situation in France, where nearly complete mating isolation between the two pheromone races was postulated. Furthermore *Tpi*-allele patterns from all populations were studied by allozyme

analyses because previous analyses had shown distinctly different *Tpi*-allele patterns than described by Glover (1990, 1991).

4. 2 Materials and Methods

4. 2. 1 ECB Sampling and Rearing

Field samples of ECB for this study consisted either of diapausing larvae, pupae, adults or of egg masses collected in light trap cages. In total 19 European ECB populations were tested, originating from Germany (6 populations), Italy (5), France (2), Spain (2), Greece (2), Serbia (1) and Croatia (1). Larvae from Pola (Serbia), Serres and Komotini (both Greece) were provided by local scientists. All populations were collected from maize plants, exept for two German populations, one from mugwort and one from hop. The location of sampling sites is illustrated in Figure 16. For ECB rearing procedures see Chapter 2.

Pupae collected from Camposampiero were kept individually in plastic boxes. After emerging, one male and one female each were transferred into small mating cages for single matings to obtain egg masses for GC-analyses of the F_1 generation. Populations solely collected for GC-analyses were tested using the parental or/and F_1 generation, populations which were first used for other analyses, were tested by GC using the F_2-F_4 generation, and populations already present in the laboratory for some time were analysed using advanced generations (Bonn maize and Bonn mugwort, both F_{35}; Ebro, $F_{6\text{-}8}$; Oderbruch, F_{11}). The population from Padua was tested twice using the $F_{2\text{-}4}$ and the F_{11} generation. The population from Pombia was collected twice (2006 and 2007) and tested both using the parental and parental/F_1 generation, respectively.

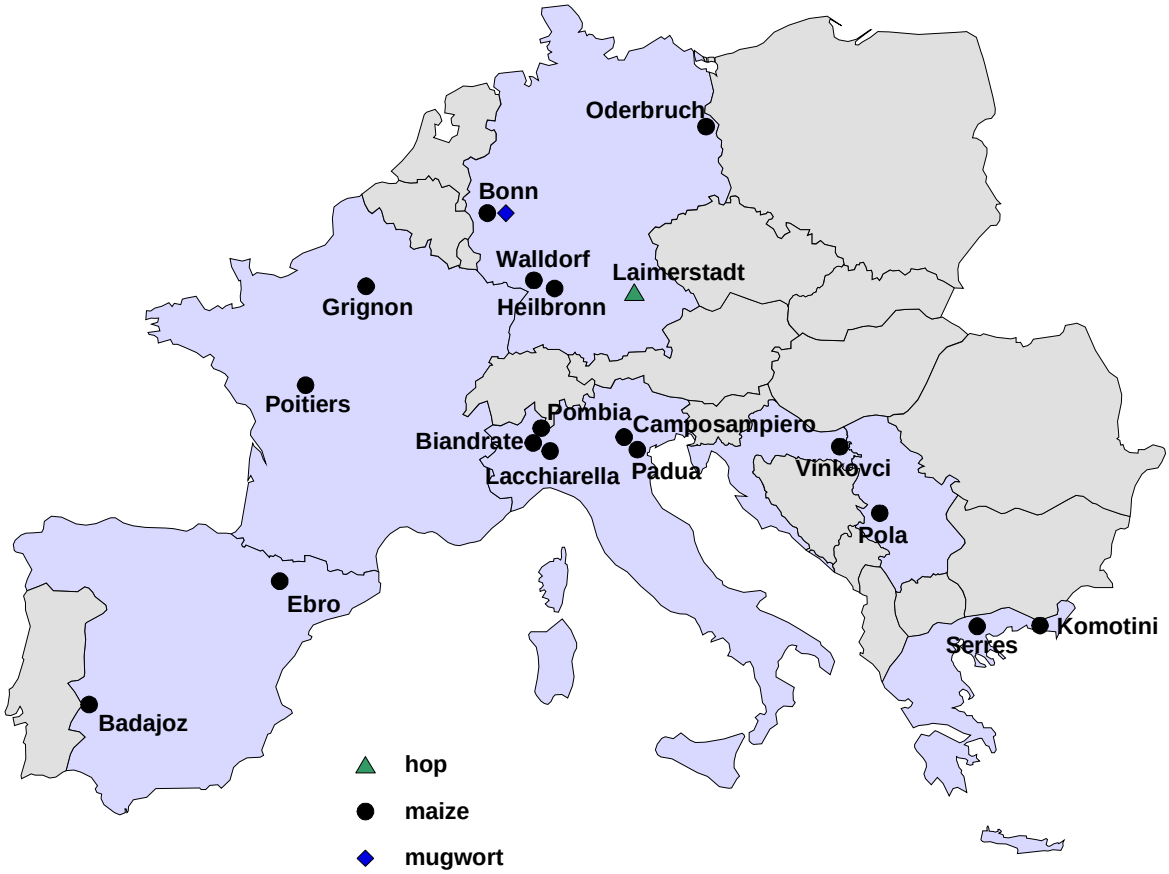

Figure 16. ECB sampling sites

4. 2. 2 Gas Chromatographic Analyses

(See Chapter 2).

4. 2. 3 Allozyme Analyses (TPI)

Horizontal starch gel electrophoresis was used to study the allele pattern of triose phosphate isomerase (TPI) in the different pheromone types of ECB in different geographic and host plant populations. TPI is located on the sex chromosome Z (Glover et al. 1990). Consequently females are hemiploid (ZW) for this locus, whereas males are diploid (ZZ). As TPI is a dimer, it exhibits a three-band pattern in heterozygous males, and a single-band pattern in females and homozygous males.

For electrophoresis each moth was homogenized in 100µl Tris-EDTA buffer (pH 6.8) after removing the head and the wings. Electrophoresis of the homogenates was carried out using a Tris-borate-EDTA (pH 8.6) buffer system (Pasteur et al. 1987). TPI allele patterns were revealed by staining with 1.5 ml TRIS (pH 8.6), 15 mg EDTA, 5 ml distilled H_2O, 1.2 ml

NAD (1%) and 1 ml sodium arsenate acid (1%) 500 ml MTT, 300 µl PBT and glyceraldehyde-3-phosphate dehydrogenase. (For details see Chapter 2).

4. 3 Results

4. 3. 1 Pheromone patterns

The GC pheromone results for all European ECB populations are given in Table 10. ECB individuals from maize populations in Italy and Greece were predominantly E-race, whereas those from German, French, Spanish, Serbian and Croatian maize populations were mostly Z-race. Z-race individuals also dominated a German hop population with only 1 hybrid out of 23 individuals. In the German mugwort population from Bonn all three pheromone types were present. Because these first analyses were done with a very advanced generation (35^{th} generation), new larvae were sampled in 2005 to analyse parental individuals. Thereby the previous result could be confirmed although, for technical reasons and a limited number of collected larvae, reliable results were only obtained for six individuals (Table 10). By contrast, individuals collected from maize approximately 5 km from the mugwort population were predominately Z-race.

Nearly all populations showed a considerable number of EZ-hybrids, except for those where only a small number of females of the parental generation could be analysed due to difficulties in breaking diapause. In the case of the population of Pombia (Italy), which were assigned to the E-race by analysing nine females in 2006, a second sampling one year later resulted in 28.6 % hybrids and even 2.9 % Z-pheromone composition by analysing a larger amount of females. Only the Pola (Serbia) and Poitiers (France) population gave a clear (100%) Z-pheromone result. The second French population (Grignon) showed 28.6 % hybrids and 4.8 % E-individuals.

Table 10. Pheromone frequencies of all tested ECB populations together with geographic and host plant origin and sampling year (la: larvae; em: egg masses; pu: pupae; N: number; x: unknown number as population was collected by a third party).

Population	Host plant	Year	N collected & life stage	Tested generation	N tested	Pheromone distribution in %		
						E	EZ	Z
Germany								
Bonn	mugwort	2001	x la	F_{35}	57	21.1	42.1	36.8
		2005	30 la	P	6	33.3	33.3	33.3
Bonn	maize	2001	x em	F_{35}	66	3.0	28.8	68.2
Oderbruch	maize	2004	1000 la	F_{11}	43	4.7	18.6	76.7
Heilbronn	maize	2004	200 em	F_{2-4}	37	0	27.0	73.0
Walldorf	maize	2005	1100 em	P; F_1	91	1.1	8.8	90.1
Laimerstadt	hop	2004	55 ad	P	23	0	4.3	95.7
Spain								
Ebro	maize	2004	200 la	F_{6-8}	56	3.6	12.5	83.9
Badajoz	maize	2004	1000 la	F_2	41	0	14.6	85.4
France								
Grignon	maize	2005	500 la	F_2; F_3	42	4.76	28.6	66.7
Poitiers	maize	2005	500 la	F_2; F_3	32	0	0	100
Italy								
Padua	maize	2005	300 pu	F_{2-4}	109	69.7	26.6	3.7
				F_{11}	115	62.6	33.0	4.4
Lacchiarella	maize	2005	500 la	F_2; F_3	74	71.6	25.7	2.7
Biandrate	maize	2006	145 la	P	7	100	0	0
Pombia	maize	2006	60 la	P	9	100	0	0
		2007	210 la	P; F_1	35	68.6	28.6	2.9
Camposampiero	maize	2007	300 pu	P; F_1	26	65.4	26.9	7.7
Serbia								
Pola	maize	2005	x la	F_4	16	0	0	100
Greece								
Serres	maize	2005	x la	F_4	29	62.1	34.5	3.4
Komotini	maize	2006	40 la	P	7	100	0	0
Croatia								
Vinkovci	maize	2006	60 la	P	4	0	0	100

Fifty individuals of the Camposampiero population collected in the field were used to set up 25 single matings, fourteen of which produced egg masses. The resulting pheromone patterns of the individuals in the F_1-generation is illustrated for each successfully mating pair in Figure 17. Six ECB pairs produced only E-individuals (mating number 2, 3, 8, 9, 14 and 16), six pairs produced E-individuals and hybrids (mating number 4, 7, 13 and 20) or only hybrids

(mating number 5 and 11) and two pairs produced hybrids and Z-individuals (mating number 22) or only Z-individuals (mating number 21). Consequently all pheromone types were present in the field population.

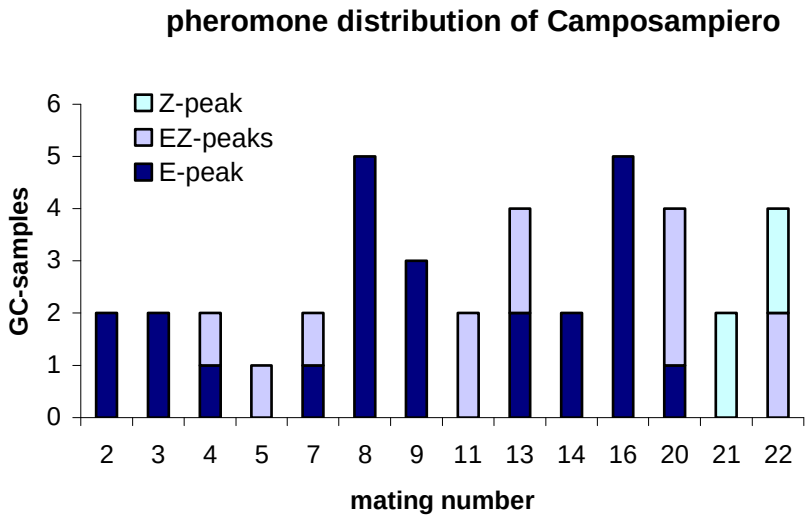

Figure 17. Pheromone distribution of the F_1-offspring from single matings of the Camposampiero field population

4. 3. 2 *Tpi*-allele frequencies

The distribution of alleles at the *Tpi* locus of ECB males is shown in Table 11. For the most populations more than 26 individuals were investigated, except the populations of Biandrate (17 individuals), Oderbruch (19) and Komotini (22). Individuals predominately belonging to the Z-race as classified by GC-analyses, were mainly heterozygous regarding the *Tpi* locus (*Tpi-1*/*Tpi-2*) and homozygous for the *Tpi-2* allele. However, some individuals were homozygous for the *Tpi-1* allele. The population from Pola showed more homozygous individuals for the *Tpi-1* allele than for the *Tpi-2* allele. The only populations without homozygous males for the *Tpi-1* allele were the populations from Laimerstadt collected from hop and the population from Badajoz, which showed even in the F_2-generation only homozygous individuals for the *Tpi-2* allele. *Tpi* results from three German populations (Oderbruch, Heilbronn and Walldorf) revealed the existence of a third allele (Table 11 and 12), designated here as the *Tpi-3* allele, which has not been described for populations from other European countries. Some German individuals were heterozygous for the *Tpi*-1 and the *Tpi*-3 allele, one ECB from Walldorf was homozygous for *Tpi-3*, and others carry the *Tpi*-2 and the *Tpi*-3 allele. According to the GC-analysis the distribution of *Tpi*-alleles in

populations belonging to the E-race the situation is converse compared to the Z-race. Individuals from most populations are either heterozygous regarding the *Tpi* locus or homozygous for the *Tpi-1* allele. Only from the Serres and the Pombia population showed more homozygous individuals for the *Tpi-2* allele than for the *Tpi-1* allele.

Table 11. *Tpi* allele frequencies of males from different populations (120-band = *Tpi*-1 allele (E); 100-band = *Tpi*-2 allele (Z); 80-band = *Tpi*-3 allele; G: generation; N: number of individuals).

Population, G.	**N**	***Tpi***					
		80	80/100	100	100/120	120	80/120
Z-race:							
Heilbronn, F_2	30	0.000	0.167	0.300	0.433	0.033	0.067
Oderbruch, F_9	19	0.053	0.158	0.526	0.105	0.105	0.053
Walldorf, P	30	0.033	0.000	0.433	0.433	0.100	0.000
Bonn, F_{35}	32	0.000	0.000	0.750	0.188	0.062	0.000
Laimerstadt, P	30	0.000	0.000	0.767	0.233	0.000	0.000
Ebro, F_6	30	0.000	0.000	0.733	0.233	0.033	0.000
Badajoz, F_2	30	0.000	0.000	1.000	0.000	0.000	0.000
Poitiers, F_2	30	0.000	0.000	0.567	0.400	0.033	0.000
Grignon, F_2	30	0.000	0.000	0.600	0.367	0.033	0.000
Pola, F_4	30	0.000	0.000	0.200	0.567	0.233	0.000
E-race:							
Serres, F_4	30	0.000	0.000	0.267	0.600	0.133	0.000
Komotini, P	22	0.000	0.000	0.000	0.591	0.409	0.000
Lacchiarella, F_2	30	0.000	0.000	0.000	0.400	0.600	0.000
Padua, P	44	0.000	0.000	0.159	0.409	0.432	0.000
Camposampiero, P	29	0.000	0.000	0.069	0.483	0.448	0.000
Biandrate, P	17	0.000	0.000	0.059	0.706	0.235	0.000
Pombia, P	26	0.000	0.000	0.424	0.346	0.231	0.000

Table 12 shows the distribution of *Tpi* alleles for ECB females from different populations which had previously been classified according to their pheromone types (E; E/Z; Z) by GC-analyses. Because females are hemiploid (ZW) at the *Tpi* locus they exhibited only one *Tpi*-allele, either the *Tpi-1* allele or the *Tpi-2* allele, or in the case of some German populations the *Tpi-3* allele. The population from Bonn collected from mugwort is not included because of the occurrence of all pheromone types to equal rates. Therfore no pheromone race classification was possible. Females of all three pheromone types carried the *Tpi-2* allele more frequently than the *Tpi-1* allele (< 3-fold).

Table 12. *Tpi* allele frequencies of females from different populations which were classified by GC according to their pheromone (120-band = *Tpi*-1 allele (E); 100 = *Tpi*-2 allele (Z); 80 = *Tpi*-3 allele).

population	Pheromone	N	*Tpi*		
			80	100	120
Z-race:					
Heilbronn	E	-	-	-	-
	EZ	4	0.000	0.250	0.750
	Z	18	0.167	0.444	0.389
Oderbruch	E	2	0.500	0.500	0.000
	EZ	6	0.167	0.833	0.000
	Z	31	0.129	0.806	0.065
Walldorf	E	3	0.000	0.667	0.333
	EZ	17	0.000	0.765	0.235
	Z	14	0.000	0.857	0.143
Laimerstadt	E	-	-	-	-
	EZ	-	-	-	-
	Z	10	0.000	1.000	0.000
Ebro	E	2	0.000	1.000	0.000
	EZ	7	0.000	0.714	0.286
	Z	30	0.000	0.833	0.167
Poitiers	E	-	-	-	-
	EZ	-	-	-	-
	Z	28	0.000	0.607	0.393
Grignon	E	2	0.000	1.000	0.000
	EZ	11	0.000	1.000	0.000
	Z	21	0.000	0.762	0.238
Pola	E	-	-	-	-
	EZ	-	-	-	-
	Z	17	0.000	0.235	0.765
E-race :					
Serres	E	16	0.000	0.125	0.875
	EZ	7	0.000	0.571	0.429
	Z	1	0.000	1.000	0.000
Lacchiarella	E	25	0.000	0.400	0.600
	EZ	16	0.000	0.062	0.938
	Z	1	0.000	0.000	1.000
Padua	E	10	0.000	0.500	0.500
	EZ	10	0.000	0.700	0.300
	Z	4	0.000	0.500	0.500
Pombia	E	9	0.000	0.444	0.556
	EZ	-	-	-	-
	Z	-	-	-	-

In total 189 females pheromone-typed by GC as Z-race showed a *Tpi-1* to *Tpi-2* allele distribution of 1 : 3 (Table 13), whereas 98 females classificated as E-race by GC showed a *Tpi-1* : *Tpi-2* allele distribution of nearly 2 : 1 (Table 14).

Table 13. *Tpi* allele frequencies of GC-analysed females belonging to the Z-race from Heilbronn, Oderbruch, Laimerstadt, Poitiers, Grignon, Ebro, and Pola. (N: number; 120-band = *Tpi*-1 allele; 100-band = *Tpi*-2 allele; 80-band = *Tpi*-3 allele)

N of GC-analysed females	pheromone composition	N = GC-results	% *Tpi-1*	% *Tpi-2*	% *Tpi-3*	*Tpi-1*:*Tpi-2*
214			25.23	71.03	3.74	1 : 3
	EZ	45	20.00	77.78	2.22	1 : 4
	Z	169	26.63	69.23	4.14	1 : 3

Table 14. *Tpi* allele frequencies of GC-analysed females belonging to the E-race from Lacchiarella, Pombia, Padua, and Serres. (N: number; 120-band = *Tpi*-1 allele; 100-band = *Tpi*-2 allele)

N of GC-analysed females	pheromone composition	N = GC-results	% *Tpi-1*	% *Tpi-2*	*Tpi-1*:*Tpi-2*
92			65.22	34.78	1.9 : 1
	E	59	66.10	33.90	2 : 1
	EZ	33	63.64	36.36	1.8 : 1

4. 4 Discussion

In the past several studies on distribution and mating of the two pheromone races were performed using different methods like pheromone traps, flight tunnel experiments, receptor studies, allozyme studies, and/or GC-analysis. However, many GC-studies were based on the analyses of only a few individuals, and studies using pheromone traps or flight tunnel experiments did not provide unequivocal results. It has been reported that hybrid females from reciprocal crosses produced an intermediate E11-/Z11-14:OAc pheromone blend of 65:35, but that males responded equally to blends containing 3-65% of the E-isomer (Roelofs et al. 1987; Glover et al. 1991). Furthermore Glover et al. (1990) described that E-males were much less fixed in their behavioral response than were Z-males, with 30% of the E-males

responding to the hybrid blend (65:35 E/Z), whereas none of the Z-males responded to this blend. F_1 individuals (E x Z crosses) did not predominantly respond to the hybrid blend, but rather at intermediate levels to a variety of blends. A significant portion of the F_1 males will respond to the Z-blend, but very few hybrids respond to the E-blend. Consequently, because hybrid ECB males seem to be attracted to pheromone traps containing the Z-lure as well as hybrid lure, pheromone traps are no appropriate means to estimate interpopulational hybridization or to assign the pheromone-response genotype for captured individual males. Additionally, combined flight tunnel and single cell electrophysiological studies presented 'unusual' males whose antennal olfactory cells responded to the Z-blend, but whose behavioral response was that of an E-male (Cossé et al. 1995).

In this study several European ECB populations were investigated regarding their pheromone blends by GC-analysis. Most populations which were analysed with a sample size greater then 30 (Bonn, Oderbruch, Walldorf, Ebro, Grignon, Padua, Lacchiarella, Serres) contained at least a few females with the opposite pheromone blend. However these results do not clearly express the pheromone blend situation in the field because some populations reared in the laboratory were used for GC-analysis in advanced generations. Hence, the necessity for long-distance mate location by pheromone is eliminated under laboratory conditions. Laboratory conditions may even induce mating of different pheromone types. Therefore only the analysis of the parental generation gives unequivocal proof of the pheromone patterns in the field. Nevertheless, the occurrence of hybrids already in the 2^{nd} generation, is a fair indication that some hybrids or individuals of the opposite blend were already present in field populations, especially when a large amount of individuals were collected. In general the results show a higher percentage of hybrids in E-race population than in Z-race populations, when not only very few females were investigated (Table 10). An explanation for this could be that E-males were much less fixed in their behavioral response than were Z ones, as described by Glover et al. (1990). Futhermore field and laboratory data indicated that hybridization may occur when E-males court and mate with Z-females (Liebherr and Roelofs 1975; Glover et al. 1991). Noticeable is the difference of the pheromone distribution for the two French populations, which were both analysed by GC with females of the F_2/F_3 generation. The population of Grignon revealed some hybrids and even a small percentage of E-individuals in contrast to the population of Poitiers which consistently showed only the pheromone blend for the Z-isomer. Hence the distribution of E- and Z-individuals in French ECB populations varied strongly between different locations.

A reason for this could be that both, maize and mugwort, are substantially infested by ECB in Northern France, whereas infested mugwort in western parts of France is generally not present or rare (Martel et al. 2003). However, in previous studies it was postulated, that not only in the region of Grignon but throughout France, a clear separation between E- and Z-race existed, so that ECB feeding on maize predominantely belonged to the Z-race, whereas ECB feeding on the alternative host plants mugwort or hop belonged to the E-race (Thomas et al. 2003; Bethenod et al. 2004; Pelozuelo et al. 2004). A similar situation was described for the larch budmoth, *Zeiraphera diniana*. In this species, two sympatric host races have been reported, one feeding on larch (*Larix decidua*), the other feeding on cembran pine (*Pinus cembra*), (Emelianov et al. 1995). The females of each race produce a blend of E11-14OAc and E9-dodecenyl acetate (E9-12:OAc), but in inverse ratios. Larch-race females produce close to 100% E11-14OAc with traces of E9-12:OAc, whereas pine-race females produce a 1:1000 ratio of E11-14OAc to E9-12:OAc. Males responded strongest to the pheromone blend of their own race (Priesner and Baltenweiler 1987). Another example of specialization to different host plants is the apple maggot, *Rhagoletis pomonella* (Diptera: Tephritidae). Studies suggested that *R. pomonella* is undergoing sympatric speciation (i.e. divergence without geographic isolation) in the process of shifting and adapting to a new host plant (Feder et al. 2003). In North America, *R. pomonella* infested the fruits of native hawthorn species (*Crataegus spp*.), but after the introduction of cultivated apple (*Malus pumila*) in the mid-1800s the fly evolved a sympatric race on apple. Apple fly adults eclose on average 10 days earlier than sympatric hawthorn lies, reflecting the approximately three-week-earlier mean fruiting phenology of apples compared to haws (Feder et al. 1993; Feder 1995). Differences in emerging times of ECB regarding “host-plant races” were also observed in France (Thomas et al. 2003), which could be an explanation for local assortative mating of ECB. The French “mugwort-race” moths emerged on average 10 days earlier than the “maize-race” moths. It was also observed that males of both races emerged earlier than females, so that there may be an overlapping time period for E-race females to mate with Z-race males. However, an argument against would be that Z-males are attracted only by the Z-pheromone and not by another pheromone blend (Roelofs 1987; Glover 1990). An interesting difference regarding the fitness of ECB and *R. pomonella* hybrids is reported by Linn et al. (2004). Reciprocal F_1 hybrids between apple and hawthorn host races of *R. pomonella* do not respond to host fruit volatiles in wind-tunnel assays at doses that elicit maximal direct flight in parental flies. The reduced ability of hybrids to respond to fruit volatiles could result from a conflict between neural pathways for preference and advoidance behaviors, and it suggests that hybrids might

suffer a fitness disadvantage in finding fruits in nature. By contrast ECB hybrids did not exhibit any obvious reduction in fitness in the laboratory (Liebherr and Roelofs, 1975).

GC results in this study for the German populations from mugwort and hop displayed a completely different situation in comparison with the French populations. There was no maize field in the vicinity of the area where stalks from mugwort infested with ECB were collected. Differences in moth emergence pattern could not be observed in German populations. Moths from mugwort emerged at the same time as other moths of German populations collected from maize fields and showed all three pheromone types (Table 10). Another surprising result was the detection of the Z-phenotype in hop. Individuals from hop were collected using a light-trap and thereafter females were analysed by GC. Although maize was cultivated close to the hop cultivation area, and ECB individuals from the maize plants could also be attracted, it was obvious that ECB infested both host plants, maize and hop, in equal measure without a host preference. This is confirmed by historical evidence. From 1880 to 1942 *O. nubilalis* caused great damage in hop in Germany (Andersen 1943), whereby it was reported that maize cultivated next to the hop fields was not infested by ECB. At that time, scientists already pointed out that two races of ECB exist, one of which infested hemp (*Cannabis sativa*) and hop, but not maize, and vice versa (Zwölfer 1928; Schlumberger 1931; Zattler 1939; Hampp and Jehl 1940). Since 1953, no further reports regarding the infestation of hop by ECB are known. But in 2002 hop was again infested by ECB which caused between 5 and 25% yield loss. It was observed, that hop was only infested when maize was infested by ECB in close vicinity. In the region where samples for this study were collected, farmers reported that ECB damage started when maize cultivated less than 500 m from the hop fields was not ploughed after harvest. Hence in contrast to past hop infestation it seems that the previous host preference changed in German ECB populations. However, in southern France hop plants were infested by the E-strain of *O. nubilalis*, even if maize was found to be exclusively infested by the Z-strain (Pelozuelo et al. 2004). But it is to be considered that in France ECB individuals were collected at wild hop plants, in contrast to the German population which originated from a cultivated hop area. Population genetic studies using allozymes or microsatellites on ECB populations feeding on different host plants showed genetic differentiation between most populations on the respective hosts (e.g. Martel et al. 2003; Bontemps et al. 2004, Leniaud et al. 2006). But recently, in one region in southern France the genetic structure of populations found on hop plants displayed intermediate allele frequencies (Malausa et al. 2007), which was explained by the presence of both peromone races (E and Z)

on the same host. The *R. pomonella* apple-infesting race showed consistent allele frequency differences compared to the hawthorn-infesting race at six allozyme loci mapping to three different chromosomes. Alleles at all six of these allozymes correlated with the timing of adult eclosion, an event dependent on the duration of the overwintering pupal diapause (Feder et al. 2003).

In the case of ECB it has been postulated that E-strain populations are fixed for the *Tpi-1* allele, whereas Z-strain populations are segregating for both *Tpi-1* and *Tpi-2* at intermediate frequencies, with *Tpi-2* being the more common allele (Glover et al. 1991). Consequently it was assumed that the *Tpi* gene may be a "speciation gene". The *Tpi* allozyme locus and the locus for male pheromone response (*Resp*) are both Z-linked (Glover et al. 1990). Therefore the two loci may map close to each other on the Z chromosome. Dopman et al. (2004) reported that close physical linkage and/or low recombination of loci could explain the apparent reduction of introgression in spite of an ongoing hybridization. Furthermore, a recent selective sweep at the locus for the male response could initially have driven differentiation at the *Tpi* locus via genetic hitchhiking. Therefore it was expected that females for example belonging to the E-race would carry the E *Tpi*-allele which consequently their progeny would inherit. Most of our results did not show this affiliation. Only a trend of a higher frequency of the *Tpi-1* allele in E-race individuals and a higher frequency of the *Tpi-2* allele in the Z-race was confirmed. On average a ratio of 2 : 1 for *Tpi-1* : *Tpi-2* resulted for the E-race (Table 15). This was not due to testing advanced generations produced under laboratory conditions, because mostly individuals of the parental generation were analysed for the *Tpi* allele except for Serres (F_4) and Lacchiarella (F_2) (Table 11). In summary, there was no obvious pattern indicating a correlation between the *Tpi* allele predicting male behavioral response and the pheromone blend of females.

A consistent result was obtained for French populations (Martel et al. 2003). *Tpi* analyses of French populations did not show a genetic differentiation of the two host groups mugwort and maize but displayed a highly significant deficit of heterozygotes at 16 sampling sites (Martel et al. 2003). Dopman et al. (2004) mapped *Tpi* at an area of reduced recombination 28.1 ± 4.1 cM from the gene for male response (*Resp*), which was located close to the opposite end of the Z chromosome in an area of higher recombination. This distance contradicted the hypothesis that patterns of differentiation at *Tpi* are explained by tight linkage to this "speciation gene". Furthermore *Tpi* allele analyses in this study revealed the existence of a

third allele in several German populations (Oderbruch, Heilbronn and Walldorf) (Table 11 and 12), designated here as the *Tpi-3* allele, which has not been described in populations from other European countries. According to Dopman et al. (2005) ECB moths carrying the *Tpi-1* allele (which moves faster than the *Tpi-2* allele during electrophoresis) have got an asparagine at amino acid residue 189, whereas moths carrying the *Tpi-2* allele have a lysine in this position. They suggested that the *Tpi-2* allele may be recently derived within the Z-strain because related moth species (Asian corn borer and *H. virescens*) also have an asparagine at residue 189. The *Tpi-1* allele is therefore held to be the original allele. In this study another modification of the enzyme must have taken place which caused an even slower migration on the gel than the *Tpi-2* allele. In France only the mugwort race showed a third *Tpi* allele. However, this allele does not seem to be equivalent to the German *Tpi*-3, because Thomas et al. (2003) described it as a (faster) 130-band and not a 80-band as found for the German populations.

Conclusion

Overall our results, and also the results of some French ECB populations (Martel et al. 2003), indicated that the *Tpi* locus cannot be used reliably as a marker to distinguish the two different pheromone races. Especially not in the case of the German populations which revealed a third, so far unknown *Tpi* allele. It has to be established whether this mutation is due to another substitution at amino acid residue 189 or whether a substitution at another amino acid residue took place. Especially investigations regarding pheromone races in ECB populations of hop seemed to be interesting because according to reports of the "Bavarian regional authority of farming" (LfL 2003) heavy damage of ECB in hop in the "Hallertauer" region, the importantest hop region in Germany, were caused by insects which originated from maize plants in 2002. Furthermore in a flight tunnel study by Bengtsson et al. (2006) with different host plants, significantely more Z strain females landed on a hemp (*Cannabis sativa*) plant than on an adjacent corn plant. This is very interesting, because in the past ECB infested hop and hemp, but not maize in the German "Hallertauer" region.

In general, E-/Z-strain differentiation can be confirmed, except for the German population from mugwort, but mating between the two races is more frequent in some EU areas than origianally postulated. Therefore, in areas of mating, the corresponding race from other host

plants than maize may act as reservoir of Bt susceptible individuals, regarding the high-dose/refuge strategy.

The GC-results in this study showed that it is insufficient to determine the pheromone composition of only a few females per population for a general conclusion. In Table 10 it is shown that nine females of the Pombia population expressed exclusively the E-pheromone in 2006, but in a sample just one year later at exactly the same site the P/F_1 generation contained a considerable number of hybrids and even one female with the opposite Z-phenotype. In France ECB females from pepper and cocklebur produced exclusively the Z-pheromone blend (Leniaud et al. 2006), but only a few females of each of these host plants were tested. The only reliable way to a better idea of the mating behaviour of ECB and the distribution of the pheromone types on different host plants is to analyse a reasonably large number of individuals of the parental generation collected from field populations.

Chapter 5: Gene flow in the European corn borer *Ostrinia nubilalis*

5. 1 Introduction

Resistance evolution in pests is a significant and increasing problem in crop cultivation. Insects have evolved resistance to all classes of synthetic chemical insecticides (Georghiou 1986), causing significant social cost including increased use of environmentally harmful pesticides (Pimentel et al. 1980).

The European corn borer, *Ostrinia nubilalis*, one of the most important maize pest in Europe and the USA causes high yield losses. Since 1996 an established method to control the European corn borer in North America is the cultivation of Bt-maize, which produces its own lepidoteran-specific toxin. However, insects are also expected to evolve resistance to these transgenic insecticidal crops (Gould 1998). Diminished susceptibility of this pest could be observed under high selection pressure in the laboratory (Bolin et al. 1999; Chaufaux et al. 2001; Siqueira et al. 2004; Pereira et al. 2008) but not in the field until now. Nevertheless methods should be developed to delay or avoid a possible resistance development in the field. In way of an insect resistance management (IRM) the high-dose/refuge (HDR) strategy currently seems to be the best method to delay resistance of pest insects in crops producing *Bacillus thuringiensis* toxins (Bt-crops), (Alstad and Andow 1995; Andow 2008). The strategy requires high levels of gene flow between pest individuals feeding on transgenic and those feeding on refuge non-Bt-plants (Bourguet et al. 2000a, 2000b). This way potential resistant individuals from Bt-crops will most likely mate with susceptible individuals from non-Bt crops. A crucial question in this context regards the level of gene flow between the two different pheromone races of *O. nubilalis*, the E- and the Z-race. Previously, it has been postulated that strong assortative mating occurs between the respective pheromone race individuals (Bethenod et al. 2004; Malausa et al. 2005), which should result in a corresponding pattern of genetic differentiation. Population genetic patterns can be revealed by analysing the allozyme polymorphism in a species. So far the population structure of several migrant Noctuidae species has been investigated (Daly and Gregg 1985; Pashley et al. 1985; Korman et al. 1993; Buès et al. 1994), but few studies concerned the allozyme variability of ECB (Harrison and Vauter 1977; Cardé et al. 1978; Glover et al. 1991). Bourguet et al. (2000a and 2000b) carried out a detailed study on the genetic differentiation and the rates of gene flow among ECB populations in France. They found little genetic differentiation among ECB populations from northern to southern France which may

therefore constitute a single large panmictic unit with high rates of gene flow. Consequently, the probability of resistance, which was estimated to be less than 9.2×10^{-4} with 95% probability, may be characteristic of the entire *O. nubilalis* population in France (Bourguet et al. 2003). However, as the above study covered only a limited geographical range of the distribution of ECB, the results should be confirmed on a much larger scale within Europe. In this study allozyme analyses of ECB have been carried out testing populations from different European countries and different pheromone races in order to illustrate the patterns of genetic differentiation on different spatial scales and of different pheromone races.

5. 2 Materials and Methods

5. 2. 1 ECB Sampling and Rearing

Field samples of ECB consisted either of diapausing larvae, pupae and adults, or of egg masses collected in light trap cages. All sampling sites are illustrated in Figure 18. Larvae from Muret, Pola, Komotini, Serres and Georgia were provided by other scientists. In total 22 ECB populations were tested, originating from Germany (N = 6), Italy (5), France (3), Spain (2), Greece (2), Serbia (1), Croatia (1), Austria (1) and Georgia, Asia (1). All populations were collected from maize plants, except for the Laimerstadt population which was collected from hop. As an "outgroup" for genetic distance and genetic identity estimates, one population of *Sesamia nonagrioides*, the Mediterranean corn borer (MCB, Lepidoptera: Noctuidae), was collected from Poitiers (France). At this location both, ECB and MCB, could be found in the same maize field. For the ECB rearing procedures see Chapter 2.

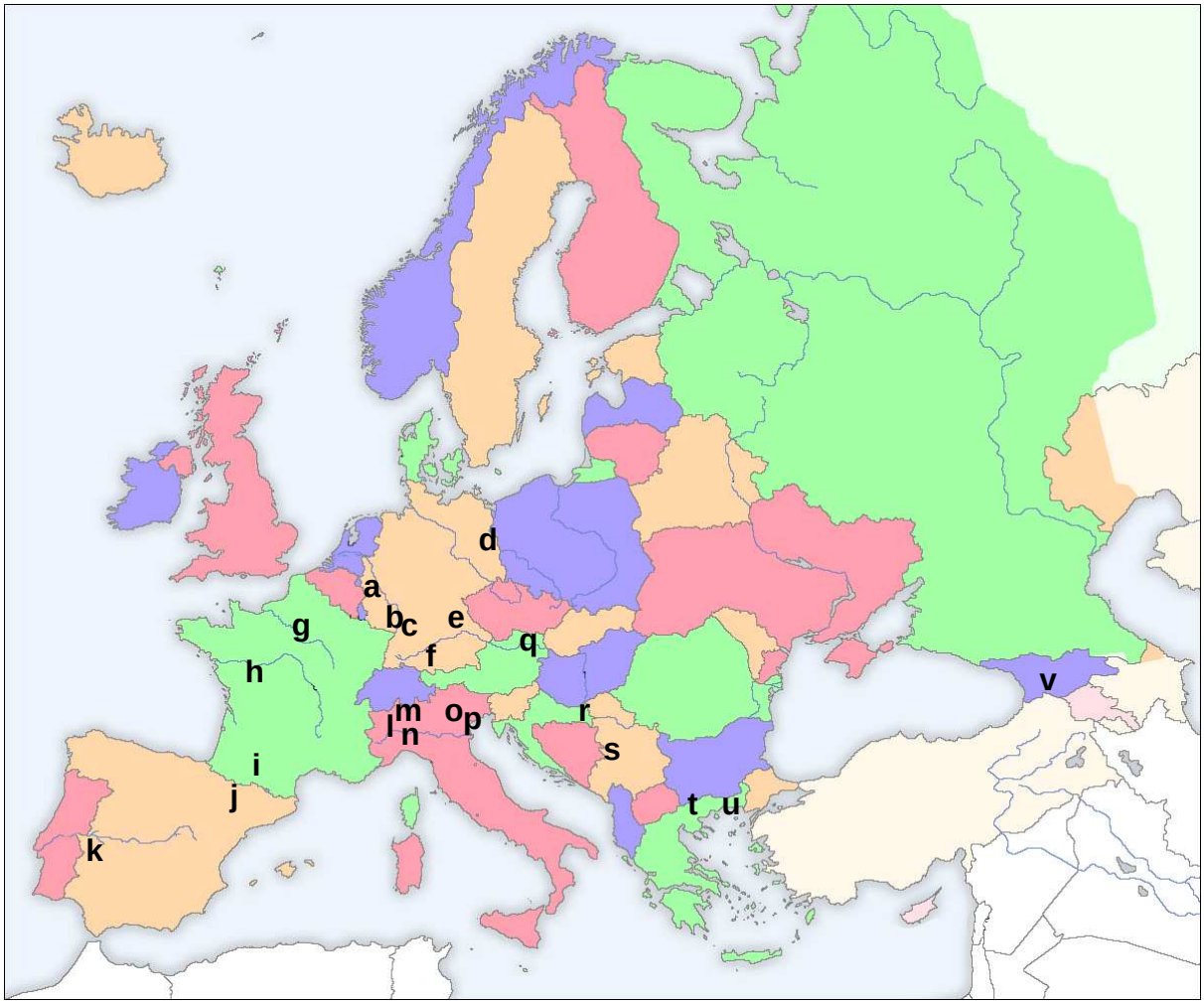

Figure 18. ECB sampling sites, (a: Bonn, b: Walldorf, c: Heilbronn, d: Oderbruch, e: Laimerstadt, f: Bodensee, (all Germany); g: Grignon, h: Poitiers, i: Muret, (all France); j: Ebro, k: Badajoz, (both Spain); l: Biandrate, m: Pombia, n: Lacchiarella, o: Camposampiero, p: Padua, (all Italy); q: Tulln (Austria); r: Vinkovci (Croatia); s: Pola (Serbia); t: Serres, u: Komotini, (both Greece); v: Georgia)**. Source:** www.vivasalsa.eu (Christian Domingo Hamann).

5. 2. 2 Allozyme Analyses

Horizontal starch gel electrophoresis was carried out to study ECB allozyme patterns using five polymorphic enzyme systems: TPI (triose phosphate isomerase), GPI (glucose-phosphate-isomerase), PGM (phosphogluco-mutase), AAT (aspartate-amino-transferase) and HBDH (3-Hydroxybutyrate-dehydrogenase) in the different geographic populations. TPI is located on the sex chromosome Z (Glover et al. 1990), so that ECB females are hemiploid (ZW) at this locus, whereas males are diploid (ZZ). Therefore only males were used for allozyme analysis. After removing the head and the wings each moth was homogenized in 100µl Tris-EDTA buffer (pH 6.8). Electrophoresis of the homogenates was carried out using

Tris-borate-EDTA (pH 8.6) buffer systems (Pasteur et al. 1987). For methodological details see Chapter 2.

5. 2. 3 Data Analyses

For each population the allele frequencies, the mean number of alleles, the observed heterozygosity, the gene diversity and the F_{is}-values were estimated using FSTAT 2.9.3. P-values referring to deviations from the Hardy-Weinberg equilibrium (HWE, probability test) were obtained with GENEPOP 4.0.7 (Raymond and Rousset 1995; Rousset 2008). This probability test is the "exact HW test" (Haldane 1954; Weir 1996; Guo and Thompson 1992). The F-statistics of Wright (1965) subdivides the total genetic variability into the two components F_{is} (variability within a population) and F_{st} (variation among populations) (Goudet 2001). Thus, F_{is} is a measure for a heterozygosity deficit (positive values) or a heterozygosity excess (negative values). The genetic structure of the total population, for each of the five loci and over all loci, was analysed by testing for genotypic differentation by using exact tests and computing the estimator θ of F_{st} according to Weir and Cockerham (1984) using GENEPOP 4.0.7 (Raymond and Rousset 1995). The among-sites analysis were carried out at different geographical areas: Germany (Heilbronn, Walldorf, Oderbruch, Bonn, Bodensee, Laimerstadt), Italy (Pombia, Camposampiero, Padua, Lacchiarella, Biandrate), France (Grignon, Poitiers, Muret), Spain (Ebro, Badajoz) and Greece (Serres, Komotini). A dendrogramm based on Nei's (1978) genetic distance was calculated by the UPGMA method ("unweighted pair-group method using arithmetic averages") with POPGENE (modified from the NEIGHBOR procedure of PHYLIP 3.5, Felsenstein 1993).

Nei's index for genetic distance:

$$I_n = -\log_e \frac{[\sum_{i=1}^{m} (P_{ix} P_{iy})]}{[(\sum_{i=1}^{m} P_{ix}^2) \cdot (\sum_{i=1}^{m} P_{iy}^2)]^{(\frac{1}{2})}}$$

P_{ix} = frequency of allele *i* in population *x*
P_{iy} = frequency of allele *i* in population *y*
m = number of alleles at the specific locus

Nei's index for genetic identity:

$$D_n = e^{-I_N}$$

As an "outgroup" MCB was included into the analysis. The utilization of an "outgroup" is essential to obtain a "rooted tree" (Koch and Jung 1997; Hedrick 2000). Pairwise comparisons

of all populations to each other were performed to calculate estimates of genetic identity and genetic distance.

5. 3 Results

Three alleles were observed for the *Tpi* locus, four for *Gpi*, three for *Aat*, four for *Pgm* and two for *Hbdh*. The overall allele frequencies are given in Table 15. There were several rare alleles. The first of the *Tpi* alleles was only present in three German populations (Heilbronn, Walldorf and Oderbruch), the fourth *Gpi* allele was carried by only one individual of the Pombia population. The fourth *Pgm* allele was observed only in two individuals of the Walldorf population and in one of the Camposampiero population. At the *Hbdh* locus the second allele was only observed in two populations (Padua and Muret).

Table 15. Overall allele frequencies, weighted by sample size (all W) and non-weighted (all UW).

Locus	allele	all W	all UW
TPI	1	0.012	0.014
	2	0.580	0.603
	3	0.408	0.383
GPI	1	0.089	0.091
	2	0.643	0.639
	3	0.267	0.269
	4	0.001	0.001
PGM	1	0.015	0.016
	2	0.940	0.936
	3	0.043	0.045
	4	0.002	0.002
AAT	1	0.134	0.142
	2	0.771	0.768
	3	0.094	0.091
HbdH	1	0.997	0.997
	2	0.003	0.003

The mean number of alleles per population obtained in this study ranged from 1.2 to 2.6 (Table 16). Except for the Georgian population, the observed average degree of heterozygosity and of gene diversity were similar for all tested population and ranged from 0.167 to 0.380 and 0.164 to 0.380, respectively. The population from Georgia was very homogenous with no polymorphism except for the *Gpi* locus where five of 51 individuals

were heterozygous with a second allele. This is reflected in the lowest mean number of alleles of all populations, and a very low observed heterozygosity (0.021) and gene diversity (0.019) (Table 16). The highest values of gene diversity and observed heterozygosity (both 0.380) were observed for the population from Heilbronn (Germany).

The estimated F_{is}-values did not indicate a large excess or deficit in heterozygotes in any of the populations. Across the five loci only two of 22 populations showed significant deviations from Hardy-Weinberg expectations (Padua: p = 0.027; Komotini: p = 0.008, Table 16).

Table 16. Within-sample polymorphism (n: sample size; A: mean number of alleles; H_0: observed heterozygosity; H_s: gene diversity; F_{is} –values; and p-values referring to Hardy-Weinberg equilibrium (HWE) probability test).

Population	**n**	**A**	**H_o**	**H_s**	**F_{is}**	**p**
Germany:						
Heilbronn	30	2.2	0.380	0.380	-0.029	0.806
Walldorf	30	2.6	0.267	0.257	0.015	0.379
Oderbruch	19	2.2	0.256	0.258	0.124	0.371
Bonn	32	2.2	0.233	0.260	0.141	0.576
Bodensee	20	2.2	0.210	0.270	0.147	0.358
Laimerstadt	30	2.2	0.313	0.287	-0.091	0.126
Italy:						
Pombia	26	2.6	0.338	0.304	-0.005	0.364
Camposampiero	29	2.4	0.253	0.284	0.040	0.981
Padua	44	2.6	0.314	0.328	0.122	0.027
Lacchiarella	30	1.6	0.287	0.247	-0.192	0.608
Biandrate	17	2.0	0.346	0.294	-0.125	0.664
France:						
Grignon	30	2.0	0.253	0.214	-0.109	0.999
Poitiers	30	2.0	0.273	0.230	-0.248	0.434
Muret	29	2.0	0.206	0.296	0.242	0.080
Spain:						
Ebro	30	1.8	0.253	0.238	-0.055	0.442
Badajoz	30	1.6	0.089	0.087	0.001	0.831
Greece:						
Serres	30	1.6	0.276	0.222	-0.199	0.529
Komotini	22	2.0	0.309	0.294	0.001	0.008
Serbia:						
Pola	30	1.8	0.167	0.164	0.052	0.381
Croatia:						
Vinkovci	19	2.2	0.207	0.252	0.165	0.215
Austria:						
Tulln	28	2.4	0.237	0.237	0.182	0.096
Georgia:	51	1.2	0.021	0.019	-0.044	-

In Table 17 the θ-values over all loci estimated at a regional scale are listed. The estimates ranged from 0.060 (German populations) to 0.363 (Spanish populations). Negative θ-values resulting in two cases reflect minor inaccuracies of the algorithm and should be interpreted as no genetic differentiation between the respective populations. In a few cases (*Pgm*, *Hbdh*) no θ-values were given because only one allele occurred in all individuals of the group.

Table 17. Within- and between-country genetic differentiation of ECB populations estimated as θ (= estimator of F_{st}) for each locus.

	Tpi	*Gpi*	*Pgm*	*Aat*	*Hbdh*	all
All populations						
θ	0.361	0.178	0.173	0.161	0.023	0.234
German populations						
θ	0.059	0.059	0.037	0.109	-	0.069
Italian populations						
θ	0.076	0.145	0.047	0.167	-0.0074	0.123
French populations						
θ	0.322	0.131	0.023	0.191	0.035	0.206
Spanish populations						
θ	0.134	0.466	-	0.273	-	0.363
Greek populations						
θ	0.126	-0.012	0.089	0.107	-	0.071

All estimated values for the genetic identity and genetic distance (Nei, 1978) are listed in Appendix A. As an "outgroup" the Mediterranean corn borer *S. nonagrioides* was used. The lower the values for the genetic identity, the more remotely the populations are related. By contrast, the lower the values for the genetic distance, the closer the populations are related. For *O. nubilalis* the values for the genetic identity ranged from 0.804 (Muret / Ebro) to 0.996 (Poitiers / Bodensee and Georgia /Badajoz), the values for the genetic distance ranged from 0.004 (Badajoz / Georgia) to 0.219 (Muret / Ebro). In comparison the values of the estimates for *S. nonagrioides* were much lower, and higher, respectively (0.191 to 0.411, mean value: 0.279; 0.889 to 1.655, mean value: 1.297). Mean values of genetic distances were 0.040 for all German populations, 0.069 for all Italian populations, and 0.085 for all German populations compared with all Italian populations.

In Figure 19 a dendrogramm is given based on Nei's (1978) genetic distances between all tested ECB populations. Overall there was low genetic differentiation of the *O. nubilalis* populations, which did not result in a pattern reflecting their geographical location.

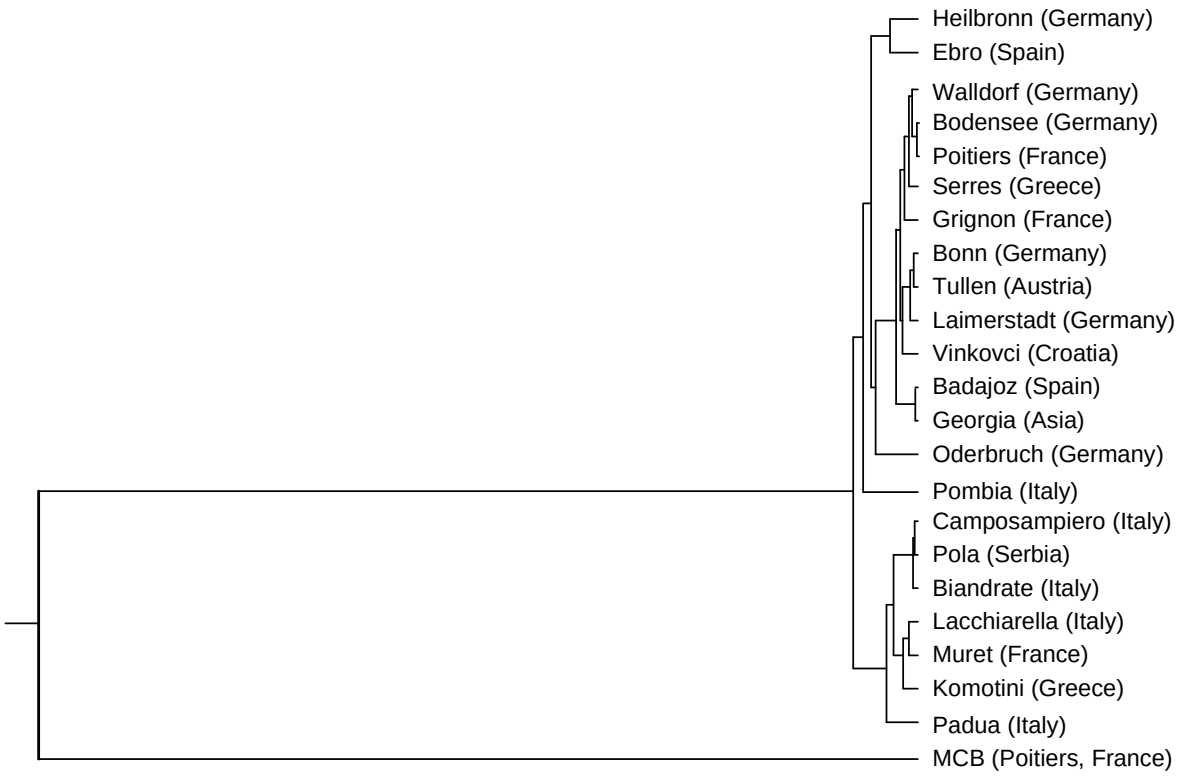

Figure 19. Dendrogramm according to the UPGMA method (Nei 1978), whereby horizontal lines illustrate the relative genetic distances of the populations to each other.

5. 4 Discussion

All genetic estimates obtained from the allozyme data in this study indicated a low genetic differentiation of *O. nubilalis* populations in Europe. This is visible in the regional and overall θ-estimates for the population groups given in Table 17, which were low considering the wide geographic range of the populations. In the dendrogram derived from the genetic distances of the populations no geographic clusters of populations from the same country appeared, which suggested that there was no geographic differentiation. Therefore it can be concluded that there are high rates of gene flow between the populations. The θ-value (0.206) obtained for the French populations in this study cannot be compared with previously reported θ-estimates (Bourguet et al. 2000a, 2000b), because in their study populations from many areas were pooled for the θ-estimates. Furthermore, the number of alleles for the examined loci were not only consistently higher in the Bourguet et al. (2000a) studies than the number identified in our study, but differed amoung the two French allozyme studies (Bourguet et al. 2000a, 2000b).

In this study no obvious difference in the observed heterozygosity, the gene diversity (Table 16) and the θ-values (Table 17) could be attributed to a differentiation of the two known sex pheromone races in *O. nubilalis*. The choice of tested populations included individuals from both, the E- and Z-race from maize and one population from hop, which according to previous studies is pure E-race (Malausa et al. 2005). Previous gas chromatographic analyses (see Chapter 4, all populations except Tulln and Georgia) indicated that the Italian and Greek populations have to be assigned to the E-race, whereas all other populations belonged to the Z-race, even the German hop population. This confirmed the results of several other allozyme studies on the genetic variation within and between the E- and Z-race of ECB in the United States, that there is a very small amount of differentiation across the genome (Harrison and Vawter 1977; Cianchi et al. 1980; Glover et al. 1990). Furthermore it was postulated that *Tpi* was the only allozyme locus displaying obvious differences in the allele frequencies of the pheromone races (Glover et al. 1990).

However, the above results are not consistent with those of previous allozyme studies for French ECB populations, which revealed a genetic differentiation at the *Tpi* locus along the different pheromone races (whereby E-race individuals originated exclusively from mugwort or wild hop plants, Bourguet et al. 2000b). It was therefore suggested that there is restricted gene flow between the pheromone races. But even among the French studies, results regarding a genetic differentiation at the *Tpi* locus in the different pheromone races, were not consistent (e. g. Martel et al. (2003) in contrast to Bethenod et al. (2004)).

These discrepancies in the results on pheromone race differentiation may partly be due to different allele patterns at the *Tpi* locus for European and American ECB populations. For the USA it is reported that E-strain populations were fixed for the *Tpi-1* allele, whereas Z-strain populations were segregating for both *Tpi-1* and *Tpi-2* at intermediate frequencies, with *Tpi-2* being the more common allele (Glover et al. 1990, 1991). This is rarely the case in European populations (see Chapter 4). In terms of the genetic distances (Nei 1978) no significant differences could be observed for populations of different pheromone races or different host plants. Harrison and Vawter (1977) found that Nei's genetic identity for the two pheromone strains was 0.977, which matches the range of the results in this study.

The conclusion resulting from this study that there is high gene flow between ECB populations corresponds to estimates on the dispersal capacity of *O. nubilalis*. Release-

recapture experiments with marked adults demonstrated, that ECB males and females were able to disperse 23 to 49 km, whereas some of them were recovered 14 km from the release site within 100 min after release (Showers et al. 2001).

The critical issues concerning gene flow between the pheromone races of ECB might also concern their origin from different host plants. All used E-race populations in this study originated from maize plants, in contrast to the French studies where E-race populations were taken from mugwort or hop. Therefore, for further studies, it will be of interest to explore in more detail whether there is a difference in gene flow rates of Z-race populations compared to E-race populations originating both from maize plants, and in gene flow rates of Z-race populations originating from maize plants compared to verified E-race populations originating from other host plants, e. g. from hop or mugwort. The results obtained in this study could not detect a significant difference in the genetic identity and genetic distance (Nei 1978) between Z- and E-race populations both originating from maize and E-race populations originating from maize and the Z-race population originating from hop (Laimerstadt).

Gene flow within and between ECB populations is a key component for the sustainability of transgenic insecticidal maize. For a successful implementation of the HDR strategy it is necessary that resistant individuals of Bt-crops will mate randomly with susceptible individuals of the non-Bt-crops. In this study an overall high gene flow could be confirmed. Considering these results, there seems to be no support for the concern of Gould (1998) that the spatial distribution of Bt-crops and refuges may be too large relative to adult movements so that most resistant individuals surviving on Bt-plants might not move far enough to mate with susceptible moth and conversely.

Appendix A

Nei's measures of genetic identity (above diagonal) and genetic distance (below diagonal), (Nei, 1978), (blue marked values = highest values; red marked values = lowest values).

	Hei 1	**Wall 2**	**Oder 3**	**Bonn 4**	**Bod 5**	**Laim 6**	**Pom 7**	**Cam 8**	**Pad 9**	**Lacc 10**	**Bian 11**	**Gri 12**
1	---	0.976	0.921	0.944	0.970	0.959	0.925	0.962	0.934	0.904	0.960	0.946
2	0.024	---	0.926	0.965	0.983	0.973	0.938	0.982	0.918	0.890	0.975	0.978
3	0.082	0.077	---	0.961	0.948	0.924	0.863	0.929	0.944	0.866	0.904	0.908
4	0.058	0.036	0.039	---	0.989	0.986	0.929	0.952	0.900	0.842	0.920	0.975
5	0.030	0.017	0.054	0.011	---	0.985	0.937	0.972	0.910	0.876	0.945	0.984
6	0.042	0.028	0.079	0.014	0.015	---	0.954	0.940	0.883	0.815	0.915	0.985
7	0.078	0.064	0.148	0.074	0.065	0.048	---	0.937	0.893	0.852	0.911	0.972
8	0.039	0.018	0.074	0.050	0.028	0.062	0.065	---	0.946	0.952	0.990	0.964
9	0.069	0.086	0.058	0.105	0.094	0.125	0.113	0.055	---	0.951	0.947	0.881
10	0.101	0.117	0.144	0.173	0.133	0.204	0.160	0.050	0.050	---	0.960	0.847
11	0.041	0.025	0.101	0.083	0.057	0.089	0.093	0.010	0.055	0.042	---	0.933
12	0.056	0.022	0.097	0.025	0.016	0.015	0.029	0.037	0.126	0.167	0.069	---
13	0.025	0.006	0.068	0.024	0.005	0.022	0.069	0.021	0.095	0.125	0.041	0.017
14	0.108	0.099	0.091	0.137	0.117	0.182	0.162	0.042	0.031	0.016	0.037	0.150
15	0.045	0.099	0.136	0.095	0.060	0.070	0.110	0.117	0.134	0.189	0.149	0.096
16	0.079	0.036	0.076	0.016	0.015	0.028	0.107	0.059	0.170	0.212	0.097	0.026
17	0.024	0.012	0.083	0.057	0.024	0.051	0.064	0.012	0.062	0.076	0.022	0.034
18	0.054	0.065	0.131	0.131	0.088	0.133	0.090	0.027	0.039	0.017	0.023	0.102
19	0.064	0.026	0.076	0.067	0.045	0.090	0.107	0.007	0.067	0.057	0.013	0.058
20	0.047	0.039	0.047	0.015	0.012	0.039	0.110	0.037	0.102	0.123	0.064	0.046
21	0.040	0.032	0.031	0.009	0.010	0.015	0.070	0.049	0.076	0.160	0.081	0.030
22	0.093	0.041	0.077	0.028	0.026	0.049	0.145	0.034	0.179	0.214	0.097	0.045
23	**0.889**	**1.148**	**1.120**	**1.401**	**1.310**	**1.169**	**1.258**	**1.417**	**1.090**	**1.500**	**1.300**	**1.379**

	Poi 13	**Mur 14**	**Ebro 15**	**Bada 16**	**Ser 17**	**Kom 18**	**Pola 19**	**Vin 20**	**Tul 21**	**Geo 22**	**MCB 23**
1	0.975	0.897	0.957	0.924	0.977	0.948	0.938	0.954	0.961	0.911	**0.411**
2	0.994	0.906	0.906	0.965	0.989	0.937	0.984	0.962	0.969	0.960	**0.317**
3	0.934	0.913	0.873	0.927	0.920	0.877	0.927	0.955	0.970	0.926	**0.326**
4	0.976	0.872	0.909	0.984	0.944	0.877	0.936	0.985	0.991	0.972	**0.246**
5	**0.996**	0.889	0.942	0.985	0.976	0.916	0.956	0.989	0.990	0.974	**0.270**
6	0.979	0.834	0.933	0.972	0.950	0.876	0.914	0.962	0.985	0.952	**0.311**
7	0.933	0.851	0.896	0.898	0.938	0.914	0.899	0.896	0.932	0.865	**0.284**
8	0.979	0.959	0.890	0.943	0.989	0.973	0.993	0.963	0.952	0.938	**0.243**
9	0.909	0.970	0.875	0.844	0.940	0.962	0.935	0.903	0.927	0.836	**0.337**
10	0.883	0.984	0.828	0.809	0.927	0.983	0.945	0.884	0.852	0.807	**0.223**
11	0.960	0.964	0.862	0.907	0.978	0.978	0.987	0.938	0.923	0.907	**0.273**
12	0.983	0.861	0.909	0.975	0.967	0.903	0.943	0.955	0.970	0.957	**0.252**
13	---	0.896	0.931	0.981	0.988	0.927	0.968	0.978	0.980	0.973	**0.286**
14	0.110	---	**0.804**	0.838	0.931	0.967	0.965	0.899	0.880	0.844	**0.226**
15	0.071	**0.219**	---	0.887	0.925	0.882	0.847	0.918	0.940	0.860	**0.378**
16	0.020	0.176	0.120	---	0.942	0.851	0.937	0.978	0.970	**0.996**	**0.207**
17	0.012	0.071	0.078	0.060	---	0.967	0.979	0.951	0.960	0.935	**0.310**
18	0.076	0.034	0.125	0.162	0.033	---	0.958	0.902	0.897	0.842	**0.289**
19	0.033	0.036	0.166	0.066	0.022	0.043	---	0.951	0.935	0.942	**0.219**
20	0.022	0.107	0.086	0.023	0.050	0.103	0.050	---	0.976	0.973	**0.220**
21	0.020	0.128	0.062	0.030	0.041	0.109	0.068	0.025	---	0.958	**0.308**
22	0.027	0.170	0.151	**0.004**	0.068	0.173	0.060	0.027	0.043	---	**0.191**
23	**1.251**	**1.487**	**0.972**	**1.576**	**1.170**	**1.241**	**1.517**	**1.515**	**1.178**	**1.655**	---

Chapter 6: General discussion

The cultivation of Bt-plants offers several advantages in comparison to other methods of controlling the ECB, e. g. less or no non-target organism will be killed due to reduced insecticide spraying, lower costs, higher effectiveness and the avoiding of disadvantages caused by other methods. Mechanical methods such as ploughing to destroy the larvae and bury the maize splits deeply into the soil are also effective, but frequently the local soil or weather conditions do not allow effective tillage operations. Disadvantages of Bt-spraying formulations are the quick degradation of the Bt-toxins and the spores through UV-light, the easy elution by rain and the low effectiveness against larvae which already entered the stem (High et al. 2002). In the German Oderbruch area different control methods (Insecticide, *Trichogramma*, Bt-maize) referring to the local conditions were tested in 2000-2002. Bt-maize lines achieved the highest efficiency close to 100% in controlling the European corn borer. Insecticide treatment reached effiency rates ranging from 60% to 90%. The application of *Trichogramma* under local conditions in the Oderbruch area was not satisfactory at all (Schröder et al. 2006).

Plant transformation with *Cry* genes provides an exciting new approach to insect control in which transgenic plants produce their own protective proteins (Van Rie et al. 1989). But obviously, the extent to which insects are able to develop resistance to Bt-toxins will be an essential condition for the sustainable use of these insecticides. Therefore effective insect resistance management (IRM) is essential to prevent the resistance development of the target insects to Bt-toxins in the field. IRM contains detecting changes in susceptibility through regular monitoring and to apply resistance management strategies to prevent or delay adaption by pests (Gould 1998). One of the major strategies of Bt resistance management is the “high-dose/refuge” strategy. Until now in North America the high-dose/refuge strategy seems to be the most promising method to delay a resistance development of target insects to Bt-crops. The IRM plan is working and therefore the advantages of Bt-plants can be used.

6. 1 Baseline susceptibility to Bt-toxin Cry1F

In this study the baseline susceptibility against the Bt-toxin Cry1F has been established for ECB populations from main European maize cultivation areas. Such data are necessary for a sustainable cultivation of Bt-maize expressing the Cry1F toxin in Europe. To detect a development of resistance it is essential to know beforehand how susceptible insects are for

the specific toxin before they become exposed to it. Therefore baseline susceptibility bioassays need to be performed for each toxin with a range of different populations. The results of Cry1F susceptibility in Chapter 3 showed small, but biologically irrelevant differences in the susceptibility between populations. The variation in susceptibility can be due to different factors (e.g. methodical causes, different toxin batches and stability of Bt-toxins, natural genetic variation):

1. Methodical factors, such as the use of specific mortality criteria (because lower LC_{50} estimates might result if larvae with severe growth inhibition are considered as dead (Marçon et al. 1999)), and changes of the abiotic conditions which can also happen in the laboratory. Different mortality endpoints are very critical for a comparison of the susceptibility data from different studies. The assessment of when a larvae is considered lethally affected is also critical. The common rule, that larvae are lethally affected when they do not show any reaction when prodded does not always lead to a correct result. Furthermore neonate ECB larvae can have different degrees of fitness. Larvae with a lower fitness than others can be recognized by a slower movement and will have a lower chance to survive during the bioassay. Additionally it is not known, if neonates may be affected by being placed into the wells by a brush. One of the major critical issues concerning Bt-protein bioassays is the used protein application method. In this study surface application was used as opposed to the alternative, the incorporation method. For the latter, the toxin was mixed in the just prepared heated diet, which means great care has to be taken that the temperature of the diet will not affect the insecticidal activity of the incorporated protein. In the diet overlay approach of the surface method, protein mixtures can be applied at room temperature avoiding the above problem. Additionally, the amount of protein used in the diet overlay is much lower, enabling sustainable and cost-effective use of protein batches. One of major drawbacks of surface method, however, is the ability of some larvae to bore through the thin toxin layer thus avoiding its insecticidal effect. Whichever approach is used, constant attention to quality and consistency in the methodology is vital for comparisons across bioassays in a monitoring program designed to detect changes in sensitivity through time. In any case, for a world-wide comparison of baseline susceptibility data to Cry-toxins it is necessary to rely on one method. Only then it will be possible to restrict the number of population analyses by bioassays, when the baseline susceptibility of different populations falls into a similar range.

2. Protein source factors: Different batches of Bt-toxin with different toxin concentration and quality could cause quite large differences in the baseline susceptibility estimates, as reported by Saeglitz et al. (2006). In the case of the Bt-toxin Cry1F the possibility of instability during storage is given, because Hernandez and Ferré (2005) reported that the Cry1F toxin deteriorated during storage. Crucial for the durability of the Cry-toxins are not only the storage conditions during the tests, but already the conditions during the transport to bioassay laboratory.

3. Biological factors: The natural genetic variation in and among the tested populations causes a certain degree of variability in the LC-values, particularly when the populations cover a large geographic range.

Susceptibility differences similar to this study were observed in bioassays for the Cry1Ab toxin. González-Nunez et al. (2000) established the baseline susceptibility to Cry1Ab toxin of several ECB populations, including populations from Madrid and Ebro. The obtained results present a LC_{50} value of 109 ng/cm^2 (Ebro). Farinos et al. (2004), however, obtained LC_{50} values in the range of 9-34 ng/cm^2 for the Ebro population. This difference between the two studies was explained by using different toxin batches. The order of magnitude of the latter Spanish values (Farinos et al. 2004) were comparable to LC_{50} values obtained in bioassays with the Cry1Ab toxin for US populations, which ranged from 2.22 to 7.89 (Carozzi and Koziel 1997; Marçon et al. 1999), and also to German populations which resulted in a LC_{50} of 3.61 with the Cry1Ab toxin obtained from the Monsanto company (Saeglitz et al. 2006). By comparing the North American LC_{50} values (1.23 – 6.23) in this study for Cry1F with LC_{50} values (2.22 to 7.89) obtained with Cry1Ab of Marçon et al. (1999), it seems that there is no biologically relevant difference in the level of toxicity, as resistance development is defined by an increase of the 500- to 1000-fold susceptibility.

A standard laboratory ECB strain should be established and provided to each lab involved in the testing for a future standardisation of susceptibility testing. This lab-strain should be tested at certain time intervals parallel to field-collected populations. Emerging fluctuations between estimates from different laboratories can then be included as a factor, with the result that different laboratories can be compared. A condition for this would be, that this lab-strain needs to be reared for several generations, and susceptibility testing shall be performed with the same toxin batch. Bioassay data from the laboratory at Aachen (RWTH) University

showed no changes in the susceptibility even in the 35th generation. Additionally, as mentioned in Chapter 3, it is required to perform the bioassays under strictly standardised conditions (temperature, bioassay procedure, toxin charges and criteria for mortality) to allow a comparison of different populations and different Cry-toxins.

In conclusion, the baseline susceptibility in all ECB populations studies is high enough to apply the HDR strategy with the given (high) amount of Bt-toxin expression in Cry1F maize event 1507.

6. 2 Pheromone races differentiation

This study confirmed that the two known pheromone races do not show strict preference for a single host plant. Instead, there seems to be considerably variation in host plant / pheromone race interaction across locations in Europe. In particular, this study investigated the population structure of the ECB regarding 1. the different pheromone races, 2. their spatial distribution, 3. the adaptation of the races to specific host plants and 4. their suitability to serve as an alternative refuge apart from non-Bt-maize. In principle, refuges should decrease the exposure of target organism to Bt and therefore minimize an adaption of the insects to transgenic maize through selection. Hence a development of resistant insects would be delayed (McGaughey and Whalon 1992; Tabashnik 1994; Ramachandran et al. 2000).

Regarding the distribution of the different pheromone races it was postulated that the Z-race predominates in most of the range in Europe and North America, whereas the E-race is found in Switzerland, Italy and Eastern North America, (Klun and Huettel, 1988; Mason et al. 1996). In North America (e.g. Glover et al. 1990) and Switzerland (Pena et al. 1988) both E- and Z- populations infested maize plants. In France, it is reported that ECB feeding on maize predominantely belonged to the Z-race, whereas ECB feeding on e.g. mugwort or hop belonged to the E-race (Thomas et al. 2003; Bethenod et al. 2004; Pelozuelo et al. 2004).

This study demonstrated that the present ‘ECB isolation’ situation given in France regarding so-called “host-races” cannot be conferred to other ECB infesting areas. By means of gas chromatographical analyses, the ECB population of Laimerstadt (Germany) sampled from a hop-infested area belonged to the Z-race and not to the E-race as in France, whereas ECB in France were colleced from wild hop plants in contrast to the German ECB, which were

collected from cultivated hop. But recently even in one region in France the genetic structure of populations found on hop plants displayed intermediate allelic frequencies (Malausa et al. 2007) which was explained by the presence of both pheromone-races (E and Z) on the same host. Additionally host-plant experiments with a pure Z-race confirmed the acceptance of cultivated hop plants (Gaspers et al.; in preparation). Prelimary plant choice experiments with three different plants (maize, mugwort and hop) showed that Z-females of *O. nubilalis* use maize and hop plants to nearly the same degree for oviposition. By contrast the mugwort plant was nearly ignored by the females and few egg masses were found. On the other hand females of a predominant E-population, collected from maize in Italy, prefered the cultivated hop plants for oviposition. Maize and mugwort were chosen in a similar range. In contrast, host plant experiments in France showed that individuals of the E-race collected from presumably wild hop prefer mugwort for oviposition when maize and mugwort were offered together (Bethenod et al. 2004). If these data were regarded necessary for further development of HDR strategies, it would be necessary to perform detailed experiments with pure E- and Z-race individuals, verified by GC, e.g. on the mating behavior and the possible specialization on different host plants. Especially the finding that ECB belonging to the Z-race infest cultivated hop plants in Germany, but not wild hop plants in France has to be investigated.

In conclusion, it can be assumed with high scientific certainty that the ECB populations are polyphagous on maize, hop and mugwort host plants. This polyphagous nature supports the possibility of delaying resistance development against Bt-maize if mating between maize populations and other host plant populations is more widespread than assumed until now.

6. 3 Gene flow

In principle, the HDR strategy will only be successful if crucial assumptions are met. Resistance must be inherited as a recessive trait (Sayyed et al. 2000) and resistance alleles must be rare ($p < 10^{-3}$) in the insect population (Andow et al. 2000), so that there will be only few homozygous survivors ($p^2 < 10^{-6}$) (Roush 1994) which will mate with the susceptible insects of non-Bt-refuges. Therefore, for delaying the development of resistance of the ECB to the *B. thuringiensis* toxins in Bt-maize a high level of gene flow between ECB feeding on transgenic and refuge plants is required (Bourguet et al. 2000a, 2000b).

To estimate the frequency of resistance alleles in an insect population the F_2-screen is a flexible used methodology. This screening method involves 1. sampling mated adult females from natural populations and establishing isofemale lines; 2. rearing and sib-mating F_1 progeny in each isofemale line; 3. screening F_2 neonates for survival on Bt-maize leaf tissues; and 4. confirming the resistance on commercial Bt-maize plants. For some European countries the frequency of resistance alleles to Bt-maize (Cry1Ab) of *O. nubilalis* was determined by means of the F_2-screen by Engels et al. (in preparation), which yielded frequencies below 4×10^{-4} for Germany, below 3×10^{-4} for France, below 3.1×10^{-3} for Italy, and below 9×10^{-3} for Slovakia. For other insect pest species such as *Heliothis virescens* (Lepidoptera: Noctuidae; Gould et al. 1997) and *Pectinophora gossypiella* (Lepidoptera: Gelechiidae; Tabashnik et al. 2000) higher initial frequencies of 1.5×10^{-3} were estimated regarding Cry1Ac cotton. The higher initial frequency in the case of *H. virescens* emphasises the need for caution in deploying transgenic cotton to control insect pests (Gould et al. 1997).

Gene flow within and between populations of *O. nubilalis* represents a key factor in the sustainable use of transgenic insect-resistant maize plants (Bourguet et al. 2000a). The gene flow between the two pheromone races, the E- and the Z-race, is of particular significance regarding potential refuges. In this study the genetic estimates obtained from the allozyme data indicated a low genetic differentiation of *O. nubilalis* populations in Europe and therefore a high gene flow can be assumed, even between populations of the different pheromone races. According to this result each wild or cultivated plants attacked by *O. nubilalis* regardless their pheromone composition could serve as refuge areas or at least complete refuges of non Bt-maize.

One difficulty in estimating gene flow by allozyme analyses is, among others, the subjective interpretation of allele banding patterns. This was obvious in the studies of Bourguet et al. (2000a, 2000b) which described deviating allele patterns. Both studies were done with ECB from several areas in France, but revealed different numbers of alleles for the same allozymes, (first study: number of alleles: TPI (4), GPI (7), PGM (5), AAT (5), MPI (8), HBDH (4); second study: TPI (2), GPI (4), PGM (6), AAT (4), MPI (4), HBDH (2)). There is a number of problems advising caution for the interpretation of the results of protein electrophoresis. Banding patterns on gels can sometimes be complicated by the formation of conformational allozymes that are actually multiple forms of the same gene product. These subbands have different mobilities because they differ in secondary or tertiary structure (Richardson et al.

1986). The electrophoretic mobilities of protein products can also be affected by polymorphic modifier loci, the physiological condition of the organism, or by environmental effects (Conkle 1971; Harry 1983; Lebherz 1983; Womack 1983). An additional technical source of variation is often the varying quality of the starch matrix provided by chemical companies, which is well known amoung electrophoresis experts.

A pervasive problem of protein electrophoresis is that it fails to detect even a majority of the genetic variation existing at the nucleotide level, as only about one-third of nucleotide substitutions result in amino acid changes (of which about 25 % are detectable by elecrophoresis; Nei 1987). For populations and species that have diverged recently or that have undergone recent or recurrent bottlenecks, a major limitation of protein electrophoresis can be that insufficient polymorphism exists for an accurate assessment of population structure (Baker 2000).

In conclusion, allozymes techniques still have value for studies on gene flow, but results do not show a high resolution and might be negatively affected by transferability between different test laboratories. However, there is sufficient evidence from this study that gene flow is high enough in ECB populations to to support the HDR strategy.

6. 4 Importance of and requirements on the HDR strategy

As stated above, for the high-dose/refuge strategy to be effective, specific assumptions must be met. 1. Plant tissue must express high toxin concentrations during the whole growing season to ensure that insect genotypes heterozygous for the Bt resistance are killed. 2. Resistance must be inherited as a recessive trait (Sayyed et al. 2000) and the frequency of resistance alleles must be low, less than 1×10^{-3} (Andow et al. 2000). 3. A refuge of non-Bt-plants provides enough homozygous susceptible individuals to mate randomly with the presumably few homozygous resistant individuals (Alstad and Andow 1995). The strategy works best if the dose of toxin ingested by insects on Bt-plants is high enough to kill all or nearly all of the hybrid progeny of matings from the rare resistant individuals from Bt-plants with the abundant susceptibile individuals from nearby refuges of host plants without Bt-toxins (Gould 1998; Tabashnik 2004). If a high dose is not achieved, resistance can be delayed by increasing refuge abundance, which lowers the proportion of the population

selected for resistance to compensate for survival of hybrid progeny on Bt-plants (Gould 1998; Tabashnik 2004).

Greenhouse experiments showed that without refuges and the high-dose standard, the western corn rootworm, *Diabrotica vergifera vergifera* rapidly evolved resistance to transgenic corn producing Bt-toxins Cry34Ab and Cry35Ab (Meihls et al 2008; Lefko et al 2008). Recently, field-evolved resistance to Bt-corn has been reported for 2 additional caterpillar pests: *Busseola fusca* resistance to Cry1Ab-producing corn in South Africa (Van Rensburg 2007) and *Spodoptera frugiperda* resistance to Cry1F-producing corn in Puerto Rico (Matten et al 2008). In these cases, it seems that failure to achieve the high-dose standard and to implement adequate refuges may have hastened resistance (Van Rensburg 2007; Matten et al 2008; Tabashnik 2008). For example, Van Rensburg (2007) reported that some maize producers in South Africa for practical reasons are reluctant to include refugia inside irrigated plantings and regard susceptible plantings provided under rain fed conditions in the immidiate vicinity of irrigated plantings as refugia. Moths possible give preference to irrigated maize, which could have contributed to increased selection pressure towards the development of resistance to the Bt-toxin (Van Rensburg 2007).

This African case and also the greenhouse experiments document how important it is to maintain high-dose and refuge standards and to public and share new information to improve the HDR-strategy to guarantee a delaying of the development of resistance. The case of the American cotton bollworm *Helicoverpa zea* to Bt-cotton producing Cry1Ac is consistent with the theory underlying the refuge strategy because this resistance is not recessive (Tabashnik et al. 2008), and therefore it was expected that *H. zea* evolved resistance faster than other pests. This nonresessive inheritance of resistance means, the concentration of Cry1Ac in Bt-cotton is not high enough to kill the hybrid offspring produced by matings between susceptible and resistant *H. zea* and thus, the “high-dose” requirement is not met (Gould 1998; Tabashnik et al. 2008).

Regarding a cultivation of Cry1F expressing maize it was shown, that the HDR strategy is essential for delaying an adaption by *O. nubilalis*. Pereira et al. (2008) selected a laboratory strain of *O. nubilalis* which displayed a more than 3000-fold resistance to Cry1F and concentration-response bioassays of reciprocal parental crosses indicated that the resistance is autosomal and recessive. Furthermore greenhouse experiments with Cry1F-expressing maize,

showed that the F_1 progeny of susceptible by resistant crosses had fitness close to zero. Therefore it is of enormously importance to provide refuge areas.

Bt-toxin-free refuge areas, where alleles conferring susceptibility to the Bt-toxin would be kept at high frequency in pest populations not exposed to the toxin's selection pressure should be set in close enough vicinity to Bt-fields (<800 m; United States Environmental Protection Agency, 2001), so that random mating among adults from Bt-fields and refuges will dilute resistance alleles within the global population (Leniaud 2006). Refuges can be Bt-toxin-free maize fields or alternative host plants. However, the density reached by the ECB hosted on alternative plants should be approximately 500 susceptible individuals for every resistant moth that might emerge from Bt-maize fields – according to the United States Environmental Protection Agency (2001). The results in this study (Chapter 4) show that theoretically cultivated hop plants could be the first alternative host plant which could provide the desired ratio of susceptible over resistant ECB and therefore serve as a suitable refuge. Furthermore, the pheromone classification of the analysed hop population (Z-race) confirms that cultivated hop host individuals which will mate freely with adults emerging from maize infested with ECB of the Z-race which is a condition for the success of the HDR strategy (Gould, 1998; Losey et al. 2001). However, in the absence of protection against pest attacks, hop yield may be expected to be low and not cost-effective for the hop farmers. Wild hop plants can not be considered as refuges, even if as it transpired that wild hop could also be infested by the Z-race. Wild hop is indeed common in almost whole middle-Europe, but forms only larger groups of plants, but no stocks. Hence single groups of wild hop will host only low densities of ECB larvae compared with maize. Therefore, they may not replace non-Bt-maize refuges in the framework of the HDR resistance-delaying strategy, but merely complement them. The same is assumed for further altenative host plants (e. g. sunflower, sorghum, cocklebur, and pepper) which are also hosts for the Z-race (Leniaud 2006).

In conclusion, there is sufficient evidence and supporting data available to recommend strongly the application of HDR strategy for the cultivation of Cry1 F maize in the EU.

6. 5 Prospects

The goal of insect resistance management is to delay or prevent the occurence of control failures from resistance by delaying or preventing the evolution of resistance (Andow 2008).

The most favoured strategy for managing resistance to Bt-crops is the combination of two independet concepts to delay development of resistance: high expression of the cry gene and the use of refuges. For this strategy to be effective resistance has to be recessive, the susceptible individuals have to outnumber the resistant survivors, the refuges have to be at an appropriately close distance from all Bt-plants, sexual maturity of resistant and susceptible insects must be reached more or less synchronically and mating between them must be at random, the initial frequency of resistance alleles must be low, and the toxin concentration in plants has to be high enough to kill all resistant heterozygous insects (Andow 2002). If these conditions are fulfilled, practically all resistant individuals will mate with susceptible ones, producing heterozygous offspring which will die upon exposure to the Bt-plants (Ferré et al. 2008).

The difficulty is to make IRM practicable for every farmer who is cultivating Bt-crops, especially in small-scale cropping systems characteristic of many developing or emerging countries. Small scale farmers have little land, capital or economic flexibility to bear the costs of IRM individually. That means, the costs associated with implementing IRM must be considered in setting the IRM strategy (Andow 2008). This was possibly the problem in the case of the field-evolved resistance of *B. fusca* in South Africa. Refuge areas were not include inside irrigated plantings maybe to reduce costs.

To monitor insect resistance it is necessary to observe areas of cultivated Bt-crop. Unexpected damage to a Bt-crop could result from a number of different factors, with insect resistance development being but one of the possibilities. Any unexpected damage report must be carefully investigated. Yearly monitoring will be able to detect small shifts in resistance gene frequency prior to the oneset of wide spread crop failures (Ferré et al. 2008). Baseline data is collected on the target field insect population, preferable before the first introduction of a Bt-crop, but at least during the initial years of launch prior to high market penetration (Sims et al. 1996; Siegfried et al. 2000; Siegfried and Spencer 2000). Following the establishment of the baseline data, annual sampling of field insect populations should be linked to regions of the high product sales, which would present the highest risk of resistance development. (Ferré et al. 2008).

If a development of resistance can be detected early, measures can be taken in sufficient time to avoid an expansion of developed resistance alleles in insect populations. Control of *H. zea*

has been augmented by insecticide sprays and by rapidly increasing use of transgenic cotton that produces the Bt-toxins Cry2Ab as well as Cry1Ac (Tabashnik et al. 2008; Gassmann et al. 2009). In the case of the evolved-field resistance of *S. frugiparda*, Bt-corn producing Cry1F has been voluntarily withdrawn from the market in Puerto Rico (Tabashnik et al. 2008).

All insights which were gained in the past regarding effectivness of IRM and regarding the first documented cases of field-evolved resistance to Bt-crops can help to keep a development of insect resistance in check and to sustain the effiancy of current and future generations of transgenic crops. Furthermore resistance management has to remain adaptive to provide a way to change IRM strategies and tactics as new information and experience becomes available, so that resistance risks can be managed to preserve the usefullness of transgenic insecticidal crops into the future (Andow 2008).

Resistance management strategies, relying on a 'high dose/refuge strategy', have been endorsed in several countries (Alstad and Andow 1995; Bates et al. 2005; Andow 2008; Bravo and Soberón 2008). Until now, the EFSA GMO Panel considers that appropriate insect resistance management strategies are capable of delaying possible onset of resistance in field conditions (EFSA 1507 opinion for cultivation). Looking at the data generated in this study, there is no reason to alter the successful HDR strategy.

7. Conclusions

Currently the most effective method to avoid high yield losses in maize caused by *O. nubilalis* seems to be the cultivation of Bt-maize. Controlling *O. nubilalis* with transgenic insecticidal maize plants provides considerable advantages compared to other methods. But a sustainable use of Bt-toxin expressing crops is only possible when insect resistance will be avoided for a long period. Therefore, an insect resistance management (IRM) is required to delay or even to avoid the development of resistance in *O. nubilalis.* In this study important scientific data on the baseline susceptibility of *O. nubilalis* towards the Bt-toxin Cry1F, its population structure concerning pheromone races and alternative host plants, and on the rates of gene flow between different populations of *O. nubilalis* were compiled, which are conductive to a successful resistance management and support the application of the HDR strategy.

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This study provides part of the scientific background for a possible cultivation of Bt-maize expressing Cry1F toxin in Europe. Although the LC-values, obtained for the European populations in baseline susceptibility bioassays were slightly higher than those for the American populations applying the same mortality criteria, the overall differences in Cry1F baseline susceptibility were low and were probably due to the natural variation among the different populations. Considering that resistance development is defined by an increase of the 500- to 1000-fold susceptibility, the observed differences are biologically meaningless. It is therefore expected, that the use of standard procedures will allow baseline susceptibility testing on only a small number of European populations for an efficient monitoring of the resistance development to Bt-toxins.

The analysis of the population structure of ECB by means of GC and allozyme analyses was performed to explore its mating system (random or assortative mating), to examine the postulated relationship of pheromone races with specific host-plants and to estimate gene flow between the populations of *O. nubilalis*. These genetic and biological aspects are important to ensure that the HDR strategy will provide a successful control of ECB. For that, one of the most important requirements constitutes, that a refuge of non-Bt-plants needs to provide enough homozygous sensitive individuals to ensure random mating with the presumably few homozygous resistant individuals. Only in this case the resulting progeny would be heterozygous resistant, and thus susceptible to Bt. Important findings from this study are:

1. A sex-linked control of the sex pheromone behavioural responses in *O. nubilalis*, as described by Glover et al. (1990), cannot be confirmed in this study with the TPI

marker gene for the tested populations (Chapter 4). Thus, it is not possible to determine an affiliation to a specific pheromone race by means of the banding pattern of the *Tpi* allele. These results have implication for the HDR strategy in terms of monitoring as the *Tpi*-locus can not longer be taken as an exclusive monitoring tool to determine the pheromone race regarding the mating behaviour of individuals from Bt-fields and refuges.

2. ECB individuals collected on hop were determined to be Z-race, and ECB individuals collected on mugwort exhibited all three pheromone types (see Chapter 4). Therefore alternative host plants like hop are likely to be considered as refuges for a resistance management. However, as long as quantitative measures of the role of wild and cultivated hop plant refuge are not known, there is no reason to decrease the use of conventional maize as refuge for Bt-maize. These results support for the HDR strategy as wild and cultivated hop may act as additional 'safety margin' for hosting susceptible ECB individuals ready for mating with resistant individuals.
3. The GC-results from this study confirm that the two pheromone races of ECB cannot be designated as "host plant races". Although a number of studies were done on the mating system of *O. nubilalis* during the last decades, the question if there is random or assortative mating remains unsolved. However, from this study it is obvious that the mating behaviour cannot be strictly assortative. These results support the use of the HDR strategy in terms of delaying resistance development since susceptible individuals living on other host plants (even from the opposite pheromone blend) may mate with possible resistant individuals from Bt-maize fields.
4. Allozyme studies of several European and one Asian ECB populations revealed an overall high gene flow. Hence the requirement of the HDR strategy for delaying the resistance development to Bt-toxins produced by transgenic maize, which needs a high level of gene flow between individuals feeding on transgenic and refuge plants, are complied with.

8. Summary

One of the most promising and effective method for a control of the dominant maize pest, *Ostrinia nubilalis*, is the cultivation of Bt-maize, which expresses lepidoteran-specific toxins. For a sustainable use of this technology an insect resistance management (IRM) is required to delay resistance development of the target pest, because in the past insects have developed resistance to all major classes of chemical insecticides. As a consequence of the increasing cultivation of Bt-plants since 1996 in the USA and since 1998 in Europe (Spain) it is suspected that resistance against several Bt-toxins is likely. The most promising strategy to recognize and counter such a development seems to be a monitoring of the baseline susceptibility of the target pest and the implementation of the high-dose/refuge (HDR) strategy, which entails the cultivation of Bt-maize expressing high toxin concentrations in combination with setting up refuges. For the HDR strategy to be effective, further requirements (low incidence of resistance, recessive inheritance of resistance) are essential.

Baseline studies are an efficient approach to observe a development of resistance world-wide provided that standardised procedures will be followed. Bt-maize producing the Cry1F protein was commercially available in 2003 in the USA and is pending regulatory approval in the EU. Therefore, in this study the baseline susceptibility of ECB to Cry1F prior to a widespread commercial use of Cry1F Bt-maize in Europe and the USA was established for 11 European (EU) and, in cooperation with Prof. Blair D. Siegfried (Nebraska), for 24 North American (US) populations of ECB. Mortality and growth inhibition were ascertained for each population. Inter-population variation in the susceptibility of EU and US ECB neonates to Cry1F protein and intra-population variation of the European ECB populations with regard to the development of effective resistance monitoring methods were characterized. The results showed small, but meaningless differences (for the HDR strategy) in the observed susceptibility between the European and North American ECB (up to 3-fold higher LC_{50} values for the European populations), among the various populations within each continent (up to 3-fold), and in two cases among replicates for the same European population (up to 7-fold). Relevant differences in susceptibly levels would be a factor >100 e.g. regarding the level of high Bt-toxin expression in 1507 maize. The source of the differences in susceptibility between EU and US borers is most likely due to the natural variation, differentiation in the test protocols applied, or the use of different toxin batches for different bioassays. Differences among and within populations are probably due to the bioassay procedure, which was performed as the surface application method. It was observed for the

European bioassays that temperature conditions may influence mortality and growth of ECB larvae.

Another research area in this study was the population structure of ECB regarding the different sex pheromone races, their spatial distribution and their association with specific host plants. For a successful implementation of the high-dose/refuge (HDR) strategy for resistance management reliable data are needed on these aspects of ECB genetics and biology. Any resistant insect emerging from the Bt-crop should be able to mate with susceptible adults emerging from the refuge. The resulting offspring will be heterozygous in terms of their resistance genes, and thus susceptible to Bt. GC-analysis was used to assess the distribution of the two pheromone races of the ECB in several European countries and from different host plants. The results indicated that ECB individuals from maize populations in Italy and Greece were predominantly E-race, whereas those from Germany, France, Spain, Serbia and Croatia were mostly Z-race, including a German hop population. In a German mugwort population all three pheromone types were present. Nearly all populations contained a considerable number of hybrids (EZ) except for Pola (Serbia) and Poitiers (France) with pure Z-race. After race-determination, *Tpi* (Triose-phosphate-isomerase) allozyme analyses were performed with males and with GC-analysed females. The obtained results indicated that the *Tpi* locus cannot be reliably used as a marker for the different pheromone races as described previously.

Allozyme electrophoresis was used to examine the genetic variability of the ECB for surveying the sustainability of Bt-maize, because the strategy for delaying resistance to Bt-toxins requires high gene flow between ECB from transgenic and refuge plants. ECB samples collected from 22 locations in Europe and one from Asia (Georgia) were investigated for their allozyme variation at five loci, which were known to be polymorphic in *O. nubilalis*. All populations were collected from maize, except one which was collected on hop. The overall genetic differentiation was low, suggesting a high and homogeneous gene flow. No obvious genetic differences could be detected between the ECB population on hop and those collected on maize, nor between the two pheromone race populations collected on maize. However, a limitation of protein electrophoresis could be that insufficient polymorphism existed for an accurate assessment of the population structure in this study.

The overall results of this study support strongly the application of the HDR strategy to counteract and delay a pest resistance development of ECB in Europe and the US. The risk of resistance development is real but it can be managed.

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10. Abbreviations

AAT	Aspartate-amino-transferase
APN	Aminopeptidase N
BBMV	brush border membrane vesicle
Bt	*Bacillus thuringiensis*
DHAP	Dihydroxyacetone phosphate
EC	effective concentration
ECB	European corn borer
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EFSA	European Food Safety Authority
GC	gas chromatography
GMO	Genetically modified organism
GPI	Glucosephosphate-isomerase
HBdH	3-Hydroxybutyrate-dehydrogenase
HDR	high-dose/refuge
i.d.	inner diameter
IRM	insect resistance management
LC	lethal concentration
L:D	Light:Dark
MCB	Mediterranean corn borer
$MgCl_2$	Magnesium chloride
MTT	Thiazolyl Blue
NAD	Nicotinamid Adenine Dinucleotid
NADP	Nicotinamid Adenine Dinucleotid phosphate
NBT	Nitroblue Tetrazolium
PAT	Phosphinotricin N-acetyltransferase
PGM	Phosphogluco-mutase
PMS	Phenazin Methosulfat
RH	relative humidity
RT	room temperature
SIM	single ion modus
Tris	Tris(hydroxymethyl)aminomethane
TPI	Triose-phosphate-isomerase

USDA NASS	United States Department of Agriculture National Agricultural Statistics Service
WCR	Western corn rootworm

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